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EVALUATION OF THE EFFECT OF BIOLOGICAL ELICITORS ON THE RESISTANCE TO SALINITY STRESS IN THE DATE PALM (*Phoenix dactylifera* L., cv. Stameran)

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ABSTRACT

To evaluate the effect of biological elicitors, including fungi and bacteria, on resistance to salinity stress in the date palms. The experiment was conducted in a factorial, completely randomized design (CRD) with two factors, i.e., salinity with three levels (0, 150, and 300 mM) and the fungal and bacterial elicitors at five levels. The elicitors treatments were comprised of fungal consortium (BFC), fungal elicitor at 1,000 ppm concentration (EL1), fungal elicitor at 2,000 ppm concentration (EL2), and the bacteria (BS), *Bacillus safensis*, and *Bacillus pumilus*. The results showed that the lowest hydrogen peroxide content (278 µmol/g) was found in the seedling treated with elicitor of *B. safensis* at the zero salinity level. Catalase enzyme activity was higher in plants treated with fungal elicitor at 2,000 ppm concentration and the zero salinity level, *B. safensis* at the 150 mM salinity level, and fungal consortium at the 300 mM salinity level. The hydrogen peroxide content in the plant decreased as the activity of PAL and PPO enzymes increased. Applying an elicitor may reduce the effects of salinity stress in the date palm, but the stress level could determine the impact of each elicitor.

Key words: antioxidant enzymes, bacteria, concentration, fungus, hydrogen peroxide

INTRODUCTION

Iran is one of the largest producers of date palms (*Phoenix dactylifera*), which ranks third in the world for production after Egypt and Saudi Arabia [FAOSTAT 2021]. Salinity stress is one of the factors limiting the growth and yield of plants in Iran. About 50% of the country's lands are salinin [Yousefi et al. 2020] Under salinity conditions and saline soils, high osmotic suction reduces water absorption by plant roots, which leads to osmotic stress, imbalance, and ionic toxicity, as well as a lack of nutrients in plants [Kabiria and Hogue 2019]. Salinity stress also causes the accumulation of sodium and chlorine ions in plant tissues in soils with high concentrations of sodium and chlorine, followed by the nutritional balance being lost and their excessive absorption causing severe physiological disorders. The main metabolic processes and some cellular structures, such as biological membranes, would be damaged following the salinity stress in plants. In saline soils, the high concentration of sodium ions by reducing the absorption of potassium ions leads to a decrease in growth and yield and even drying plants. There is severe competition between the absorption of sodium and potassium [Hazzouri et al. 2020]. Soil microbial compounds play a significant role in the growth and development of host plants under biotic and abiotic stress factors. Soil fungi may be

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effective in plant growth and development [El Kinany et al. 2022]. The flexibility of soil fungi by multiple options is required to regulate plant growth, and the production of plant growth-regulating substances such as auxin [Rashid et al. 2012], cytokinin, and gibberellin are among those noticeable options [Bhore et al. 2010]. Most of these substances prevent ethylene production produced when faced with environmental stress conditions. Soil fungi also produce secondary metabolites, directly provide food, i.e., nitrogen and phosphate, break down waste materials resulting from plant metabolism and convert them into usable materials by plants such as ammonium ions, and also transfer carbon in two directions [Indeiragandhi et al. 2008]. Among different bacteria and fungi, PGPRs play a significant role in managing biotic and abiotic stress factors [Kumar et al. 2020]. PGPRs are a large group of bacterial microorganisms that live in the plant rhizosphere and colonize the plant root system. Others are endophytes in seeds and other plant organs. In addition to controlling pathogens, these factors increase root growth, leaf area, chlorophyll, protein, nitrogen, and magnesium levels. In this regard, these bacterial factors increase the resistance to drought and salinity stress, increase the branch weight, and delay the aging of the leaves [Singh et al. 2022]. In a research conducted by El Kinany et al. [2022] on the use of organic fertilizers and microbial agents in increasing the growth of date palms, they found that the treatments of compost, mycorrhiza (MycENA16), and Pantoea agglomerans in the presence of each other are effective in stimulating the growth of date palms. Yais et al. [2015] reported that growth-promoting bacteria in date palms could change the level of internal ethylene production and the IAA content, affect the absorption of nutrients by the roots, and improve the growth and development of date palms under salinity stress conditions. Evaluation of the activities of defense enzymes has shown different activity patterns of oxidase, peroxidase, and polyphenol oxidase enzymes under stress conditions. The enzyme phenylalanine ammonia-lyase is a central component in the biosynthesis of propanoids, and these compounds play a crucial role in the challenges of salinity stress [Gholizadeh et al. 2010]. The current research aimed to obtain an effective elicitor in increasing the resistance and efficiency of date palms to salinity stress.

MATERIAL AND METHOD

Isolation of fungal and bacterial agents: preparation of fungal consortium (bioactive)

First, soil sampling was done in Abadan County from five different rhizosphere regions to collect fungal isolates. Fungal isolates collected from soil in Dox agar medium, containing concentrations of 0, 5, 10, 15, and 20 g/l of sodium chloride prepared by mixing with the culture medium, were cultivated to isolate salinity-resistant fungal isolates. The isolates that had the highest growth rate and the lowest inhibition percentage at the highest salinity concentrations were used as representative isolates for investigation in *in vitro* studies. 500 g of pearled barley was poured into a 500 ml Erlenmeyer flask and sterilized by autoclave to prepare the inoculum stock. After sterilization of pearled barley, five pieces of the desired fungus colony were inserted into the Erlenmeyer flask and incubated for seven days. During this time, the Erlenmeyer flask was stirred every 24 hours to spread the fungi evenly. After the preparation of the inoculum stock, it was added to the soil.

Preparation of fungal elicitor

We extracted fungal elicitors from the method of Farakya et al. [2005]. First, the selected isolates were added to a 250 ml Erlenmeyer flask containing 50 ml of culture medium (three g/l of malt extract, 3 g/l of yeast extract, five g/l of peptone, and ten g/l of glucose) with pH 2.6 and placed on a shaker for six days at a speed of 200 rpm and a temperature of 25°C with a cycle of 16 hours of light and eight hours of darkness. Then, we collected the liquid phase of the culture medium by passing through the Whatman filter paper grade 1 and centrifuged the resulting solution at 5,000 rpm for 15 minutes. The obtained solution was divided into two parts. The first part of the solution passing through a 0.22 mm filter was used as a fungal elicitor. The second part was autoclaved at a pressure of 15 psi and 121°C for 20 minutes, and then it was considered a fungal elicitor. The fungal body was also washed several times with distilled water, dried in an oven at 40°C, and crushed in a mortar. Then, we dissolved ten grams of it in 100 ml of distilled water, autoclaved at a pressure of 15 psi and 121°C for 20 minutes, and centrifuged at a speed of 5,000 rpm for ten minutes.

Table 1. Fungal primer sequences

Its1	TCCGTAGGTGAACCTGCGG
Its4	TCCTCCGCTTATTGATATGC

Table 2. Sequences of general primer pair used in molecular analysis of bacteria

5'-CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACGCT-3	P1
5'-CGGGATCCTACGGCTACCTTGTTACGACTTCACCCC-3'	P6

We collected the supernatant as a cell extract and used the obtained solution as a fungal elicitor. The obtained fungal elicitor was kept at 4°C.

Bacterial elicitor

Fresh leaf and root samples were prepared from date palms. The samples were washed with tap water for 20 minutes to separate the soil particles attached to the sample's surface. The washed samples were surface sterilized with 0.5% sodium hypochlorite solution for 30 minutes. Then, they were rinsed at least three times with sterile distilled water and then cut into 5- to 8-mm pieces. After being pressed with sterile forceps, these pieces were transferred to tubes containing nutrient agar (NA) culture medium and incubated for two to three days. Selected bacterial strains were purified and kept in slanted agar tubes for further studies [Yaish et al. 2015, Rashid et al. 2012].

The selected bacteria were cultured on NA medium containing different levels of salinity, i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg of NaCl/100 ml culture medium. The Petri plates were placed in the incubator at 37°C. The growth rate of bacteria was investigated. Also, the suspension of the mentioned bacteria was prepared at a concentration of 106 cfu/ml, and the roots of the seedlings were placed inside them for some time, and then they were cultivated.

Salinity stress (greenhouse experiment)

Palm seedlings (cv. Stameran), which were prepared by tissue culture method at six leafs stage, were used to examine the activity of bio-elicitors to reduce the salinity stress. The greenhouse experiment was conducted in factorial completely randomized design (CRD) with two factors (salinity × bio elicitors). It includes i) salinity stress at three levels of NaCl (zero, 150, and 300 mM), ii) different types and concentrations of bio elicitors (bacterial and fungal elicitors) (Tab. 3). Three replications were used in this experiment, where a total of forty-five units experiment, each unit included three palm seedlings.

PCR-based identification

Bacterial DNA was extracted by the alkaline lysis method [Elboutahiri et al. 2009], and fungal DNA was extracted by the methods of Walsh et al. [1991], Suenaga et al. [2005], and Conlon et al. [2021]. Fungal primer sequences and sequence of general primer pair used in the molecular analysis of bacteria are in Table 1 and Table 2.

Plant sample collection

Plant samples were collected in two stages of growth: the first stage, one month after the application of elicitors, and the second stage, three months after salinity. The plant samples were kept in a freezer at –80°C until the time of content measurement and enzyme evaluation.

Measurement of hydrogen peroxide content

The hydrogen peroxide content was determined based on the reaction of hydrogen peroxide with potassium iodide (KI) [Loreto et al. 2001]. In this method, 0.5 g of fresh leaf tissue was macerated in 0.1% TCA. The resulting extract was centrifuged at 13,000 rpm for 15 minutes. Then, 500 µl of 100 mM potassium phosphate buffer (pH = 7) and 2 ml of 1M potassium iodide were added to 500 µl of the supernatant solution. The reaction mixture was placed in the dark at room temperature for one hour, then the absorbance of the samples was measured at 390 nm with a spectrophotometer. A standard curve was used to calculate the hydrogen peroxide content.

Table 3. Factors and treatments applied in the research

Factors		Treatments
Elicitors	BFC	1. Fungal suspension
Elicitors	Fungal	2. Concentration number 1: 1,000 ppm (EL1)
		3. Concentration number 2: 2,000 ppm (EL2)
Elicitors	Bacteria	4. Selected bacterium from sample 1: BP
		5. Selected bacterium from sample Root 2: BS
Stress level	Salinity stress treatment at three levels	1. Concentration: Control
		2. Concentration: 150 mM
		3. Concentration: 300 mM

Assay of defense enzyme activities

The frozen leaves were poured into a porcelain mortar, and liquid nitrogen was added to extract and measure enzymes. Then, the leaves were beaten well until they were thoroughly crushed. One gram of the ground powder was transferred to 2 ml microtubes and vortexed by adding two ml of phosphate buffer with pH = 7.6 and immediately centrifuged at 14,000 rpm for 15 minutes at 4°C. After centrifugation, extract samples were prepared.

Phenylalanine ammonia-lyase (PAL) activity

We used the method of Beaudion-Eagan and Thrope [1985] to measure the PAL enzyme. The reaction mixture was 0.5 mM Tris-buffer with pH = 8 and 6 µM phenylalanine and 200 µl of enzyme extract, and it was kept at 37°C for 60 minutes. The reaction was stopped after 60 minutes by adding 50 µl of standard HCl. Then, the absorbance of the samples was determined spectrophotometerly at 290 nm. PAL activity is determined by cinnamate production. Enzyme activity was expressed in µmol per milligram of fresh weight per minute.

Peroxidase (POX) activity

We used the method of Hammerschmidt et al. [1982] to assay POD activity. 490 µl of 225 mM hydrogen peroxide solution and 490 µl of 45 mM guaiacol solution were mixed, and 20 µl of plant sample extract was added. Absorbance changes were read at 470 nm using a spectrophotometer. In the blank solution, 50 mM phosphate buffer was used instead of the extract. The activity of this enzyme was calculated using Beer–Lambert's law and with the quenching coefficient of guaiacol peroxidase equal to 26 $mM^{-1}cm^{-1}$, and, finally, it was expressed in µmol per gram of fresh tissue per minute.

Catalase (CAT) activity

The method of Luck [1974] was used with some modifications to assay CAT activity: 20 µl of enzyme extract was mixed with 980 µl of phosphate buffer containing 2-mM hydrogen peroxide, and their absorbance changes were recorded at 240 nm with a spectrophotometer as absorbance changes over time (OD/min). Enzyme activity was calculated using Beer–Lambert's law and with the quenching coefficient of catalase equal to 39.4 $mM^{-1}cm^{-1}$ and, finally, was expressed in µl per gram of fresh tissue.

Polyphenol oxidase (PPO) activity

The amount of PPO was measured by Gregory and Bendali's method [1966]. The reaction medium contained 50 µl of 0.02 pyrogallol, 3,000 µl of phosphate buffer (pH = 7), and 50 µl of enzyme extract. Enzyme activity was calculated based on pyrogallol oxidation values at 420 nm. Finally, the enzyme activity was reported in terms of absorbance changes of the samples at 420 nm per minute per milligram of fresh weight.

Data analysis

The data were recorded in Excel software and then analyzed by using SAS 9.1 statistical software. The Duncan's multiple range (DMR) test at a 5% level was used to compare the means of the treatments.

RESULTS

The results of PCR product sequences of representative fungal isolates selected in the NCBI BLAST database: *Aspergillus tubingensis*, *Aspergillus niger*, *Penicillium chrysoyenum*, and *Fusarium solani*.

Bacteria identified: *Bacillus pumilus* and *Bacillus safensis.*

Analysis of variance of the data

The results showed that the individual effects of elicitor as well as salinity were significant effects on hydrogen peroxide, polyphenol oxidase, phenylalanine ammonia-lyase, catalase, and peroxidase activity. Furthermore, there was a significant interaction between the elicitor and salinity on the mentioned enzymes at $p < 0.01$ (Tab. 4).

Hydrogen peroxide

The interaction of salinity levels and elicitors on hydrogen peroxide showed that in bacterial elicitors (BS and BP), hydrogen peroxide first increased and then decreased with increasing salinity. In fungal elicitors (EL1 and EL2), hydrogen peroxide first decreased and then increased with increasing salinity so that the highest amount of hydrogen peroxide was found in the fungal elicitor (EL1) at 1000 ppm (945 µmol/g) at a salinity of 300, which was significantly different from other elicitors. The lowest amount was observed in BS elicitors at the zero salinity level and in BFC at Zero and 150 mM salinities (278, 268, and 260 µmol/g, respectively) (Fig. 2).

Antioxidant enzyme activities

Catalase (CAT). The interaction of salinity levels and elicitors on CAT showed that the amount of CAT In BFC and fungal elicitors (EL1 and EL2), the amount of CAT decreased first and then increased with increasing salinity. Thus, the highest amount of CAT was observed at zero salinity, related to EL2 (0.211 µmol/mg). It should be noted that in these three treatments at 300 salinity, the amount of CAT increased significantly compared to the control (Fig. 3).

Production of peroxidase (POX). The interaction of salinity levels and elicitors on POX showed that the amount of POX did not change in the EL2 elicitor with increasing salinity. In EL2 and BFC, we were able to significantly increase the amount of POX compared to the control so that the maximum amount of POX was

Table 4. Probability of analysis of variance of the data regarding the effect of elicitors on the traits under salinity conditions

	Trait	PPO	PAL	CAT	POX	Hydrogen peroxide
Sources of variation (mean squares/MS)	Elicitor (A)D.F = 4	1.857**	0.440**	0.713**	14.472**	1340.6**
	Salinity (B)D.F = 2	0.878**	0.138**	0.500**	7.162**	890.9**
	Interaction (AB)D.F = 8	0.577**	0.078**	1.143**	15.637**	629.7**
	Error D.F = 30	0.002	0.003	0.003	0.004	1.9
Coefficient of variation/ CV%		2.4	8.5	10.1	1.9	3.2

** shows significance at a 1% level

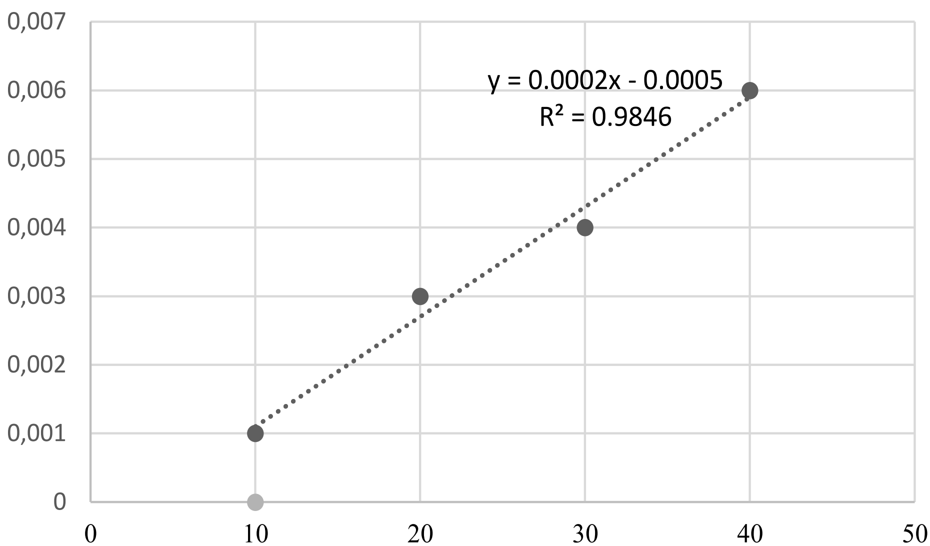

Fig. 1. Hydrogen peroxide standard curve

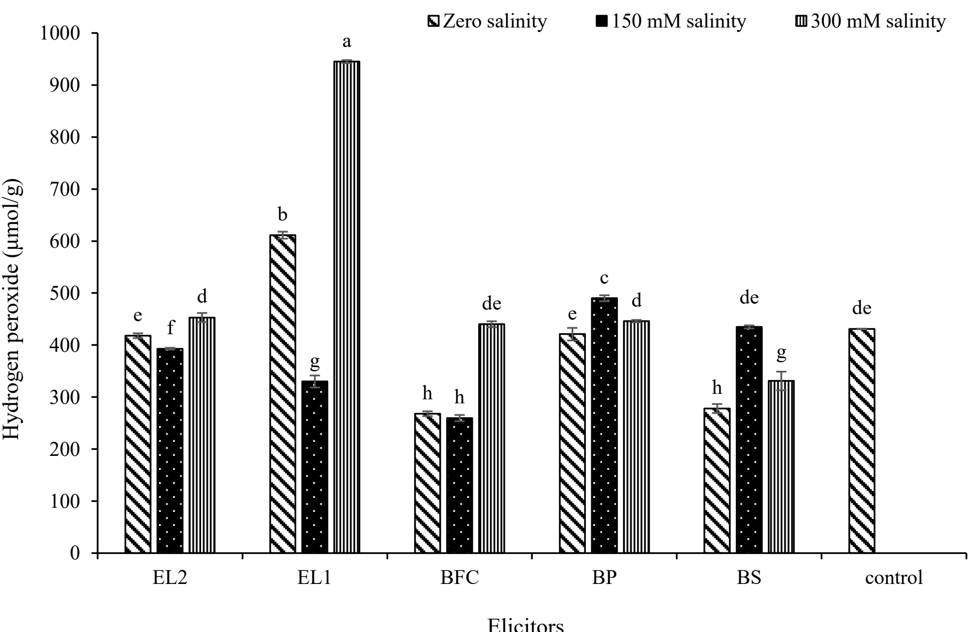

Fig. 2. Interaction effect of salinity and elicitors on the level of hydrogen peroxide. Columns with different alphabet letters are considered significant differences at a level less than 0.01 according to the DMR test

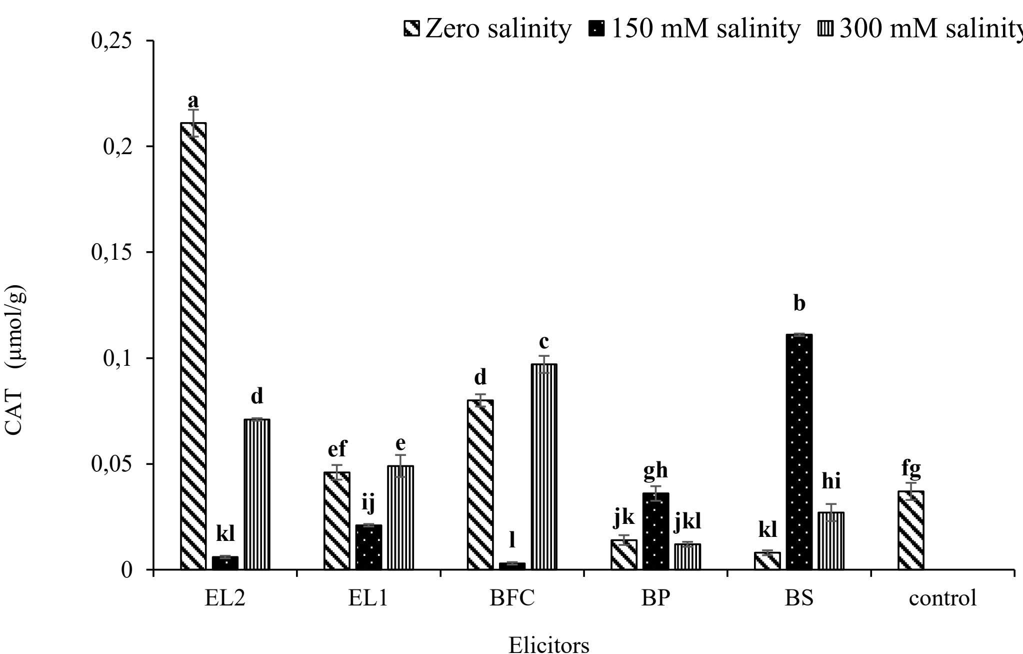

Fig. 3. Interaction of salinity levels and elicitors on the amount of CAT. Columns with different alphabet letters are considered significant differences at a level less than 0.01 according to the DMR test

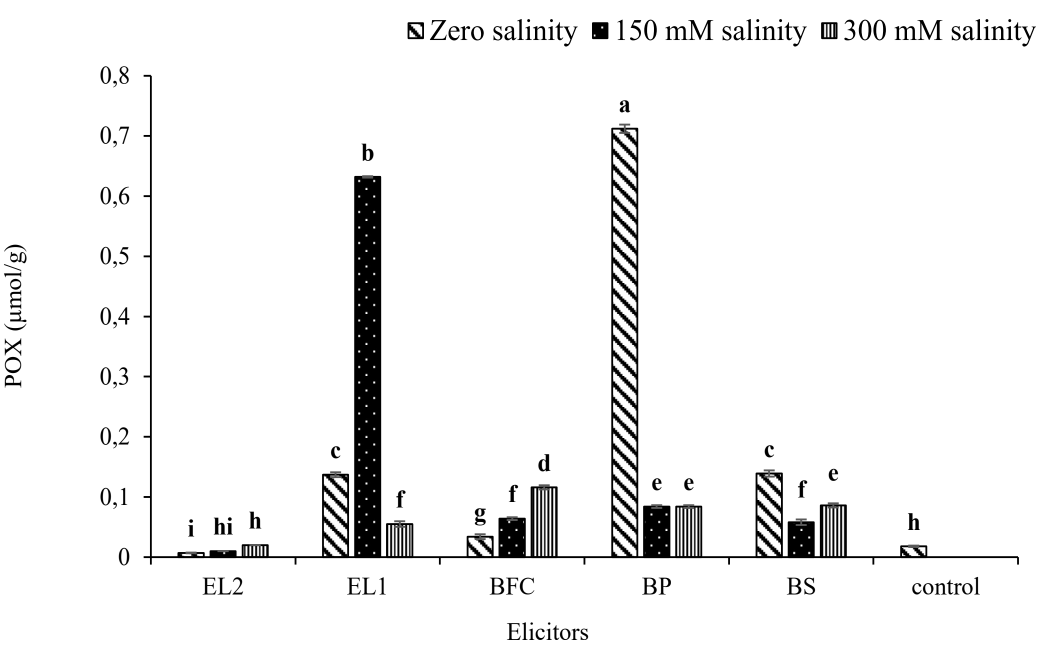

Fig. 4. Interaction of salinity levels and elicitors on the amount of CAT. Columns with different alphabet letters are considered significant differences at a level less than 0.01 according to the DMR test

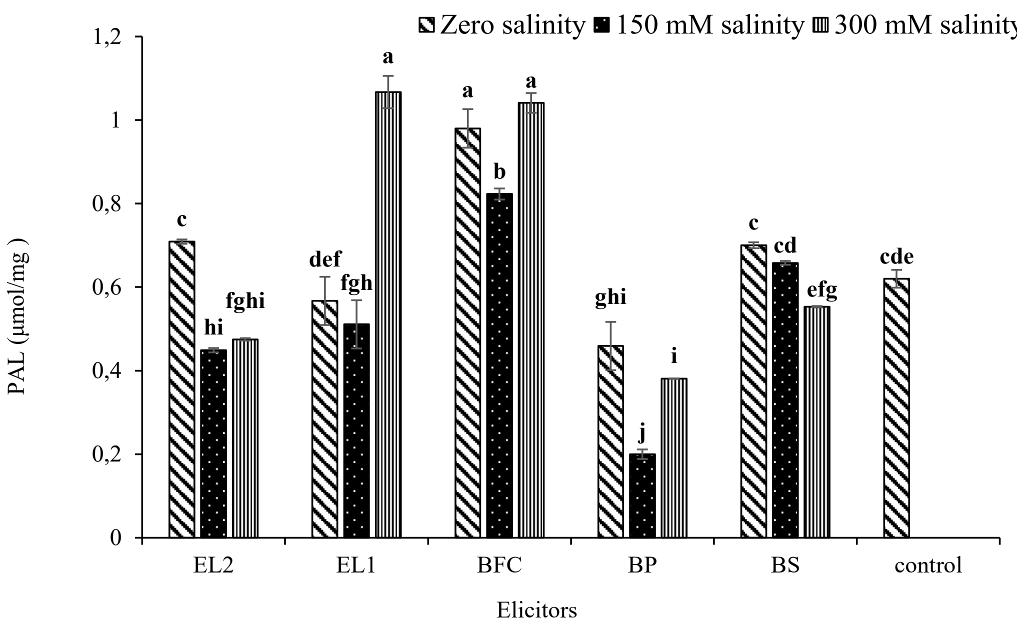

Fig. 5. Interaction of salinity levels and elicitors on the amount of PAL. Columns with different alphabet letters are considered significant differences at a level less than 0.01 according to the DMR test

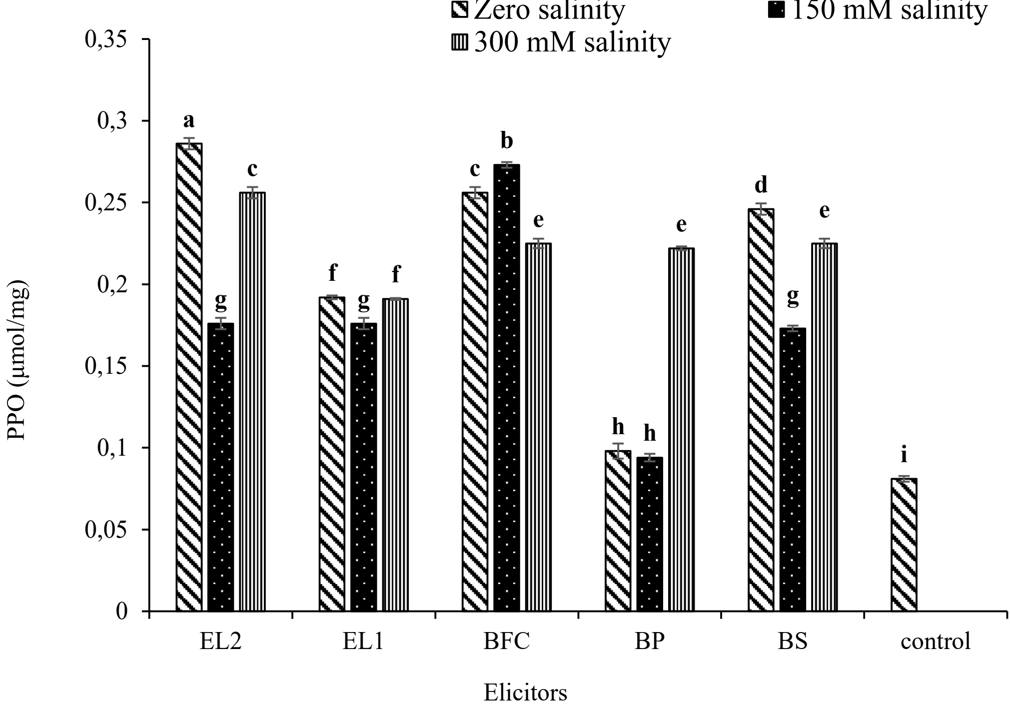

Fig. 6. Interaction of salinity levels and elicitors on polyphenol oxidase. Columns with different alphabet letters are considered significant differences at a level less than 0.01 according to the DMR test

observed at zero salinity related to BP (0.712 µmol/mg), followed by EL1 at 150 mM salinity (0.632 µmol/mg) (Fig. 4).

Phenylalanine ammonia-lyase (PAL). The interaction of salinity levels and elicitors on PAL showed that in BS and BP elicitors and the fungal elicitor EL2, with increasing salinity, the amount of PAL did not change and even decreased. The fungal consortium and the fungal elicitor EL1 were able to significantly increase the amount of PAL at 300 mM salinity so that the highest amount of PAL was found at 300 mM salinity for 1000 ppm fungal elicitor (EL1) and fungal consortium (1.067 and 1.041 µmol/mg, respectively) (Fig. 5).

Polyphenol oxidase (PPO). The interaction of salinity levels and elicitors on PPO showed that in *Bacillus safensis* and fungal elicitors (EL1 and EL2), with increasing salinity, the amount of PPO first decreased and then increased. The fungal consortium (BFC) was the opposite of this situation. The highest amount of PPO was observed at zero salinity for the treatment EL2 (0.286 µmol/mg) (Fig. 6).

DISCUSSION

We measured the PPO, PAL, CAT, and POX activities. The results showed that the amount of PPO activity increased significantly under stress. Under salinity stress, the activity of this enzyme was the highest in the treatments of EL2 at salinity levels of zero and 300 mM and BFC at salinity levels of zero and 150 mM. The amount of PAL activity before and after applying stress in the presence of different elicitors did not change significantly, but its amount in the treatment EL1 at 300 mM salinity level and BFC in all three salinity levels showed the highest activity of this enzyme. In addition, the treatment BP at zero salinity level, EL1 at 150 mM salinity level, and BFC at 300 mM salinity level had the highest POX activity. As can be seen, at each stress level, a specific elicitor reduces the effect of salinity stress better than other treatments.

The activity of antioxidant enzymes in response to environmental stress factors differs depending on plant species, genotype, and stress duration and intensity. In stresses that lead to plant stomatal closure, such as drought and salinity stress applied to the plant, Rubisco oxygenation (i.e., photorespiration) increases and leads to increased production of H_2O_2 in peroxisomes. Since catalase activity is crucial in H_2O_2 removal, an increase in catalase activity under stress conditions may be related to the need to deal with the production and inhibition of H_2O_2 caused by photorespiration. Catalase is an enzyme in almost all organisms that uses oxygen and converts hydrogen peroxide into water and oxygen. Catalase is one of the enzymes with the highest catalytic efficiency (Kcat/Km), and one molecule of catalase can convert millions of hydrogen peroxide molecules into water and oxygen. This enzyme is a tetramer whose chain has about 500 amino acids and contains four heme porphyrin groups that allow it to react with hydrogen peroxide. Any heavy metal ion (such as the copper cation in copper sulfate) can react with catalase as a non-competitive inhibitor. Cinnamide, a toxic substance, can competitively react with catalase and stop enzyme activity by forming a strong bond with the heme catalase center [Mane et al. 2011]. In the present study, catalase activity was higher in the treatment EL2 (zero salinity), *Bacillus safensis* (150 mM salinity), and BFC (300 mM salinity). At any salinity level, if the goal is to increase the catalase activity, these elicitors can be used. The results of this research are consistent with studies conducted by Bagheri and Khosrovinejad [2017]. Their results showed that endophytic fungi increased the activity of antioxidant enzymes such as ascorbate peroxidase, catalase, and superoxide dismutase and decreased malondialdehyde production in rice-inoculated plants compared to control plants at all salinity levels. Therefore, it can be concluded that the endophyte fungus moderates the risks caused by the effect of salinity stress on rice plant growth in terms of improving the antioxidant defense system. The cause of the increase in antioxidant enzyme activities in the presence of endophytic fungi has not yet been determined. However, these fungi have likely caused a positive rise in morphological, physiological, and biochemical characteristics of symbiotic plants by activating various mechanisms whose nature has not yet been determined [Rodriguez et al. 2004].

CONCLUSIONS

Salinity stress is one of the factors limiting the growth and yield of plants in Iran. In this research, we concluded that some bacterial elicitors extracted from roots and leaves of date palms and soil fungi, with increasing antioxidant enzyme activities, can play a significant role in the salinity of seedlings. Unknown mechanisms of these bacteria and fungi function towards salinity stress could not also be denied. It requires further research on all host plant endophytes.

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