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Changes of Vascular Tissue Structure and *PAL* Gene Expression in Bell Pepper Var California Wonder under Zinc Stress

Helal Nemat Farhazadi[1*], Sedigheh Arbabian[2]

[1]PhD in Cellular and Developmental Plant Biology, Department of Biological Sciences, Technical and Vocational University (TVU), Teheran, Iran.
And Department of Biology, Faculty of Sciences, Islamic Azad University, North-Tehran Branch, Tehran, Iran.
[2]Assistant Professor, Department of Biology, Faculty of Sciences, Islamic Azad University, North-Tehran Branch, Tehran, Iran.

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***Corresponding Author:**
Helal Nemat Farhazadi
Email:
helalfarhzadi@yahoo.com

ABSTRACT

Zinc, a co-factor in numerous enzymatic reactions and an essential micronutrient is involved in the synthesis of protein as well as the metabolism of carbohydrates, nucleic acids, and lipids. Nevertheless, it could be harmful when the plants are exposed to elevated Zn levels of the element. Bell pepper (*Capsicum annuum* L.) is one of the most economically significant herb species that is grown and consumed around the world. It has high nutritive values as a result of having antioxidant capacity and phytochemicals. This study was undertaken to assess the effect of different concentrations of Zn on the growth parameters, the root and shoot anatomical structure, and the phenylalanine-ammonia-lyase gene (*PAL*) expression in *Capsicum annuum* L. var California Wonder. The plants were cultivated with different concentrations of zinc nitrate, including 2.5, 5, 7.5, 10, and 15 mM in a greenhouse under greenhouse conditions. The untreated plant group was considered as a control. The results showed that the elevated levels of Zn meaningfully reduced the length of shoots, petiole length, total leaf areas, and leaf frequency, particularly at the highest concentration of Zn ($P<0.05$). The high levels of Zn also caused microscopic structural changes in the vascular tissues of roots and shoots. The gene expression of bell pepper *PAL* (*CapPAL*), analyzed by quantitative real-time PCR (qRT-PCR), showed that the highest expression levels of *CapPAL* were found when plants were treated with 7.5 mM and 10 mM zinc nitrate.

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Introduction

Sweet (bell) pepper, *Capsicum annuum* L., is a member of the Solanaceae family and originated in tropical America, where various species were used centuries before Columbus got there [1]. Currently, sweet and hot peppers are commonly grown worldwide and constitute an important source of human diets, particularly in Asia [2]. Sweet (bell) pepper contains a high level of beneficial phytochemicals such as phenolic compounds, capsaicinoids, and vitamins [3]. Results of some studies revealed that capsaicin, an active pepper ingredient, has anti-diabetic [4], anti-bacterial [5], analgesic [6] and anti-carcinogenic properties [7].

The genetical, physiological, chemical, and biological effects of heavy metals exposed to plants have attracted the attention of many investigators since they can accumulate in plants and finally enter the food chain [8-11]. Several lines of evidence suggest that the heavy metals accumulated in plant tissues can result in decreased seed germination, plant biomass, root length, and biosynthesis of chlorophyll [12]. Depending on plant species, type of metal, and exposure conditions, there are different physiological responses to environmental stress induced by heavy metals in plants [13].

Zinc acts as a cofactor and is incorporated into several metalloenzymes such as oxidase dehydrogenases, anhydrases, and peroxidases. It plays an essential role in regulating energy transfer, nitrogen metabolism photosynthesis, cell multiplication, stabilization of ribosomal fractions, synthesis of cytochromes, auxin synthesis, and pollen formation in plants [14]. Furthermore, Zn contributes to the production of proteins and nucleic acids. Zn can be involved in the formation of the seeds in the presence of nitrogen ions and phosphorus [14]. Nevertheless, the high concentrations of this element are potentially toxic [15]. Excessive zinc levels can lead to cellular oxidative damage by affecting the activity of antioxidant agents in plants [16].

Different plant secondary metabolites (estimated 200,000), have unknown effects on the maintenance and growth of the cells; however, they may be bioactive and protect plants against different types of stressful factors. Thus, secondary metabolites have significant roles in the plants' acclimatization to their environment [17]. It is noteworthy that the levels of these metabolites such as phenylpropanoids are altered in response to environmental stress [18]. Phenolic compounds (such as flavonoids, hydroxycinnamic acids, coumarins, and stilbenes) are synthesized along the phenylpropanoid pathway [19]. This pathway starts with the deamination of L-Phe into cinnamic acid by the phenylalanine ammonia-lyase (PAL) (PAL EC 4.3.1.5) enzyme that catalyzes a critical formation step of many

phenylpropanoid compounds [20]. The expression of the phenylalanine ammonia-lyase gene is altered in response to a variety of environmental insults [21].

Taking into consideration the economic and nutritional importance of pepper in recent decades in the world, the increasing rate of heavy metal contamination in the environment, the significant features of zinc toxicity in plants, and the lack of reports on the impact of zinc intoxication on *PAL* expression in *Capsicum annuum* L., it was essential to evaluate the impact of zinc on the growth parameters, anatomical features, and the expression of the *PAL* gene in *Capsicum annuum* L. var California Wonder.

Methodology

Plant materials, treatments, and growth condition

Seeds of *Capsicum annuum* L. var California Wonder were purchased from the plant gene bank of the Seed and Plant Improvement Institute of Karaj, Iran. Following the sterilization of the seeds, they were cultured in a sterilized soil (obtained from Behkam Company, pH = 7.43, Zn = 137 mg/Kg, Fe = 6177 mg/Kg, Cu = 25.08 mg/Kg, EC $_{1:10}$= 3.72). Six treatment groups in the form of applying different concentrations of zinc [0 (control), 2.5 (treatment 1), 5 (treatment 2), 7.5 (treatment 3), 10 (treatment 4), and 15 mM (treatment 5)] were selected, and the plants were watered for 14 weeks with these treatments. During the propagation stage, the plants were grown under standard conditions at a temperature of 25 ± 2 °C, humidity of 75-80%, 16/8 h photoperiod, and light intensity of 125 µmol/sm^2.

Anatomical and morphometrical growth parameters study

For the anatomical analysis of root and shoot parts of the plant (at the end of the vegetative growth period, i.e. 14 weeks after the treatment course), samples were incubated in 100% ethanol and glycerin (1:1 v/v) for two weeks before being sectioned. The shoot and root sections at a 5-mm length were cut out from 2.5 cm above and 2.5 cm below the shoot-root intersection. The sections were stained with methyl green and Carmen. Specimens were observed under a light microscope connected to the OLYMPUS digital camera. At least 10 specimens for each tissue were analyzed, and the best samples were chosen for the preparation of photographs.

The plant samples were collected after 14 weeks for the measurement of various morphometrical growth parameters. Five plants from each replicate were analyzed to measure various parameters, and the average values of each parameter were calculated. The mean values were used for the statistical analysis. The difference between the treatments was analyzed by one-way analysis of variance (one-way ANOVA) followed by Turkey's post hoc test. Data are reported as means

± standard deviation. The significant difference was set at $P < 0.05$. The various growth parameters such as shoot length (mm), petiole length (mm), total leaf areas (LA) (mm^2), and the number of leaves per plant were determined for all samples. Shoot length was measured from the soil level to the tip of the shoot), while petiole length was calculated with a simple ruler. The number of leaves was simply counted manually by count, and the LA of each leaf was estimated according to the Licow model, expressed as mm^2.

Molecular study

The total RNA contents were extracted from the fresh leaves (100 mg) harvested after all treatments using the TRIzol reagent (Isogene Lab. Ltd. Cat No: T1044, USA) and then treated with DNase I (RNase-free DNase I, Fermentas, USA) to remove residual genomic DNA based on manufacturer's instructions. A nanodrop spectrophotometer (NanoDrop 2000c Thermo Scientific, USA) was utilized to verify the concentration and integrity of the extracted RNA by measuring the absorbance at 230, 260, and 280 nm. First-strand cDNA was synthesized from 1 µg of the extracted RNA with the Oligo d (T) 18 primer in a final reaction volume of 20 µL using the Reverse Transcription Kit (first-strand cDNA synthesis Kit, Fermentase, USA) based on the manufacturer's recommendations. The synthesized cDNA samples were stored at –20°C.

The primers for phenylalanine-ammonia-lyase and 18S RNA genes (length *PAL*= 300 bp, *18s rRNA* = 250 bp) were designed by the Gene Runner software based on conserved regions, and sequences of the *PAL* gene of *C. annuum* L. (NM_001324603.1) registered in NCBI. Then, the specificity of the designed primers was checked by the Primer-BLAST online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The 18S ribosomal RNA (*Capsicum* L.) gene was employed as a control gene to normalize the gene expression of all samples (Table 1). For the detection of the expression of the *CapPAL* gene in the leaf tissue, RT-PCR was performed using a PCR kit (CinnaGen, Iran). Thermal conditions for the amplification of *PAL* were performed in 30 cycles; each cycle consisted of denaturing at 95°C for 50 s, annealing at 58°C for 50 s, extension at 72°C for 50 s, an initial denaturing step at 95°C for 5 min, and a final extension step at 72°C for 10 min. Polymerase chain reaction products were visualized on a 2% agarose gel after electrophoresis.

The amplification was carried out by a real-time rotary analyzer (Rotor-Gene 6000, Corbett, Australia) to evaluate the expression level of the *PAL* gene in all samples. The relative expression analysis of the gene was conducted according to the $-2^{\Delta\Delta Ct}$ method. The relative quantification was analyzed according to the comparative $2^{\Delta\Delta Ct}$ method. The amplification of the synthesized cDNA was performed in a final reaction volume of 20 µL containing 4 µL master mix, 0.4 µL

forward primer, 1 µL target cDNA, 0.4 µL reverse primer (Light Cycler Fast Start DNA Master Syber Green I, Roche, Germany) and 14.2 µL PCR-grade water. The housekeeping gene *18SrRNA* was used to normalize the expression of the analyzed gene. In addition, for each primer pair, a control PCR reaction was utilized, lacking nucleic acid templates. The properties of amplified segments were controlled by the melting curve analysis. Cycling parameters were as follows: 98°C for 15 s (denaturation), 58°C for 15 s (primer annealing), and 72°C for 15 s (primer extension) for 40 cycles followed by 72°C for 15 s (final extension). All reactions were run in triplicates.

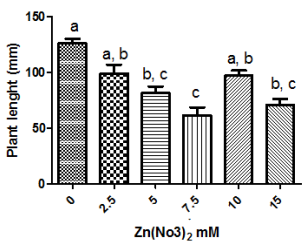
Table 1. Primer sequences were used for the sequence of the *PAL* gene belonging to *C. annuum* L. and primers were used for the gene expression analysis using the qRT-PCR technique.

	Location	Forward Primer	Reverse Primer	PCR Product (bp)
PAL-Ca	NC_029985.1	GGTTTTGGTGCAACATCACATA GGAG	ATTGTCAAAGTTCTCTTAGCTA CTTGGC	1200 bp
PAL	NC_029985.1	ACTACTGGTTTGGTGCAACATG	GACAACGGGACAAGATCACC	300 bp
18S rRNA	NC_024624.1	AAACGGCTACCACATCCAAG	CCCATCCCAAAGTCCAACTA	250 bp

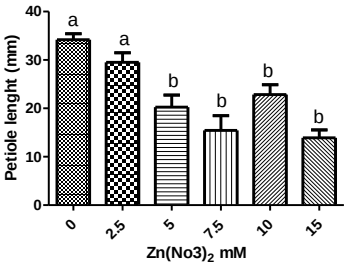
The multiple-alignment analysis was performed by the Cluster W. The phylogenetic analysis was carried out using the MEGA software version 6 by the neighbor-joining method. One-way analysis of variance (ANOVA) was used to determine significant differences between treatments (version 9.4; SAS Institute Inc. Cary, NC, USA).

Results and morphometrical growth parameters change in response to different concentrations of Zn

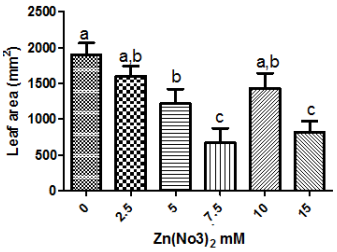
The shoot length, petiole length, total leaf areas, and the leaf number of the bell pepper in response to exposure to different concentrations of zinc are shown in Table 2 and Figure 1 (a, b, c, d).



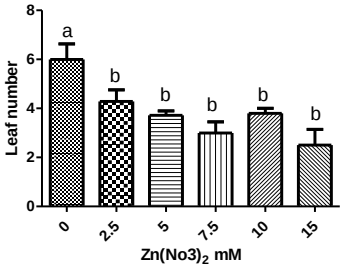
a) Shoot length (mm)



b) Petiole length (mm)



c) Total leaf areas (mm^2)



d) Leaf numbers

Figure 1. The effects of Zn $(NO_3)_2$ on the growth parameters: shoot length (mm) (a), petiole length (mm) (b), total leaf areas (mm^2) (c), and leaf numbers (d) of the sweet (bell) pepper.

There was no significant difference between the control and treatment 1 in terms of shoot length, petiole length, LA, and the number of leaves, while a significantly decreasing trend was found in the growth parameters of plants when treated from treatment 2 to treatment 5 (Table 2). Shoot length, petiole length (mm), total leaf areas (mm^2), and the number of leaves were markedly decreased when exposed to a gradual increase in the concentration of Zn, as compared with the control (Figure 2). Minimum shoot length was detected in plants treated with treatment 3, whereas the maximum shoot length was found in plants exposed to treatment 1 (Table 2, Figure 1a). Petiole length was decreased in various levels of Zn (Table 2, Figure 1b). The highest and the lowest petiole lengths were obtained when plants were treated with treatment 1 and treatment 5, respectively (Table 2, Figure 1b). The LA was diminished upon exposure to treatments 3 and 5 (Table 2, Figure 1c). The number of leaves declined in parallel with an increase in the concentration of zinc (Table 2, Figure 1d). The statistical analysis revealed significant ($P<0.05$) differences in plant growth parameters in response to different doses of zinc nitrate (Table 2). The excessive amounts of zinc nitrate are characterized by decreased growth and morphological alterations in response to Zn toxicity that includes stunting of the shoot, rolling of young leaves, the appearance of chlorotic spots on the leaves, and weaker development of shoot branches. The results indicated no significant difference in the growth parameters when the control and treatment 1 were compared (Table 2) as they showed a lack of morphological symptoms in response to zinc toxicity.

Table 2. The impact of Zn $(NO_3)_2$ on shoot length (mm), petiole length (mm), total leaf areas (mm2), and the number of leaves in the sweet (bell) pepper. Different letters indicate statistically significant (P< 0.05) differences (n=5).

$Zn(NO_3)_2$ concentration (mM)	leaf areas (mm^2)		petiole length (mm)		shoot length (mm)		leaf numbers	
	Mean ± SE	P value	Mean ± SE	P value	Mean ± SE	P value	Mean ± SE	P value
2.5	1586.80±547.37	.196	29.66±11.24	.606	126.06±10.21	1.000	4.29±1.25	.087
5	1271.83±702.94	.001	21.22±10.85	.001	81.49±15.96	.000	3.71±0.49	.010
7.5	768.47±523.83	.000	15.85±10.14	.000	61.73±17.36	.000	3.00±1.10	.001
10	1450.36±543.31	.049	22.75±10.50	.006	97.18±10.27	.024	3.80±0.45	.027
15	871.72±473.79	.000	14.64±6.51	.000	70.97±11.43	.000	2.50±1.29	.000

Figure 2. The impact of different concentrations of Zn $(NO_3)_2$ [0 (control), 2.5, 5, 7.5, 10, and 15 mM] on the sweet pepper.

Anatomical changes in response to different concentrations of Zn

The bell pepper plants show various degrees of detrimental changes in the structure of their root and shoot structures in response to an increase in the concentration of zinc nitrate in comparison with the control plants. The deleterious effects of zinc nitrate were dose-dependent. In contrast to the vascular bundles present in the root of control plants, in Zn-treated roots, a significant reduction in the xylem tissue and a marked increase in vascular elements in the metaxylem areas were observed. The phloem vessels were also morphologically altered (slightly reduced) in response to applying different doses of Zn (Table 3, Figure 3 A-F). The microscopic analysis of the shoot showed a considerable increase in the xylem and phloem tissues (Table 3, Figure 4A-F). Generally, plants treated with different doses of zinc nitrate exhibited higher vascular tissues in addition to an increased number of vessels than the control plants except for treatment 5 in which the vascular tissues and the number of vessels were dramatically lowered (Table 3, Figure 4A-F).

Table 3. The effect of Zn $(NO_3)_2$ on the xylem and phloem tissues of the root and shoot of the sweet (bell) pepper.

$Zn(NO_3)_2$ concentration (mM)	Root		Shoot	
	diameter of xylem (µm)	diameter of phloem (µm)	diameter of xylem (µm)	diameter of phloem (µm)
2.5	198.64	75.68	154.68	42.64
5	183.21	68.68	167.85	49.41
7.5	168.36	62.62	178.65	54.68
10	159.60	58.87	189.79	68.97
15	148.68	51.54	34.54	32.68

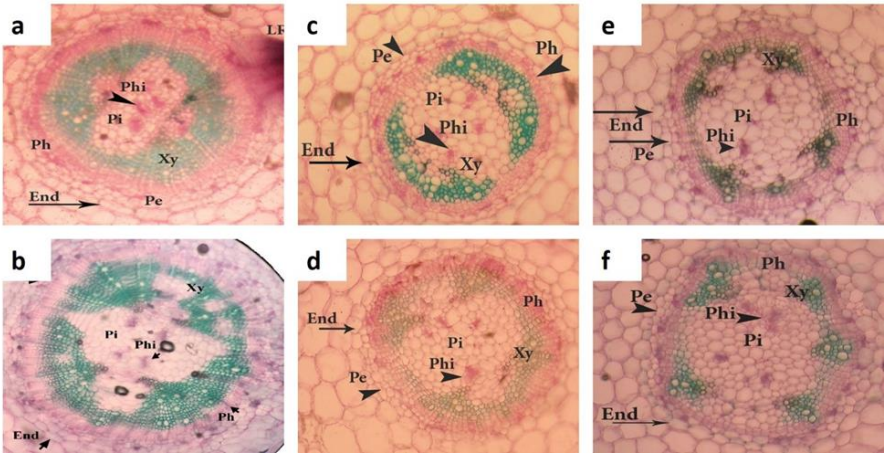


Figure 3. The cross-section of the root of the sweet pepper seedlings (40X) stained with Carmen and methyl green, (a) control, (b) treatment 1, (c) treatment 2, (d) treatment 3, (e) treatment 4 (f) treatment 5. End; Endoderm, Pe; peripheral circle, Xy; xylem, Ph; phloem, Phi; internal phloem, Pi; pith, LR; lateral root.

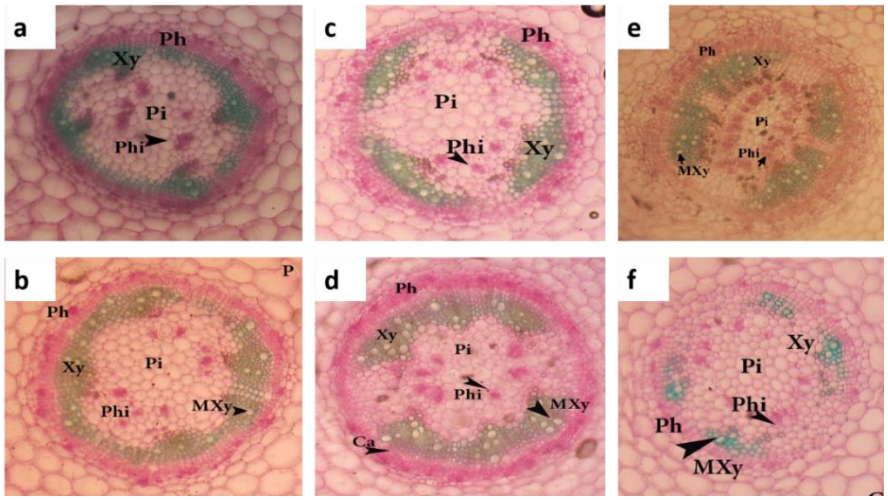


Figure 4. The cross-section of the shoot of the sweet pepper seedlings (40X) staining with Carmen and methyl green, (a) control, (b) treatment 1, (c) treatment 2, (d) treatment 3, (e) treatment 4, (f) treatment 5. P; cortex parenchyma, Xy; xylem, Ph; phloem, Phi; internal phloem, Mxy; metaxylem.

Sequencing and phylogenic-tree analysis of Cap PAL

Partial sequencing of the *PAL* gene with a length of 1200 bp in leaves of *Capsicum annuum* L. was performed. The similarities between the sequences of the PAL gene of *Capsicum annuum* L. and some Solanaceae species are presented in Table 4 and Figure 5**,** as analyzed by the ClustalW BLAST alignment method. The maximum nucleotide sequence similarity was found when the *PAL* gene was compared between *Solanum tuberosum* (potato) and *Solanum melongena* (eggplant). The nucleotide sequence of the *PAL* gene demonstrated an 85-92% identity to that of some Solanaceae species (Figure 5). The alignment of the *PAL* gene, belonging to *Capsicum annuum* L. with that of *Solanum tuberosum PAL* (potato) (NM_001318638.1), *Solanum melongena PAL* (eggplant) (KT259041.1), *Solanum Lycopersicum PAL* (tomato) (NM_001320609.1), and *Nicotiana tabacum PAL* (Tobacco) (NM_001325544 .1) revealed 92%, 92%, 91% and 85% sequence identities, respectively (Table 4). In the *PAL* gene of *Capsicum annuum* L., a conserved motif with the sequence of GTITASGDLVPLSYIA was identified in the active site of the *PAL* protein (Figure 5).

Table 4. The similarity in the sequence of the *PAL* gene in *Capsicum annuum* L. with other species belonging to Solanaceae using the ClustalW BLAST alignment.

Gene Bank accession No	Species	sequence similarity %
NM_001318638.1	*Solanum tuberosum* (potato)	92%
KT259041.1	*Solanum melongena* (eggplant)	92%
NM_001320609.1	*Solanum lycopersicum* (tomato)	91%
NM_001325544.1	*Nicotiana tabacum* (tobacco)	85%

Figure 5. Multiple alignments of the amino acid sequences of the *Cap PAL* gene with that of some Solanaceae species. The compared sequences belong to *Solanum tuberosum* (NM_001318638.1), *Solanum melongena* (KT259041.1), *Solanum Lycopersicum* (NM_001320609.1), *Nicotiana tabacum* (NM_001325544 .1). The active catalytic sites are underlined in red. The digits on the left side denote the order of amino acid sequences.

The phylogenetic analysis was conducted by the MEGA software version 6 through the neighbor-joining method. The phylogenetic tree was divided into two central branches, comprising dicotyledons and monocotyledons (Figure 6) according to the traditional taxonomic classification. Our results demonstrated that *CapPAL* was the closest gene to the *PAL* gene belonging to *S. tuberosum*, *S. melongena*, *S. Lycopersicum*, *N. tabacum,* and *S. pennellii*, and also the gene *PAL* of the above species originated from the same ancestor.

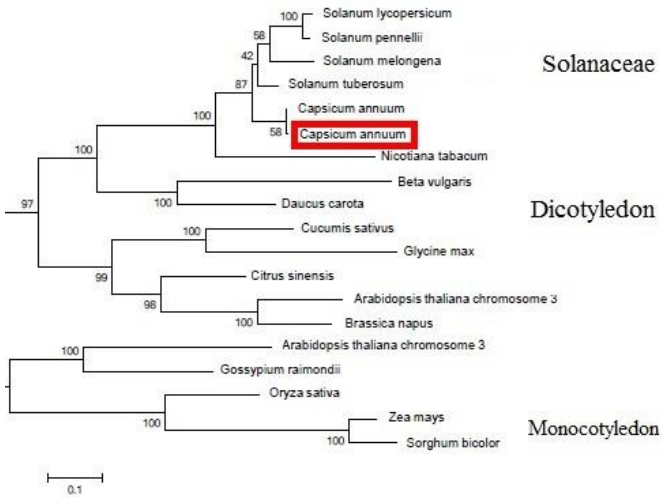


Figure 6. The phylogenetic tree depicts a genetic relationship between the *Cap PAL* gene and the *PAL gene* in other plants. The name of the sequence and Gene Bank Accession Number in Figures are shown as follows: *Solanum tuberosum* (NM_001318638), *Nicotiana tabacum* (M84466.1), *Solanum melongena* (KT259041.1), *Arabidopsis thaliana PAL2* (NM_115186.4), *Arabidopsis thaliana PAL4* (NM_111869.4), *Solanum Lycopersicum* (NM 001320040.1), *Zea mays* (NM 001111864.2), *Oryza sativa* Japonica Group (XM 015771243.1), *Capsicum annuum* (NM 001324603.1), *Cucumis sativus* (NM 001308910.1), *Brassica napus* (NM 001315686.1), *Gossypium raimondii* (XM 012579736.1), *Sorghum bicolor* (XM 002454153.2), *Solanum pennellii* (XM 015232558.1), *Beta vulgaris* (XM 010685777.2), *Citrus sinensis* (XM 006481430.2), *Glycine max* (NM 001250027.1), *Daucus carota* (XM 017403043.1).

The expression levels of Cap PAL in response to different concentrations of Zn

RT-PCR was carried out to confirm the expression of the *CapPAL* gene in leaf tissue. When the expression of *CapPAL* was corroborated in leaf tissue, the relative gene expression of *Cap PAL* was analyzed in the presence of the different concentrations of Zn performed by the qRT–PCR technique. The qRT–PCR analysis showed that the expression of the *CapPAL* gene was altered in response to the

application of various levels of Zn. The expression levels of the *Cap PAL* gene in treatments 4 and 5 were significantly higher than other treatments as well as control ($P < 0.05$). Compared with the control plants, the expression levels of the *Cap PAL* gene were increased by 2 and 3 folds upon the exposure of the plants to treatments 4 and 5, respectively. The highest expression of *CapPAL* was observed at the concentration of 15 mM (treatment 5), while the lowest expression was detected at the concentration of 7.5 mM (treatment 1) (Figure 7).

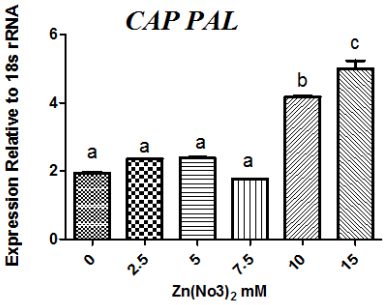


Figure 7. The effect of Zn $(NO_3)_2$ on the expression of the *Cap PAL* gene in *Capsicum annuum* L. Vertical bars implies the means ± SE (n = 3).

Discussion

Growth parameters are the best indices for the analysis of stress, and their changes could be identified after the exposure of plants to different types of heavy metals [22]. In the present study, a marked reduction was found in shoot length, petiole length, leaf area, and the number of leaves in response to increasing concentrations of Zn in comparison with the control plants. Growth inhibition is one of the most common symptoms of Zn toxicity in plants. Excessive amounts of Zn inhibit many metabolic activities in plants by degrading the mitochondrial structure in cells [14]. The elevated accumulation of Zn in the plant cells' cytosol also results in impaired function and inhibition of the respiratory reactions related to the process of cell growth. It finally leads to reduced growth and development of the whole plant [23]. This essential micronutrient, in excessive amounts, can decrease the growth by disturbance in cell division and cell elongation or even the combination of both by interrupting the interphase, prolonging the stage of prophase and G2 (mitotic activity) as well as stopping the synthesis of proteins required for the cell cycle and nucleic acid production [24]. The decrease in growth parameters was also reported in radish (*Raphanus raphanistrum*) [25], tomato (*Solanum Lycopersicum* L.) [26], and wheat (*Triticum aestivum* L.) [27] when exposed to high levels of Zn. The present study results also support these reports. The heavy metals affect the root, the first organ of the plant, by different mechanisms. According to the present research findings, in treated roots, a significant decrease was observed in xylem tissue. This occurrence might confine the translocation or uptake of heavy metals from the root to the shoot and leaves

[28; 29]. In agreement, [30] examined the effect of Pb on the growth rate of chamomile (*Matricaria chamomilla* L.).

Heavy metal stress causes deleterious changes in the anatomical structure of shoots. These alterations are dependent on the plant species and heavy metals applied [31]. The current study data revealed an increase in the vascular network system in shoots. The increased vascular system in Zn-treated plants indicates their resistance to Zn toxicity, adaptation, and tolerability [32]. It is also suggested that Zn might have positive effects on plant growth as it is involved in the formation of tryptophan which is a precursor of indole-3-acetic acid (IAA), contributing to the process of growth stimulation in plants [33]. It is now known that the cell wall would be lignified when the cell expansion is decreased, particularly when the cells are under stress conditions and when they differentiate into particular specialized tissues, such as xylem [34].

It appears that the bell pepper is not able to tolerate high concentrations of Zn (15 mM) as the maximum lignin biosynthesis would not be able to neutralize the toxicity of this element. Excessive stress beyond the permitted limit of the endurance of plants usually results in the development of cell death [35]. In chamomile [30], a substantial decrease was detected in the vascular system, which is not in agreement with the current research results. Genetic differences between different species in addition to different reactions of plants to different heavy metals might be reasons for this incompatibility.

The gene expression of phenylalanine ammonia-lyase and the synthesis of phenylpropanoids could be influenced by environmental factors and stress conditions [36]. Molecular genetics methods have been used to knock-down or knock-out the *PAL* genes for the analysis of their functionality in plant development and their responses to external stimuli [21]. In the present study, it was demonstrated that zinc affected the *PAL* gene expression in response to treatment with the bell pepper. The inhibition of *PAL* expression in response to stress conditions is a less known plant response but observed in Chicory (*Cichorium intybus*) exposed to thermal shock [37] as well as the tea plant (*Camellia sinensis*) under drought stress [38]. However, stress factors mostly increase the transcription activation of the *PAL* gene [39; 40].

It is generally accepted that the common plant responses to stress factors involve an increase in PAL activity and lignin content [41]. Numerous genes with different expression patterns appear to be involved in abiotic stress responses to lignin biosynthesis when differentially expressed genes were compared between *Arabidopsis thaliana* and *Thlaspi caerulescens* [32]. High expression of genes responsible for lignin biosynthesis is associated with the deposition of lignin in the endodermis. The endodermis consists of two layers in the root of *T. caerulescens* and only one layer in the root of *A. thaliana* [32]. In soybean, the treatment of plants with Cd and Pb elevated the expression of the *PAL* mRNA level, but in lupine, the changes in the expression of *PAL* depended on the

concentrations of metal used. It has been shown that Cd can decrease while lead can increase the expression of the *PAL* gene. The lignin content was increased in soybean in response to treatment with Cd and Pb, while in lupine, the effects were the opposite. Consequently, increased *PAL* expression in lupine in the presence of heavy metals could result in the synthesis of secondary metabolites that are not involved in the lignin formation [39].

Conclusion

The findings of the present study also confirmed these views. The analysis of the sequence of the *PAL* gene belonging to the sweet pepper (*Capsicum annuum)* indicates that this gene is evolutionally conserved. In the *Cap PAL* gene, a conserved motif with the sequence of GTITASGDLVPLSYIA was identified in the active site of the *PAL* protein [42]. Furthermore, the *PAL* gene in *Capsicum annuum* originates from the same ancestor when compared with other species.

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