Research article

Chitosan-induced enhanced expression and activation of alternative oxidase confer tolerance to salt stress in maize seedlings

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ABSTRACT

This study aimed to investigate the possible alleviating effect of chitosan on salt-induced growth retardation and oxidative stress and to elucidate whether this effect is linked to activation of mitochondrial respiration on the basis of alternative respiration in maize seedlings. Salt stress significantly reduced root length and plant height in comparison to the control, whereas foliar application of chitosan ameliorated the adverse effect of salinity to a certain degree. Moreover, chitosan resulted in plant growth promotion as compared to unstressed seedlings. The separate applications of chitosan and salt had a stimulatory effect on the activities of antioxidant enzymes; however, combined application of chitosan and salt were more effective than that of chitosan or salt alone. Similarly, mitochondrial total respiration rate (V_t) and alternative respiration capacity (V_{alt}) were increased by separate applications of chitosan and salt; however, the combination of chitosan and salt gave the highest values for these parameters. The highest values of V_{alt}/V_t was recorded at seedlings treated with salt plus chitosan. Similarly, cytochrome respiration capacity was also increased by chitosan in both stress-free and stressed conditions. In addition, *AOX1*, encoding alternative oxidase, was significantly upregulated by chitosan and/or salt. The maximum transcript level was recorded at seedlings treated with salt plus chitosan. Chitosan also significantly decreased superoxide anion and hydrogen peroxide contents and lipid peroxidation level under normal and the stressed conditions. These results suggest that the mitigating effect of chitosan on salt stress is linked to activation of alternative respiration at biochemical and molecular level.

1. Introduction

Salinity is one of the most severe environmental stresses limiting the growth and productivity of plants. Its detrimental effect on agricultural production is increasing day by day all over the world due to a variety of reasons, such as natural phenomena, improper irrigation practices and high evaporation in arid and semi-arid areas (Tuteja, 2007).

Salt stress leads to many metabolic disorders such as nutrient imbalance, oxidative stress, and ionic toxicity and, eventually, affects negatively plant growth and productivity (Jacoby et al., 2011; Parida et al., 2004). These metabolic disorders manifest itself by triggering the excessive production of reactive oxygen species (ROS), which causes serious damages on membrane lipids, proteins, nucleic acids, and photosynthetic apparatus (Erdal and Turk, 2016; Gorcek and Erdal, 2015; Turk et al., 2018). Plants have several defense strategies in response to excessive level of ROS. ROS are neutralized by an effective defense system that consists of includes enzymatic (superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), etc.) and non-enzymatic (glutathione, vitamin C, *etc.*) antioxidants. However, plant tolerance to environmental stresses is not only due to the prolonged activation of this defense system, it is also linked to integration capability of different metabolic processes such as photosynthesis and mitochondrial respiration. Possible degradation in any of these complex metabolic processes brings unfavorable increase of ROS (He et al., 2019).

Plant mitochondria and chloroplasts, the energy transducing organelles, are inevitable producers of ROS since ROS formation is a natural result of electron leakages formed in protein complexes in electron transport chain in even unstressed conditions (Erdal and Genisel, 2016; Erdal et al., 2015; Siedow and Moore, 1993). In mitochondria, these protein complexes locate inner membrane and the major production sites of superoxide (O_2^-) anion are complex I and ubisemiquinone in complex III (Moller and Kristensen, 2004). In addition to this normal cytochrome pathway where both electrons are transported from ubiquinone to oxygen via complex IV, plant mitochondria also have an alternative electron transport chain in which alternative oxidase (AOX) enzyme functions and electrons are directly transported from ubiquinone to oxygen thorough AOX. Alternative respiration pathway, therefore, causes to heat production instead of ATP and reduction of ROS formation because electrons bypass two (Complex III and IV) of the

three sites of energy conservation (Erdal et al., 2015; He et al., 2019; Jacoby et al., 2011). Moreover, AOX prevents the blockade of electron flow and helps maintain the intracellular oxygen-water concentration, thus provides optimization of respiration metabolism under normal and stressed-conditions (Chien et al., 2011; Erdal and Genisel, 2016; Moller and Kristensen, 2004). AOX also plays important roles in plant acclimation process to environmental stresses by modulating the balance of carbon and nitrogen, carbon-use efficiency and the ratios of ATP/ADP and NAD(P)H/NAD(P)$^+$ (Finnegan et al., 2004; Hu et al., 2017). Several studies have exhibited that AOX has an important role in the adaptation of plants to environmental stresses, such as salt, cold, drought, and high light (He et al., 2019). These studies have shown that salt stress-tolerance abilities of plants are linked to increasing in gene expression and enzymatic activity of AOX (He et al., 2019; Jacoby et al., 2011). Moreover, recent studies have focused on encouraging alternative respiration pathway and exogenous application of plant growth regulators and various signal compounds such as salicylic acid (Belozerova et al., 2014), hydrogen peroxide (Feng et al., 2008), respiration inhibitors (Erdal et al., 2015), and progesterone (Erdal and Genisel, 2016) have been determined to improve plant stress tolerance by stimulating the activation of AOX at the level of biochemical and/or molecular.

Chitosan, one of the plant growth regulators and stress tolerance inducers, has recently been used in plant protection studies. Chitosan (2-amino-2-deoxy-b-D-glucosamine) is a partially deacetylated form of chitin, a natural degradable biopolymer, which is main component of exoskeletons of crabs, shrimp, lobster, and insects, cell walls of fungi, nematode eggs and gut linings. It was firstly identified as an elicitor of plant responses because it incited phytoalexin production as a proteinase inhibitor (Walker-Simmons et al., 1983). Since then, chitosan has been demonstrated to have significant effects in the stages of germination, growth, and flowering of various crop species, including cereal, fruit, ornamental, and medicinal crops. These studies exhibited that in addition to its antifungal (Ma et al., 2013), antibacterial (Sudarshan et al., 1992), and antiviral (Davydova et al., 2011) effects, chitosan promotes germination index, root growth, shoot height, and seedling vigor in begonia and maize (Chen et al., 2016; Mondal et al., 2013); reduces flowering time in several ornamental plants (Amiri et al., 2016; Safikhan et al., 2018); increases fresh and dry weights in potato (Asghari-Zakaria et al., 2009); reduce pathogen attack and infections to plants (Reddy et al., 1999); improves activities of antioxidant enzymes in many plants (Guan et al., 2009; Hong et al., 2012; Jabeen and Ahmad, 2013); decreases electrolyte leakage and lipid peroxidation level in apple seedlings under drought stress (Yang et al., 2009); induces the synthesis of indole-3-acetic acid in tobacco (Guo et al., 2009); and the expressions of genes related to vegetative growth, development, and primary metabolism in wheat (Zhang et al., 2017). Despite all these knowledge, there are still many points that need to be clarified about the effects of chitosan on plant metabolism.

The specific objectives of the current study were to elucidate whether chitosan-induced stimulation of plant growth and stress tolerance is linked with the activation of mitochondrial respiration pathway, in particular alternative respiration, in addition to enzymatic antioxidant system. Our findings are expected to provide a new insight into the physio-biochemical and molecular mechanisms of chitosan as a growth promotor and stress tolerance inducer, which will provide an important guidance for its use in agriculture applications.

2. Materials and methods

2.1. Experimental design and treatments

The seeds of maize (*Zea mays* cv. Arifiye) were surface sterilized with 3% H_2O_2 for a short time and %5 NaOCl solution for 5 min and then thoroughly rinsed a few times with distilled water. After the sterilization, the seeds were incubated in petri dishes including wet filter paper for 72 h at 25 °C in a germination cabinet. Uniform seedlings were selected and transferred into plate holes on hydroponic media containing a half-strength Hoagland nutrient solution. After 2 d of cultivation, the medium was changed to full-strength Hoagland solution. The seedlings were grown in a growth chamber (14 h photoperiod at 25 $\pm$ 1 °C, and 10 h dark at 20 $\pm$ 1 °C, relative humidity was approximately 70%). The eleven-day-old seedlings were divided into four groups: (1) control, foliar spray with distilled water; (2) salt, NaCl (100 mM) addition to the Hoagland solution + foliar spray with distilled water; (3) salt + chitosan, NaCl (100 mM) addition to the Hoagland solution + foliar spray with chitosan (0.1% w/v); (4) chitosan, foliar spray with chitosan (0.1% w/v). The seedlings were grown for three days more before harvesting, and then collected to assess the changes occurring. Chitosan (Sigma-Aldrich, USA) was dissolved in 1% acetic acid to get desired concentrations of (0.1% w/v). The pH of the solution was adjusted to 6.5 with NaOH. The chitosan concentrations were screened from a preliminary study comprising different levels of chitosan.

2.2. Growth characteristics

After harvesting, roots and leaves of the seedlings were washed. The root length and plant height were measured by using a meter scale. The results were presented as the mean of the values obtained.

2.3. Extract preparation

Leaf samples (0.5 g) were homogenized in 5 ml of 0.1 M phosphate buffer (pH 6.75) containing 0.3% (w/v) polyvinylpyrolidone and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15 000 rpm for 15 min at 4 °C. The supernatant was used as the source of enzyme and protein.

2.4. Protein content and antioxidant activity

The amount of total soluble protein was determined by using the bicinchoninic acid assay according to Smith et al. (1985) at 562 nm. The protein values corresponding to absorbance values were obtained utilizing the standard chart in which bovine serum albumin was used as a standard. Superoxide dismutase (SOD) activity was registered according to method of Agarwal and Pandey (2004) by the inhibition of the photochemical reduction of nitroblue tetrazolium chloride (NBT). The 3 ml of reaction mixture contained 50 mM potassium phosphate (pH 7.8), 13 mM methionine, 63 μM NBT, 100 μM EDTA, 390 μl riboflavin, and 30 μl supernatant. The reaction was started by using light source, 15 min later the reaction was ended by switching off the light and the absorbance was read at 560 nm. One unit of enzyme activity was described as the quantity of the enzyme that leads to 50% inhibition of NBT reduction.

Guaiacol peroxidase (GPX) was determined according to method of Ye et al. (2003) by measuring the increase in absorption and color development at 470 nm. The reaction mixture (3 ml) included 10 μl enzyme extract, 0.1 M sodium phosphate buffer (pH 5.5), 5 mM guaiacol (hydrogen donor) and 5 mM H_2O_2. One unit of GPX activity was defined as the amount of enzyme that causes a change of 0.01 in absorbance per mg protein and per minute.

The activity of catalase (CAT) was detected by Gong et al. (2001) by monitoring the consumption of H_2O_2 at 240 nm for 1 min. The reaction medium (3 ml) contained 100 mM potassium phosphate buffer (pH 7.5), 40 mM H_2O_2 and 50 μl enzyme extract. One unit of CAT activity was described as the amount of enzyme required to decompose 1 μmol of H_2O_2 per minute at 25 °C.

Ascorbate peroxidase activity (APX) was measured following the decrease in A290 nm due to H_2O_2-dependent ascorbate oxidation according to Nakano and Asada (1981). The assay mixture (3 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2,

250 μM ascorbate, 0.1 mM EDTA and 100 μl enzyme extract. The APX activity was calculated by using the molar extinction coefficient of 2.8/mm/cm.

2.5. Reactive oxygen species (ROS) and lipid peroxidation degree

The production rate of superoxide anion ($O_2^{-\cdot}$) in leaves was determined according to the method of Elstner and Heupel (1976) as a spectrophotometrically at 530 nm. The amount of superoxide was calculated from a calibration curve prepared using sodium nitrite as standard. The contents of hydrogen peroxide (H_2O_2) was measured according to Velikova et al. (2000). The values of absorbance were determined at 390 nm. The formation of H_2O_2 was calculated by using a standard calibration curve.

Lipid peroxidation was measured by estimating malondialdehyde (MDA), a product of lipid peroxidation, using a thiobarbituric acid reaction Velikova et al. (2000). The changes of absorbance were recorded at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. The content of MDA (ng g^{-1} FW) was calculated by using the molar extinction coefficient 155 (mM cm^{-1}).

2.6. Mitochondria isolation

The mitochondria isolation was performed according to the methods of Plummer (1980) and Chien et al. (2011) with some modifications. Plant leaves were extracted with homogenization buffer (0.4 M mannitol, 50 mM MOPS (pH 7.2), 2 mM EGTA, 4 mM L-cysteine, 20 mM β-mercaptoethanol, 0.6% (w/v) PVP and, 0.5%, (w/v) BSA) at ratio to 1:4 (sample weight: buffer volume). Firstly, the plant extract was centrifuged at $400 \times g$ for 5 min at 4 °C, and then supernatant was centrifuged again $2000 \times g$ for 15 min. The portion of supernatant was taken and centrifuged again at 10 000 × g for 30 min. The supernatant was spilled and the pellet in the bottom of the tube was extracted in a wash buffer including 0.3 M mannitol, 20 mM MOPS (pH 7.2), 0.1% (w/v) BSA and 1 mM EGTA and then centrifuged at $2000 \times g$ for 10 min. The supernatant portion was centrifuged at 15 000 × g for 15 min and the crude mitochondrial pellet was gently extracted in wash buffer, and thereafter mitochondria were purified in Percoll gradient according to Grabel'nykh et al. (2011). Suspension of washed mitochondria (60–90 mg of protein) was resuspended in 1–1.5 ml of medium containing 300 mM sucrose and 40 mM MOPS-KOH buffer (pH 7.4), layered on Percoll gradient, and centrifuged at $24500 \times g$ for 45 min. After centrifugation, intact mitochondria were collected from the interface between 23% and 35% Percoll. Mitochondria were washed twice with the resuspension medium, recovered by centrifugation ($24000 \times g$, 10 min). The pellet of purified mitochondria was washed again with 10 vol of suspending medium and precipitated at $24000 \times g$ for 10 min. The obtained the suspension of mitochondria was stored on ice to use respiration parameter.

2.7. Total mitochondrial respiration rate, cytochrome respiration capacity, and alternative respiration capacity

The rates of total mitochondrial respiration rate (V_t), cytochrome respiration capacity (V_{cyt}) and alternative respiration capacity (V_{alt}) (expressed as AOX activity) were determined using an oxygen electrode. Mitochondria samples were placed in oxygen electrode cuvettes and were incubated in a standard reaction medium containing 0.1 M ATP, 0.5% BSA, 1 mM DTT, 0.15 mM pyruvate at the room temperature. Oxygen consumption was identified by adding 2 mM KCN (cytochrome pathway inhibitor) and 4 mM SHAM (AOX inhibitor) in medium for 2 min intervals and the values of oxygen consumption were recorded. V_{cyt} was presented as the difference between V_t and V_{alt} (Borecky and Vercesi, 2005).

2.8. RNA isolation and analysis

RNA isolation was performed by using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. RNA concentrations of the samples were determined using the Nanodrop apparatus (Thermo Scientific μDrop Plate). The cDNAs of each samples were synthesized using 2 μg of RNA by ThermoScript™ RT-PCR System with cDNA Synthesis Kit (Quantiscript Reverse Transcriptase) and they were kept at −20 °C for further molecular analysis.

The gene expression levels of AOX were determined using quantitative real-time PCR. Specific gene primer and housekeeping gene (18S rRNA) were obtained from Qiagen. The quantitative real-time PCR reactions were designed as reaction mixture including 5 μl cDNA solutions, 1 μl each primer, 6.5 μl dH₂O and 12.5 μl Mastermix (Rotor-Gene Probe PCR Master Mix, 2x). PCR amplification reactions were carried out by a Thermal Cycler (Qiagen, Rotor-Gene Q); as follows; 15 min initial denaturation at 95 °C, 41 cycles denaturation at 94 °C of 15 s, annealing/extension at 60 °C and 1 min. The obtained Ct (Cycle threshold) values were used based on the 2ΔCT method with raw data normalization using the 18S rRNA reference gene as the control and fold change criteria to analyse real-time PCR data. Analysis of the relative gene expression was occurred based on Qiagen Data Analysis Center. The used amplification primers for RT-PCR in this study were shown in Table 1.

2.9. Statistical analysis

The results presented in the manuscript are the average of nine values that obtained from three independent experiments with three repetitions for each sample. Comparison of the significant differences between the samples was performed by using the SPSS 20 package software with single variant analysis (ANOVA). Statistical significance was described as $P < 0.05$ by using Duncan's multiple comparison test and standard errors were displayed at all figures and tables in results sections.

3. Results and discussion

3.1. Effect of chitosan and salt on root length and plant height of maize seedlings

The increase of salt concentration in plant growth media severely reduces germination rate, seedling establishment, growth, development, and survival, which are critical parameters in determining plant productivity (Tuteja, 2007). These deleterious effects of salinity are resulted from its potential to form osmotic and ionic stress that ultimately trigger oxidative stress in plants (Jacoby et al., 2011; Parida et al., 2004). In literature, there are a lot of studies exhibiting the inhibitory effect of salt stress on growth and productivity of plants (Erdal et al., 2012; Genisel et al., 2015). In accordance with prior reports, this study showed that salt application caused to significant reductions by 24.4% for root length and 20.8% for plant height in 14-day-old maize seedlings (Fig. 1). This growth reduction could be due to deceleration in elongation and division of cells in addition to salt-induced dysfunction in various biosynthesis reactions. Similarly, many prior researchers

Table 1
Amplification primers for RT-PCR in this study.

Primer	Direction	Sequence
AOX1	Forward	AGGTGCTTTTCTGGCGTTT
AOX1	Reverse	CGATATTAGCGAGCCCAATTC
rrn18	Forward	AAAGTGGCGGAATGCTCTC
rrn18	Reverse	TCTACGGATTTCACCCCTACA
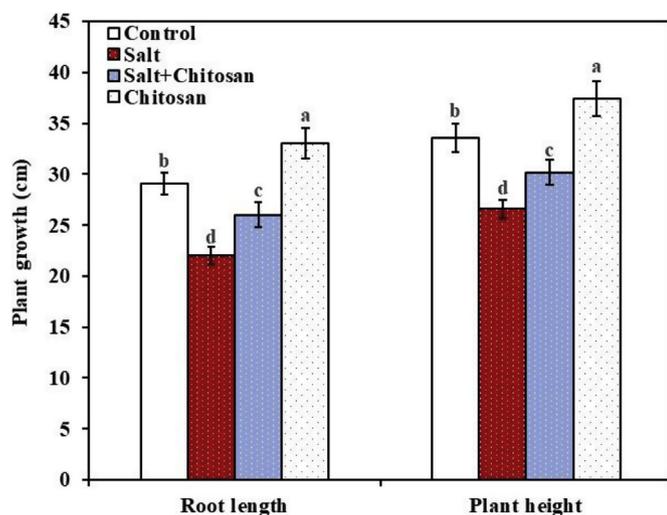
Fig. 1. Effects of chitosan and salt on root length and plant height of maize seedlings. 11-days-old maize seedlings were treated with chitosan and salt, separately or in combination, for 72 h. Experimental data are presented as means ± SE for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.

expressed that the salinity-induced growth retardation is associated with inhibiting synthesis reactions, slowing cell division and expansion, and declining carbon assimilation due to stomatal limitation and/or metabolic deterioration (Erdal, 2012b; Genisel et al., 2015; Hajiboland et al., 2014; Murata et al., 2007). It has been firmly appointed that

activities of some key enzymes (*e.g.* Rubisco and nitrate reductase) involved in primary metabolic pathways are reduced under salinity conditions (Erdal, 2012a; Sharwood et al., 2014). Exogenous chitosan application exhibited greater root length (by 13.5%) and plant height (by 11.3%) as compared with the control plants without the stress. The present findings agree with the findings of earlier studies, in which chitosan has been shown to have a growth enhancement effect in several plants, such as tomato (El-Tantawy, 2009), maize (Guan et al., 2009), sweet pepper (Ghoname et al., 2010), coffee (Nguyen Van et al., 2013), turmeric (Anusuya and Sathiyabama, 2016), and begonia (Chen et al., 2016). However, it should be noted that plant response to chitosan might vary depending on the type of the used chitosan and plant species and developmental stages (Pichyangkura and Chadchawan, 2015). The growth-promoting effect of chitosan was highly likely due to chitosan-induced enhancement in primary metabolic pathways, such as photosynthesis, glycolysis, and nitrogen assimilation. Zhang et al. (2017) reported that chitosan regulated a series of primary metabolic pathways including carbon and nitrogen metabolisms in wheat leaves and thus contributed to plant growth promotion. In addition, it is possible that this effect of chitosan is linked to stimulation of some signaling pathway related to plant hormones such as gibberellins and auxin (Safikhan et al., 2018). We also found that chitosan application compensated to some extent for growth reduction of maize exposed to salinity conditions (Fig. 1). Compared with the salt-stressed seedlings alone, chitosan supplementation increased root length by 13.7% and plant height by 10.7%, respectively. These results were consistent with some previous findings, in which it was reported that chitosan ameliorated the negative effects of salt stress on the growth of safflower and sunflower (Jabeen and Ahmad, 2013), wheat (Zou et al., 2015), milk thistle (Safikhan et al., 2018), and maize genotypes (Al-Tawaha et al., 2018). Studies regarding with the mode of action of chitosan on plant
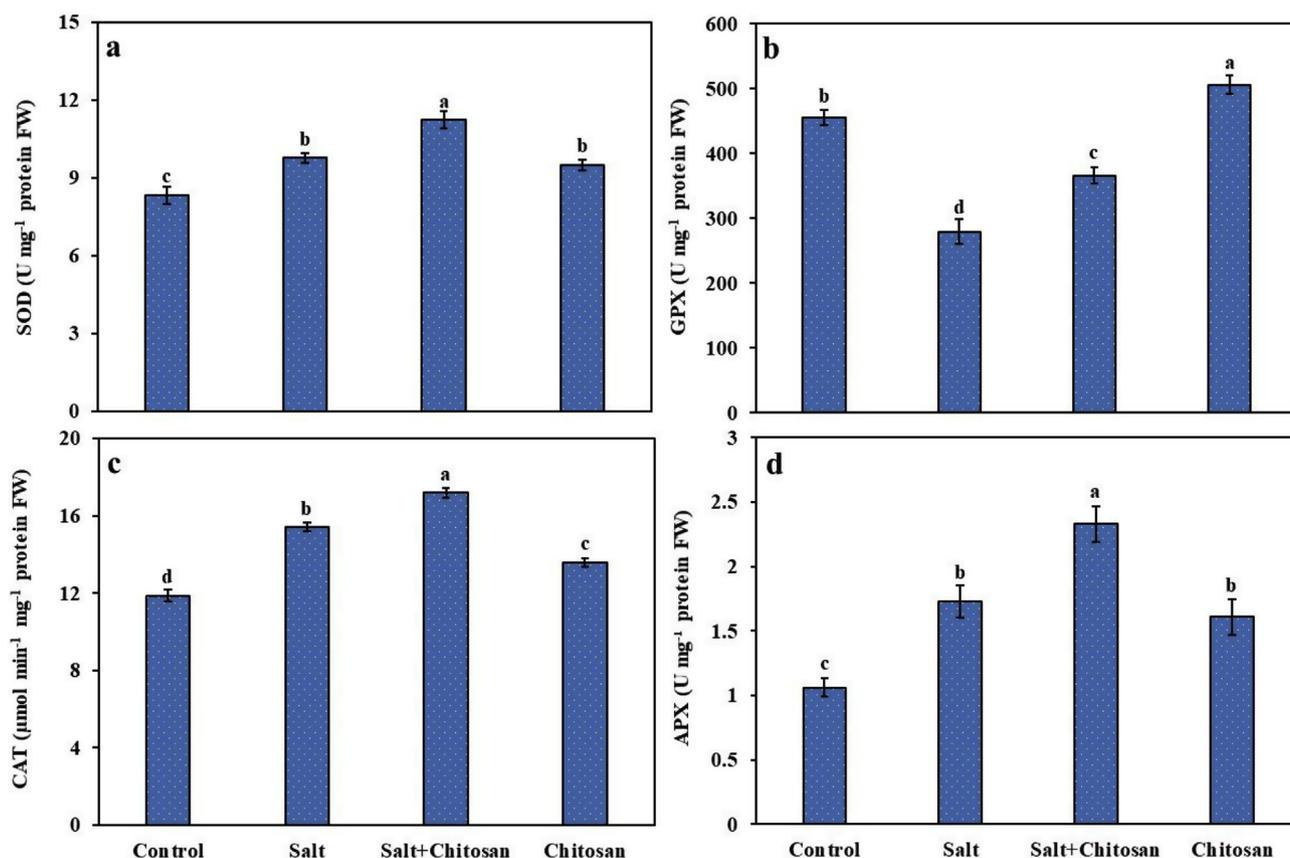
Fig. 2. Effects of chitosan and salt on antioxidant enzyme activity (**a** SOD; **b** GPX; **c** CAT; **d** APX) of maize leaves. 11-days-old maize seedlings were treated with chitosan and salt, separately or in combination, for 72 h. Experimental data are presented as means ± SE for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.

growth demonstrated that chitosan causes to a number of physiological, biochemical and molecular changes in plant metabolism through stimulation of various biological processes (Limpanavech et al., 2008; Nguyen Van et al., 2013). Chitosan performs these effects via distinct signaling pathways involving different second messengers (Malerba and Cerana, 2016).

3.2. Effect of chitosan and salt on antioxidant enzyme activities of maize leaves

Plants are equipped with enzymatic and non-enzymatic antioxidant systems, which play critical role in detoxifying ROS. SOD is regarded as the most important enzyme in cellular defense mechanisms against ROS and directly dismutases O_2^- to H_2O_2. The formed H_2O_2 is eliminated by converting to water in subsequent reactions. GPX, CAT, and APX are main enzymes responsible for H_2O_2 scavenging. Earlier researchers have reported that significant increases happen in the activities of antioxidant enzymes' in plants grown under salt stress (Erdal et al., 2011, 2012; Gorcek and Erdal, 2015). In parallel with previous studies, we found that salt stress significantly elevated the activities of SOD, CAT, and APX activities by 17.4%, 30.1%, and 63.2%, compared to their controls, respectively, whereas GPX activity exhibited a drastic decline by 38.7% under the stress (Fig. 2). In a similar manner, chitosan application significantly induced the activities of SOD, GPX, CAT, and APX as compared to their controls, as well. The increase ratios were 14.1%, 11.2%, 14.7%, and 51.9%, respectively. When combined with salt, chitosan application resulted in further increases by 35%, 45.1%, and 119.8% in the activities of SOD, CAT, and APX in comparison to control seedlings. In addition, it declined significantly salt-induced reduction in GPX activity by 20.1% relative to the stressed-seedlings alone (Fig. 2). These findings were in harmony with those of reported by earlier studies. They showed that chitosan could regulate the activities of antioxidant enzymes and increase plant tolerance to biotic and abiotic stresses (Chen et al., 2016; Guan et al., 2009; Mondal et al., 2016; Pirbalouti et al., 2017; Safikhan et al., 2018; Zong et al., 2017).

3.3. Effect of chitosan and salt on mitochondrial respiration pathway in maize leaves

The data regarding with the effect of salt stress on the mitochondrial respiration are quite controversial. Some of the studies reported that salt stress increased the respiration rate as a result of adaptation to salinity; however, the others informed it decreased or did not affect (Jacoby et al., 2011). These contradictory results were possibly due to differences of the studied plant species, studied tissues, and time and severity of the stress. In the present study, salt stress increased the total respiration rate (V_t) by 27.4% in comparison to control. Chitosan application also resulted in significant increments by 32.9% and 86.3% in comparison to control under normal and the stressed conditions, respectively (Fig. 3). This is the first data exhibiting effect of chitosan on total respiration rate in plants and this data revealed that chitosan has a stimulating effect on total respiration rate of plants under normal and the stressed conditions. A supporting study demonstrated that chitosan application stimulated expression levels of glycolysis-related genes and thus provided fuels to plant respiration (Chamnanmanoontham et al., 2015). It is well known that plants need to high amount of energy to maintain their growth and cope with the stress under stressed conditions. In this study, the enhanced mitochondrial respiration rate might indicate an increase in energy status of cells; however, to interpret correctly the changes in total respiration rate, it is needed to determine the changes occurred in cytochrome respiration capacity (V_{cyt}) and alternative respiration capacity (V_{alt}).

The cytochrome pathway is an indicator of the energy state of the cell, while the activity of alternative pathways is more related to the tolerance to stress (Del-Saz et al., 2018). Although salt stress led to a marked elevation in V_t, it had no important effect on V_{cyt}. However, V_{alt}
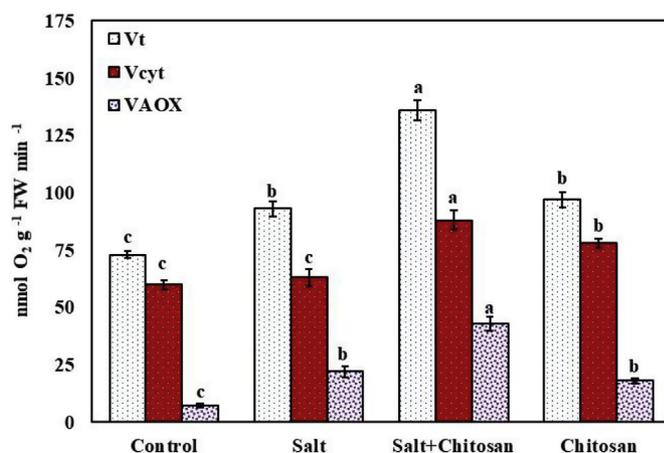

Fig. 3. Effects of chitosan and salt on mitochondrial respiration (total respiration rate, V_t; cytochrome respiration capacity, V_{cyt}; and alternative respiration capacity, V_{alt}) of maize leaves. 11-days-old maize seedlings were treated with chitosan and salt, separately or in combination, for 72 h. Experimental data are presented as means ± SE for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.

was significantly augmented by 206% in salt-applied seedlings in comparison to control. V_{alt} was assessed via measurement of activity of AOX, which is responsible for alternative respiration pathway. On the other hand, chitosan application enhanced both V_{cyt} and V_{alt} under control and saline conditions. The increase ratios were 30 and 150%, respectively (Fig. 3). This meant that while salt application shifted the way of mitochondrial respiration from normal cytochrome pathway to alternative respiration to contribute to coping with the stress, chitosan stimulated both cytochrome pathway to maintain the plant growth and alternative respiration to overcome the stress way under normal and the stressed-conditions. Considering high amount of energy needed for plant growth and stress tolerance, the data obtained from root length and plant height strongly supported this assumption. As mentioned above, both the maximum growth was recorded at chitosan-treated seedlings and salt-induced growth retardation was markedly alleviated by chitosan. Moreover, the changes determined in the ratios of V_{cyt} to V_t and V_{alt} to/V_t was agreement this assumption. In spite of high increases recorded at V_t of salt-applied seedlings compared to the control, the ratio of V_{cyt} to V_t significantly decreased (by 14.5%); however, the ratio of V_{alt} to V_t was higher (by 13.8%) than that of the control (Fig. 4). While V_{cyt}/V_t was not significantly affected by chitosan, V_{alt}/V_t showed an important increase of 88.2% related to control. The highest ratio of V_{alt} to/V_t was recorded as 31.6% in chitosan together with salt-treated seedlings.

3.4. Effect of chitosan and salt on relative expression of AOX1 in maize leaves

Chitosan might be performed its effect on mitochondrial respiration via either its possible allosteric role on AOX enzyme or by stimulating signaling pathways responsible for gene expression of AOX. Some studies demonstrated that chitosan could specifically bind to miscellaneous cell membranes and incite a series of defense reactions by a receptor mode or dependent on its cationic property (Guo et al., 2009; Miya et al., 2007; Zhang et al., 2018). This knowledge suggest that chitosan might have directly affected the activity of AOX in biochemical level. However, considering its inducing role on signaling cascades in plants (Chamnanmanoontham et al., 2015), we also investigated the effect of chitosan on gene expression level of AOX. As well-known that the transcriptional response was faster than the changes in enzyme
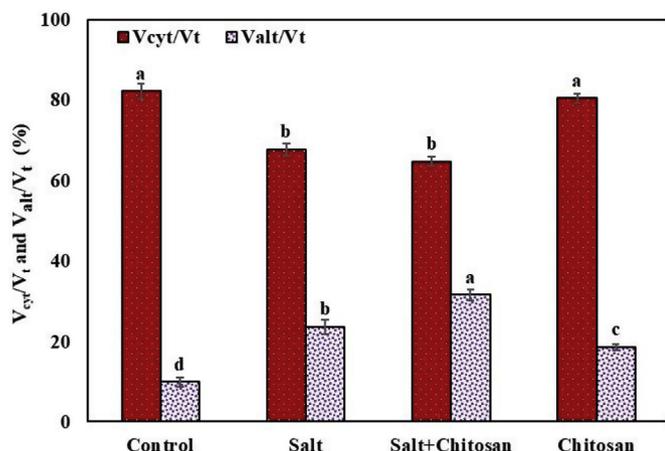
Fig. 4. Effects of chitosan and salt on the ratios of V_{cyt}/V_t and V_{alt}/V_t of maize leaves. Experimental data are presented as means ± SE for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.

Fig. 5. Effects of chitosan and salt, separately or in combination, on the relative expression of AOX1 in leaves of maize seedlings. Experimental data are presented as means ± SE for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.
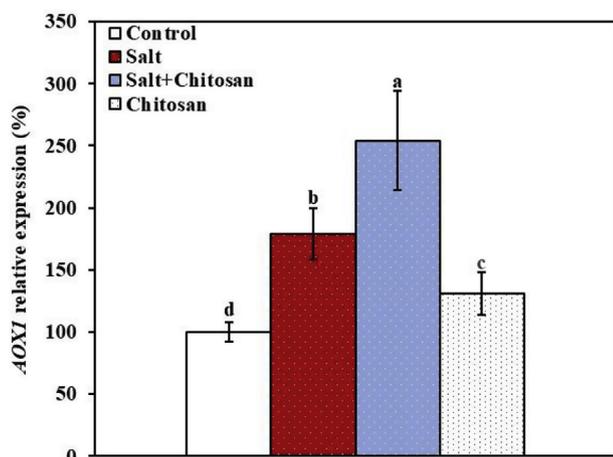
activities (Gibon et al., 2004). A small family of nuclear genes in plants encodes AOX and these genes are highly responsive to abiotic stresses. There are three AOX genes identified in the maize (*AOX1a*, *AOX1b*, and *AOX1c*). While *AOX1* is present both monocot and eudicot plant species, *AOX2* is absent all monocot species (Considine et al., 2002; Feng et al., 2013). Among these genes, *AOX1* is the most intensively studied and is the most responsive to stress. There have been many studies reporting an increase in the expression level of *AOX1* under abiotic

stresses such as salt stress (Arnholdt-Schmitt, 2009; Jacoby et al., 2011), chilling stress (Purvis and Shewfelt, 1993; Vanlerberghe and Mcintosh, 1992), and pathogenic attack (Lennon et al., 1997). In accordance with previous data, we found that the expression level of *AOX1* was significantly up-regulated by 79% under salt stress (Fig. 5). This significant elevation put forward that plant activated alternative respiration pathway to counteract the stress. Similarly, the relative expression of the *AOX1* was higher (31%) in chitosan-applied seedlings than control seedlings. In stress-free medium, the chitosan-induced these increases are a clear indicator of its stimulating effect on signaling pathways responsible for gene expression of AOX. The highest level of gene expression (154%) was observed in seedlings treated with chitosan and salt (Fig. 5). This data meant that chitosan modulated alternative respiration by regulating both activity and gene transcription level of AOX in normal and salinity conditions.

3.5. Effect of chitosan and salt on reactive oxygen species and oxidative damage parameters in maize leaves

At this point, to support the chitosan-induced increase in activities of AOX and antioxidant enzymes, the level of ROS, (O_2^- and H_2O_2) and the degree of lipid peroxidation, which is an indicator used to determine oxidative stress, and MDA level were also measured. Salt-treated seedlings demonstrated a marked elevation by 40.6% in O_2^- content compared to control (Table 2). Previous studies showed that salt stress causes significant elevations in O_2^- content (Erdal, 2012a; Genisel et al., 2015; Gorcek and Erdal, 2015). In spite of high activities of SOD and AOX in salt-treated seedlings, determining high content of O_2^- put forward that plants tried to withstand the stress, but its defense ability was not adequate to achieve it. In chitosan-treated seedling without the stress, as expected, the lowest value of O_2^- content as a natural result of high activities of AOX and SOD were determined. Here, it can be easily said that chitosan-induced high activity of AOX lowered ROS generation by preventing the over-reduction of ubiquinone. This interpretation is strongly supported by the studies of Wang et al. (2011), which reported that transgenic (without AOX) tobacco suspension cell cultures had a high ROS content relative to control. In addition, the mitigating role of AOX on ROS generation has been reported by many investigators (Cvetkovska and Vanlerberghe, 2013; Vanlerberghe et al., 2009). Combined application of chitosan and salt at which the highest activities of AOX and antioxidant enzymes were recorded showed that chitosan significantly ameliorated the inducing effect of salt on O_2^- content. As in O_2^- content, similar changes were determined in H_2O_2 content, as well. Salt-induced increment in H_2O_2 content was significantly reduced by chitosan application (Table 2). This reduction might be attributed to chitosan-induced high activities of GPX, CAT, and APX. In addition, determining the highest activities of AOX in these seedlings meant that chitosan has contributed to keep H_2O_2 content in lower level by prohibiting generation of O_2^-, precursor of many other toxic ROS. The lowest value of H_2O_2 content was recorded at chitosan-applied seedlings in which the activities of GPX, CAT, and APX as well as AOX were higher than those of control seedlings. This data indicates that chitosan improves plant defense by reducing ROS level both in generation stage via activation of alternative respiration and scavenging them through activation of antioxidant

Table 2
Effects chitosan and salt on the contents of superoxide anion, hydrogen peroxide, and malondialdehyde in 14 days old maize leaves. Experimental data are presented as means for three independent experiments with three repetitions. Significant differences were determined by one-way ANOVA with Duncan's multiple range test at $p < 0.05$ and marked with different small letters.

Treatments	Superoxide anion (nmol.min^{-1}.g^{-1} FW)	Hydrogen peroxide (μmol.g^{-1} FW)	Malondialdehyde (nmol.ml^{-1} FW)
Control	3.52c	0.939c	1.64c
Salt	4.95a	1.170a	2.12a
Salt + Chitosan	4.13b	1.060b	1.89b
Chitosan	3.06d	0.901c	1.40d

system.

It is well-known phenomenon that salt stress severely increased the content of MDA, which is an indicator of lipid peroxidation in membranes (Rahman et al., 2016; Shtereva et al., 2015). Our findings were similar to those reported in the current literature. As in O_2^- anion production and H_2O_2 content, MDA content was also increased by salt application. MDA content was recorded by 2.12 nmol ml^{-1} in the control seedlings, while it was recorded as 2.12, 1.40, and 1.89 nmol ml^{-1} in salt-applied seedlings, chitosan-applied seedlings and their combination, respectively (Table 2). High content of MDA in salt-treated seedlings might be attributed to high level of ROS. Chitosan application significantly lowered MDA content under normal and salt-stressed conditions. This reductive effect of chitosan was likely to due to its stimulating effect on alternative respiration and antioxidant system. Our findings are consistent with these earlier reports. Guan et al. (2009) reported that chitosan reduced MDA content in maize under low temperatures. Zong et al. (2017) informed that chitosan treatment lowered MDA content in edible rape leaves under cadmium toxicity by activities of inducing antioxidant enzymes'.

4. Conclusions

In this paper, for the first time it was investigated the effect of chitosan along with and without salt stress on mitochondrial respiration and evaluated the findings together with the changes in activities of antioxidant enzymes'. The results illustrated that chitosan application could enhance plant growth and improve plant tolerance to salinity by increasing total respiration rate, cytochrome pathway, and alternative respiration as well as the activities of antioxidant enzymes'. This study also showed that chitosan carried out this effect at the level of gene expression as well as in biochemical level. These findings will provide a new insight for elucidating the mode of action of chitosan-induced growth- and tolerance-enhancing effect and will be an important resource for the future usability of chitosan in agriculture.

Conflicts of interest

Author declare no conflict of interest.

Author contribution statement

Dr. Hulya Turk designed the experimental framework and performed all the experiments, the statistical analysis of data, and the paper writing.

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