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RESEARCH PAPERS

Azospirillum brasilense Can Modulate Salt Stress in *Triticum aestivum* via *MN052803-LTP* Regulation and Phosphatidylcholines Content

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Abstract—*Azospirillum brasilense* may regulate *MN052803-LTP* expression and the activity of defensive enzymes in wheat (*Triticum aestivum* L.) cultivars to improve salinity tolerance. In a primary experiment, germination indexes of 18 wheat cultivars were measured and Sorkhtokhm and Qods were selected as tolerant and sensitive cultivars to salinity, respectively. Selected cultivars inoculated with *A. brasilense* (Sp245 produce more ABA and Sp7 as standard strain) and grown-up to five days, then salinity (200 mM NaCl) was applied to seedling via Hoagland's nutrient solution. The relative expression of *MN052803-LTP* (authors recorded in the Gen Bank) of roots and shoots was measured at 12, 24, and 48 hours after salinity applied. The results showed that *MN052803-LTP* expression increased in the order of salinity, inoculation, and inoculation plus salinity. Meanwhile, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activity increased in the same order in 12 days-old seedlings. In a similar experiment, 10 mM dithiothreitol (DTT) was used as a reducer and inoculation as a stimulator of *MN052803-LTP* expression, then the relative expression and phosphatidylcholines (PC) content were measured. Although the *MN052803-LTP* expression and PC was reduced due to the application of DTT, inoculation eliminates its inhibitory effect. The highest amount of PC was observed in inoculated plants, and the lowest in the plants treated with DTT. Probably, *A. brasilense* improves salt tolerance of wheat cultivars through *MN052803-LTP* expression, and PC content via repairing the membrane damages by supplying the membrane phospholipids, such as phosphatidylcholine, and accumulation of antioxidant compounds by activating PAL and TAL via membrane lipid-dependent signaling cascades.

Keywords: *Azospirillum brasilense*, *Triticum aestivum*, phosphatidylcholines, dithiothreitol, inoculation, salinity stress, phenylalanine ammonia-lyase, tyrosine ammonia-lyase

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INTRODUCTION

Unwanted salt in the rhizosphere decreases the plant's water uptake and consequently with the usual leaf transpiration result in a reduction in the plant's water content. Following dehydration, cells shrink and lose their turgor pressure leading to changes in morphology, physiology, and gene expression of plants. The consequence of the adverse effect of salinity is a reduction of quality and quantity of plant production. One alternative strategy for improving the tolerance of a plant to stress is using the beneficial microorganisms present in the rhizosphere. *Azospirillum brasilense* is one of the plant growth-promoting rhizobacteria (PGPRs) that can associate with wheat and increase crop quality and quantity through suitable physiological and biochemical modification in plants [1].

Accumulation of antioxidant compounds such as flavonoids and other phenolic compounds is one of the general responses to abiotic stresses including salinity, which induce oxidative stress due to the formation of ROS and free radicals [2]. The activation of PAL and TAL enzymes stimulates the biosynthesis of the phenolic compounds of the phenylpropanoid pathway that have strong ROS scavenging activity and assist plants to overcome oxidative stress [2]. Besides, phenolic compounds are important in plant defensive mechanisms and play a crucial role in the interactions of plants with microbes [2]. PAL also plays an important role in the synthesis of chemical signals such as phenols, phytoalexins, and lignin that they decrease

Abbreviations: DTT—dithiothreitol; NFB—nitrogen-fixing bacteria; PC—phosphatidylcholine; PGPRs—plant growth-promoting rhizobacteria.

cell wall expansion and cell extensibility as well as limit water loss and prevent cell collapse due to dehydration. The activation of the plant defense system against bacteria as a physiological response of a host plant is so important. In this regard, the utilization of plant defense systems (PAL and TAL enzymes) would facilitate or limit the anchoring process of bacteria on the root surface. Benizri et al. [3] reported that the anchoring procedure in the inoculation condition is an important step in a successful association between microorganisms and plant cells.

One of the major inducible plant defense responses is the accumulation of plant defense proteins. In this regard, plant lipid transfer protein (LTPs), previously thought to be involved only in the transfer of a broad range of lipids between membranes [4], while they also implicate in the plant defense system. The defensive role of the plant LTPs was found because of the response of *LTP* genes expression to biotic and abiotic stresses [5]. Overexpression of *LTP* genes in different plants indicated that they significantly enhance tolerance of rice (*OsDIL*) and pepper (*CALTP1)* to drought as well as Arabidopsis (*AZI1*), *Tamarix hispida (ThLTP), Nicotiana tabacum* (*NtLTP4*), and rice (*Os11g24070, Os04g33920* and *Os05g06780*) to salinity. Interestingly, *LTP* in *N. tabacum* regulating transcription levels of *NHX1* and *HKT1* to alleviate the toxicity of salinity stress [6].

Kader [4] reported that *LTP* has a main role in the transport of phospholipids (that are a major component of the plasma membrane, PM) between membranes within the cell. They maintain cell function and mediating responses to stress during plant growth and development. Furthermore, the *LTP* could activate the plant phenylpropanoid pathway genes [7] that they defend against free radicals and may also provide tolerance to a wide array of stresses. Not only *LTPs* are involved in stress conditions, but they were also transiently expressed during the inoculation process [4]. In Chinese milk vetch (*Astragalus sinicus*), *LTPAsE246* has been shown to participate in the transport of plant lipids to symbiosome membranes and nodule organogenesis associated with infection thread formation [8]. In the inoculation of *Oryza sativa* roots with *mycorrhizal,* the expression of *LTP* and *Pal* was increased, these genes are involved in the plant response to the environment stress [7]. This simply means that PGPRs can affect the expression of stress-responsive genes and modulate plant responses to stress.

LTP transfer activity inactivated with increasing specific concentration of reduced-dithiothreitol [9] while this inactivation-transfer was accompanied by the change of the protein conformation. Dithiothreitol (DTT) reduces the expression of *LTPs*, via the change in the α-helix proportion of disulfide bridges [4]. Kader [4] reported that the α-helix proportion of disulfide bridges of *LTPs* decreased in wheat and maize from 40 to 25% under treatment with dithiothreitol (DTT) which confirms the effect of DTT on *LTPs* indirectly. In addition, the reduction of maize LTPs was absorbed by DTT, which prevents lipid transfer activity [9].

Azospirillum brasilense reduces the adverse effects of salinity of wheat cultivar [10]. Meanwhile, the effect of some PGPRs such as *Rhizobium* spp. on *LTP* expression and improvement of plant tolerance to abiotic stress has been reported [8]. Be noted, the effect of *A. brasilense* on the expression *of MN052803-LTP* (Authors recorded in the Gen Bank) of wheat (*T. aestivum* L.) cultivars under salinity condition and also the effect of dithiothreitol on *MN052803-LTP* is not yet reported. Therefore, the objective of this study was (a) to evaluate the effects of *A. brasilense* strains and/or salinity on PAL and TAL enzyme activities (two enzymes involved in the phenylpropanoid pathway), (b) to assay the effects of *A. brasilense* strains and/or salinity on the expression of *MN052803-LTP* of tolerant and sensitive wheat cultivars, and (c) to check the effects of *A. brasilense* strains as a stimulator and dithiothreitol as a reducer on the *MN052803-LTP* expression, and PC content under salinity condition.

MATERIALS AND METHODS

Plant and bacteria materials. Seeds of 18 common wheats (*Triticum aestivum* L.) cultivars (Bezostia, Hamun, Sivand, Kaveh, Sardary, Kaskogen, Azady, Gaspard, Karaj, Sorkhtokhm, Qods, Dez, Sepahan, Roshan, Zarin, Shoele, Bam, and Navid) were obtained from Institute of Agricultural and Research, Isfahan, Iran. Sterilized seeds were transferred into an autoclaved Petri dish containing 8 mL of saline water (0, 100, 150, and 200 mM NaCl) in each Petri dish. During 12 days, germination rate, seedling vigor, salinity tolerance index, and germination stress index were measured or calculated every day. On day 12th the average root length, shoot length, and the weight of roots and shoots were measured. According to the results of germination indexes, the concentration of 200 mM NaCl was the more effected concentration, and two cultivars of wheat named Sorkhtokhm and Qods were the most tolerant and sensitive cultivars to salinity, respectively. Therefore, according to the result of the preliminary experiment, two cultivars of Qods and Sorkhtokhm were selected as the sensitive and tolerant cultivars, respectively to salinity, and the concentration of 200 mM NaCl (as the salt stress condition), to perform the main experiments.

Bacteria culture. Two strains of *Azospirillum brasilense* including Sp7 (standard), and Sp245 (produce more ABA) were obtained from NCIMB Ltd, Germany. *A. brasilense* strains (Sp7 and Sp245) cultured in Nitrogen free basal (NFb) medium [11] supplemented with NH4Cl (0.25 g/L) at 30°C in Erlenmeyer flasks for 48 h and shaken in a rotary shaker at 0.56 *g*. The growth was harvested by centrifugation (1000 *g*, 10 min), washed with sterile saline phosphate buffer

and then re-suspended in phosphate buffer at a concentration of 10^8 CFU/mL of *A. brasilense* [1].

Inoculation of wheat seedlings and induction of salt stress. Seeds of tolerant and sensitive wheat cultivars (Sorkhtokhm and Qods) were sterilized, then the sterilized seeds were incubated at room temperature for 3 h. Wheat seeds were shaken in high phosphate NFb liquid medium enriched with 0.1% ammonium chloride contain 10^8 CFU/mL of *A. brasilense* strains and was shaken at 0.28 *g* for 3 h (Shaker Model INFORS AG, BOTTMINGEN, Japan). The inoculated and none inoculated seedlings were transferred into the sterilized pots filled with perlite. The pots (with 6 seedlings) irrigated with 1/4 strength of Hoagland's nutrient solution [12]. The pots kept at 25/18°C (day/night) and 16/8 h (light/dark) photoperiod using white light (photon density 650 μmol/(m^2 s)) for 5 days. Then, Hoagland's nutrient solution with two levels of salinity (0 and 200 mM) applied to pots as irrigated water. After 0, 12, 24, and 48 h of exposure of plants to salt stress, the roots and the shoots of inoculated and non-inoculated cultivars were collected and stored at -80°C in ultra-freezer for real-time quantitative PCR. Furthermore, some of the treated plants kept for 7 more days, and then PAL and TAL enzyme activity of shoots were measured.

Determination of PAL and TAL enzymatic activities. The activities of PAL and TAL in the shoots of wheat cultivars were determined using the method of Beaudoin-Eagan and Thorpe [13]. All steps of enzyme extraction were carried out at 0 to 4°C. One gram fresh tissues of shoots were homogenized with 3 mL of 0.05 M Tris-HCl buffer (pH 8) (Sigma-Aldrich, Germany) containing 15 mM of 2-mercaptoethanol (Sigma-Aldrich). Then the homogenates were centrifuged for 20 min at 4025 *g*. The protein content of each extract was measured. The enzymatic activities of PAL and TAL of shoots were assayed by measuring the amount of trans-cinnamic acid at 290 nm and p-coumarate at 333 nm for PAL and TAL, respectively.

RNA extraction and real-time quantitative PCR. The total RNA was isolated from frozen shoots and roots, using Trizol reagent (RNA Biotech, Iran). The extracted RNA was treated with DNase (Fermentas, United States). Then, the first stranded cDNA was synthesized using the M-MLV reverse transcriptase (Fermentas). Real-time PCR was performed in triplicate using SYBER Green Master Mix (RNA biotech). Gene-specific primers were designed for a 101 bp fragment of *LTP2*. The primer pair was 5'-CTCGTGCTGGTCGCCCTGGTG-3' in sense direction and 5'-TGGGAATCAAGGGTGGACG-3' in anti-sense direction. The primers pair for the housekeeping gene, actin, (Gen Bank Accession No. GI:48927617) were designed as 5'-GTTCCAATCTATGAGGGATACACGC-3' in sense direction and 5'-GAACCTCCACTGAGAACAACATTACC-3' in anti-sense direction [14]. Serial dilutions of cDNA were used to obtain optimized standard curve amplification efficiency and the best cDNA concentration for real-time PCR was obtained. The relative expression ratio of target and reference genes was calculated based on its real-time efficiencies (*E*) and crossing point difference (ΔCp) of the sample versus control as well as reference versus control, respectively [15]. Finally, cDNA of *LTPs* was sequencing and the results were examined by CLC sequences viewer software. The final file was prepared for gene registration, which was ultimately recorded at Gene Bank as *MN052803-LTP* (https://www.ncbi.nlm.nih.gov/nuccore/MN052803). Homology searches were carried out using the NCBI blast Email server. The total volume of the real-time qPCR was 12.0 μL, containing 6.25 mL SYBER Green Master Mix (RNA Biotech), 0.25 mL of 10 mM each primer (forward and reverse), 1.0 mL cDNA (1 : 25 dilutions, as previously defined), and 4.25 mL ultra-pure water. The protocol for PCR was 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 1 min. Three technical repetitions were performed (triplicate) for each biological repetition. The relative quantification of expression (RQ) was calculated using the comparative threshold cycle method [16], using the equation $RQ = 2^{-\Delta\Delta Ct}$, based on the RQ values.

Determination of effective DTT concentration and use of DTT as a treatment. According to Miroliaei et al. [17] different concentrations of dithiothreitol (DTT) had different effect on the amount of protein activity, therefore, as a primary experiment the effect of different concentrations of DTT (5, 10 and 15 mM) on the expression of *MN052803-LTP* (Authors recorded at the Gen Bank) was evaluated. DTT applied in Hoagland's nutrient solution as irrigated water. After 0, 12, 24, and 48 h that wheat cultivars (tolerant and sensitive to salinity) exposed to DTT, the roots and the shoots of both cultivars were collected and stored at −80°C in ultra-freezer for real-time quantitative PCR. The result showed that 10 mM DTT reduced the expression of *MN052803-LTP*. In contrast, the other concentrations of DTT increased the gene expression. Therefore, 10 mM DTT was selected as a reducer of *MN052803-LTP* expression for further experiment. Then the effect of 10 mM DTT on the expression of *MN052803-LTP* and phosphatidylcholine (PC) content of inoculation and non-inoculated cultivars were evaluated in a separate experiment. To do so, inoculated and none inoculated seedlings irrigated with Hoagland's nutrient solution for 5 days, and then 10 mM of DTT (as a reducer-concentration) added to nutrient media. After 0, 12, 24, and 48 h of exposure to DTT, the roots and the shoots of both cultivars were collected and stored at −80°C in an ultra-freezer for real-time quantitative PCR. Furthermore, some of the treated plants kept for 7 more days after the application of DTT and phosphatidylcholine (PC) content were measured in 12 days old seedlings.

Chromatographic assay. Phospholipid extracts were obtained using the method reported by Folch et al.

[18] which modified to extract phospholipid from whole plant (roots and shoots), the homogenate tissue was transferred to a graduated glass tube. Subsequently, chloroform-methanol (2 : 1, v/v) was added to the glass tube at twice the volume as that of the used extract. Then strongly oscillated for 1 min and centrifuged at 2500 *g* for 10 min. After centrifugation, the supernatant was discarded, but the boundary layer was not. The methanol-water solution (1 : 1, v/v) was added to the glass tube at a quarter of the volume as that of the subnatant, and strongly oscillated for 1 min then, centrifuged at 2500 *g* for 10 min. The supernatant and the boundary layer were then discarded. Finally, the subnatant was transferred to another glass tube, dried under a stream of the nitrogen, and stored at −20°C. Before HPLC analysis the extracted phospholipid was dissolved in a mobile phase solvent containing 20% chloroform. HPLC analyses were performed on a KNAUER/AZURA using 100 mm Agilent Zorbax $C_{18}H_{10}$ 5 μm particle size analytical UV-vis spectra recorded on a Lambda 25 UV-Vis spectrometer using a 1 cm quartz cuvette**.** The mobile phase solvent acetonitrile, methanol, and 85% phosphoric acid (90 : 3 : 1, v/v) was thoroughly mixed in advance, filtered through a microporous membrane (0.2 μm) and degassed, and then, delivered to the column by a computerized solvent delivery system at the flow rate of 0.80 mL/min. The sample volume injected for HPLC analysis was 20 μL. The effluent was detected by a UV detector at 203 nm. The data were analyzed by computer-based on the model DL-800 chromatographic workstation.

Statistical analysis. All experiments were carried out with three replicates. The biochemical and gene expression parameters were statistically analyzed using ANOVA and the mean values were compared using Duncan's multiple range tests. Excel 2016 was used to draw the necessary graphs. Sequence data and its analyses were carried out with CLC sequences viewer programs and then Sequin software was used to record the gene in the NCBI gene bank.

RESULTS

A brief explanation of the results of the primary experiment (germination of 18 common wheat cultivars) indicated that the seedling vigor, germination percentage, and germination rate in all cultivars were significantly affected by salinity stress (details in Supplementary Tables S1-S4). However, the lowest and the highest seedling vigor, germination percentage, and the rate of germination were observed in Qods and Sorkhtokhm cultivars, respectively under 200 mM of NaCl of the growth media. Therefore, Qods and Sorkhtokhm cultivars were selected as sensitive and tolerant cultivars, respectively to salinity for further experiments.

The Symbiotic Effects of A. brasilense Strains on Growth Parameters

The results of growth parameters in wheat cultivars, including the fresh, and dry weight of plants as well as the shoot length, and the average plant root lengths, showed that salt stress (200 mM) reduced these parameters, while inoculation of wheat seedlings with *A. brasilense* (Sp245 and Sp7) improved these parameters, even under salt stress. The results also revealed there were significant differences between the two cultivars. In fact, the levels of measured parameters in salt-tolerant cultivar (Sorkhtokhm) were higher than salt-sensitive one (Qods) in all salinity levels (Fig. 1).

The Activities of PAL and TAL Enzymes

Analysis of variance (details in Supplementary Table S5) showed that salinity and inoculation had a significant effect on the PAL and TAL enzyme activity of the shoots, while cultivar and their interactions did not affect PAL activity. Under the control condition, the highest activity of PAL in the shoots of tolerant and sensitive cultivars was 4.04 and 3.14 U/mg proteins, respectively. Simultaneously, TAL activity was lower in different cultivars (1.9 U/mg proteins) but almost the same in the sensitive and tolerant cultivars (Fig. 2). Inoculation increased the activity of PAL in both cultivars by almost 40% but TAL activity increased differently in the tolerant and sensitive cultivars (22 and 10%, respectively). Although salinity increased PAL and TAL enzymes activity, the dual effect of inoculation and salinity increased much higher the activity of these enzymes (50% more for PAL in both cultivars, and 16 and 8% more for TAL activity in the tolerant and sensitive cultivars, respectively) compared to non-inoculated plants under salinity condition.

Effect of Salinity and Inoculation on MN052803-LTP Expression

The highest *MN052803-LTP* expression was observed at 12 hours during the course of the experiment in all cases. This means that the *MN052803-LTP* expression in the roots and the shoots were increased after the plants subjected to salinity up to 12 hours then decline or stay as constant in the sensitive and tolerant cultivars (Fig. 3). Twelve hours after salinity imposed, *MN052803-LTP* expression of non-inoculated plants was increased in the roots and the shoots (6 and 2.6-fold in the tolerant and 3 and 1.2-fold in the sensitive cultivars, respectively), and afterward their values were reduced but still was higher than that in control plants. Meanwhile, inoculation caused a significant increase in the expression of *MN052803-LTP* and reached its values to 6 and 2.9-fold in the tolerant and 3.9 and 2.4-fold in the sensitive cultivars for the roots and the shoots, respectively. Under dual treat-

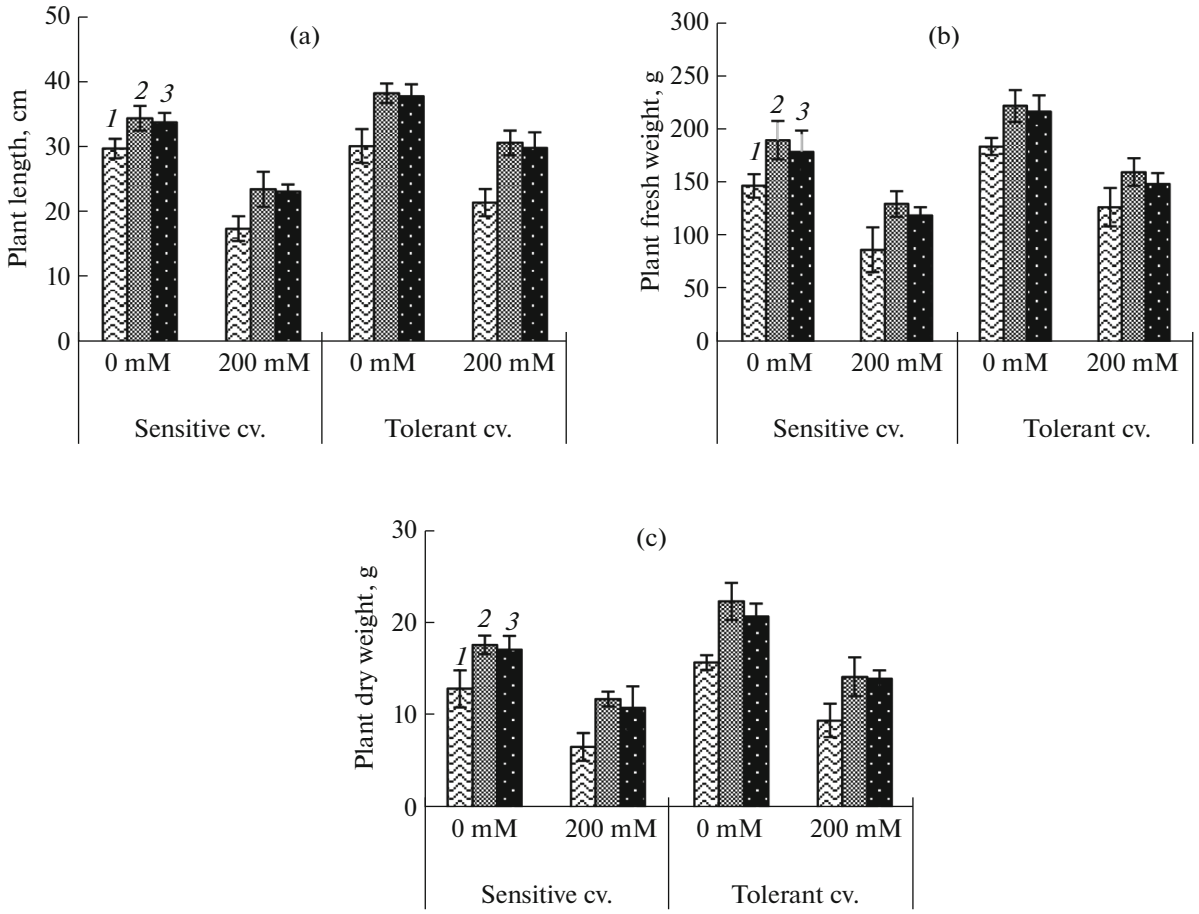

Fig. 1. Growth parameters (plant length (a), fresh (b) and dry (c) weight) of sensitive (Qods) and tolerant (Sorkhtokhm) wheat cultivars to salinity when inoculated with *Azospirillum brasilense* (Sp245 and Sp7) strains under salt stress (200 mM NaCl). The values related to non-inoculated (*1*) and inoculated wheat cultivars (using 10^7 CFU/mL) with Sp245 (*2*) and Sp7 (*3*) strains. Each value represents the mean of three replicates ± SD.

ments (salinity and inoculation), the maximum relative expression of *MN052803-LTP* was observed as compared to other treatments. The *MN052803-LTP* expressions of the roots were 7.7 and 5.9-fold in the tolerant and sensitive cultivars, respectively, and 3-fold in both cultivars for the shoots when compared to their corresponded control plants at 12 h. However, the relative expression was higher in the tolerant cv. than the sensitive one.

Effect of Dithiothreitol (DTT) and Inoculation on MN052803-LTP Expression

Different concentrations (5, 10, and 15 mM) of DTT (as described in part 2 of the primary experiment) on the expression of *MN052803-LTP* had different effects (Fig. 4). Although the trend of the relative expression of *MN052803-LTP* was similar for 5 and 15 mM of DTT in roots and shoots of both cultivars, the *MN052803-LTP* expression was much higher, and looks likes that these concentrations stimulated this gene to express more. In contrast, 10 mM of DTT caused a reduction of *MN052803-LTP* expression in the roots and the shoots (on average 0.7 and 0.5-fold, respectively) of both cultivars. Although the positive or negative effect of DTT concentrations was much higher in the tolerant cultivar than the sensitive one, the concentration of 10 mM of DTT was considered as a reducer of *MN052803-LTP* activity.

Using 10 mM of DTT (as a reducer of *MN052803-LTP* expression) and inoculation (as stimulator) on the *MN052803-LTP* expression showed that the *MN052803-LTP* expression changed according to the type of treatment applied (Fig. 5). The higher expression of *MN052803-LTP* was observed in the roots and shoots of inoculated plants. Meanwhile, 10 mM of DTT caused a reduction of *MN052803-LTP* expression in the roots and the shoots (on average 0.7 and 0.5-fold, respectively) of both cultivars. In the dual effect of treatments, inoculation reduced the inhibitory effect of DTT on the *MN052803-LTP* expression. In another point of view, it looks like 10 mM DTT also unaffected the role of *A. brasilense* in an association system on the *MN052803-LTP* expression.

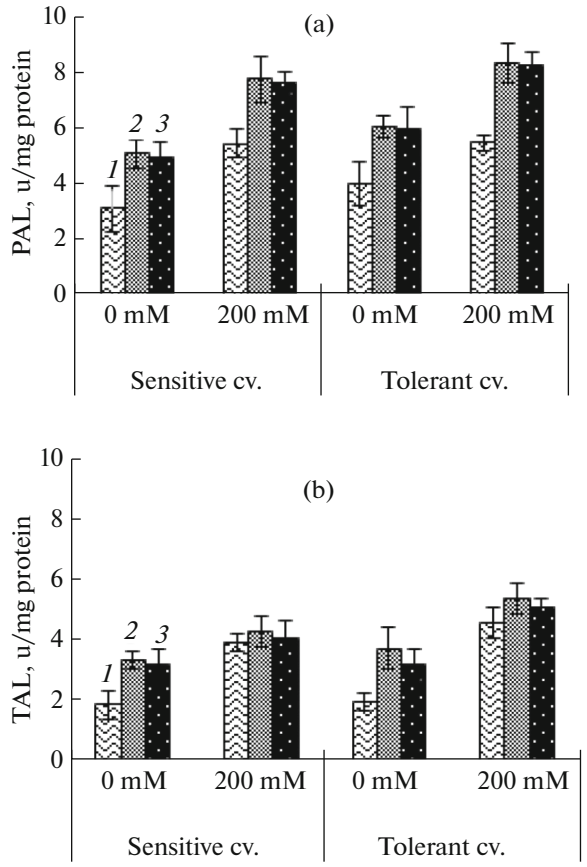

Fig. 2. PAL (a) and TAL (b) shoots enzymes values of sensitive (Qods) and tolerant (Sorkhtokhm) wheat cultivars to salinity when inoculated with *Azospirillum brasilense* (Sp245 and Sp7) strains under salt stress (200 mM NaCl). The values related to non-inoculated (*1*) and inoculated wheat cultivars (using 10^7 CFU/mL) with Sp245 (*2*) and Sp7 (*3*) strains. Each value represents the mean of three replicates ± SD.

Variation of Phosphatidylcholine Content

The result showed that phosphatidylcholine (PC) content was significantly linear with the peak area within the wide linear range. The optimum resolution (separation and detection) of the PC was achieved within 6.7 min (Fig. 6a). The results (Fig. 6b) showed that phosphatidylcholine content increased in the order of salinity (200 mM NaCl), *A. brasilense* (Sp245 strain), and their dual effects (salinity and inoculation) in comparison to the control plants. Moreover, there were significant differences between the effect of salinity and inoculation on the PC content of the salt-sensitive and salt-tolerant cultivars. The PC content of the salt-tolerant cultivar (Sorkhtokhm) was higher than the salt-sensitive cultivar (Qods). In contrast, adding DTT led to a reduction in the amount of PC. Therefore, the highest PC content (3.4 μg/g fr wt) was measured in the inoculated salt-tolerant cultivar treated with 200 mM NaCl, while the minimum amount of PC (0.3 μg/g fr wt) was observed in the plants exposed to 10 mM DTT.

DISCUSSION

Salt stress is one of the abiotic stresses that negatively affect plant growth and development. Our results showed that 200 mM NaCl could reduce the growth parameters of wheat cultivars, including the average plant root lengths, shoot length, fresh, and dry weight of plants. Results of different studies also reported a significant reduction of root length, dry and fresh weight under salinity conditions [1]. However, inoculation with *A. brasilense* (Sp245 and Sp7) could reduce the adverse effects of salinity and consequently improved growth parameters. This positive effect of *A. brasilense* under salinity conditions and inducing a better growth environment for plants could be related to the useful substances produced by symbiotic bacteria such as growth regulators [1].

There was a positive correlation between the measured parameters and the compatibility of tolerant and sensitive wheat cultivars with the *A. brasilense* strains. Inoculation of wheat cultivars with *A. brasilense* Sp245 and Sp7 strains showed different physiological responses to salinity conditions due to the compatibility of a strain of *A. brasilense* and wheat cultivar. In this experiment, Sp245 and Sp7 work well on both cultivars, but Sp245 showed more positive effects as compared to Sp7. However, not only different strains of bacteria showed different plant responses but also different wheat cultivar showed different responses. Improved growth patterns of inoculated seedlings under salinity conditions are a substantial result of salt tolerance due to inoculation. Indeed, our results indicated that using *A. brasilense* (at a concentration of 10^8 CFU/mL) as a natural and eco-friendly substance can be beneficial to improve salt tolerance in wheat cultivars. Previous studies also showed that both strains of *A. brasilense* (Sp7 and Sp245) caused an increase in physiological parameters of wheat seedlings under non-saline and saline conditions [19]. However, our result originated from different wheat cultivars. It looks like the use of inoculation is an effective strategy to enhanced salinity stress tolerance in plants.

In an inoculation process, the reaction of plant defense enzymes is important in the establishment of a successful association between plant host and bacteria. The application of PGPRs significantly increases some plant defense-related enzymes like PAL and TAL enzymes [4]. These enzymes are involved in the anchoring process and their variation could limit or stimulate their partnership. Zeffa et al. [20] reported that *A. brasilense* may enhance the defensive mechanism of maize genotypes via changing the activities of these enzymes same as the other PGPRs. The establishment of a symbiosis relationship between plant and bacteria can stimulate the biosynthesis pathway of phenolic compounds as a plant defense mechanism. Therefore, an increase in the activity of TAL and PAL can cause more phenolic compounds in inoculated plants especially with a compatible strain which is an

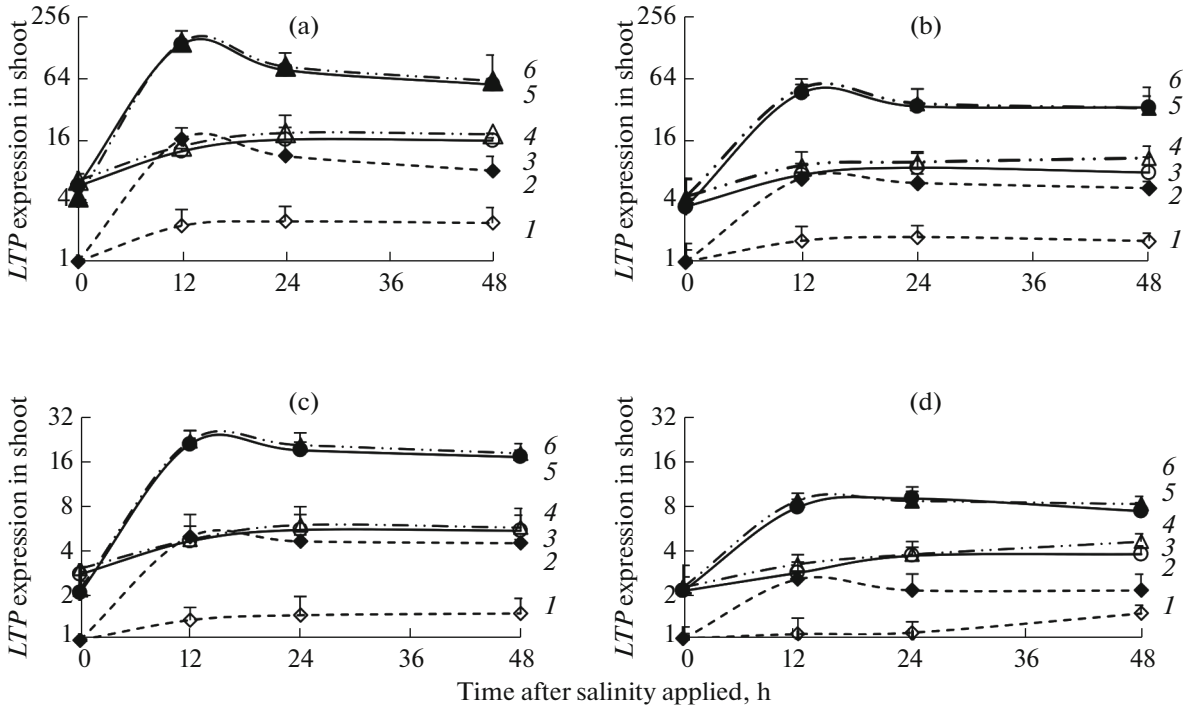

Fig. 3. *MN052803-LTP* expression of roots (a, b) and shoots (c, d) of the sensitive and tolerant (Qods and Sorkhtokhm, respectively) wheat cultivars to salinity when inoculated with *Azospirillum brasilense* (Sp245 and Sp7) strains under salt stress (200 mM NaCl). The values related to the relative expression of *MN052803-LTP* of non-inoculated (NI) and inoculated wheat cultivars (using 10^8 CFU/mL) with Sp7 and Sp245 strains at 0, 12, 24 and 48 hours after salinity started. (*1*) NI-0 mM NaCl, (*2*) NI–200 mM NaCl, (*3*) Sp7-0 mM NaCl, (*4*) Sp245-0 mM NaCl, (*5*) Sp7-200 mM NaCl, (*6*) Sp245-200 mM NaCl. Each value represents the mean of three replicates ± SD.

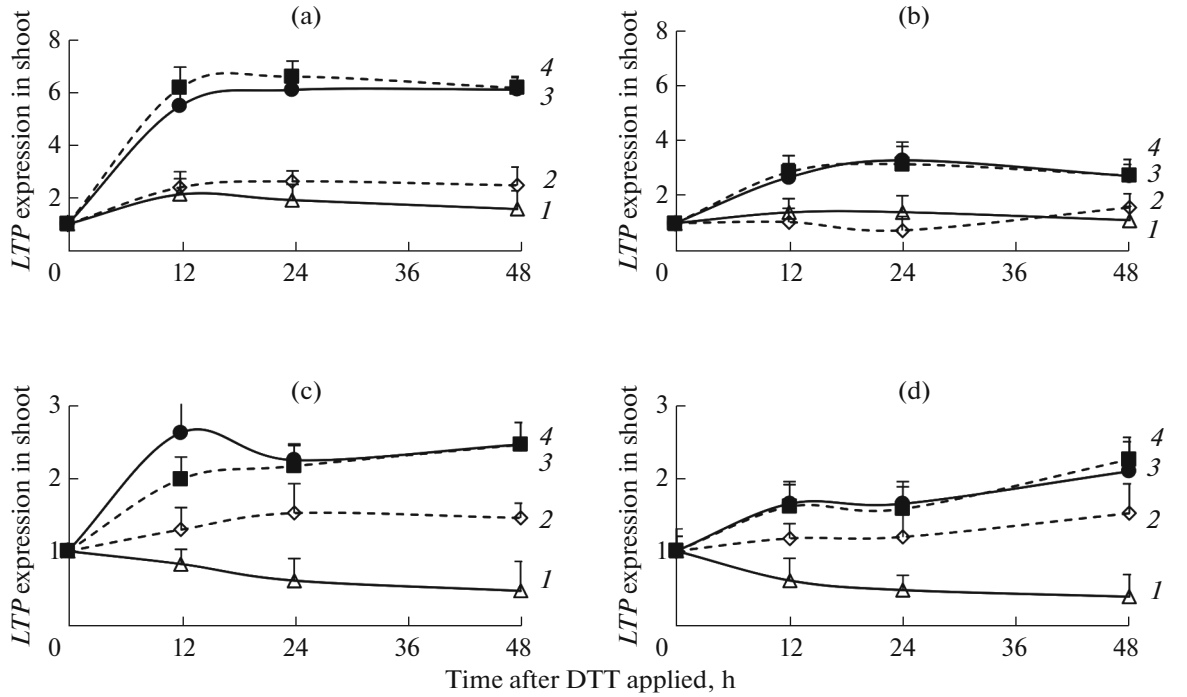

Fig. 4. *MN052803-LTP* expression of sensitive (Qods) and tolerant (Sorkhtokhm) wheat cultivars to salinity when treated with different concentration of DTT ((*1*) 10 mM DTT, (*2*) 0 mM DTT, (*3*) 5 mM DTT, (*4*) 15 mM DTT). The values related to the relative expression of *MN052803-LTP* of roots (a, b) and shoots (c, d). Each value represents the mean of three replicates ± SD.

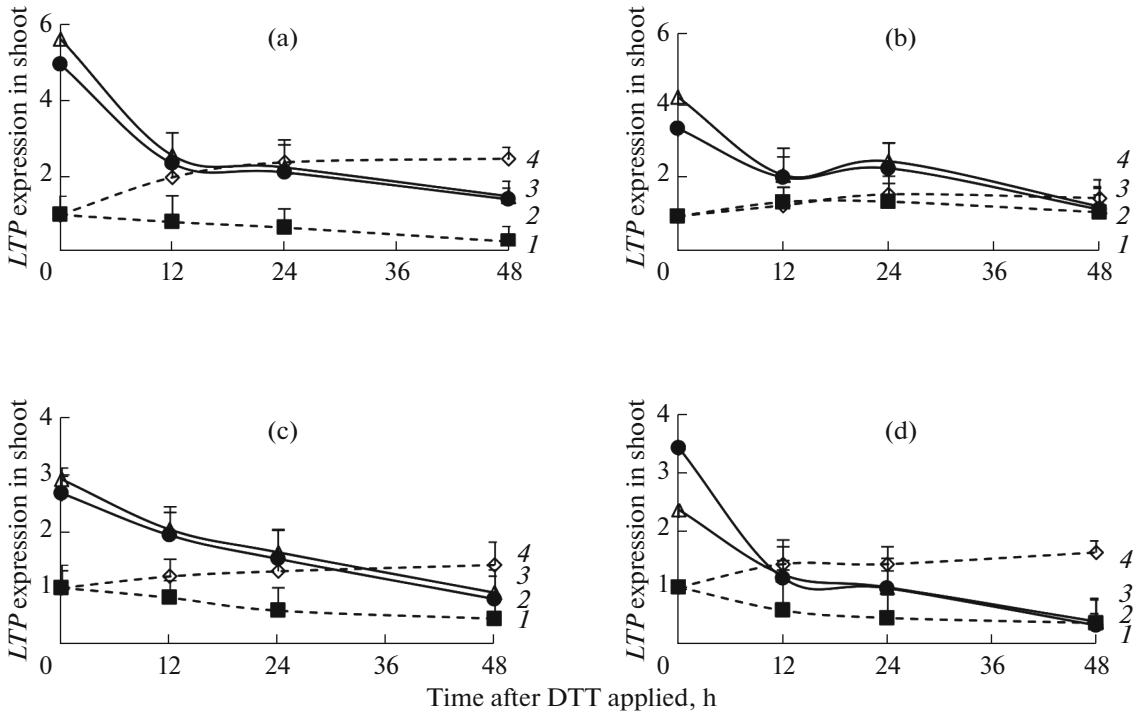

Fig. 5. *MN052803-LTP* expression of inoculated (Sp7 and Sp245) and non-inoculated (NI) wheat cultivars when treated with 10 mM DTT. The values related to the relative expression of *MN052803-LTP* of roots (a, b) and shoots (c, d) of the sensitive and tolerant (Qods and Sorkhtokhm, respectively) wheat cultivars to salinity. (*1*) NI—10 mM DTT, (*2*) Sp7—10 mM DTT, (*3*) Sp245—10 mM DTT, (*4*) NI—0 mM DTT. Each value represents the mean of three replicates ± SD.

efficient mechanism to deal with the adverse effects of reactive oxygen species during the infection process. As also Singh et al. [2] reported, the above explanation means that the microbial inoculation enhanced polyphenolic accumulation and improved PAL and TAL enzyme activity.

Feduraev et al. [21] reported that PAL and TAL enzymes also have the main role in salinity tolerance in wheat plants through the accumulation of phenylpropanoid compounds. This means inoculation influences the accumulation of polyphenolics and activity of PAL enzyme since polyphenolics compounds are strong antioxidants and PAL is a defense-related enzyme. Therefore, high accumulation of polyphenolics and enhanced PAL enzyme activity in the leaves are supposed to strengthen plants under salinity challenged conditions.

Our result showed that PAL and TAL activity in the inoculated wheat cultivars was higher than the plant exposed to salinity condition, in the dual effect of salinity and inoculation, their activities were much higher than the salinity condition alone. Meanwhile, much higher activities of PAL in inoculated tolerant wheat cultivar than the sensitive one indicated that this positive effect is mostly related to compatibility of wheat cultivar and bacterial strain. However, the dual effect of inoculation and salinity represents the interaction effect on the properties of the association between wheat cultivar with the strain of bacteria. It seems that accumulation of PAL and TAL enzymes is mostly related to compatibly of wheat cultivar and strain of bacteria. However, the effect of environmental factors such as salinity on this process should be considered. It looks like that the higher salinity tolerance could be obtained via the addition of TAL and PAL enzyme activities in a symbiosis process especially between a compatible association of wheat cultivar with *A. brasilense* strain.

Under salinity stress, the formation of reactive oxygen species in plants damages the plants' lipids [22]. In this regard, plasma membrane lipids play a crucial role in determining cell structures, regulating membrane fluidity, and transducing signals. Kader [4] reported that plant lipid transfer proteins (LTPs*)* were thought to participate in membrane biogenesis and regulation the intracellular fatty acid pools. Nevertheless, further isolation and analysis of *LTP* genes have revealed roles for *LTPs* including the adaptation of plants to various environmental conditions. *LTPs* are abundant and involved in various physiological processes in plants and be implicated in abiotic stresses in various species, such as drought, cold, and salt stresses [23]. Almost 49, 52, and 156 members of *LTPs* have been identified in *Arabidopsis*, rice, and wheat, respectively [23]. Wang et al. [24] based on gene expression data showed that *LTPs* are somehow involved in adaptation to salt

stress. In this study, we identified a novel *LTP* gene from wheat cultivar registered as *MN052803-LTP*, which was dramatically induced and up-regulated rapidly by abiotic and biotic stresses. The result of our study showed that the inoculation of wheat cultivars with *A. brasilense* (Sp245 and Sp7 strains), and salinity increased the *MN052803-LTP* expression. However, the *MN052803-LTP* was expressed at a higher level in the inoculated cultivar especially in the tolerant cultivar under salinity condition. Similar to our results, Benitez et al. [25] reported the up-regulation of *oso3go251000-LTP* in salt-tolerant cultivars of rice subjected to salt stress for 12 hours. Therefore, it is possible to say that this gene (*MN052803-LTP*) not only has an important role in the modification of salinity tolerance, the inoculation could positively contribute to this process.

Notable differences in the *MN052803-LTP* expression of the roots and the shoots of both cultivars were observed in the control condition, however, the high expression of *MN052803-LTP* in the root might be related to the organ-dependent or due to the priority of receiving signals from the rhizosphere. Therefore, this might be an indication of higher activity and/or physiological importance of *MN052803-LTP* in the root tissues. Wang et al. [26] reported that *ThLTPs* of *Tamarix hispida* showed different relative abundance in different tissues. The contribution of *MN052803-LTP* in the modification of salinity adverse effects in one hand and higher expression of this gene in inoculated condition especially with *A. brasilense* Sp245 (according to our result) in the other hand, proposed a suggestion that inoculation of a wheat cultivar with a compatible strain can provide a proper tool to improve tolerance of wheat to salinity via regulation of *MN052803-LTP* expression.

To facilitate endoplasmic reticulum (ER) stress-related research, in most cases, DTT is used as a stress activator [27]. DTT is a redox reagent that can destroy the oxidation conditions required for the formation of disulfide bonds. The important genes involved in ER stress responses were identified and analyzed comprehensively to determine a possible mechanism of stress regulation in plants. However, different gens exhibited a different expression pattern under DTT treatment. Yu et al. [28] reported that *DEG* genes were significantly up-regulated under DTT (7.5 mM), while *GRP94* expression was down-regulated under DTT (7.5 mM) treatment in wheat at 48 h. Probably there might be a relationship between DTT and the expression of the *LTPs* gene. Our results showed that the concentration of 10 mM DTT significantly decreased the expression of *MN052803-LTP*, while 5 and 15 mM DTT up-regulated this gene. Importantly, the higher expression of *MN052803-LTP* in inoculated plants with *A. brasilense* (Sp245 and Sp7) under 10 mM DTT may indicate the role of *A. brasilense* in partial removal of the inhibitory effect of DTT. Reduction in the expression of *MN052803-LTP* in inoculated wheat cultivars under 10 mM of DTT is also induction of the adverse effect of DTT on the symbiotic effect of *A. brasilense*.

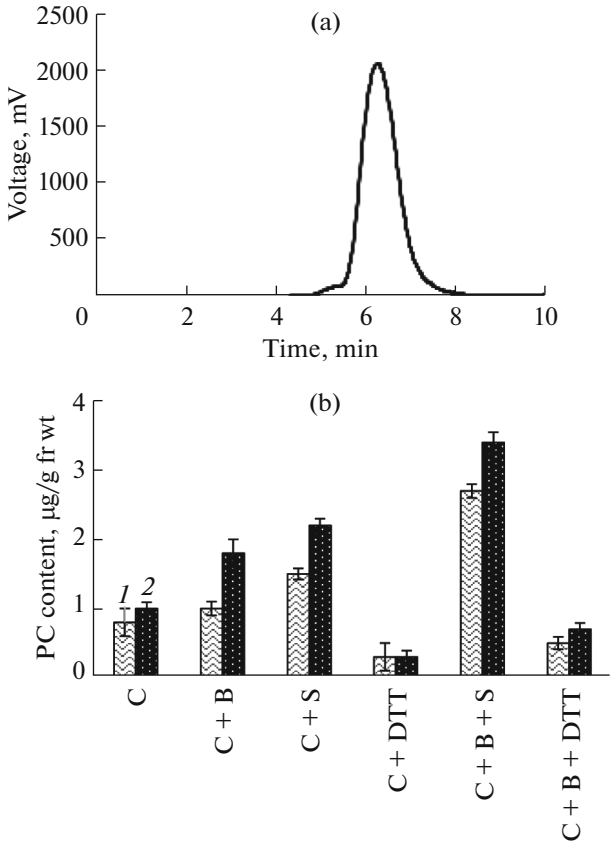

Fig. 6. The curve of standard chromatogram of phosphatidylcholine (a), and phosphatidylcholine (PC) content (b) of the salt-sensitive (Qods, *1*) and salt-tolerant (Sorkhtokhm, *2*) cultivars under inoculation with *Azospirillum brasilense* stain (Sp245), DTT (10 mM) and salt stress (200 mM NaCl). The values related to the phosphatidylcholine content of whole plant. The abbreviated letters C, B, S, and DTT stand for cultivar, Sp245, salinity, and DTT, respectively. Each value represents the mean of three replicates ± SD.

Several studies have shown that *LTPs* are able to transfer all common lipids and to bind Acyl-CoA like phospholipid [4]. Phosphatidylcholines (PC) are a class of phospholipid that is a major component of biological membrane. In addition, PC also has a regulatory role in most signaling pathways in plants [30]. Under salinity stress and inoculation, the PC content and transcription level of *MN052803-LTP* was increased, whereas under 10 mM of DTT, the PC content and transcription level of *MN052803-LTP* was decreased. Deng et al. [30] demonstrated the role of *GhLTPG1* in phospholipids transport as well as the relationship between *GhLTPG1* expression and phospholipid content. Since LTPs mediate transfer of phospholipids (like PC) to membranes, the similarity of patterns of changes in PC and *LTP* expression in response to different treatments could be logical.

Therefore, it can be said that increase in the expression of *MN052803-LTP* by *A. brasilense,* probably can be lead to changes in composition and density of PC content of membrane. In conclusion, although salinity usually increases PAL and TAL enzyme activities in plants, the MN052803-*LTP* mRNA expression, and phosphatidylcholine content of the wheat cultivars increased in this experiment due to the salinity condition. The higher PAL and TAL enzyme activities, MN052803-*LTP* mRNA expression, and phosphatidylcholine content in the inoculated plants with *A. brasilense* under salinity conditions can be considered as a positive role of bacteria for improving salt tolerance. The result of the present study demonstrated that one of the mechanisms of inducing salt tolerance in the wheat cultivar could be through the *MN052803-LTP* expression, which would be provided by symbiosis with *A. brasilense*. This gene can be involved in salinity tolerance by supplying the required phospholipids for membranes, such as phosphatidylcholine, activation of the biosynthesis of antioxidant compounds, and the phenylpropanoid pathway.

In conclusion, although salinity usually increases PAL and TAL enzyme activities in plants, the MN052803-*LTP* mRNA expression, and phosphatidylcholine content of the wheat cultivars increased in this experiment due to the salinity condition. The higher PAL and TAL enzyme activities, MN052803-*LTP* mRNA expression, and phosphatidylcholine content in the inoculated plants with *A. brasilense* under salinity conditions can be considered as a positive role of bacteria for improving salt tolerance. However, 10 mM DTT could reduce the effect of *A. brasilense* on the LTP expression. The result of the present study demonstrated that one of the mechanisms of inducing salt tolerance in the wheat cultivar could be through the *MN052803-LTP* expression, which would be provided by symbiosis with A. *brasilense*. This gene can be involved in salinity tolerance by supplying the required phospholipids for membranes, such as phosphatidylcholine, activation of the biosynthesis of antioxidant compounds, and the phenylpropanoid pathway.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving animals performed by any of the authors.

AUTHOR CONTRIBUTIONS

Dr. A. Mostajeran contribution: the idea, design and conducting the project, and manuscript editing. M. Riahi: layout and performing the experiment, preparing the manuscript draft. Dr. M. Miroliaei: adviser in the LTPs subject.

SUPPLEMENTARY INFORMATION

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