ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 19(4) 2020, 21–30

https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2020.4.2

ORIGINAL PAPER

Accepted: 19.07.2019

THE EFFECT OF CHITOSAN ON GENE EXPRESSION, SOME MORPHOLOGICAL AND PHYSIOLOGICAL TRAITS OF SWEET BASIL (*Ocimum basilicum* L.) UNDER SALINITY STRESS

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ABSTRACT

Sweet basil is an important medicinal plant belonging to Lamiaceae family. In this plant, Phenylpropanoid pathway possesses some enzymes involving in generating suitable essential oil constituents. The main purpose of conducting this study was to investigate the effects of chitosan on sweet basil's growth and physiological parameters as well as gene expression subjected to salinity stress. After employing a foliar-spray of chitosan at 0 (as control) and 0.2 gl^{-1}, the plants were subjected to salinity treatments at 0, 25, 50, 100, and 150 mM NaCl. The results of this research revealed that chitosan, compared to the controls, improved growth parameters under stressed or non-stressed conditions. In this regard, chitosan increased protein and chlorophyll contents as well as the expression of PAL and COVMT genes leading to an increase in phenolic compounds. To sum up, chitosan improved sweet basil tolerance to salinity through influencing the genes involved in the pathway of phenylpropanoid so as to produce secondary metabolites.

Key words: chlorophyll, elicitor, phenol, phenylalanine, phenylpropanoid

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is one of the most important medicinal plants belonging to Lamiaceae family. For a long time, sweet basil has been used as a medicinal plant to cure coughing, headache, diarrhea, parasites, wart, and kidney malfunctions [Labra et al. 2004]. This plant possesses a large number of aromatic compounds with different properties such as repelling insects, anti-parasite, virus, fungus as well as antioxidant activities [Labra et al. 2004, Juliani and Simon 2002].

An accumulation of secondary metabolites in medicinal plants is usually affected and regulated by environmental factors [Wang et al. 2016]. Similar to other environmental stresses, salinity adversely affects the growth and development of plants [Carillo et al. 2011]. Lowering water potential in rhizosphere, enhancing toxicity of sodium and chlorine, and an imbalance in nutrient elements are of main factors leading to a reduction in plants' growth and development under salinity stress [Munns et al. 2002]. In some medicinal plants, however, salinity results in an increase in their secondary metabolites [Wang et al. 2016]. In plants, tolerance to salinity is a complex characteristic involving harmonizing the activity of different genes responsible for ionic secretion, metabolic adjustment, osmotic adjustment, and antioxidant defenses [Munns et al. 2002].

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Using different elicitors is one of procedures employed by plants for reducing the adverse effects of salinity. An elicitor can act as an environmental factor or a signaling molecule transducing a signal or involving biotechnologically in expression of genes, dependent on the biosynthesis of secondary metabolites [Kumar 2000, Rahman et al. 2003]. Based on their origins, elicitors are categorized into three groups: biological, chemical, and physical. The biological elicitors are often derived from composites of microbes' cell walls (i.e. Chitin, Chitosan, and Glucan) as well as carbohydrates (i.e. Oligosaccharides) or the plants' cell walls (i.e. pectin, pectic acid, and cellulose) [Sheikha and AL-Malki 2011]. Chitosan is a prime-derived and deacetylated molecule of chitin resulted from deacetylation of alkaline chitin. Also, it is produced not only by some fungi naturally that its content is lower than that yielded by chitin [Kumar 2000], but also from the cell walls of fungi, the external skeleton of crustaceans, insects' cuticle, and some alga. This compound is biodegradable and fully nontoxic. It has been reported that this compound and other similar compounds are able to induce defensive responses in plants [Falcón-Rodríguez et al. 2009].

Methyl chavicole is one of the most important composites of essential oil of sweet basil. The early precursor of methyl chavicole is phenylalanine. In the pathway of phenylalanine, two paths lead to two products, at first path chavicole and methyl chavicole and in second path eugenol and methyl eugenol are produced [Naderi et al. 2014]. Therefore, the conversion of phenylalanine to cinnamic acid is performed by a phenylalanine ammonia lyase (PAL) enzyme through deamination of phenylalanine. Then, by adding a hydroxyl group (OH) to cinnamic acid, para-coumaric acid is produced and eventually chavicole is created. Finally, chavicole is methylated by chavicole O-methyltransferase (CVOMT) enzyme and eventually methyl chavicole is yielded [Gang et al. 2002]. In this research, the effect of chitosan on: 1) expression of CVOMT and PAL genes and 2) some physiological and morphological sweet basil responses under salinity stress were evaluated.

MATERIALS AND METHODS

Experimental design and treatments. This experiment was conducted in greenhouse and research laboratories of Islamic Azad University in 2017. Sweet basil seeds were prepared from Karaj seed and plant improvement institution. The same and homogenous seeds were selected. The seeds disinfected for 10 minutes by 1% sodium hypochlorite. Then, the seeds were placed on Whattman No. 4 filter paper in sterilized 10 cm diameter petri dishes and covered by aluminum foil. After germination, the homogenous germinated seeds were selected and were replaced to pots containing distilled water moisted sand. The pots were irrigated by Hoagland solution for 20 days [Hoagland and Arnon 1950]. Then Hoagland irrigated basil were foliar sprayed by 0.2% chitosan two times every other three days. Three days after foliar spray, plants were treated with 0, 25, 50, 100 and 150 mM NaCl solutions. During NaCl solution treatments, the pots were irrigated three times a week by Hoagland solution. The plants were kept in greenhouse. The periodically replacement of pots in greenhouse for microclimatic effects avoidance were carried every day. Forty days old plants were picked up for biochemical and physiological assessments.

Total chlorophyll. To assess the total chlorophyll content, a series of samples of fresh leaves were taken and according to method described by Stoeva et al. [2005]. Acetone extractions of fresh plants for pigment extracting were used. The sample absorptions in three wave lengths 646.6 and 663.6 by spectrophotometer spectra 22 USA were achieved. The chlorophyll concentrations in mg g^{-1} fresh plant have been calculated.

Total phenols. The assessments of total phenolic contents were conducted by folin-ciocalteu reagent according to Singleton and Rossi [1965]. The amounts of phenolic constituent were achieved by virtue of Gallic acid standard curve in 765 nm wavelength. Total phenolic contents in plant extracts were expressed in gallic acid equivalents (GAE).

Total protein. For total protein content assessment of treated basil Bradford method [1976] was used. The protein extractions prepared by phosphate buffer containing polyvinyl pirolidine and EDTA. Then Bradford indicator added and amounts of light absorption at 595 nm were measured. Total protein content was determined using bovine serum albumin as a standard.

Total RNA extraction and cDNA synthesis. Total RNA extraction from sweet basil leaves by Cinna Gen kit was accomplished according to manufacturer's instructions. First 0.2 g of leaves was ground in liquid

nitrogen and carried to a vial. Then 600 µl of RNAX Plus added to leaves tissue. After mixing, a clear and homogenized mixture is achieved. The vial was preserved at 4ºC on ice and 16–17 times in several stages was mixed. Then 200 µl chloroform was added to mixture and homogenized. The sample was centrifuged at 12000 rpm, at 4ºC for 15 min. Supernatant was carried to a new vial. Equal volume of isopropanol was added and vial preserved in –20˚C for 20 min. The sample was centrifuged in 12000 rpm, at 4ºC for 15 min. After decanting the supernatant, sediment washed in 1 ml of 70% ethanol. The sample was centrifuged in 7500 rpm, at 4ºC for 8 min. After removing supernatant, sediment preserved in room temperature until ethanol was evaporated. About 10–30 µl of DEPC water added to sediment and sample preserved at 56ºC in hot block (Techno) for 10 minutes. Sample was preserved at –20ºC. If long term preserving is needed, sample should be preserved at –80ºC. Quality of extracted RNA was considered by agarose gel electrophoresis [Sambrook et al. 1989].

cDNA synthesis. First-strand cDNA synthesis from extracted RNA was performed. Complementary DNA was synthesized through the addition of 200 U μl^{-1} (RevertAid™ M-MuLV) reverse transcriptase (fermentase) and 0.5 µg μl^{1} unspecified oligo-dT-primer (MWG-Biotech AG) to other components of mixture [Sambrook et al. 1989]. Then mixture was heated to 42ºC for 60 min and 70ºC for 10 min by Hot block (Techno). At last, the total RNA was used to cDNA amplification by RT-PCR. After making suitable concentration, cDNA samples stored at –20ºC for short term and −80ºC for long term.

Primer designing. Genes expression analysis were conducted by semi-quantitative RT-PCR. These two genes were PAL and CVOMT. The sequence of the genes of studied sweet basil, were achieved in NCBI (www.ncbi.nlm.nih.goc). Primers designing were carried out by Oligo5 and Gene runner software. Primer designing of CVOMT gene by accession number (AB530137) was accomplished. In this research, Tubulin gene was used as internal control. The sequence of above mentioned gene was achieved from NCBI site. Used primers in this research are mentioned in Table 1. The fragments length of gene replication of PAL and CVOMT were 480 and 494 bp, respectively. High Purified Salt Free (HPSF), lyophilized primers were made by Korean Bioneer Co.

Table 1. Primer designing for PAL and CVOMT genes

Gene name	Primer sequence
Forward PAL	5'-GGCTACTCCGGCATAAGATTC-3'
Reverse PAL	5'-GTACGAGCTTCCGTCGAGGATG-3'
Forward CVOMT	5'- CCAATTTCTTCATAGAAGAAAACTC-3'
Reverse CVOMT	5' -GATAAGCCTCTATGAGAGACCTC-3'
Forward Tub	5'GGGGCGTAGGAGGAAAGCA3'
Reverse Tub	5'GCTTTCAACAACTTCTTCAG3'

Table 2. PCR reaction condition

Cycles	Time	Temperature
Predenaturation	4 min	94°C
Denaturation	30 sec	94°C
Annealing	1 min	52–63°C
Final Extension	1 min	72°C
Extension	10 min	72°C

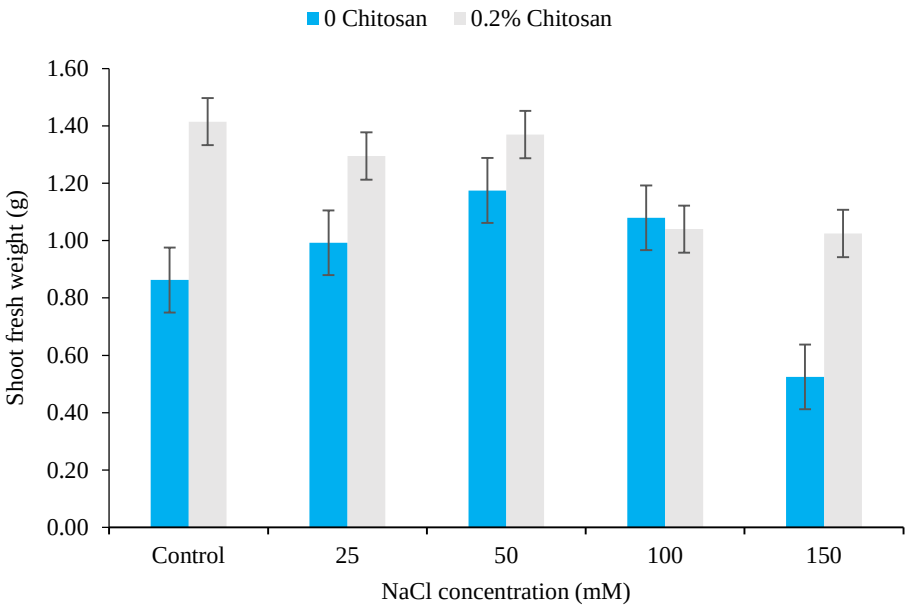

Fig. 1. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray on shoot fresh weight in sweet basil. Bars represent standard errors of three replication. Values with the different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.05$

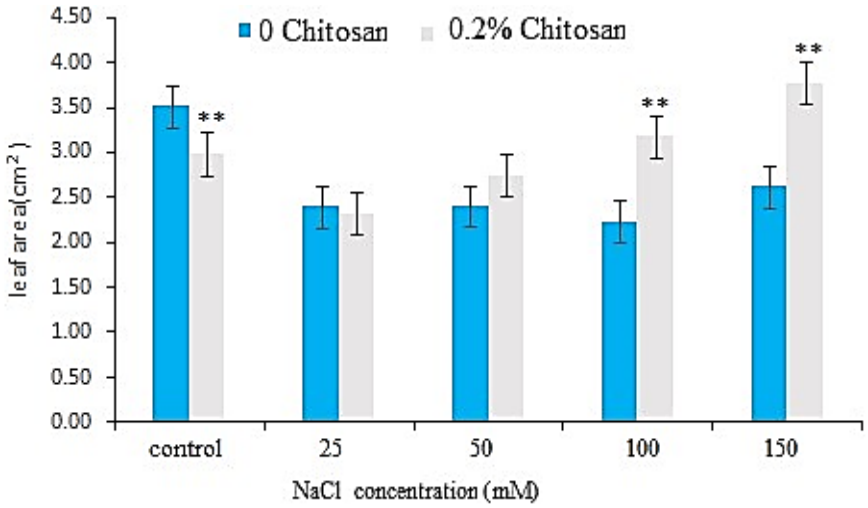

Fig. 2. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray on leaf area in sweet basil. Bars represent standard errors of three replication. Values with the different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.05$

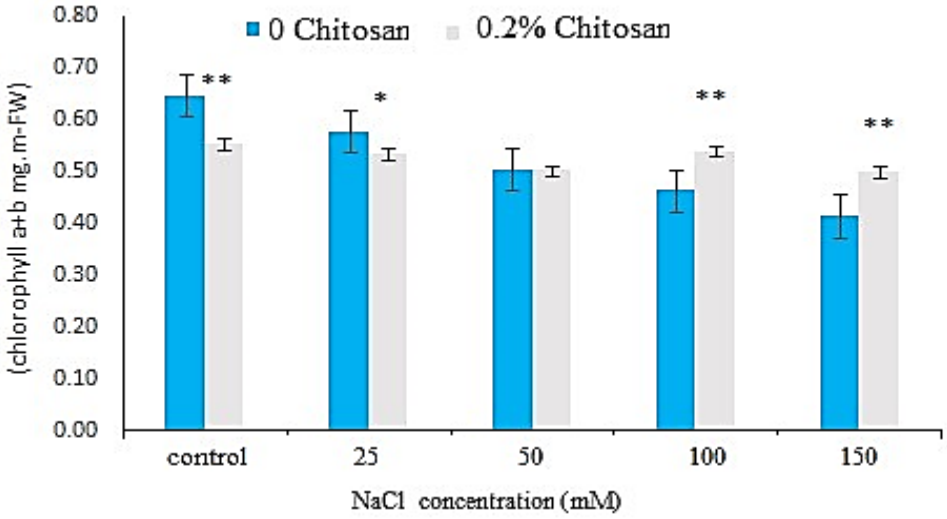

Fig. 3. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray on total chlorophyll in sweet basil. Bars represent standard errors of three replication. Values with the different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.05$

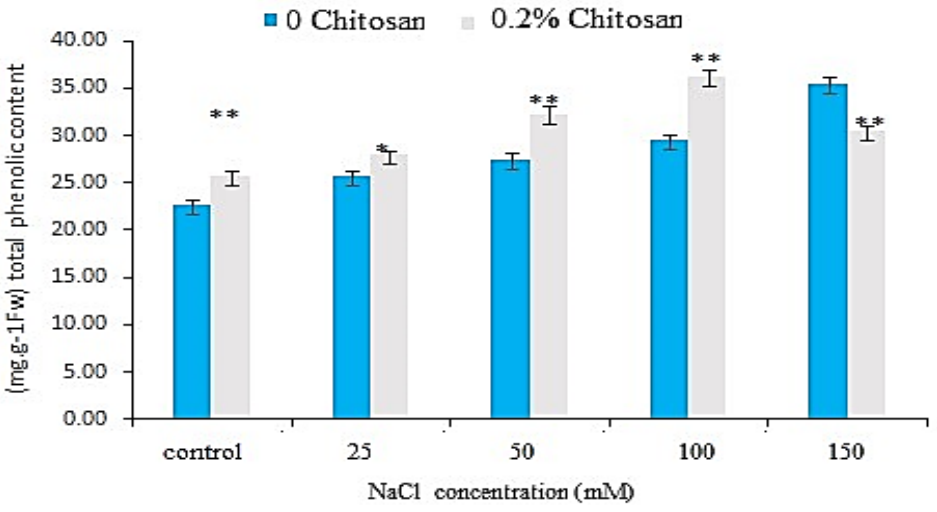

Fig. 4. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray on total phenol in sweet basil. Bars represent standard errors of three replication. Values with the different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.05$

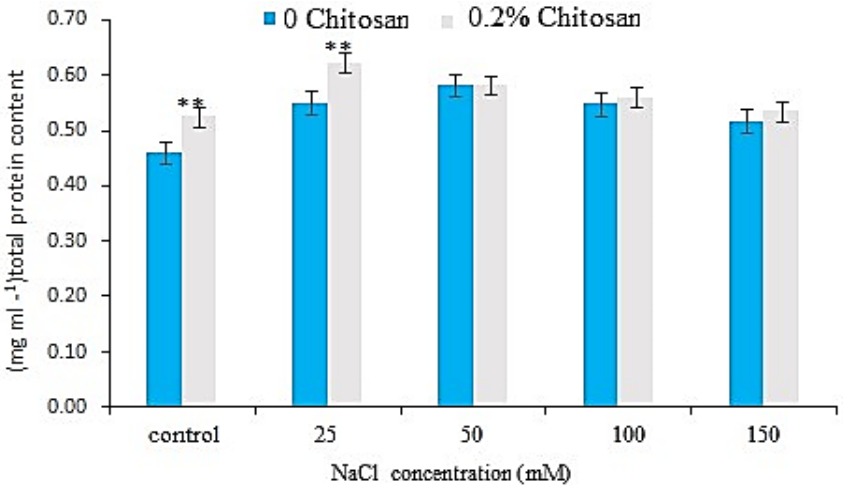

Fig. 5. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray on total protein in sweet basil. Bars represent standard errors of three replication. Values with the different letters are significantly different according to Duncan's Multiple Range Test at $P < 0.05$

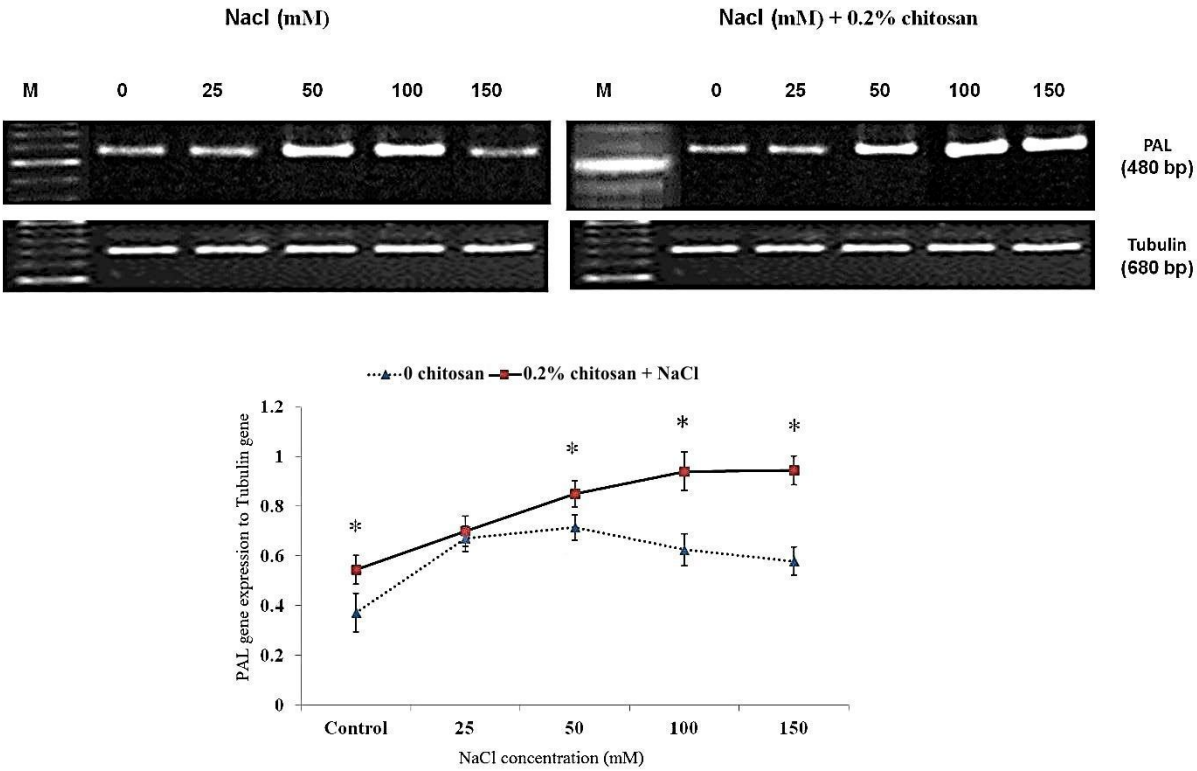

Fig. 6. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray PAL gene expression in sweet basil. Bars represent standard errors of three replication

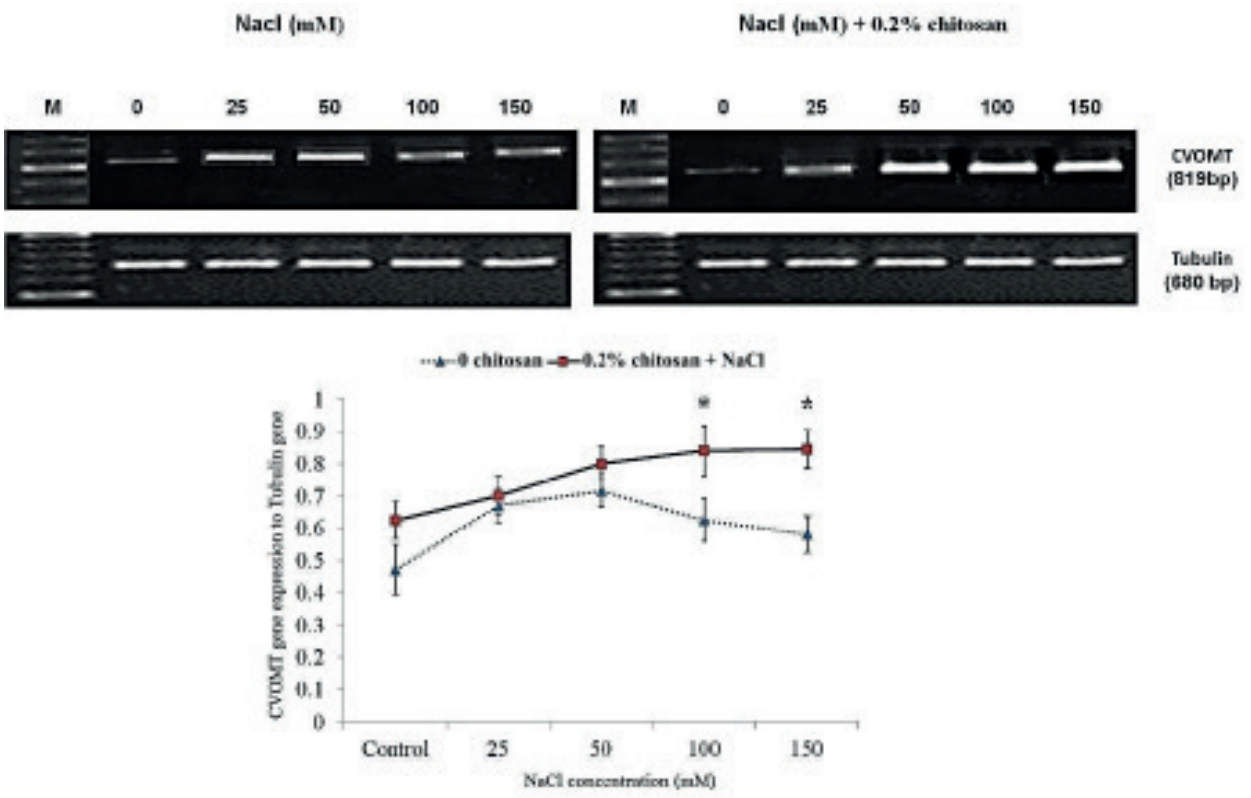

Fig. 7. Effect of different concentration of sodium chloride and 0.2% chitosan foliar spray CVOMT gene expression in sweet basil. Bars represent standard errors of three replication

PCR reaction. After RT-PCR reaction, PCR reaction for favorite pieces and tubulin as internal control were carried out (Tab. 2). The PCR products were used for gel electrophoresis and the reminders were conserved in –20˚C. Standard ladder was used as reference for relative quantification of bands.

Statistical analysis. Experiment results were the mean of three replicated treatments. The data were subjected to analysis of variance (ANOVA) using SAS software to assess the significant differences between treatments. Means comparisons were assessed by Duncan's range test with differences being considered significant at $P < 0.05$.

RESULTS

Shoot fresh weight. The results of this research showed that salinity significantly decreased shoot fresh weight (SFW) at 150 mM NaCl compared to controls. Likewise, application of chitosan increased SFW at all levels of salinity in comparison to controls. The highest SFW was gained under employing chitosan under control and a severe salinity stress (150 mM NaCl) (Fig. 1).

Leaf area. Effects of salinity and chitosan on leaf area (LA) was given in Fig 2. At control (without chitosan), all salinity levels significantly reduced LA. Moreover, application of chitosan significantly increased LA, especially at higher levels of salinity. The highest LA was gained under application of chitosan and 150 mM NaCl (Fig. 2).

Chlorophyll. The results showed that salinity under chitosan and without chitosan significantly reduced chlorophyll content. Application of chitosan at control and 25 mM NaCl reduced total chlorophyll. However, at higher levels of salinity (100 and 150 mM NaCl), chitosan was found to increase chlorophyll compared to control (Fig. 3).

Total phenol. The results showed that the treatments had a significant effect on total phenol (TP). Under either using chitosan or without chitosan, TP was increased as the level of salinity was increased. However, at all levels of salinity (except for 150 mM NaCl), TP was increased as compared to controls (Fig. 4).

Total protein. The results showed that under both chitosan and without chitosan conditions, the salinity increased total protein as compared to controls. Meanwhile, the highest total protein was obtained at 25 mM NaCl treatment. However, chitosan increased total protein at control and 25 mM NaCl conditions. At 50, 100, and 150 mM salinity treatments, chitosan did not have a significant effect on total protein (Fig. 5).

PAL gene expression. The results showed that PAL gene expression was affected by salinity and chitosan. PAL gene expression was increased as the level of salinity was increased. Also, the results of this experiment showed that chitosan at all levels of salinity, except 25 mM NaCl, increased the expression of PAL gene. Moreover, the effect of chitosan on PAL gene expression was observed when level of salinity was increased up to 150 mM NaCl (Fig. 6).

CVOMT gene expression. The effect of treatments on CVOMT gene expression was presented in Figure 7. As salinity increased, the expression of CVOMT significantly increased under control and 0.2 % chitosan conditions. At control level (without chitosan), all salinity treatments did not have a significant different with each other. However, chitosan application, rather than without-chitosan condition, increased the expression of CVOMT gene (Fig. 7).

DISCUSSION

This experiment was conducted to investigate the effect of foliar application of chitosan on sweet basil, as an important medicinal plant. The results showed that sweet basil is unable to tolerate salinity especially at higher levels of salinity, because its growth was depressed due to salinity. A reduction in plants growth under salinity is expectable and a great deal of reports have been substantiated this hypothesis [Munns 2002]. The rate and type of salt and duration of stress elicit plants response to salinity stress [Çulha and Çakirlar 2011]. There is a remarkable difference between genotypes in terms of tolerating salinity; accordingly the genetic criteria of plants in response to salinity and also their tolerant mechanism has not fully been understood yet [Carillo et al. 2011]. Under salinity, the secondary stresses such as oxidative stress lead to generate extraordinary reactive oxygen species (ROS); these molecules are so toxic that they can impair cells through their higher oxidative effects on proteins and lipids [Ray et al. 2015]. Under oxidative stress, chlorophyll is disintegrated and the rate of protein is increased in favor of osmotic adjustment. In our research, salinity caused a reduction in chlorophyll content as well as an accumulation of protein in sweet basil, which are in agreement with those obtained by Ayala-Astorga and Alcaraz-Meléndez [2010] who stated that salinity led to a reduction in chlorophyll and an increase in protein accumulation.

The result of this experiment showed that TP was significantly increased in sweet basil under salinity. An increase in TP content of salinity-experienced plants was previously reported by some researchers and its measurement can serve as a defensive mechanism under environmental stresses [Rezazadeh et al. 2012]. Phenol compounds are one of the most important antioxidant compounds in plants. The antioxidant characteristics of phenolic compounds stem from their reductant property and chemical structures enabling them to nullify free radicals in cells. These compounds depress the lipid oxidation through donating electrons to free radicals [Ahmadi et al. 2007]. It seems that an increase in TP content of stressed plants can be taken into account as one of mechanism effective on growing tolerating stressful condition.

The results of this research showed that using chitosan, rather than without it, caused a significant increase in growth parameters, chlorophyll, protein and TP. This compound has been reported to increase the production of secondary metabolites. For instance, Chakraborty et al. [2009] reported that chitosan increased production of phenylpropanoid derivatives in suspension culture of *Cocos nucifera*. Rahman et al. [2003] reported that using this compound improved an accumulation of limonene and linalool in *Citrus grandis*. Different mechanisms have been introduced over how chitosan affect metabolite production in plants. For instance, Vasconsuelo et al. [2004] stated that signaling approaches (cellular transduction) stimulated

by chitosan can increase anthraquinone production in *Rubia tinctorum* L. These researchers showed that effect of this substance may work through activating phospholipase (PLC)/ protein kinase C (PKC). Also, Falcón-Rodríguez et al. [2009] reported that using chitosan, as an elicitor, increased the defensive activities of peroxidase, phenylalanine ammonia lyse (PAL), and Glucan in leaf and root of tobacco.

It has been reported that chitosan, through activation of physiological processes, improves vegetative growth of plants [Ray et al. 2015]. As a plant elicitor, chitosan is bonded with cellular membranes and serve as a secondary messenger, and it also manage the production of some hormones such as ABA [Iriti and Faoro 2008]. This hormone causes stomatal closure and manages other plant defensive mechanisms and gene expression [Pichyangkura and Chadchawan 2015]. The results of this research confirmed that chitosan significantly increased the expression of the genes of PAL and CVOMT. It has been demonstrated that an increase in biosynthesis of PAL, as a most important enzyme involving in biosynthesis of phenolic compounds, due to chitosan stimulation [Chakraborty et al. 2009]; this is in relation with an increase in the biosynthesis of phenolic compounds [Pichyangkura and Chadchawan 2015]. In current research, the results of this research revealed that the expression of CVOMT was significantly increased due to chitosan treatment. This gene performs the methylation of chavicole and is one of enzyme family of O-methyltransferase. Deschamps et al. [2008] figured out that chemical treatments such as methyl jasmonate and methyl salicylate enhanced the expression of CVOMT in sweet basil. Naderi et al. [2014] reported the expression of CVOMT gene by different concentrations of chitosan which is in agreement with ours. Also, there are some reports over the role of PAL and other enzymes involving in phenylpropanoid pathway, as the main plants responses to stresses, through improving production of phenolic compounds [Wen et al. 2008]. This result showed that using chitosan can mitigate the negative effects of salinity on plants.

SUMMARY

To sum up, the results of this research showed the negative effects of salinity on growth and physiological responses of sweet basil. Under salinity in sweet basil, chlorophyll content was decreased and inversely protein and TP were increased. Application of 0.2% chitosan could somewhat alleviate the adverse effect of salinity on sweet basil through affecting PAL and CVOMT and increasing phenolic compounds.

ACKNOWLEDGEMENTS

Authors are grateful to Islamic Azad University, Tehran, Iran, for providing necessary facilities to carry out the research.

Authors are grateful to Islamic Azad University, Tehran Branch, Tehran, Iran, for financial assistance.

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