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RESEARCH PAPERS

# Response of *Triticum aestivum* to Boron Stress[1]

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**Abstract**—Despite the demonstration that proline accumulation and gene expression of $\Delta^1$-pyrroline-5-carboxylate synthase (*p5cS*) increased under osmotic stress, the impact of excess boron on proline metabolism is not well known. Therefore, we investigated the effect of different boron concentrations (10, 50, 70, 140 and 200 ppm) on seedlings root growth, lipid peroxidation rate, antioxidant enzyme activity (glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT)), proline accumulation and transcription level of *p5cS* gene in *Triticum aestivum* L. AK-702. It was observed that seed germination and root growth in *T. aestivum* decreased depending on the concentration of boron. Our results indicated that boron toxicity induced lipid peroxidation and decreased GR activity under a high concentration of boron. However, the APX activity did not significantly change under high concentrations of boron (70, 140 and 200 ppm), while it increased under the lower levels of boron (10 and 50 ppm). In addition, excess boron enhanced CAT activity in the 200 ppm boron treated groups. Proline accumulation increased 2.25 and 1.45 fold in the 140 and 200 ppm boron applications. In addition, analyses of the mRNA transcription level using the semi-quantitative RT-PCR results showed that excess boron increased the *p5cS* mRNA transcript levels and showed a positive correlation of these levels with proline accumulation in *T. aestivum* roots.

*Keywords: Triticum aestivum*, boron, proline, *p5cS* gene

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## INTRODUCTION

*Triticum aestivum* L. is the most widely grown food crop in the world. Wheat's (*T. aestivum*) places of origin are Israel, Jordan, Lebanon, Syria, Turkey, Iraq and Iran. Wheat is a source of flour for bread, chapattis, semolina, biscuits and other confectionary products. The high protein content in wheat grain makes it a very important source of human nutrition [1]. Wheat is also an important staple crop in Turkey in terms of the economy, nutrition and employment. Wheat is grown in all regions of Turkey, mostly under rain fed conditions [2].

Boron is an essential element for higher plants, however a toxic concentration of boron has a negative effect on the development and growth process in higher plants [3]. Boron toxicity is caused by increased stomatal resistance, reduced biosynthesis of photosynthetic pigments, increased membrane permeability and decreased seed yield and quality [4]. In addition, genotypic differences in tolerance to boron toxicity have been reported within cereal varieties [5]. Despite the fact that most research to date has focused on the role of boron in plant development, the mechanism of boron tolerance or toxicity is not well understood.

Reactive oxygen species (ROS) are mainly produced at low concentrations in chloroplasts, mitochondria and peroxisomes throughout the plant's life [6]. However, their production level is dramatically increased under various environmental stresses. Many photosynthetic organisms have very efficient antioxidant systems, including antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POX) and ascorbate peroxidase (APX), and antioxidants to scavenge ROS as a protection against oxidative stress. Moreover, the defense activity of antioxidant enzymes is found in many plant species under various stress conditions [7]. Although the effect of boron toxicity on antioxidant enzymes is not clearly understood, it is known that excess boron inhibited the enzymatic activity of glutathione synthase and decreased glutathione pools in *Helianthus annuus* leaves [8]. Cervilla et al. [9] also showed that boron toxicity increased the ascorbate pool size and antioxidant enzyme activity in *Solanum lycopersicum*. In con-

[1] The article is published in the original.

*Abbreviations:* APX—ascorbate peroxidase; CAT—catalase; GR—glutathione reductase; MDA—malondialdehyde; POX—peroxidase; p5cS-$\Delta^1$—pyrroline-5-carboxylate synthase; SOD—superoxide dismutase; TBARS—thiobarbituric acid reactive substances.

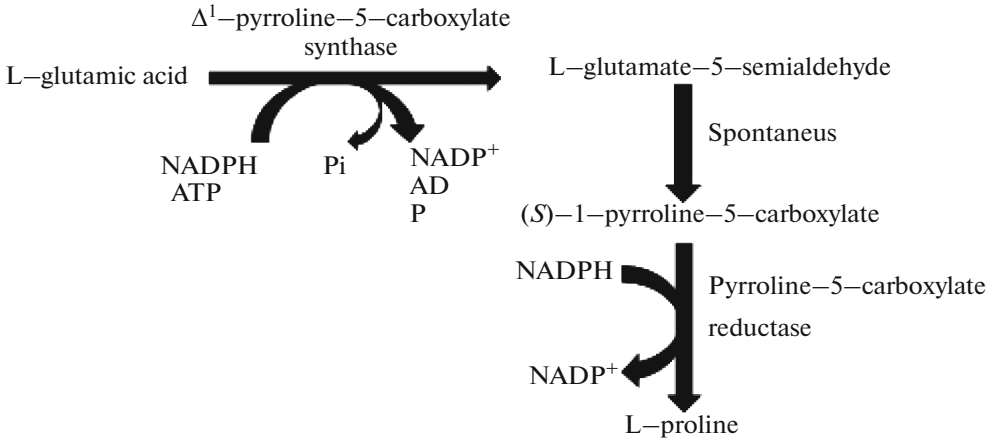


**Fig. 1.** Metabolic pathway of proline biosynthesis.

trast, Gunes et al. [10] demonstrated that boron toxicity caused a decrease in the activity of APX in *Vitis vinifera*.

Proline is an osmolyte that accumulates in plants and algae in response to different environmental stress condition [11, 12]. Physiological studies in higher plants demonstrated that proline accumulation may also be related to an adaptation to osmotic, drought and salt stresses [11, 13, 14]. Proline is derived from either the glutamate or ornithine biosynthetic pathways; however, the biosynthesis pathway via glutamate appears to be more predominant than via the ornithine pathway, especially under environmental stress conditions. In the glutamate pathway, the reduction of glutamate to semialdehyde is catalyzed by the enzyme $\Delta^1$-pyrroline-5-carboxylate synthase (Fig. 1), and the *p5cS* gene has been characterized from several plants [13, 15]. Molecular physiology studies showed that *p5cS* has a key role in proline accumulation under osmotic stress [11, 16].

Our knowledge on boron toxicity in cereal is very limited and needs more studies to understand the physiological response to boron toxicity/tolerance. Hence, in the present study we investigated the effect of (i) boron toxicity on seed germination and root growth, (ii) the impact of boron on the antioxidant defence mechanism and lipid peroxidation and (iii) proline accumulation and the gene expression of *p5cS* in *T. aestium* AK-702 plants.

## MATERIALS AND METHODS

**Plant material and experimental design.** In this study seeds belonging to one variety of *Triticum aestivum* L. (Poaceae) AK-702 provided by the Transitional Zone Agricultural Research Institute in Eskişehir were used. The seeds were germinated in seedbeds formed by Petri dishes containing a double layer of filter paper and were exposed to a 16 h light/8 h dark photoperiod, 25 ± 1°C, repetitions of three (100 seeds per dish × 4), control group consisting of distilled water and six different $HBO_3$ concentrations (10, 50, 70, 140 and 200 ppm). When the radicle touched the seed bed it was regarded as a germinated seed. The germination experiments were observed daily for two months, and germinated seeds were removed from the seed bed. The experiments took 15 d and the number of germinated seeds was recorded every day. After root and stem development for 15 d, the root and lengths were measured by a digital caliper, and the root weights were measured by analytical balance.

**Measurement of lipid peroxidation.** Formation of malondialdehyde (MDA) was evaluated as an indicator of lipid peroxidation. Thiobarbituric acid reactive substances (TBARS) method was used in the determination of MDA content [17]. The absorbance difference between 532 and 600 nm was used to calculate MDA formation as a product of lipid peroxidation. Each treatment comprised three replicates.

**Proline analysis.** Proline content was measured following the method of Bates et al. [18] with modification of the extraction procedure. 100 mg of homogenized wheat plant tissue was added to 10 mL of 3% (v/v) sulfosalicylic acid and the homogenate was kept under laboratory conditions for 24 h. The homogenates were filtered through six layers of cheesecloth. Two milliliters of the extracts containing proline were pipetted and subsequently treated with 2 mL of acid-ninhydrin at 90°C for 1 h. The reaction was then terminated in an ice bath and the colored complex was extracted in toluene. Absorbance was recorded at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range 0.5–10 μg/mL. Absorbance values were converted to absolute amounts using a standard curve and statistical tests were applied to the converted data.

**Antioxidant enzyme analysis.** 100 mg of homogenized wheat tissue was added to the buffer (50 mM phosphate buffer, pH 7.5 containing 1 mM EDTA, 2% TritonX-100, 2% polyvinylpyrrolidone). The homogenate was centrifuged at 12000 *g* at 4°C for 20 min and then used as an enzyme extract. The total protein contents of the enzyme extracts were analyzed according to Bradford [19] using bovine serum albumin as the standard. The specific enzyme activities were calculated and expressed as mg/protein.

The activity of CAT (E.C.1.11.1.6) was assayed in a mixture containing 100 mM phosphate buffer, pH 7.8, 6 mM $H_2O_2$ and the extract. The decomposition of $H_2O_2$ was monitored at 240 nm. CAT activity was calculated using an extinction coefficient of 39.4 M/cm [20]. The activity of APX (E.C. 1.11.1.11) was determined by monitoring the decrease in A 290 using an extinction coefficient of 2.8 mM/cm [21]. Each treatment was comprised of three replicates.

GR activity was analysed according to the protocol of the Glutathione Reductase Assay Kit (Cayman Chemical, United States). Each treatment comprised of three replicates.

**Isolation of RNA and RT-PCR analysis.** For RNA extractions, samples were ground to a fine powder in liquid nitrogen with a mortar. 1 mL of TRIZOL reagent was added and was incubated for 5 min at room temperature. Then, the samples were homogenized. 0.2 mL of chloroform was added for each 1 mL of TRIZOL reagent. The samples were vortexed vigorously for 15 s and incubated at room temperature for 3 min. The samples were centrifuged at 12000 *g* for 15 min at 4°C. Then 0.5 mL of isopropanol was added for each 1 mL of TRIZOL used, then mixed gently by inverting the sample for five times and incubated at room temperature for 5 min. The samples were then centrifuged at 10000 *g* for 10 min at 4°C. The RNA pellet was washed once with 75% ethanol by adding at least 1 mL of 75% ethanol per 1 mL of TRIZOL reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuged at 7500 *g* for 5 min at 8°C. The washing procedure was repeated once, and any leftover ethanol was removed. The RNA pellet was air-dried for 5–10 minutes. Partially dissolved RNA samples have an $A_{260}/A_{280}$ ratio < 1.8. Reverse Transcriptase-PCR was performed using a High Capacity cDNA Reverse Transcription Kit (Invitrogen, United States).

**Semi-quantitative RT-PCR.** To determine the total RNA isolates, a routine PCR protocol was applied to the cDNAs. The following primer sequences were used for the *p5cS* sense (GGATTCATCTGGTATATTCTGG) and antisense (GTCTCTGCTACTTTATCATCTG) orientations. The oligonucleotide primers were designed from *T. aestivum p5cS* sequence (NCBI, GenBank: AF022914.1) obtained using the 'Primer3' software. GAPDH primers were used as an internal control. The basic protocol for the PCR was carried out using 1 μL of cDNA, 2.5 μL of 10X PCR buffer without $Mg^{2+}$, 0.65 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTP mix, 0.5 μL of each 20 μM primers and 0.25 μL of *Taq* DNA polymerase, recombinant (Thermo scientific, United States). The thermal cycle was started at 95°C for 5 min to denature the double-strand DNA. PCR cycle numbers were chosen to ensure that the amplification of the PCR products was in the exponential range: 36 cycles of denaturation at 95°C for 1 min, annealing at 51°C for 1.15 min and elongation at 72°C for 1.15 min. Amplification was ended at 72°C for 10 min and products were stored at –20°C. Aliquots of PCR products (~10 μL) were subjected to electrophoresis through 2% agarose gels containing 0.5 μg/mL of ethidium bromide. For quantification, the density of the PCR bands was estimated with the Gel Logic 212 Pro analyze system (Carestream, United States).

**Table 1.** Germination percentage (%) of AK-702 wheat variety after 10 d treatment with different concentrations of boron

| Concentration of B, ppm | Seed germination, % |
|---|---|
| Control | 98 |
| 10 | 93 |
| 50 | 93 |
| 70 | 91 |
| 140 | 89 |
| 200 | 87 |

**Statistical analysis.** Statistical analysis was performed with a one-way analysis of variance (ANOVA) and Student's t-test followed by a *posthoc* Tukey test, as appropriate (SPSS for Windows v. 11.0).

## RESULTS AND DISCUSSION

In the present study, the seed germination rate decreased significantly with an increasing concentration of boron (Table 1), which was similar to *Carthamus tinctorius* under 10 and 50 ppm boron treatments [22]. The root lengths of the seedlings were significantly decreased under 10 ppm boron application (Table 2). The maximum root length was observed in the control group. In addition, the wet root weights and dry root weights decreased within increased boron concentrations (Table 2).

The decomposition product of the polyunsatured fatty acids, MDA, is produced naturally as a result of lipid peroxidation and it is often used as an indicator of oxidative damage at the cellular level [7]. Turton et al. [23] suggested that the presence of MDA in biological systems can be related to the peroxidation of unsaturated fatty acids constituting the cellular membranes. The consequences of the changes in lipid and protein structure are the loss of membrane integrity and selec-

**Table 2.** Root lengths (cm), root fresh weighs (g) and root dry weighs (g) of AK-702 wheat variety after 10 d treatment with different concentrations of boron

| Concentration of B, ppm | Root length, cm | Root fresh weight, g | Root dry weight, g |
|---|---|---|---|
| Control | 18.4 ± 3.57 | 0.70 ± 0.13 | 0.072 ± 0.02 |
| 10 | 17.1 ± 1.37 | 0.69 ± 0.19 | 0.069 ± 0.01 |
| 50 | 16.7 ± 0.93 | 0.63 ± 0.23 | 0.067 ± 0.05 |
| 70 | 14.2 ± 0.82 | 0.55 ± 0.05 | 0.066 ± 0.41 |
| 140 | 13.2 ± 0.97 | 0.49 ± 0.05 | 0.066 ± 0.06 |
| 200 | 10.7 ± 0.38 | 0.44 ± 0.08 | 0.062 ± 0.05 |
| *P* | 0.001 | NS | NS |
| *F probably* | 8.604 | | |

tive permeability [7, 23]. Destructive effects of boron on the structure of cell membranes and membrane associated reactions have been previously shown [4, 24]. In the present study, MDA production levels between boron-treated and the control plants were significantly different, as seen in Fig. 2 ($P < 0.05$). Boron exposure of 140 and 200 ppm significantly increased the MDA content in the roots ($P < 0.01$). Similarly, Eraslan et al. [3] demonstrated that boron toxicity caused an increase in the membrane permeability in tomato and pepper plants. Previous studies also reported that high concentrations of boron induced both MDA and $H_2O_2$ production in plants [9, 10, 25]. Moreover, Karabal et al. [4] showed that boron toxicity induced lipid peroxidation in barley leaves, but there were no significantly changes in the roots. These effects of boron on the lipid peroxidation level in the roots could be different depending on the variety of the plant species.

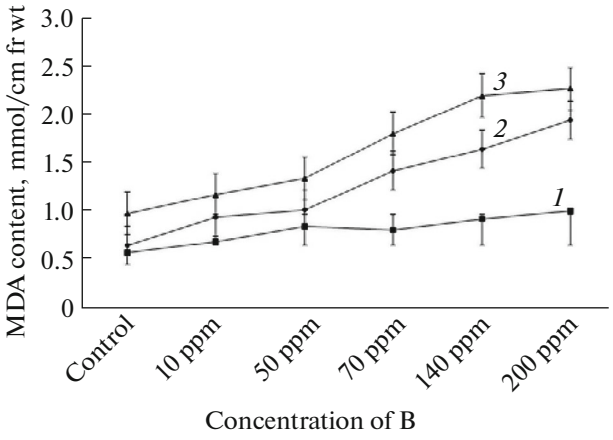


**Fig. 2.** Effects of different concentrations of boron on MDA content in *T. aestium* roots 3 days (*1*), 7 days (*2*) and 10 days (*3*).

The antioxidant defence mechanism, which includes enzymatic and non-enzymatic antioxidants, is important in response to ROS toxicity. In plants, CAT and APX have important roles in the regulation of $H_2O_2$ intracellular levels and also provide protection to cells against oxidative damage [26]. SOD is also a very important antioxidant enzyme for the dismutation of $O_2^{\bullet-}$ into $H_2O_2$ and oxygen [7]. In a previous study, an excess of boron caused ROS formation and induced oxidative damage [9], and it was mentioned that SOD and CAT had more important roles than APX in protecting cells against boron toxicity [10, 25]. APX activity in the roots of *T. aestium* increased approximately 2.8 and 2.3-fold under 10 and 50 ppm of boron applications, respectively, when compared to the control plants (Fig. 3). However, APX activity did not change significantly under 70, 140 and 200 ppm boron treatments when compared to the control plants (Fig. 3a). Similarly, Karabal et al. [4] showed that APX activity was not significantly changed in response to boron toxicity. On the other hand, the highest GR activity was found under 50 and 70 ppm boron treatments. However, GR activity decreased approximately 1.2-fold under 200 ppm of boron application when compared to the control plants (Fig. 3b). Similarly, previous studies reported that GR activity decreased in plants treated with a high concentration of boron [4]. Moreover, CAT activity was not significantly changed under any of the boron applications up to 200 ppm, and the highest level of CAT activity was found under 200 ppm of boron application (Fig. 3c). We are in agreement with previous studies [4, 9, 10] with an

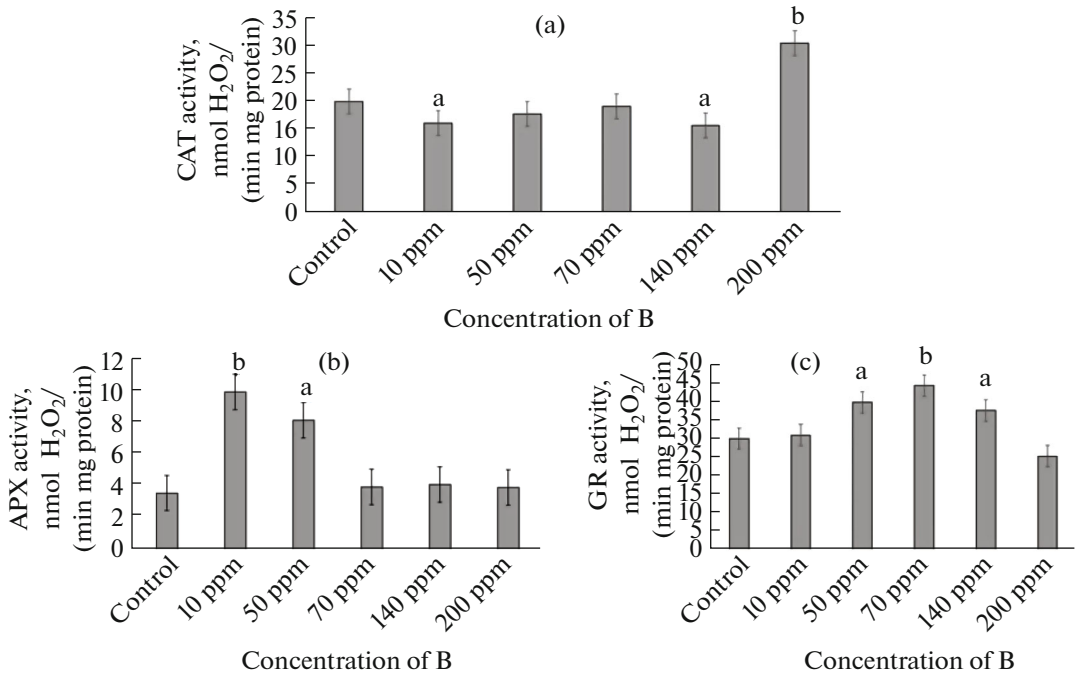


**Fig. 3.** Effects of different concentrations of boron on antioxidant enzymes CAT (a), APX (b) and GR (c) activity in *T. aestium* roots. Bars marked with (a) represent a statistically significant difference at $P < 0.05$ and with (b) represent a statistically significant difference at $P < 0.01$ when compared with the control.

increase in CAT activity under high concentration of boron treatments.

Proline content increased during salt and drought stress due to osmotic adjustment and to protect the plant cells from oxidative damage [14, 27]. In contrast, many researchers have suggested that proline accumulation is a symptom of injury that does not confer tolerance against metal or other stresses [12, 28, 29]. Similarly, under boron toxicity, an increased proline level to response to the toxicity depending on an enhanced $H_2O_2$ level from the boron treatments [4]. In the present study, the proline accumulation could be a consequence of the stress-induced damage to the roots (Fig. 4), because our proline results are in parallel with lipid peroxidation levels (Fig. 2).

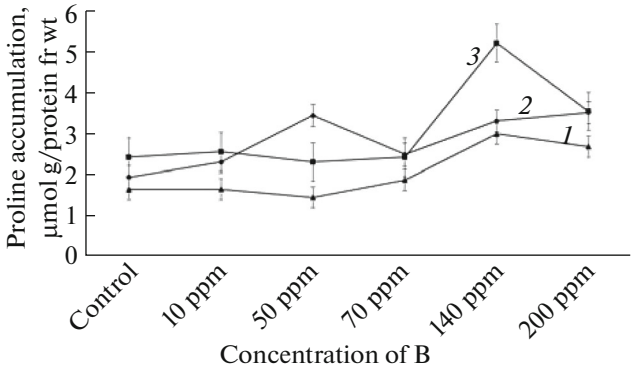


**Fig. 4.** Effects of different concentrations of boron on proline content in *T. aestium* roots 3 days (*1*), 7 days (*2*) and 10 days (*3*).

Yoshiba et al. [13] demonstrated that *p5cS* plays a central role in the accumulation of proline under osmotic stress. Hu et al. [15] showed that the *p5cS* gene transcription level was strongly enhanced by salt stress in the roots. Hmida-Sayari et al. [30] also reported the overexpression of the *p5cS* enzyme elevated proline content in transgenic tobacco plants. In the present study, our results indicated for the first time that the *p5cS* gene expression was up-regulated in response to boron toxicity. When compared with the control, the *p5cS* mRNA transcript level was increased 2.52 and 2.23-fold under the 140 and 200 ppm boron applications (Fig. 5). In addition, the comparison of the level of the proline accumulation and *p5cS* mRNA transcript level showed a positive correlation (Fig. 6).

In conclusion, the present study demonstrated that high concentration of boron could cause oxidative stress in *T. aestivum* roots. The lipid peroxidation rate increased depending on the boron concentration and the application time. Antioxidant enzyme activities (APX, CAT and GR) significantly altered during the boron stress in *T. aestivum* roots. This work also suggested that proline accumulation induced by boron stress could be related to the degree of injury caused in the roots by oxidative damage. The stress enhanced expression of the *p5cS* gene resulted in an increased proline accumulation.

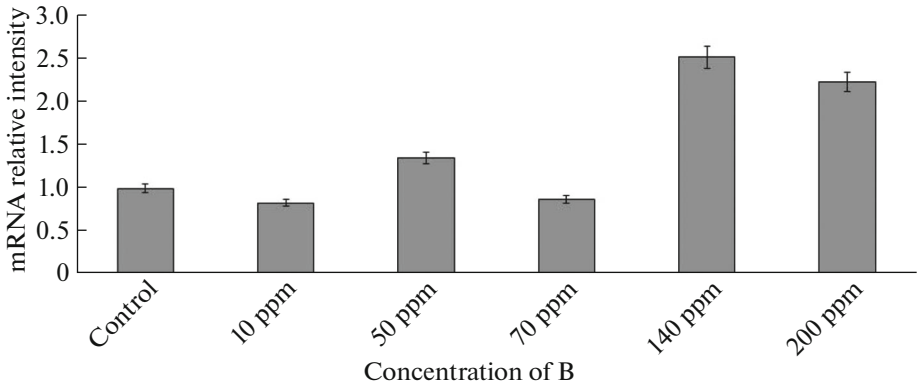


**Fig. 5.** Effects of different concentrations of boron on *p5cS* gene mRNA relative intensity in *T. aestium* roots.

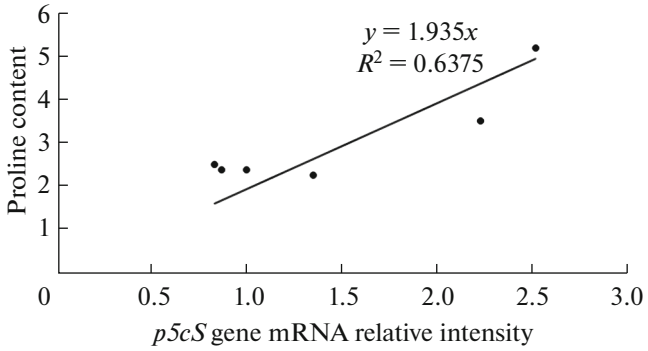


**Fig. 6.** Correlation between proline content and *p5cS* gene mRNA relative intensity.

## ACKNOWLEDGMENTS

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