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Detection of Cadmium Genotoxicity on *Fatty Acids Desaturase-2* Genes in Safflower (*Carthamus tinctorius* L.)

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Abstract

Environmental pollution negatively affects the life activities of the living things of the environment and causes structural damages on the all living and inanimate things. The extent of the damage caused by human-made pollution to nature and the environment is increasing day by day. Heavy metal pollution is one of the most important causes of environmental pollution. Cadmium, which is one of the most important pollutants, is a highly toxic metal and is not used by living things, even in trace amounts. Cadmium is also a very toxic heavy metal for plants. As with other heavy metals, it triggers oxidative stress by increasing the production of reactive oxygen species in plant cells, causing DNA damage and abnormalities in DNA and RNA production. Safflower is a very important oil plant with high economic value and intensive use as a raw material in many sectors. In this study, in experimental groups prepared at different cadmium concentrations, the negative/genotoxic effects of cadmium 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 varieties (Balci, Bdyas-04, Linas and Asol) has been determined by quantitative Real-Time PCR method. As a result, it has been detected that the decrease firstly in the expression of *FAD2* genes at increasing cadmium concentrations in all cultivars. And also, re-increase at 160 and 320 mg L^{-1} which can be considered as critical points, have been accepted as an indication that the defense mechanism against stress is activated and *FAD2* genes play a role in the defense against stress. In conclusion, the obtained data showed that *FAD2* genes in safflower cultivars not only in the conversion of fatty acids but also play a critical role in defense against cadmium heavy metal stress.

Key words: Safflower, Cadmium, *FAD2* Gene, Real-Time PCR

1. Introduction

Environmental pollution is the intense mixing of foreign substances that impair their quality and properties into the air, water and soil ecosystem. It negatively affects the life activities of the living things of the environment and causes structural damages on the inanimate elements, too. The extent of the damage caused by human-made pollution to nature and the environment is increasing day by day. It has become a clearly visible fact that some developments aimed at making life more perfect and providing a healthier and longer life have deteriorated natural resources in rural and urban areas, caused water, air and soil pollution, harmed plant and animal existence and health (Cobbett and Goldsbrough, 2002; Reddy et al., 2005; Yu, 2005; Bolukbasi, 2022).

Heavy metal pollution is one of the most important causes of environmental pollution. Although many heavy metals are naturally found in the earth's crust, pollution occurs as a result of the intense accumulation of heavy metals in nature, the use of which has increased in many areas with the developing technology (Dietz, 1999; Hall, 2002).

Cadmium, which is one of the most important pollutants (e.g: Ni, Cr, Pb, Cd, Hg, Al) among these elements, is a highly toxic metal and is not used by living things, even in trace amounts, like some elements (e.g: Fe, Mn, Zn, Cu). Cadmium is one of the toxic heavy metals, which is not found pure in nature. Cadmium is an easily processable element found in nature with zinc. It is obtained besides zinc production. It is an important pollutant because the biological half-life is very long and is extremely toxic, even at very low concentrations (Greger and Bertell 1992). It is extensively used, in nickel and cadmium battery production industry, ship industry surface coating, paint industry, PVC production, electronic industry and ceramic industry in terms of industrial use. It is also used in petroleum derivatives, detergent production and especially in the production of phosphate fertilizers. The most important sources of cadmium affecting plants are water pipes, fossil fuels, various medicines for the storage of seeds, and agricultural fertilizers used during or after planting (Nzengue et al., 2011).

Cadmium is also a very toxic heavy metal for plants. It causes inhibition of seed germination. Inhibition of chlorophyll synthesis and chlorophyll-a/chlorophyll-b protein complex affect photosynthesis, carbohydrate and nitrogen metabolism and negatively affect plant growth. It also has a negative effect on respiratory and enzyme activities. As with other heavy metals, it triggers oxidative stress by increasing the production of reactive oxygen species (ROS) in plant cells, causing DNA damage and abnormalities in DNA and RNA production (Moosavi et al., 2012; Namdjoyan et al., 2012a,b; Bolukbasi, 2021).

Safflower (*Carthamus tinctorius* L.) is a member of the Asteraceae family. There are many cultivated varieties of the safflower plant, which is represented by about 25 species around the world. It is an important oil plant with economic value. Safflower seeds contain about 30-50% quality oil. The researchers showed that the quality of safflower oil much higher than different oil crops such as soybean, sunflower and corn (Davis, 1975; Singh and Ninbkar, 2006).

Oleic acid (C18:$^{1\Delta9}$) and linoleic acid (C18: $^{2\Delta9,\ 12}$) are the two main fatty acids found in safflower oil and make up about 90% of total fatty acids. Traditional safflower oil is characterized by a relatively high linoleic acid content of around 70% compared to other oilseed products (Bayrak, 1997; Babaoglu, 2007; Ahwalat, 2008) (Figure 1).

Figure 1. General view of safflower plant, flower, field and seeds (Anonymous, 2013).

Safflower plant is most popular plant in the industry. It is used in many sectors such as medicine, paint, varnish, feed and cosmetics (Babaoglu, 2007; Sahin and Tasligil, 2016). Also its oilseed is used for biodiesel production, too. It is known that about 50 different plants used in the production of biodiesel. Among them, the most important ones are sugar cane, soybean, sorghum, canola, corn and safflower (Tortopoglu, 2011; Karabas, 2013).

In safflower plants, FAD2 enzymes encoded by *FAD2* genes, 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. Phylogenetic analysis of eleven different *FAD2* genes was performed and their genomic structural features were indicated. The expression of these *FAD2* genes in different organs of the safflower plant is given in figure 2 (Cao et al., 2013).

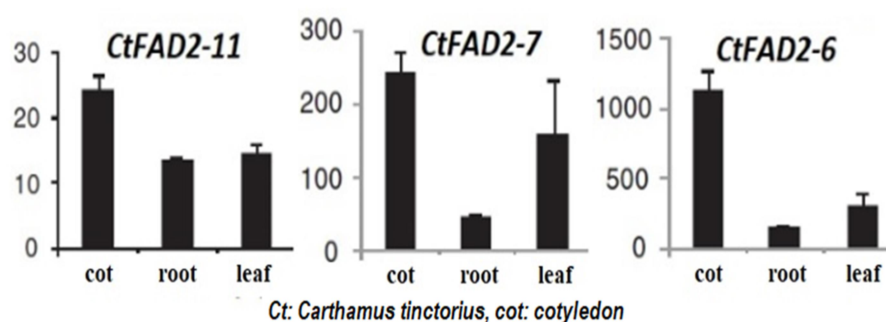

Figure 2. Comparative expression levels of the *FAD2* genes evaluated in different tissues of the safflower in the study (Cao et al., 2013).

In this current study, in experimental groups prepared at different cadmium concentrations, the negative/genetoxic effects of cadmium 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 varieties (Balcı, Bdyas-04, Linas and Asol) were determined by quantitative Real-Time PCR (qRT-PCR) method.

2. Material and Methods

2.1. Plant materials and growth conditions

Safflower varieties used in this study are nationally registered cultivars (Balcı, Bdyas-04, Linas and Asol) origin, were obtained from the "Bahri Dagdas International Agricultural Research Institute-Konya and Transitional Zone Agricultural Research Institute-Eskişehir", in Turkey. All of these selected cultivars have oil ratios ranging from 35-45%. The seeds of all safflower varieties were germinated, following the surface sterilization 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. Macro and micro nutrients were used in the preparation of Hoagland medium. Macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4$.7H2O, Ca $(NO_3)^2.4H_2O$ and KCl) and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, $ZnSO_4.7H_2O$) with a final concentration of ions as 2 mM Ca, 10^{-6} M Mn, 4 mM NO_3, 2.10^{-7}M Cu, 1 mM Mg, 10^{-8} M NH4, 2 mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-4} M Fe and 10^{-6} M B. Safflower seedlings were incubated in a controlled environmental growth chamber in the light with 250 mmol m-2s-1 photosynthetic photon flux at 25 °C, 70% relative humidity. All safflower cultivars were grown in the climatic chamber for 21 days. Within a 24-hour period, 16 hours (25 °C, 70% humidity) day and 8 hours (22 °C, 60% humidity) night cycles were applied. After growing for 21 days, the seedlings were exposed to 40, 80, 160, 320, 640 mg L^{-1} cadmium chloride ($CdCl_2$) for 24 h. 1X Hoagland solution, which does not contain any cadmium, was used as the control group. At the end of 24 hours, the seedlings taken from cadmium 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.

2.2. RNA extraction, complementary DNA (cDNA) synthesis assay

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

2.3. The qRT-PCR analyses of FAD2 genes

The primers of *Actin* (*ACT*) as housekeeping gene and *FAD2* genes used in this study were designed using the sequences of the safflower (*Carthamus tinctorius* L.) plant in the gene bank (NCBI; National Center for Biotechnology Information). *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).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 primers sequences were designed are given in Table 1.

Table 1. Information *FAD2* genes in NCBI database and sequences and melting temperatures of primers used in qRT-PCR.

Genes/ Primers name	Length	Gene Bank Number	Sequence (5'-3')	Tm (°C)
FAD2-6	1148 bp	KC257452.1	F: ACCAATGCAGTCAAGCCCAT R: TCTGCACCTTCATCTGGCTC	58-60 °C
FAD2-7	1210 bp	KC257453.1	F: CGCAAACCATTTCCTACCGC R: CGTCGATTTCAGGCCTTGGA	58-60 °C
FAD2-11	1213 bp	KC257457.1	F: ACGCCTTATTTCGCCTGGAA R: TCGCGATCTTGGACTTACGT	58-60 °C
ACTIN	1678 bp	KJ634809.1	F: GGCGTGACCTTACAGATTC R: CAAGCTCTTGCTCGTAGTC	58-60 °C

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 cadmium stress at different concentrations. PCR conditions consisted initial denaturation 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 increasing temperature 0.5 °C min^{-1}. Real-Time PCR reactions were performed in three technical repetitions using the obtained optimal conditions.

2.4. Normalization and statistical analysis of qPCR results

Gene expression results determined as Ct (Cycle Treshold) value, *ACT* (*actin*) and control conditions used in the study were normalized by considering housekeeping gene. Transcript profiles of root, cotyledon and leaf samples of safflower cultivars exposed to cadmium were compared with *actin* (*ACT*) selected as housekeeping gene. The obtained data were normalized according to the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (Livak and Schmittgen, 2001). 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, IL). 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. In previous studies in the literature, Dunnett's test is recommended to be used if a control group is compared with more than one experimental group (Dunnet, 1955). For this purpose, post-hoc Tukey HSD and Dunnett test were applied to the variables with homogeneous distribution of variances (to confirm the results), and Dunnett's T3 test was applied to the variables that did not show homogeneous distribution (Roscoe, 1975). $P < 0.05$ was considered to be statistically significant.

3. Results

Safflower varieties grown in the climatic growth chamber for 21 days were taken into the cadmium heavy metal solution prepared at different concentrations at the end of the 21st day. 1X Hoagland solution was used as the control group. Cadmium stress application was carried out in the growth chamber for 24 hours. At the end of 24 hours, sampling was carried out from 3 different tissues root, cotyledon and leaf (Figure 3).

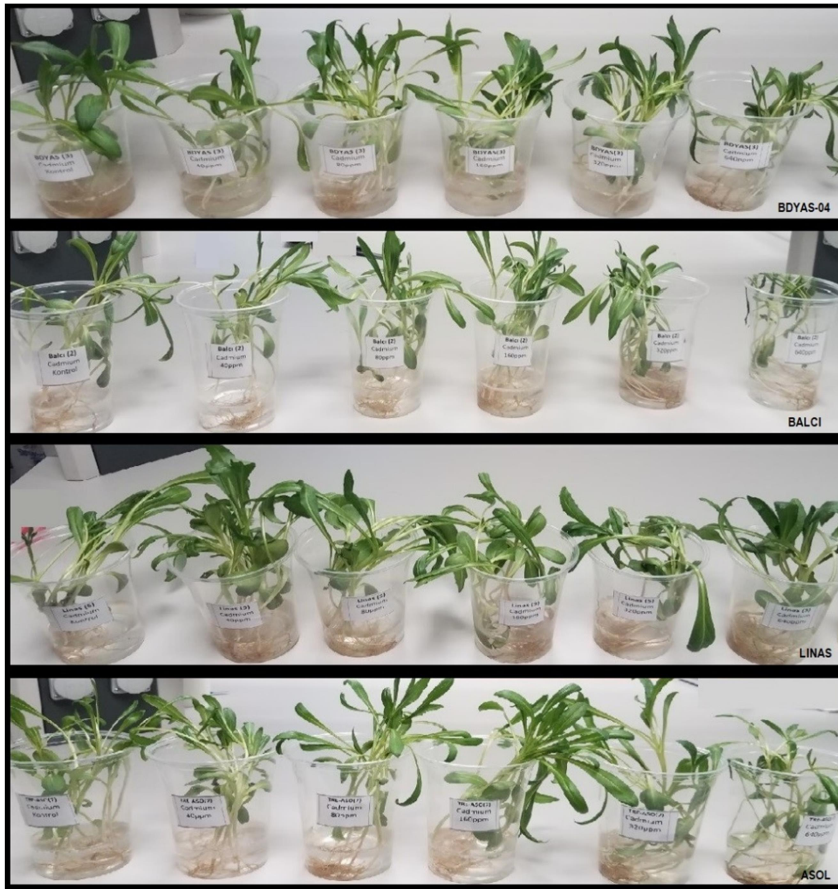

Figure 3. Safflower samples cadmium-treated for 24 hours.

Root, cotyledon and leaf tissue samples taken from safflower cultivars exposed to cadmium stress for 24 hours were stored in a deep freezer at -80 °C until the RNA isolation process. RNA isolation from these preserved samples was made according to the Trizol (TRIGent) protocol, and then the amount and purity of RNA were determined using the Nanodrop ND-Spectrometer 1000 device. Isolated RNAs were checked by running on 1.5 % agarose gel for confirmation. Gel images of some samples are given in figure 4.

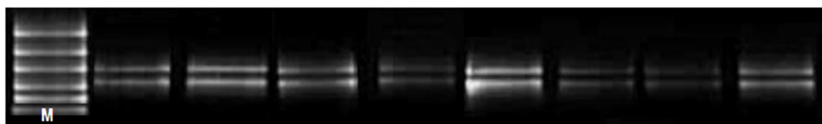

Figure 4. Agarose gel image of some RNAs isolated from samples after cadmium stress treatment.

The mRNA expression profiles of *FAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes of root, cotyledon and leaf samples of BALCI, BDYAS-04, LİNAS and ASOL cultivar with different concentrations of cadmium (Cd) stress were normalized according to the $2^{-\Delta\Delta Ct}$ method, taking into account *Actin* (*ACT*) used as a housekeeping gene and control conditions.

The mean, standard error and standard deviation of the gene expression data obtained as a result of normalization were calculated (Appendix 1-4). Normalized gene expression data were averaged and according to the results obtained, the changes in the concentration-dependent expression level of *FAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes occurring in different tissues of each safflower cultivar were shown on the separate graphs.

The expression levels of concentration-dependent *FAD2* genes in leaf samples of Balci cultivar under cadmium stress; an approximately 3,5-fold increase in *FAD2*-11 gene expression level was detected at 40 mg L^{-1} compared to the control group ($p<0,001$). While this increase decreased up to 80 and 160 mg L^{-1} ($p<0,01$), it started to increase again at 320 mg L^{-1} ($p<0,05$) concentration, reaching the highest level with an approximately 6-fold increase at 320 mg L^{-1} ($p<0,05$). The *FAD2*-7 gene expression level reached about 3,5-fold at 40 mg L^{-1} ($p<0,001$). It started to increase and reached the highest level with an approximately 4-fold increase at 80 mg L^{-1} ($p<0,001$) and then showed a decreasing trend up to a concentration of 640 mg L^{-1} ($p<0,01$). While the *FAD2*-6 gene expression level showed an decreasing graph up to 640 mg L^{-1} concentration ($p<0,05$). It shows a trend below the control group at all concentrations (Figure 5 and Appendix 1).

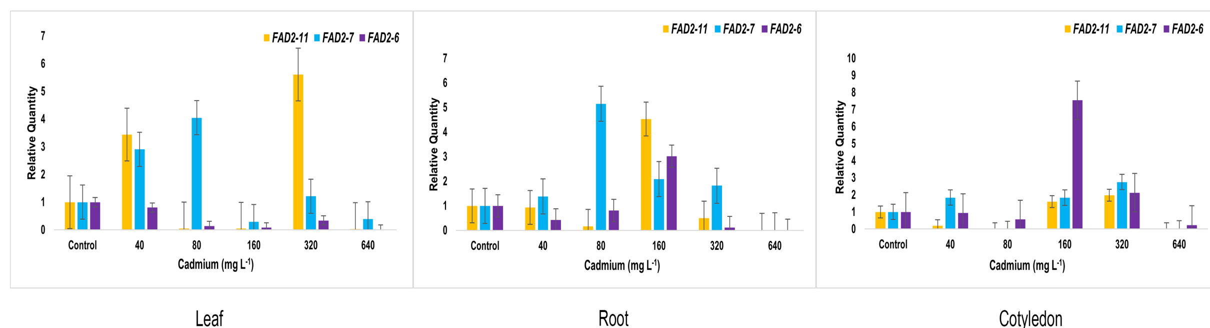

Figure 5. The expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of BALCI cultivar under cadmium stress.

Concentration-dependent changes in the expression levels of *FAD2* genes in root samples of Balci cultivar under cadmium stress; an approximately 4,5-fold increase in *FAD2*-11 gene expression level was detected at 160 mg L^{-1} compared to the control group ($p<0,01$). At other concentrations, expression levels were below the control group. The *FAD2*-7 gene expression level reached an approximately 5-fold at 80 mg L^{-1} ($p<0,05$). It decreased again at 160 and 320 mg L^{-1} concentration. However, the expression level at 640 mg L^{-1} was below the control group ($p<0,01$). An approximately 3-fold increase in *FAD2*-6 gene expression level was detected at 160 mg L^{-1} ($p<0,05$). Similarly, expression levels were below the control group at all other concentrations (Figure 5 and Appendix 1).

Changes in the expression levels of concentration-dependent *FAD2* genes in the cotyledon samples of the Balci variety applied to cadmium stress; compared to the control, the *FAD2*-11 expression levels increased only at 160 (1,8-fold) and 320 mg L^{-1} compared to the control group, while expressions were below the control group at all other concentrations. The *FAD2*-11 gene expression level was approximately 3-fold, with the highest expression at 320 mg L^{-1} ($p<0,01$) and the lowest at 640 mg L^{-1} concentration. An approximately 2-fold increase in *FAD2*-7 gene expressions level were detected at 40 and 160 mg L^{-1} compared to the control group ($p<0,05$). While this increase decreased up to 80 and 160 mg L^{-1} ($p<0,01$), it started to increase again at 320 mg L^{-1} ($p<0,05$) concentration, reaching the highest level with an approximately 6-fold increase at 320 mg L^{-1} ($p<0,05$). The *FAD2*-7 gene was expressed highest at 320 mg L^{-1} ($p<0,01$). Additionally, the highest and lowest expression concentrations of the *FAD2*-6 gene were detected 7,5-fold at 160 mg L^{-1} ($p<0,05$) and 0,2-fold at 640 mg L^{-1} ($p<0,05$) (Figure 5 and Appendix 1).

Similarly, the mRNA expression profiles of *FAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes of root, cotyledon and leaf samples of Bdyas-04, Linas and Asol cultivars with different concentrations of cadmium (Cd) stress were normalized according to the $2^{-\Delta\Delta Ct}$ method, taking into account *Actin* (ACT) used as a housekeeping gene and control conditions. Each of them was evaluated one by one as in the Balci cultivar mentioned above. The mean, standard error and standard deviation of the gene expression data obtained as a result of normalization were calculated (Appendix 2-4). Normalized gene expression data were averaged and according to the results obtained, the changes in the concentration-dependent expression level of *FAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes occurring in different tissues of each safflower cultivar were shown in separate graphs (Figure 6-8).

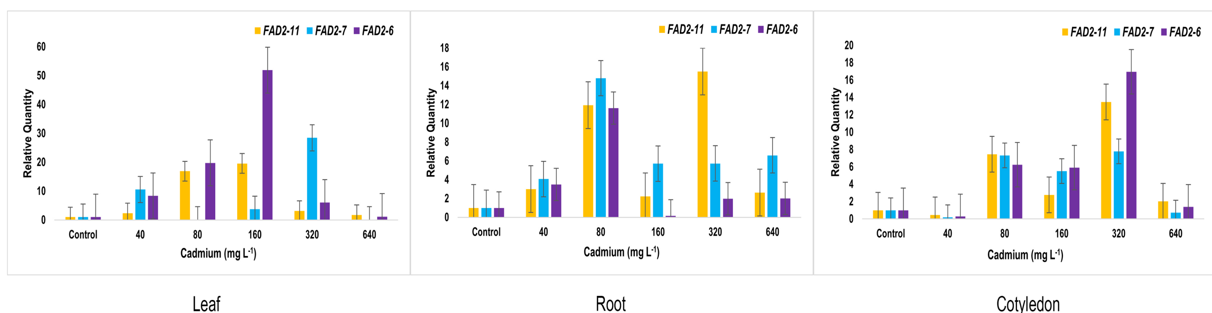

Figure 6. The expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of BDYAS-04 cultivar under cadmium stress.

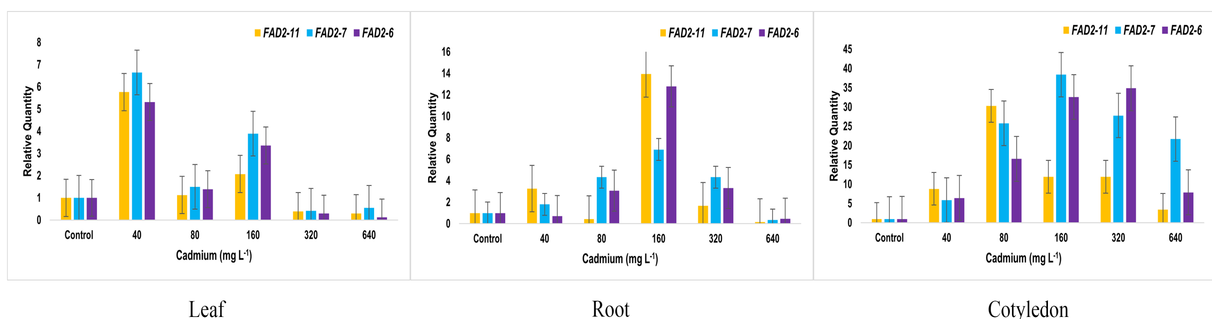

Figure 7. The expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of LINAS cultivar under cadmium stress.

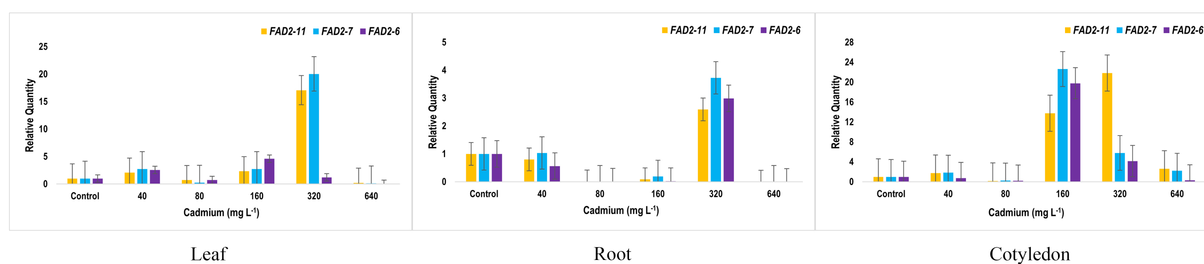

Figure 8. The expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of ASOL cultivar under cadmium stress.

3. Discussion and Conclusion

The changes in the expression levels of the targeted genes under heavy metal stress conditions were determined by Real-Time PCR method in the study. The qPCR method is a very effective method for detecting genes, determining their functions, testing and determining their relationship with various stresses (Kubista et al., 2006; Buyuk et al., 2011; Bolukbasi, 2021).

In this current study; the mRNA expression levels of the *FAD2*-6, *FAD2*-7 and *FAD2*-11 genes belonging to the *FAD2* gene family, which encode the FAD enzymes responsible for the conversion of oleic acid (C18:1) to linoleic acid (C18:2), were determined in the samples taken from root, cotyledon and leaf tissues in safflower cultivars (Balci, Asol, Linas and Bdyas-04) exposed to different concentrations (40 mg L^{-1}, 80 mg L^{-1}, 160 mg L^{-1}, 320 mg $L^{-1}$and 640 mg L^{-1}) of cadmium heavy metal stress.

FAD2 genes are one of the genes encoding the most critical desaturase enzymes and are responsible for the conversion of oleic acid (C18:1) to linoleic acid (C18:2) in non-photosynthetic tissues as well as in all tissues (Okulet et al., 1994). It has been stated that the levels of polyunsaturated fatty acids in the cell membrane provide tolerance to plants against drought, salt and cold stress through the regulation of *FAD* genes (Zang et al., 2005).

In the literature; it has been stated that *FAD* genes play a role in defense by increasing their expression levels in adverse environmental conditions (Tang et al., 2005). Overexpression of the *FAD3* gene in tomato plant increased the tolerance of tomato seedlings to salt stress (Wang et al., 2014) while overexpression of *FAD3*, *FAD8* and *FAD7* genes increased the tolerance of the tobacco plant to drought (Zang et al., 2005) and cold stress (Khodakovskaya et al., 2006). It has been determined that *FAD2* genes are involved in the defense mechanism against salt stress in sunflower (Rodríguez-Vargas et al., 2007). In addition, it was reported that the expression levels of *FAD2* and *FAD6* genes increased against salt stress in *Arabidopsis* seedlings (Zhang et al., 2012; Zhang et al., 2018). In another study, it was found that the *FAD2* gene is active and sensitive to stress factors such as darkness, heat and salt in *Arabidopsis* plant. It has been stated that the *FAD2* gene, which is expressed in various tissues of the *Arabidopsis* plant, functions during the growth and reproduction period of the plant and plays a role in defense against abiotic stresses (Yuan et al., 2012).

Feng et al. (2017) stated that the expression levels of *FAD2* genes increased in different tissues of cotton (*Gossypium hirsutum*) plant exposed to different salt and cold stress. They emphasized that the *FAD2*-3 and *FAD2*-4 genes, which are in the same gene family as the *FAD2* (*FAD2*-6, *FAD2*-7 and *FAD2*-11) genes used

in our study participate in the membrane adaptation against salt and cold stress and that the cell membrane is preserved in this way (Feng et al., 2017).

Although there are studies on other abiotic stresses related to *FAD2* genes in the literature, there is no study investigating the effects of heavy metal stress. Our study is the first of its kind on the subject, especially cadmium. Therefore, the data obtained from the study were evaluated based on the roles of the *FAD2* genes in the fatty acids mechanism. Studies have shown that *FAD* genes play critical roles in defense against salt and cold stress and take an active role in functions such as conversion, modification and restructuring of fatty acids. The data obtained as a result of the current study, support that *FAD2* genes give similar responses to heavy metal stress.

Heavy metal stress; it directly affects many biological events such as the release of protein and lipid components required for photosynthesis from thylakoid membranes in plants and metal exchange in chlorophyll (Mg^{+2}) (Maksymiec, 2007). In addition, heavy metal stress triggers the increase of reactive oxygen species (ROS). As a result of heavy metal-induced ROS accumulation and lipid peroxidase activity, polyunsaturated fatty acids in plant membrane lipids undergo peroxidation, leading to damage and loss of membrane integrity (Mithofer et al., 2004). Plants exposed to heavy metal stress try to cope with stress by making changes in the structure and amount of various lipids and fatty acids that participate in the lipid structure. In many studies; in plants, tolerance to e.g. copper (Cu) and cadmium (Cd) heavy metal stresses increases with increasing fatty acid unsaturation in cell membranes (Mithofer et al., 2004; Li et al., 2015; Gautam et al., 2016; Bolukbasi, 2021).

Namdjoyan et al. (2012a) investigated the effects of cadmium heavy metal on antioxidant compounds α-tocopherol, phytochelatin, glutathione and some non-protein thiols in different tissues of safflower plant. They stated that phytochelatin and non-protein thiol levels were increased in roots and α-tocopherol and glutathione synthesis decreased due to cadmium stress. Namdjoyan et al. in another study by (2012b), the effects on callus structure, cadmium accumulations and antioxidative responses of safflower plant exposed to cadmium heavy metal stress at different concentrations (0-100 µM) were investigated. It was stated that glutathione (GSH) and antioxidant enzyme activities increased up to 75 µM concentration and then decreased.

Moosavi et al. (2012) investigated the effects of lead (Pb) and cadmium (Cd) heavy metals, which are widely used in the electronics industry, on the germination percentage of canola, wheat and safflower seeds on the elongation of roots and shoots. According to the results, it was observed that as the concentration of heavy metal solution increased in all treatments, the percentage of seed germination, root and shoot length decreased. In the study by Moradi and Ehsanzadeh (2015), the effects of cadmium (Cd) heavy metal on photosynthesis and seedling growth in safflower plant were investigated. They stated that it negatively affects many pathways in the photosynthesis mechanism, which significantly reduces the rate of photosynthesis, and accordingly, seedling growth is reduced compared to the control groups (Namdjoyan et al. 2012a,b; Moradi and Ehsanzadeh, 2015).

In this current study the increase in the expression levels of the *FAD2* genes is thought to increase the amount of fatty acids against heavy metal stress. Li et al. (2015) investigated the effects of copper (Cu) and lead (Pb) heavy metal stresses on seedling growth and development and glutathione (*GSH*) gene expression levels of safflower plant. It was stated that seedling growth and *GSH* expression levels increased at low concentrations of

copper heavy metal. Seedling growth and *GSH* expression level decreased significantly with increasing Cu and Pb concentrations (Li et al. 2015).

The results obtained from current study support each other with the literature studies mentioned above. Increases in expression levels of *FAD* genes have been detected against various abiotic stress factors used in studies. Thus, by providing re-regulation of fatty acid metabolism, tolerance to stress is increased. Considering that the stress caused by heavy metals triggers similar mechanisms with other abiotic or biotic stress factors, the upward change in the expression levels of *FAD2* genes against the stress of heavy metals in the safflower plant shows parallelism with the studies mentioned.

The decrease in the expression of *FAD2* genes at increasing cadmium concentrations and their re-increase after 160 mg L^{-1} and 320 mg L^{-1}, which can be considered as the critical point, is accepted as an indication that the defense mechanism against the stress is activated and *FAD2* genes play a role in the defense against stress (Gautam et al., 2016).

In addition, some studies in the literature have reported that the expression profiles of various genes are tissue specific (Buyuk et al., 2011; Yang et al., 2012; Xue et al., 2017; Bolukbasi, 2021). In this article, it was determined that *FAD2* genes had different expression levels in root, cotyledon and leaf tissues of 4 different safflower cultivars exposed to cadmium heavy metal stress. When the data obtained from the study are evaluated, it has been shown that *FAD2* (*FAD2*-6, *FAD2*-7 and *FAD2*-11) genes are structurally active in root, cotyledon and leaf tissues (Cao et al., 2013) and play an active role in tissue-specific stress response. Such a study on the *FAD2* genes, which are responsible for the conversion of oleic acid to linoleic acid in the safflower plant, which has strategic importance and is an important oil plant, has not been done before, and the genetic mechanism of the response of the safflower plant to heavy metal stresses has not yet been clarified. In this study, changes in the expression of *FAD2* genes were determined in the presence of heavy metal stress factors. In this way, data that will contribute to the revealing of defense mechanisms against stress have been obtained.

As a result, when the data obtained from this study are evaluated as a whole, it was determined that the expression levels of *FAD2* genes were lower in almost all concentrations of safflower cultivars exposed to cadmium stress compared to the control group. At some concentrations, there were sudden increases in expression levels. When the expression levels of *FAD2* genes in safflower cultivars exposed to cadmium heavy metal stress were evaluated, it was determined that they responded to the stress earlier. Parallel to this, it is seen that the expression levels, which increase at 40 mg/L and 80 mg/L concentrations, reach the highest levels at 160 mg/L.

In conclusion, *FAD2* genes play a critical role in defense against heavy metal stress in safflower cultivars. With this study, it has been shown that the decrease firstly in the expression of *FAD2* genes at increasing cadmium concentrations. And also, re-increase at 160 and 320 mg L^{-1} which can be considered as critical points, are accepted as an indication that the defense mechanism against stress is activated and *FAD2* genes play a role in the defense against stress. But, the stress response against to cadmium heavy metal was insufficient.

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Conflicts of interest

The authors declares that there is no conflict of interests.

Statement contribution of the authors

This study's experimentation, analysis and writing, etc. all steps were made by the authors.

Statement of ethics

There is no need for an ethics committee decision for the studies in the article.

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Appendices

Appendix 1. The mean, standard deviation and standard error values of expression data of normalized *FAD2* genes of different tissue samples of **Balci** variety under cadmium treatments.

	Cadmium (mg L⁻¹)	Mean			Standard Deviation			Standard Error		
		FAD2-11	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*
Leaf	C	**1**	**1**	**1**	-	-	-	-	-	-
	20	3,43871	2,90775	0,80648	0,147082	0,542825	0,052017	0,084918	0,313400	0,030032
	40	0,04797	4,04993	0,14214	0,003467	0,675613	0,029766	0,002002	0,390065	0,017185
	80	0,04770	0,29799	0,08094	0,004195	0,112451	0,019643	0,002422	0,064924	0,011341
	160	5,60860	1,21289	0,34116	0,203403	0,154024	0,053486	0,117435	0,088926	0,030880
	320	0,02906	0,39503	0,00708	0,003094	0,083339	0,002335	0,001786	0,048116	0,001348
	640	3,43871	2,90775	0,80648	0,147082	0,542825	0,052017	0,084918	0,313400	0,030032
Root	K	**1**	**1**	**1**	-	-	-	-	-	-
	20	0,93407	1,37906	0,42743	0,797292	0,108121	0,289554	0,460317	0,062423	0,167174
	40	0,16452	5,14517	0,80622	0,056357	2,116881	0,196659	0,032537	1,222182	0,113541
	80	4,52571	2,08623	3,01947	0,322209	1,463939	1,070182	0,186027	0,845205	0,617870
	160	0,49641	1,82128	0,11852	0,028450	0,561154	0,026784	0,016425	0,323982	0,015464
	320	0,00367	0,00435	0,00135	0,000246	0,000330	0,000243	0,000142	0,000191	0,000140
	640	0,93407	1,37906	0,42743	0,797292	0,108121	0,289554	0,460317	0,062423	0,167174
Cotyledon	K	**1**	**1**	**1**	-	-	-	-	-	-
	20	0,18963	1,83915	0,93892	0,013122	0,016711	0,121396	0,007576	0,009648	0,070088
	40	0,00711	0,00827	0,56492	0,002875	0,000083	0,170690	0,001660	0,000048	0,098548
	80	1,60699	1,83498	7,54264	0,198058	0,212202	2,425277	0,114349	0,122515	1,400235
	160	1,97946	2,75451	2,12765	0,130804	0,085532	1,157800	0,075520	0,049382	0,668456
	320	0,00455	0,03446	0,23051	0,000372	0,002634	0,085954	0,000215	0,001520	0,049626
	640	0,18963	1,83915	0,93892	0,013122	0,016711	0,121396	0,007576	0,009648	0,070088

Appendix 2. The mean, standard deviation and standard error values of expression data of normalized *FAD2* genes of different tissue samples of **Bdyas-04** variety under cadmium treatments.

Tissue	Cadmium (mg L^{-1})	Mean			Standard Deviation			Standard Error		
		FAD2-11	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*
Leaf	C	1	1	1	-	-	-	-	-	-
	20	2,34525	10,57869	8,36358	0,02901	0,14403	2,41283	0,01675	0,08315	1,39305
	40	16,90875	0,09951	19,76175	1,17601	0,00839	3,97611	0,67897	0,00484	2,29561
	80	19,57034	3,70890	51,90747	1,00577	0,52500	4,00676	0,58068	0,30311	2,31331
	160	3,17302	28,48861	6,06166	0,06306	1,04826	1,15839	0,03641	0,60522	0,66880
	320	1,78334	0,08169	1,15709	0,06194	0,00691	0,24711	0,03576	0,00399	0,14267
	640	2,34525	10,57869	8,36358	0,02901	0,14403	2,41283	0,01675	0,08315	1,39305
Root	K	1	1	1	-	-	-	-	-	-
	20	2,99959	4,07525	3,50335	0,99109	0,97416	0,04180	0,57221	0,56243	0,02413
	40	11,91743	14,79701	11,61962	4,40722	4,46640	4,77775	2,54451	2,57868	2,75844
	80	2,23815	5,71178	0,16255	0,59536	0,32652	0,00608	0,34373	0,18851	0,00351
	160	15,50392	5,72879	1,99136	6,43655	0,88494	0,21455	3,71614	0,51092	0,12387
	320	2,62589	6,59779	2,01099	1,12944	1,15543	0,08719	0,65208	0,66709	0,05034
	640	2,99959	4,07525	3,50335	0,99109	0,97416	0,04180	0,57221	0,56243	0,02413
Cotyledon	K	1	1	1	-	-	-	-	-	-
	20	0,45782	0,19295	0,27462	0,10842	0,03814	0,11265	0,06260	0,02202	0,06504
	40	7,45974	7,32099	6,25381	1,03093	1,13264	1,19429	0,59521	0,65393	0,68952
	80	2,75794	5,51548	5,92808	0,70136	0,98448	0,71136	0,40493	0,56839	0,41070
	160	13,47890	7,78718	16,97316	0,93724	1,16514	1,95277	0,54111	0,67269	1,12743
	320	2,02799	0,71780	1,38378	0,27453	0,04656	0,30753	0,15850	0,02688	0,17755
	640	0,45782	0,19295	0,27462	0,10842	0,03814	0,11265	0,06260	0,02202	0,06504

Appendix 3. The mean, standard deviation and standard error values of expression data of normalized *FAD2* genes of different tissue samples of **Linas** variety under cadmium treatments.

	Cadmium (mg L^{-1})	Mean			Standard Deviation			Standard Error		
		FAD2-11	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*
Leaf	C	1	1	1	-	-	-	-	-	-
	20	5,766217	6,649279	5,316615	0,37102	0,757326	0,819964	0,214209	0,437243	0,473407
	40	1,130992	1,50242	1,390002	0,419665	0,64946	0,612419	0,242294	0,374966	0,35358
	80	2,074564	3,895671	3,364651	0,15074	0,441837	1,210416	0,08703	0,255095	0,698834
	160	0,395995	0,424689	0,29739	0,099987	0,115046	0,047465	0,057727	0,066422	0,027404
	320	0,302264	0,557244	0,124095	0,038993	0,12353	0,026442	0,022512	0,07132	0,015266
	640	5,766217	6,649279	5,316615	0,37102	0,757326	0,819964	0,214209	0,437243	0,473407
Root	K	1	1	1	-	-	-	-	-	-
	20	3,276751	1,801717	0,714047	0,745492	0,551439	0,127773	0,43041	0,318374	0,07377
	40	0,441941	4,332794	3,075302	0,278126	3,97908	1,201323	0,160576	2,297323	0,693584
	80	13,9582	6,912713	12,78682	3,324973	2,052503	1,156139	1,919674	1,185013	0,667497
	160	1,685655	4,331803	3,322849	0,630237	1,478849	0,379495	0,363867	0,853814	0,219101
	320	0,159873	0,352368	0,476345	0,068412	0,233767	0,093856	0,039497	0,134965	0,054188
	640	3,276751	1,801717	0,714047	0,745492	0,551439	0,127773	0,43041	0,318374	0,07377
Cotyledon	K	1	1	1	-	-	-	-	-	-
	20	8,82051	5,897346	6,438335	1,806865	1,702724	3,143134	1,043194	0,983068	1,814689
	40	30,31338	25,78899	16,59415	7,578272	4,120716	4,73708	4,375317	2,379096	2,734954
	80	11,9195	38,40436	32,62143	1,598149	5,324547	13,61705	0,922692	3,074129	7,861807
	160	11,93516	27,8158	34,90081	2,294767	3,093095	10,16685	1,324885	1,785799	5,869833
	320	3,393852	21,71542	7,900739	0,324876	1,395056	2,497038	0,187567	0,805436	1,441666
	640	8,82051	5,897346	6,438335	1,806865	1,702724	3,143134	1,043194	0,983068	1,814689

Appendix 4. The mean, standard deviation and standard error values of expression data of normalized *FAD2* genes of different tissue samples of **Asol** variety under cadmium treatments.

	Cadmium (mg L^{-1})	Mean			Standard Deviation			Standard Error		
		FAD2-11	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*	*FAD2-11*	*FAD2-7*	*FAD2-6*
Leaf	C	1	1	1	-	-	-	-	-	-
	20	2,07804	2,72871	2,55723	0,53723	0,11637	0,08920	0,31017	0,06719	0,05150
	40	0,71993	0,24223	0,71559	0,14406	0,01588	0,13455	0,08318	0,00917	0,07768
	80	2,33683	2,74464	4,62648	0,51870	0,35774	0,99145	0,29947	0,20654	0,57242
	160	17,08100	20,05714	1,22077	4,34890	1,85730	0,21663	2,51084	1,07231	0,12507
	320	0,21921	0,12651	0,03051	0,01456	0,00483	0,00815	0,00841	0,00279	0,00470
	640	2,07804	2,72871	2,55723	0,53723	0,11637	0,08920	0,31017	0,06719	0,05150
Root	K	1	1	1	-	-	-	-	-	-
	20	0,80054	1,03480	0,56620	0,00736	0,03883	0,00636	0,00425	0,02242	0,00367
	40	0,01353	0,00562	0,00401	0,00135	0,00012	0,00027	0,00078	0,00007	0,00015
	80	0,09307	0,19513	0,02395	0,01126	0,00349	0,00406	0,00650	0,00201	0,00235
	160	2,59217	3,72868	2,98993	0,27204	1,31622	1,08154	0,15706	0,75992	0,62443
	320	0,00421	0,00111	0,00177	0,00025	0,00006	0,00021	0,00014	0,00003	0,00012
	640	0,80054	1,03480	0,56620	0,00736	0,03883	0,00636	0,00425	0,02242	0,00367
Cotyledon	K	1	1	1	-	-	-	-	-	-
	20	1,77794	1,84659	0,75816	0,07636	0,19446	0,03845	0,04408	0,11227	0,02220
	40	0,17340	0,25903	0,22601	0,07587	0,02567	0,10735	0,04380	0,01482	0,06198
	80	13,77206	22,63226	19,79717	0,97197	1,56641	1,12935	0,56116	0,90437	0,65203
	160	21,82925	5,80763	4,16674	0,71812	0,29955	0,14459	0,41461	0,17295	0,08348
	320	2,64228	2,23828	0,29650	0,17555	0,16934	0,03852	0,10136	0,09777	0,02224
	640	1,77794	1,84659	0,75816	0,07636	0,19446	0,03845	0,04408	0,11227	0,02220