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Influence of boron treatments on fatty acid desaturase metabolism in different safflower cultivars

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Abstract: This study investigated the expression levels of *FAD2* genes important for the conversion of oleic acid to linoleic acid at cotyledon, root and leaf tissues of four different safflower cultivars (Olas, Remzibey, Dincer and Gokturk) subjected to B by qRT-PCR. Safflower species were grown in a controlled environmental growth chamber for 21 days and then exposed to different B concentrations ranging from 20 to 1 280 µmol for 24 h. RNA isolation, cDNA synthesis and RT-PCR analysis were performed on root, cotyledon and leaf tissues exposed to B stress for 24 h. It was determined that the expression levels of *FAD2* genes decreased in the safflower cultivars exposed to increasing B concentrations. Under B stress conditions, the expression levels of *FAD2* genes revealed an overall pattern of increase and reduction up to 160 µmol relative to the control group, and they reached the highest expression level. After 320 µmol, the activity of *FAD2* genes was almost absent at increasing concentrations. All results show that the application of B causes significant changes in the expression of *FAD2* genes and plays an important role in the defence mechanism against increased B toxicity.

Keywords: *Carthamus tinctorius* L.; boron stress; arid climate; biosynthesis; genotoxic effect

Safflower (*Carthamus tinctorius* L.) is an important commercial oil plant and one of the cultured members of the Asteraceae family. There are about 25 wild species of safflower distributed around the world. Safflower is a broad-leaved annual herb which is resistant to arid climates with its thorny and thornless forms (Babaoglu 2007) (Figure 1). The primary purpose is to use the medicinal ingredient as a food and fabric dye using flowers. It has recently been used as an oil plant due to its high oil content, and many cultivars have grown. Safflower seeds contain about 30–50% quality oil. The researchers showed that the quality of safflower oil is much higher than different oil crops such as soybean, sunflower and corn (Day et al. 2017). Also, safflower is a popular plant in many sectors of the industry, such as paint, varnish, feed, cosmetics and margarine. Additionally, as an alternative to the use of petroleum, biodiesel fuel obtained from vegetable, animal and various organic waste oils is expected to be the fuel of the future, which is becoming increasingly popular with the developing technology (Sahin and Tasligil 2016). Due to these aforementioned features, safflower cultivation has become quite widespread today, and different applications are being developed. With the intensive development of agricultural practices, the importance of some elements is increasing. One of these is boron (B) (Hua et al. 2021).

B element abundant in soil, rocks and water on earth is mostly found in Turkey and the USA with its oxygen compounds and it is found in high concentrations in regions rich in arid and volcanic land, although it

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Figure 1. General view of safflower plant, flower, field and seed

has hydrothermal activity. B is a micronutrient that must be taken in small amounts throughout life for the physiological functions of organisms and is an important element which has been used extensively in industry, agriculture and consumer markets (Yau and Ryan 2008, Hua et al. 2021).

Nowadays, B pollution arises as a result of the discharge of industrial wastes using it in different ways into lakes, rivers and streams or irrigation with common thermal waters in terms of B or the discharge of these waters into rivers. B toxicity is an important factor limiting plant growth in arid and semi-arid regions. Generally, in B toxicity, the leaf tips of old leaves turn yellow, and necrosis occurs. In addition, it is observed that B toxicity causes the formation of reactive oxygen species (ROS) and causes DNA damage/changes (Tombuloglu et al. 2012).

In higher plants, polyunsaturated fatty acids are transformed in the chloroplast and endoplasmic reticulum by a group of fatty acid desaturase enzymes. Fatty acid desaturases are enzymes that convert a single bond (C-C) to a double bond (C=C) between two carbon atoms in a fatty acid chain. The resulting double bond is generally referred to as unsaturated and desaturase enzymes catalyse this reaction. FAD2 enzymes, encoded by the *FAD2* gene, are one of the fatty acid desaturases involved in the biosynthesis pathway of polyunsaturated fatty acids. All this information includes Cao et al. (2013) isolated eleven different *FAD2* genes belonging to the *FAD2* gene family coded at different levels in different organs of the safflower plant. The expression of these *FAD2* genes in different organs of the safflower plant is given in Figure 2 (Cao et al. 2013).

Although many studies have been carried out on the functions of B in plants, its molecular biological effects in the plant are not fully understood, especially in the safflower. In this study, in experimental

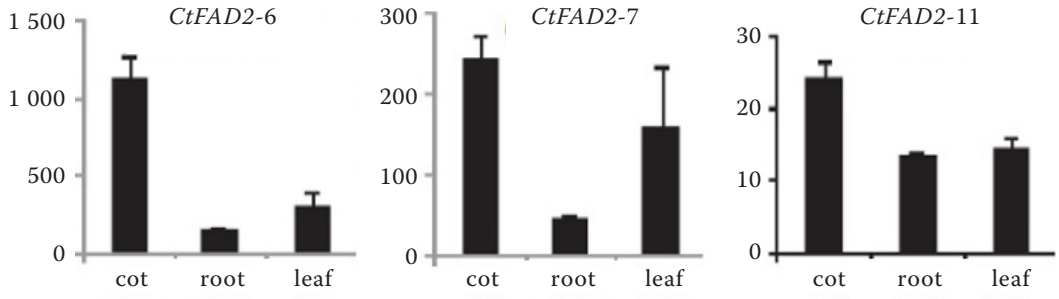

Figure 2. Comparative expression levels of the *FAD2* genes were evaluated in different tissues of the safflower cultivars in the study (Cao et al. 2013). *Ct – Carthamus tinctorius*; cot – cotyledon

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groups prepared at different B concentrations, the possible positive and negative/genotoxic effects of B on the mRNA expression levels of the *FAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes, which is responsible for the conversion of oleic acid to linoleic acid in different safflower cultivars (Olas, Remzibey, Dincer and Gokturk) were determined by the qRT-PCR method.

MATERIAL AND METHODS

Plant materials and growth conditions. Safflower cultivars used in this study are nationally registered cultivars (Olas, Remzibey, Dincer and Gokturk) origin and were obtained from the "Bahri Dagdas International Agricultural Research Institute" and "Transitional Zone Agricultural Research Institute" in Turkey. The seeds of all safflower cultivars were germinated, following the surface sterilisation in a solution containing 5% (*v*/*v*) hypochlorite for 5 min, and were grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland's solution, according to Bolukbasi and Aras (2022). Safflower seedlings were incubated in a controlled environmental growth chamber in the light with 250 mmol/m^2/s photosynthetic photon flux at 25 °C, 70% relative humidity for 21 days. Within a 24-h period, 16 h (25 °C, 70% humidity) day and 8 h (22 °C, 60% humidity) night cycles were applied. After growing for 21 days, the seedlings were exposed to 20, 40, 80, 160, 320, 640, and 1 280 µmol boric acid (H_3BO_3) for 24 h. 1X Hoagland solution, which does not contain any B, was used as the control group. At the end of 24 h, the seedlings taken from B stress were washed with distilled water and sampling was done. Sampling was carried out from 3 different tissues; root, cotyledon, leaf, and the samples were treated with liquid nitrogen and then stored in the –80 °C freezer until the RNA isolation stage.

RNA extraction, complementary DNA (cDNA) synthesis assay. Total RNA extraction of root, cotyledon and leaf samples taken from different safflower cultivars exposed to B stress for 24 h was performed according to Trizol (TRIGent) reagent according to suggested procedures by the manufacturer. Afterwards, the amount and purity of RNA were determined using the Nanodrop ND-Spectrometer 1000 device (NanoDrop Technologies, Wilmington, USA) and 1.5% agarose gel electrophoresis. Next, cDNA synthesis was performed using the ProtoScript-II First Strand cDNA Synthesis Kit (BioLabs Inc., Ankara, Turkey). Anchored-oligo(dT)18 primer was used because of the long *FAD2* and *actin* (*ACT*) gene regions.

The qRT-PCR analyses of *FAD2* genes. The primers of *ACT* as a housekeeping gene and *FAD2* genes used in this study were designed using the sequences of the safflower plant in the gene bank (NCBI; National Center for Biotechnology Information). For the design of the primers used in the study, information on fatty acids desaturase-related genes (*FAD2*) was obtained from the gene bank (NCBI). Information about these genes and the most suitable primer sequences that were designed are given in Table 1.

For quantification, analysis of *FAD2* and *ACT* genes was carried out using SYBR Green I Master dye by Light Cycler Nano (Roche) device following cDNA synthesis in samples taken from root, cotyledon and leaf tissues of safflower cultivars exposed to B stress at different concentrations. PCR conditions consisted of initial denaturation for 10 min at 95 °C (40 cycles), 95 °C for 15 s, 60 °C for 20 s, 72 °C for 20 s, and a melting analysis of 52 to 95 °C with an

Table 1. Information *FAD2* genes in the National Center for Biotechnology Information (NCBI) database and sequences and melting temperatures of primers used in qRT-PCR

Genes/primers name	Length (bp)	Gene bank number	Sequence (5'–3')	Temperature of melting (°C)
FAD2-11	1 213	KC257457.1	F: ACGCCTTATTTCGCCTGGAA R: TCGCGATCTTGGACTTACGT	58–60
FAD2-7	1 210	KC257453.1	F: CGCAAACCATTTCCTACCGC R: CGTCGATTTCAGGCCTTGGA	58–60
FAD2-6	1 148	KC257452.1	F: ACCAATGCAGTCAAGCCCAT R: TCTGCACCTTCATCTGGCTC	58–60
Actin	1 678	KJ634809.1	F: GGCGTGACCTTACAGATTC R: CAAGCTCTTGCTCGTAGTC	58–60

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increasing temperature of 0.5 °C/min. The qRT-PCR analysis contained three biological replicates, consisting of three technical replicates using the obtained optimal conditions.

Normalisation and statistical analysis of qPCR results. *FAD2*-6, *FAD2*-7 and *FAD2*-11 genes were chosen because they are transcribed in three tissues (root, cotyledon and leaf) of all safflower cultivars (Figure 2). qRT-PCR reactions of *FAD2* and *ACT* genes in samples taken from root, cotyledon and leaf tissues of safflower exposed to B stress at different concentrations were monitored simultaneously, and their peak profiles were recorded. Gene expression results determined as Ct (cycle threshold) value, *ACT* and control conditions used in the study were normalised by considering housekeeping gene. The mRNA levels of the synthesised gene products were determined quantitatively by these obtained Ct values and melting curve analysis (Kubista et al. 2006). The obtained data were normalised according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001) (Table 2). The mean, standard deviation, standard error and statistical significance of these data were calculated with the statistical program SPSS 25.0 for Windows (IBM SPSS, Inc., Chicago, USA). ANOVA, Tukey and Dunnett multiple comparison tests were performed to reveal the differences between the groups. The homogeneity of the variances was determined by the Levene test. For this purpose, post hoc Tukey *HSD* (honestly significant difference) and Dunnett tests were applied to the variables with a homogeneous distribution of variances (to confirm the results), and Dunnett's T3 test was applied to the variables that did not show homogeneous distribution (Dunnett 1955). $P < 0.05$ was considered to be statistically significant.

Table 2. The mean values of expression data of normalised *FAD2* genes (in three technical repetitions) of different tissue samples of cultivars under boron (B) treatments

	B (µmol)	cv. Olas			cv. Dincer			cv. Remzibey			cv. Gokturk		
		FAD211	*FAD27*	*FAD26*	*FAD211*	*FAD27*	*FAD26*	*FAD211*	*FAD27*	*FAD26*	*FAD211*	*FAD27*	*FAD26*
Cotyledon	C	1	1	1	1	1	1	1	1	1	1	1	1
	20	25.25	21.86	1.07	8.01	19.25	15.77	3.50	4.85	4.86	11.12	9.70	8.51
	40	17.76	26.61	9.72	17.30	19.61	18.81	4.66	3.15	4.14	9.59	5.63	4.28
	80	8.91	3.50	2.52	14.89	13.81	15.37	2.15	2.55	2.00	3.08	1.53	2.21
	160	18.84	12.13	2.24	9.75	13.39	12.03	4.89	3.37	3.17	4.94	4.05	5.90
	320	8.02	5.42	9.81	3.90	7.84	5.92	2.48	0.77	1.21	1.00	0.89	1.62
	640	3.55	5.01	1.37	3.58	3.39	1.39	0.66	0.31	0.79	0.54	0.70	1.19
	1 280	1.58	2.32	0.78	2.43	1.86	0.98	0.47	0.10	0.37	0.49	0.37	0.99
Root	C	1	1	1	1	1	1	1	1	1	1	1	1
	20	13.64	17.66	18.59	11.90	9.93	14.50	11.16	13.76	9.87	11.54	14.57	10.25
	40	10.22	7.56	15.69	4.81	12.67	10.65	3.02	7.01	3.98	9.45	5.90	3.43
	80	9.20	13.10	12.65	4.40	4.11	5.34	1.69	1.07	1.61	2.99	2.64	2.50
	160	8.60	11.48	3.08	12.08	5.69	10.07	1.07	0.81	2.56	6.74	4.16	7.70
	320	6.01	6.27	3.86	2.86	0.81	5.74	0.33	1.57	0.03	1.33	0.62	1.89
	640	2.82	2.22	2.08	1.78	0.72	2.09	0.06	0.04	0.00	1.00	0.85	1.23
	1 280	1.88	1.32	0.25	1.51	0.46	0.90	0.50	0.27	0.00	0.63	1.17	0.94
Leaf	C	1	1	1	1	1	1	1	1	1	1	1	1
	20	15.65	10.22	2.40	14.44	13.48	10.37	16.39	14.92	17.91	4.46	3.85	3.12
	40	9.53	3.26	9.79	9.38	2.64	3.28	19.91	12.34	8.53	2.33	3.19	2.72
	80	7.25	8.64	14.34	2.48	3.17	1.20	11.10	5.44	14.35	3.57	2.01	1.56
	160	14.36	4.26	0.26	6.48	5.38	2.45	7.25	11.90	2.45	0.63	1.26	2.37
	320	2.68	3.30	0.84	1.03	1.51	1.25	0.55	2.43	2.87	0.28	0.21	0.35
	640	2.32	1.73	1.22	1.05	1.73	0.94	3.18	2.15	3.42	0.49	0.06	0.12
	1 280	0.24	0.09	0.10	0.42	0.15	0.43	0.90	0.92	0.15	0.17	0.07	0.23

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RESULTS AND DISCUSSION

The B level in micronutrients necessary for plant life has been reported to be approximately 30 µmol. Above 400–500 µmol is considered toxic to all plants (Miwa et al. 2007). Therefore, in this study, the B concentration range started at 20 µmol and was increased to 1 280 µmol, which is a toxic level. In this study, seedlings grown for 21 days were then exposed to B stress and prepared at different concentrations in the hydroponic medium for 24 h. Many studies have reported that exposure to stress for 24 h is sufficient (Goupil et al. 2009).

In addition, some studies have reported that the expression profiles of various genes are tissue-specific (Xue et al. 2017, Hua et al. 2021). Consequently, in this study, it was determined that *FAD2* genes had different expression levels in the root, cotyledon and leaf tissues of 4 different safflower cultivars exposed to B stress.

According to the results of normalised gene expression data, the changes in the expression level of *FAD2* genes in different tissues according to the B concentrations for each safflower cultivar were shown on separate graphs. The data obtained from this study were evaluated as a whole, and it was determined that the expression levels of *FAD2* genes increased at low concentrations in safflower cultivars subjected to B stress (especially in 20 µmol and 40 µmol in all cultivars and tissues). For example, the highest scores at 20 µmol (4.5-3.9-3.2-fold increase) for *FAD2*-11, *FAD2*-7 and *FAD2*-6 in leaf tissue of cv. Gokturk, respectively ($P < 0.05$) (Figure 3). The highest scores at 40 µmol (17.3-19.6-18.8-fold increase) for *FAD2*-11, *FAD2*-7 and *FAD2*-6 in cotyledon tissue of cv. Dincer, respectively ($P < 0.05$) (Figure 4). Another example is the highest scores at 20 µmol (13.64-17.66-18.59-fold increase) for *FAD2*-11, *FAD2*-7 and *FAD2*-6 in the root tissue of cv. Olas, respectively ($P < 0.05$) (Figure 5). It is thought that these results, similar to the results of the studies in the literature, are due to the use of B as a micronutrient by plants. Tombuloglu et al. (2012) emphasised that although boron is used as a micronutrient by plants, its toxic effect depends on time and dose.

It is known that *FAD2* genes are responsible for converting oleic acid to linoleic acid in oil crop plants (Chen et al. 2015). And also, studies have shown that *FAD2* genes play critical roles in defence against salt and cold stress and take an active role in functions such as conversion, modification and restructuring of fatty acids. In the literature, it has been stated that *FAD2* genes play a role in defence by increasing their expression levels in adverse environmental conditions (Tang et al. 2005). Plants exposed to some stress trying to cope with stress by making changes in the structure and amount of various lipids and fatty acids that participate in the lipid structure (Li et al. 2015). It has been determined that *FAD2* genes are involved in the defence mechanism against salt stress in sunflowers (Rodriguez-Vargas et al. 2007). In another study, the *FAD2* gene is active and sensi-

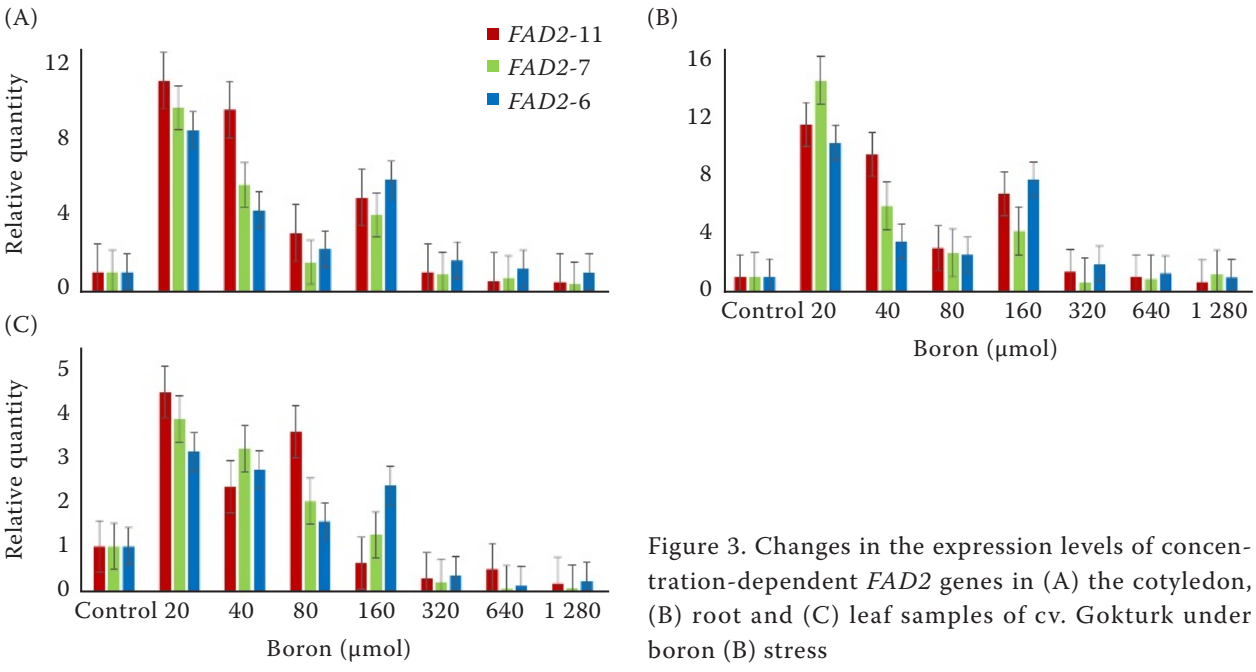

Figure 3. Changes in the expression levels of concentration-dependent *FAD2* genes in (A) the cotyledon, (B) root and (C) leaf samples of cv. Gokturk under boron (B) stress

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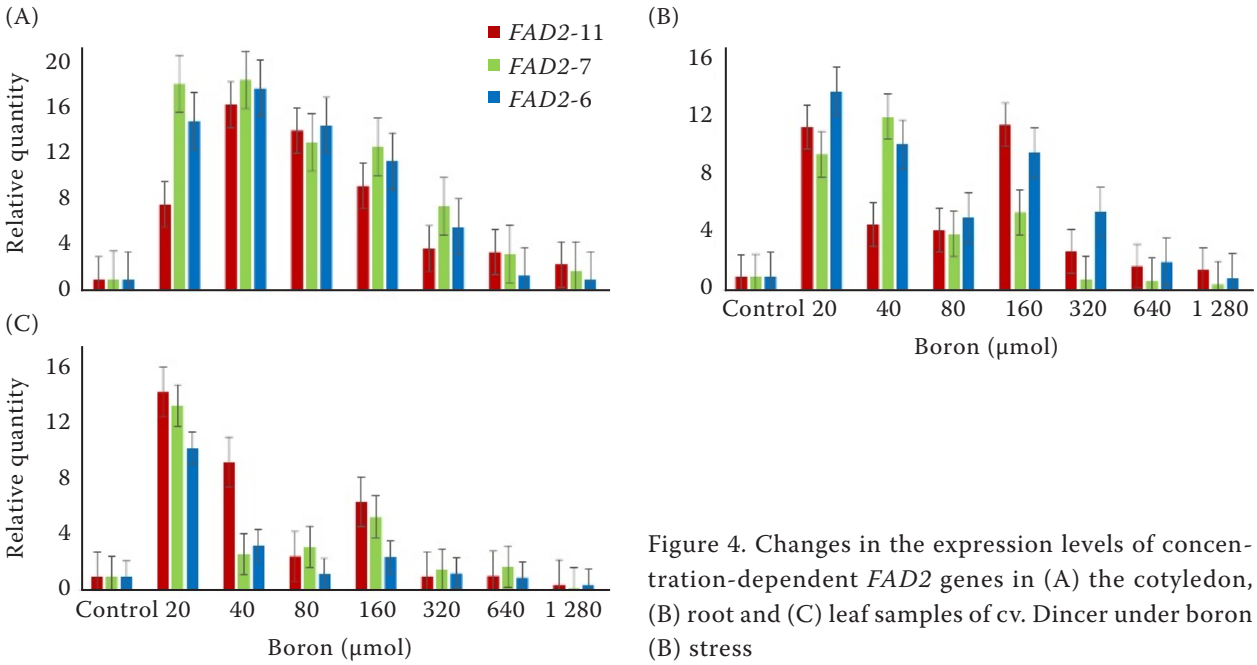

Figure 4. Changes in the expression levels of concentration-dependent *FAD2* genes in (A) the cotyledon, (B) root and (C) leaf samples of cv. Dincer under boron (B) stress

tive to stress factors such as darkness, heat and salt in *Arabidopsis* (Yuan et al. 2012). Although there are studies on other abiotic stresses related to *FAD2* genes in the literature, there is no study investigating the effects of B stress. The toxic effect of B shows its toxicity by causing the formation of reactive oxygen species, DNA damage through oxidative stress in the hereditary material and, consequently, significant changes in the expression levels (Buyuk et al. 2016, Bolukbasi 2021). In the study, we also aimed to investigate, too whether these genes can be used for the revealing of defence mechanisms against stress in safflower, especially B toxicity. The current study is the first of its kind on the subject.

The data show that the decrease in the expression of *FAD2* genes at increasing B concentrations and their re-increase after 80 µmol and 160 µmol (e.g., 4.9-3.4-3.2 fold increase) for *FAD2*-11, *FAD2*-7 and *FAD2*-6 in cotyledon tissue of cv. Remzibey, respectively ($P < 0.05$) (Figure 6). These can be considered critical points and are accepted as an indication that the defence mechanism against the stress is activated and that

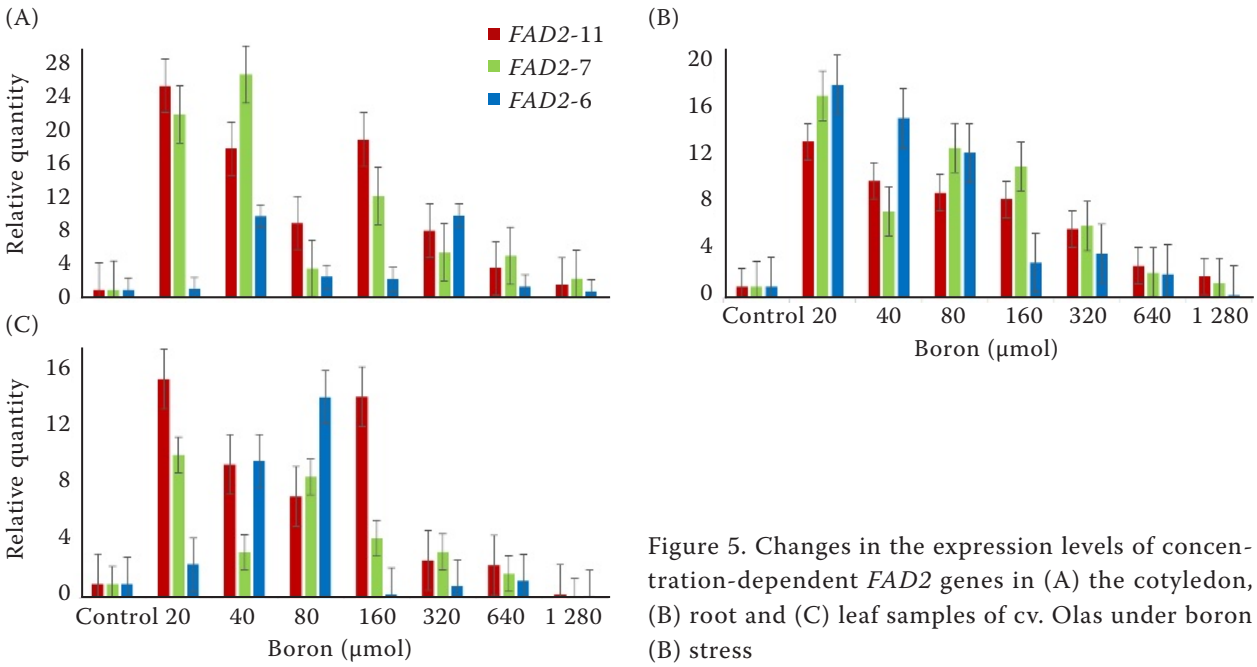

Figure 5. Changes in the expression levels of concentration-dependent *FAD2* genes in (A) the cotyledon, (B) root and (C) leaf samples of cv. Olas under boron (B) stress

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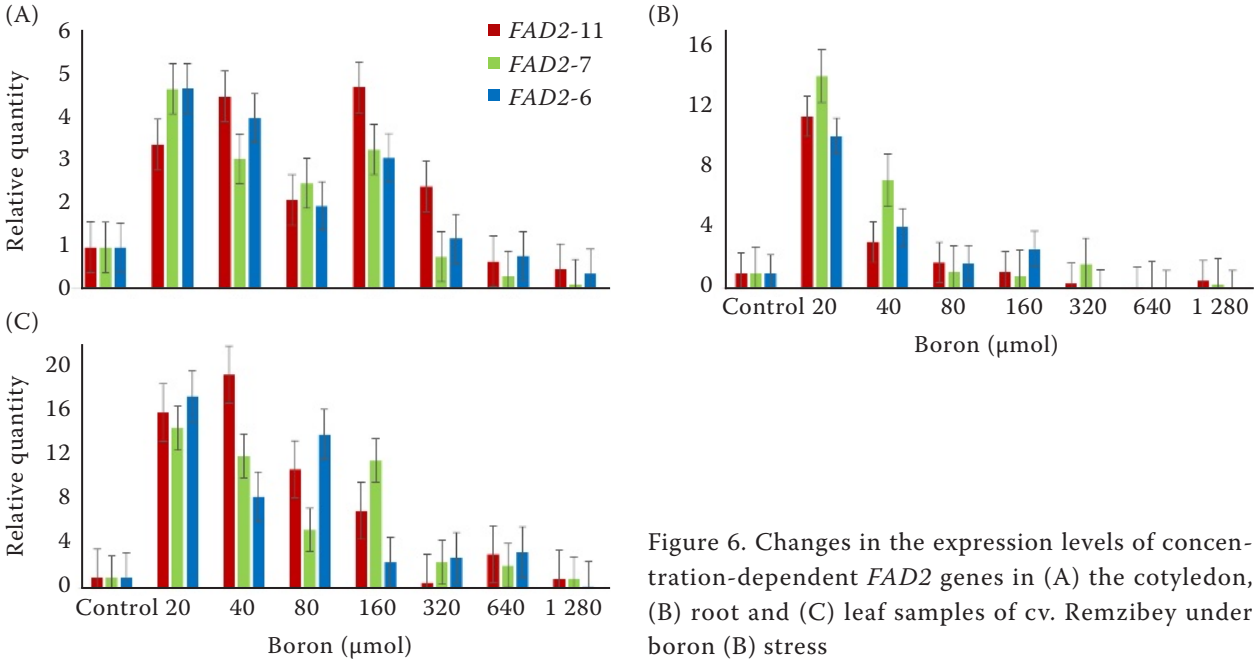

Figure 6. Changes in the expression levels of concentration-dependent *FAD2* genes in (A) the cotyledon, (B) root and (C) leaf samples of cv. Remzibey under boron (B) stress

FAD2 genes play a role in the defence against B stress. At 320, 640 and 1 280 µmol, the expression level of *FAD2* genes decreased again, as in B stress. This shows that the stress response against B is insufficient, too.

In this study, the increase in the expression levels of the *FAD2* genes is thought to increase the number of fatty acids against B stress. Tombuloglu et al. (2012) examined the expression of some stress-related genes due to B stress in tomato seedlings. While gene expression increased due to increasing concentration, a decrease was observed after specific concentrations.

The results of this study show that, according to their expression levels in tissues, *FAD2*-11, *FAD2*-7 and *FAD2*-6 genes are involved in the defence mechanism against stress in safflower cultivars and can be conveniently used in various stress-related studies. The cv. Olas has better performance than the other safflower cultivars against B toxicity. As it was expected, all *FAD2* genes revealed higher mRNA levels in cv. Olas compared to the cvs. Gokturk, Remzibey and Dincer. This result showed that cv. Olas was more resistant to B stress.

All results support each other with the literature studies mentioned above. Considering that the stress caused by B triggers similar mechanisms with other abiotic or biotic stress factors, the upward change in the expression levels of *FAD2* genes against the stress of B in the safflower shows parallelism with the studies mentioned.

As a result of this study, changes in the expression of *FAD2* genes were determined in the presence of B stress. In this way, data that will contribute to the revealing of defence mechanisms against stress have been obtained. *FAD2* genes improve plant tolerance against adverse conditions by regulating fatty acid mechanisms in membrane lipids. The regulation of *FAD2* genes is important in understanding plant growth and the response to different abiotic stresses. It has been shown that *FAD2* genes play critical role in defence against B stress in safflower cultivars. Therefore the effects of B application on the expression of *FAD2* genes responsible for the conversion of oleic acid to linoleic acid in the safflower plant, whose importance is increasing day by day, as well as the soil characteristics/content and requirements where safflower cultivation is made, were also determined in terms of molecular biology. Especially the absence of a study similar to this study in the literature will lead to both molecular biological and more efficient field studies. Hopefully, this study will be a pioneer for many studies.

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