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Determination of Expression Levels of *Fatty Acid Desaturase-2* Genes in Safflower (*Carthamus tinctorius* L.) Varieties Exposed to Copper

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Abstract: Heavy metal pollution is an important environmental problem all over the world. It is known that high concentrations of heavy metals in soils and waters cause genotoxicity in living things and damage most of the functional biomolecules. For instance, while low concentration of copper, is essential for all organisms, high concentration of copper is toxic element that negatively affect every living organism from plants to humans. Safflower is an agricultural plant with high economic value grown for its seed oil. Safflower oil is source of Omega-9 and Omega-6 used in many food and industrial applications. In this study, it investigated the expression levels of *CtFAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genes responsible for conversion of Omega-9 to Omega-6 at root, cotyledon and leaf tissues of four different safflower varieties (Balci, Asol, Linas and Bdyas-04) subjected to copper heavy metals stress by qRT-PCR. RNA isolation, cDNA synthesis and qRT-PCR analysis were performed in root, cotyledon and leaf tissues exposed to copper stress for 24 hours after cultivation for 21 days. The increases were observed at concentrations of 40 and 80 mg L^{-1}. It was determined that the expression levels of *FAD2* genes decreased at increasing copper concentrations and increased again after 160 and 320 mg L^{-1}. The decrease firstly in the expression of *FAD2* genes at increasing copper concentrations and their re-increase after 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.
In conclusion, it has been determined that *FAD2* genes, which are responsible for fatty acid desaturation in safflower cultivars exposed to copper stress, are also associated with the stress mechanism and play a role in defense.

Bakır Stresine Maruz Kalan Aspir (*Carthamus tinctorius* L.) Çeşitlerinde *Yağ Asitleri Desaturaz-2* Genlerinin İfade Düzeylerinin Belirlenmesi

Anahtar Kelimeler
Aspir,
Bakır,
FAD2 gen,
Real Time-PCR

Öz: Ağır metal kirliliği tüm dünyada önemli bir çevre sorunudur. Ağır metallerin toprak ve sulardaki yüksek konsantrasyonlarının canlılarda genotoksisiteye neden olduğu ve fonksiyonel biyo-moleküllerin çoğuna zarar verdiği bilinmektedir. Örneğin, düşük bakır konsantrasyonu tüm organizmalar için gerekliyken, yüksek konsantrasyon bakır, bitkilerden insanlara kadar her canlı organizmayı olumsuz yönde etkileyen toksik bir elementtir. Aspir, tohum yağı için yetiştirilen, ekonomik değeri yüksek bir tarım bitkisidir. Aspir yağı, pek çok gıda ve endüstriyel uygulamalarda kullanılan Omega-9 ve Omega-6 kaynağıdır. Bu çalışmada, bakır ağır metal stresine maruz kalan dört farklı aspir çeşidinin (Balcı, Asol, Linas and Bdyas-04) kök, kotiledon ve yaprak dokularında Omega-9'un Omega-6'ya dönüştürülmesinden sorumlu *CtFAD2* (*FAD2*-6, *FAD2*-7, *FAD2*-11) genlerinin qRT-PCR ile ekspresyon düzeyleri araştırılmıştır. 21 gün yetiştirildikten sonra, 24 saat

boyunca bakır stresine maruz bırakılan kök, kotiledon ve yaprak dokularında RNA izolasyonu, cDNA sentezi ve qRT-PCR analizi yapılmıştır. 40 ve 80 mg L^{-1} konsantrasyonlarında artış gözlemlenmiştir. Artan bakır konsantrasyonlarında ise *FAD2* genlerinin ekspresyon düzeylerinin azaldığı, 160 ve 320 mg L^{-1} 'den sonra tekrar arttığı tespit edilmiştir. *FAD2* genlerinin artan bakır konsantrasyonlarında ekspresyonunun önce azalması ve kritik nokta olarak kabul edilen 160 ve 320 mg L^{-1} sonrasında yeniden artması, strese karşı savunma mekanizmasının devreye girdiğinin ve *FAD2* genlerinin strese karşı savunmada rol oynadığının bir göstergesi olarak kabul edilmiştir.
Sonuç olarak, bakır stresine maruz kalan aspir çeşitlerinde yağ desatürasyonundan sorumlu olan *FAD2* genlerinin aynı zamanda stres mekanizmasıyla da ilişkili olduğu ve savunmada rol oynadığı tespit edilmiştir.

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1. Introduction

Environment is the habitat in which all living things on earth can maintain their relations with each other throughout their lives. Environmental pollution, on the other hand, is the degradation of the environment by the human hand, which is not suitable for the natural structure of the environment. Although environmental pollution first emerges as a result of the necessity of creating spaces for urban life, these unnatural deteriorations are defined as the pollution of the ecosystem. Especially after the 1950s, the rapid increase in the population and the technologies developed to respond to the needs that developed in parallel with this caused the pollution of all our natural resources even faster. In today's world, pollution of the ecosystem has unfortunately become much more serious [1].

In order to survive, living things are constantly in contact with the physical elements of the ecosystem. They may encounter negative situations against any changes that may occur in their environment. While humans and animals can react more quickly to adversity or are easier to move away from, this is the opposite of plants. It is completely vulnerable to the negative effects that may occur around the plants. They are directly exposed to the negativities that occur. Since the most important natural food sources of people in the food chain are plants, people can also suffer indirectly from these problems [2].

Plants are the living element of the ecosystem that is most affected by their nature [3]. Due to their natural structure, plants face many different stress factors and have to live under stress conditions. According to many classifications, stress factors are divided into two groups as abiotic and biotic. Abiotic factors include water pollution, air pollution, soil pollution and organic or the synthetic chemicals, various wastes, pesticides, detergents, etc., which constitute the physical elements of ecosystem [4]. Various wastes, pesticides, detergents and heavy metals in the lower step of different chemicals are the most threatening and adversely affecting ecological balance. Heavy metals are an important stress factor not only for plants but also for animals and humans. Additionally, heavy metals are important disease agents for humans [5].

Heavy metals are the most important environmental pollutants published by the United States Environmental Protection Agency (EPA). They are among the priority pollutants causing environmental pollution [4]. The list published by the EPA contains about 70 elements. Among these elements, about 20 elements (Fe, Mn, Zn, Cu, V, Mo, Co, Ni, Cr, Pb, Be, Cd, Tl, Sb, Se, Sn, Ag, As, Hg, Al) draw attention with their importance and effects. Some elements (Fe, Cu, Zn, Mn, Mo, Ni) in this list are vital for plants and animals and need to be taken as micro nutrients. These elements do not have toxic effects unless accumulation in their environment or living being exceeds certain limits [3,5]. These elements, which are included in the group of heavy metals such as iron, copper, zinc, manganese, are essential elements that should be taken up by plants at certain levels [6].

Living things need varying amounts of so-called heavy metals. In humans, especially iron, is a very important element to be taken. There is also a need for metals such as cobalt, copper, manganese, molybdenum and zinc. However, excessive intake of all of these metals can damage the organism. Heavy metals, which begin to accumulate intensively in the ecosystem, pose a danger to almost any organism from plants to animals and humans, which are living elements of the ecosystem [7]. The development of technology in recent years has led to the rapid production of industrial materials. Agricultural pesticides used against many factors in agricultural areas, various artificial organic compounds used to increase yields also have an important share [1,4,5,7]. Heavy metal pollution leads to a decrease in product quality and yield in agricultural areas [6,8]. In general, plants are the most affected by heavy metal pollution in soil or water. Heavy metals cause oxidative stress by damaging many functional biomolecules in plants. It causes the formation of reactive oxygen species (ROS) such as superoxide

anion (O^{2-}), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH^-), causing damage to genotoxicity in plants as well as all living things [5,7,8].

Additionally, heavy metal stress in plants increases the formation of reactive oxygen species causing lipid peroxidation. Lipid peroxidation, the oxidation of the structural lipids in the cell and especially the lipids (unsaturated fatty acids in the composition) of the cell membrane with oxygen is the formation of structures such as aldehyde, ketone and hydroxy acids. Lipid peroxidation, oxygen oxidation of structural lipids in the cell, and in particular lipids found in the natural structure of the cell membrane. Lipid peroxidation, oxidation of the structural lipids in the cell, and in particular lipids in the natural structure of the cell membrane, with the formation of structures such as aldehyde, ketone, and hydroxy acids. Metals catalyze this formation. Lipid peroxidation in the cell membrane can lead to reduced membrane fluidity and membrane potential, deterioration of organelles and cell integrity. Additionally, malondialdehydes (MDA) formed by lipid peroxidation lead to degradation of DNA and most structural proteins [9].

Copper is free or compound in nature, has a very good conductivity and is a preferred metal for easy processing. Copper is an important metal and micro nutrient in maintaining normal metabolism in higher plants. In many studies on the importance of copper for plants, it was stated that copper acts as a controller in metabolic events by forming compounds with organic substances and vitamins. In particular, it is added to the enzyme structure as a cofactor. Heavy metals such as copper and zinc function as cofactors in many proteins and enzymes involved in metabolic pathways in plant growth and development. Photosynthesis, which is vital for plants, plays an important role in metabolic and physiological events such as respiration and cell wall. The transport of water in xylem pipes takes place under the control of copper heavy metal. Additionally, it controls the production of cellular hereditary materials such as DNA and RNA through the enzymes it serves. In the case of deficiency, DNA and RNA production may be hampered or may also stop [6,8,9].

Although copper is so important for plants, the mentioned above metabolic events fail in case of deficiency. Likewise, although it is involved in a wide variety of biochemical and physiological processes, the accumulation of excess Cu causes these metabolic events to stop functioning. Because copper is a highly toxic metal. It causes many tissue damages in plants, in case of excess. As a result of excessive accumulation in the roots, it negatively affects the exchange of water and ions and causes events such as growth disorder and regression in the roots. Reactive oxygen species (ROS) due to the toxic effect of excessive accumulation, cause damage to DNA and the death of plants in general [6-9].

Safflower (*Carthamus tinctorius* L.) is a member of the Asteraceae family. It is an important oil plant with economic value. There are many cultivated varieties of the safflower plant, which is represented by about 25 species around the world. Safflower is a broad leaved annual herb with yellow, red, orange, white and cream color flowers. This plant, resistant to arid climate, can be sized between 50-100 cm with its thorny and thornless forms [10-12]. (Figure 1).

Figure 1. General view of safflower plant, flower, field and seed [13]

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 [12]. 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 [12,14]. Safflower flowers are used for medicinal purposes in the treatment of many diseases, as well as consumed as tea. Safflower flower is mainly preferred as tea because of some amino acids, minerals and especially vitamins B1, B2, B12, C and E in its essence [11,12,14]. Apart from the use of the safflower plant for tea and medicinal purposes, different parts of it are used as foodstuffs in Middle East and Asian countries [12]. Safflower plant is popular plant in the industry because it is used in many sectors such as paint, varnish, feed, medicine, cosmetics and margarine. Shells of safflower seeds are preferred in the paper industry, especially in packaging, brick and ceramic production [12,15]. It is known that the main raw material of biodiesel production is oilseed plants [16]. About 50 different plants can be used in the production of biodiesel. Among them, the most important ones are sugar cane, soybean, sorghum, canola and corn [15].

Safflower does not need as much water as other oil crops such as soybean, rapeseed, canola, sunflower and peanut, and is suitable for agriculture in many areas. One of the most important factors in safflower cultivation is the weed control in the field to be planted. This plant, which has relatively low competition with weeds, is very sensitive to weeds existing in the field during the 3-4 weeks period, which is the first stage of development. For this reason, it is recommended to apply herbicides with highly effective chemical formulas such as trifluralin, metolachlor, EPTC, barban, profluralin and paraquat to the soil before planting safflower. The disadvantages of the chemicals in the basal structure of these herbicides (copper ethylenediamine sulfate salts, copper triethanolamine complex, copper hydrazinium sulfate, copper sulfate, leadarsenite, copper arsenite, etc.) are that they adversely affect living things [17].

In high plants, polyunsaturated fatty acids are transformed in chloroplast and endoplasmic reticulum by a group of fatty acid desaturase enzymes. This transformation occurs by different desaturases in different organs of plants. For example, FAD3 (fatty acid desaturase-3) in safflower plant performs the conversion of fatty acids in the flower part. FAD7 and FAD8 desaturases are enzymes responsible for desaturation in leaves, roots and, to a lesser extent, flower parts [18,19]. 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 [20].

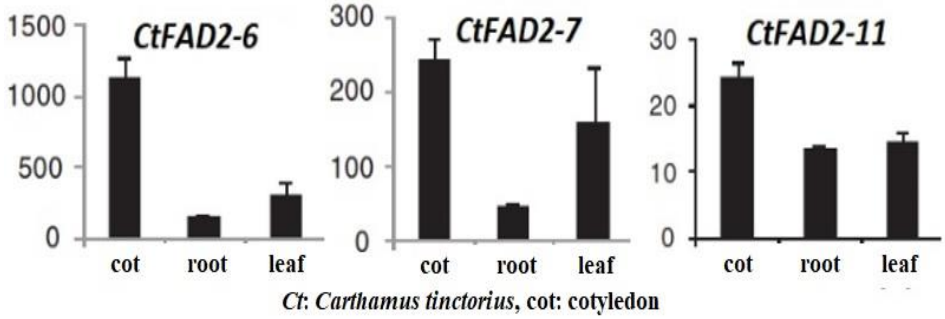

Figure 2. Comparative expression levels of the *FAD2* genes evaluated in different tissues of the safflower in the study [20]

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

2.1 Materials and Methods

2.1 Plant materials and growth conditions

Safflower varieties used in this study are nationally registered cultivars (Balci, Asol, Linas and Bdyas-04) 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 a spiny structure and their 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} Copper (I) sulfate ($Cu_2SO_4 \cdot 5H_2O$) for 24 h. 1X Hoagland solution, which does not contain any copper, was used as the control group. At the end of 24 hours, the seedlings taken from copper 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 copper 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.

2.3The 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	58-60 °C
			R: TCTGCACCTTCATCTGGCTC	
FAD2-7	1210 bp	KC257453.1	F: CGCAAACCATTTCCTACCGC	58-60 °C
			R: CGTCGATTTCAGGCCTTGGA	
FAD2-11	1213 bp	KC257457.1	F: ACGCCTTATTTCGCCTGGAA	58-60 °C
			R: TCGCGATCTTGGACTTACGT	
ACTIN	1678 bp	KJ634809.1	F: GGCGTGACCTTACAGATTC	58-60 °C
			R: CAAGCTCTTGCTCGTAGTC	

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 copper 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 copper 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 [21]. 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. 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. $P < 0.05$ was considered to be statistically significant.

3. Results

Root, cotyledon and leaf tissue samples taken from safflower cultivars exposed to copper 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 1.2 % agarose gel for confirmation. Gel images of some samples are given in figure 3.

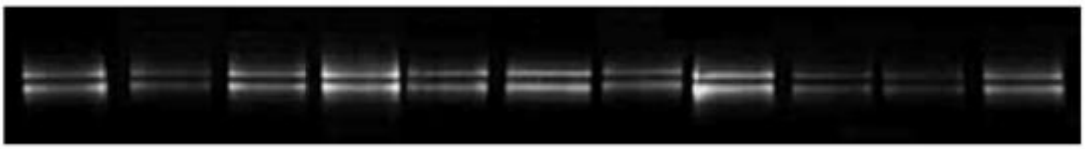

Figure 3. Agarose gel image of some RNAs isolated from samples after copper stress treatment

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 copper (Cu) 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 A-D). 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.

Changes in the expression levels of concentration-dependent *FAD2* genes in leaf samples of Balci cultivar under copper stress; an approximately 15-fold increase in *FAD2*-11 gene expression level was detected at 40 mg L^{-1} compared to the control group ($p<0,05$). While this increase decreased up to 160 mg L^{-1} ($p<0,01$), it started to increase again at 320 mg L^{-1} ($p<0,001$) concentration, reaching the highest level with an approximately 18-fold increase at 640 mg L^{-1} ($p<0,05$). The *FAD2*-7 gene expression level reached a spike of about 10-fold at 40 mg L^{-1} ($p<0,001$), and then showed an increasing and decreasing trend up to a concentration of 320 mg L^{-1} ($p<0,05$). It reached the highest level at 640 mg L^{-1} ($p<0,001$). While the *FAD2*-6 gene expression level showed an increasing graph up to 160 mg L^{-1} concentration ($p<0,05$), it decreased significantly with a sudden decrease at 320 mg L^{-1} ($p<0,05$) compared to the control group and increased again at 640 mg L^{-1} ($p<0,05$) (Figure 4 and Appendix A).

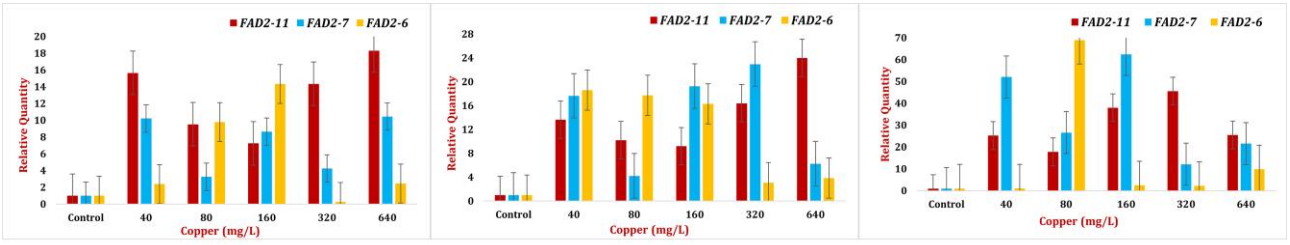

Figure 4. Changes in the expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of BALCI cultivar under copper stress.

Concentration-dependent changes in the expression levels of *FAD2* genes in root samples of Balci cultivar under copper stress; an approximately 13-fold increase in *FAD2*-11 gene expression level was detected at 40 mg L^{-1} compared to the control group ($p<0,05$). While this increase decreased up to 160 mg L^{-1} ($p<0,05$), it increased approximately 16 times at 320 mg L^{-1} concentration ($p<0,001$) and reached the highest level with a 24-fold increase at 640 mg L^{-1} ($p<0,05$). The *FAD2*-7 gene expression level reached an approximately 18-fold spike at 40 mg L^{-1} ($p<0,001$). It decreased again at 80 mg L^{-1} concentration. However, it increased up to 320 mg L^{-1}, reached approximately 23 times ($p<0,001$), and decreased again at 640 mg L^{-1}. An approximately 19-fold increase in *FAD2*-6 gene expression level was detected at 40 mg L^{-1} ($p<0,05$), and this level gradually decreased with increasing concentrations (Figure 4 and Appendix A).

Changes in the expression levels of concentration-dependent *FAD2* genes in the cotyledon samples of the Balci variety applied to copper stress; compared to the control, the *FAD2*-11 gene expression level was approximately 45-fold, with the highest expression at 320 mg L^{-1} ($p<0,05$) and the lowest at 80 mg L^{-1} concentration,

approximately 18-fold. The *FAD2*-7 gene is expressed highest at 160 mg L^{-1} ($p<0,001$) and lowest at 320 mg L^{-1}, while the highest and lowest expression concentrations of the *FAD2*-6 gene are 80 mg L^{-1} and 320 mg L^{-1}, respectively (Figure 4 and Appendix A).

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 copper (Cu) 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 B-D). 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 5-7).

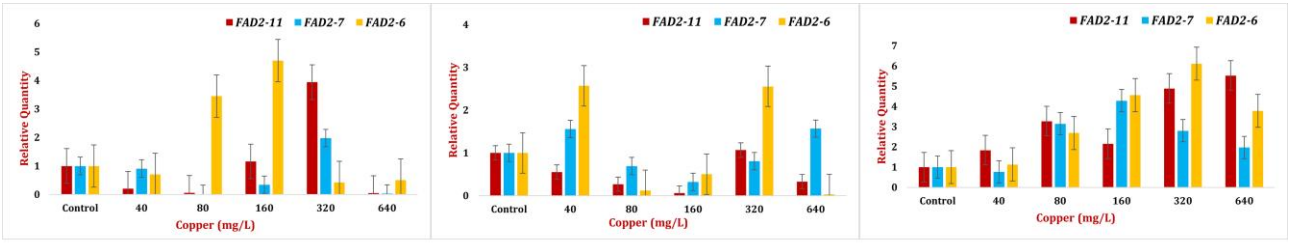

Figure 5. Changes in the expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of BDYAS-04 cultivar under copper stress.

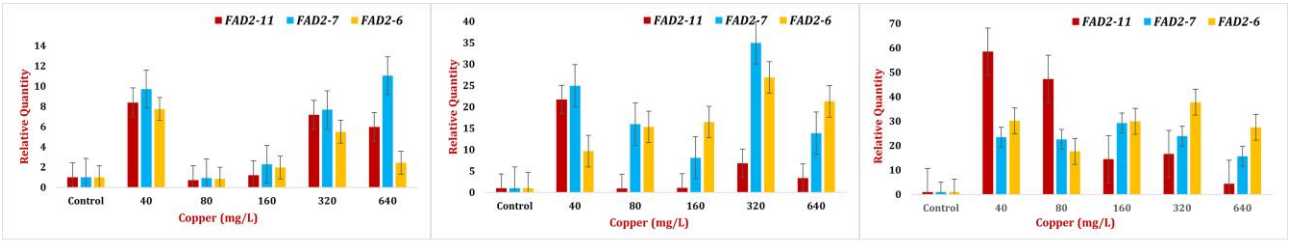

Figure 6. Changes in the expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of LINAS cultivar under copper stress.

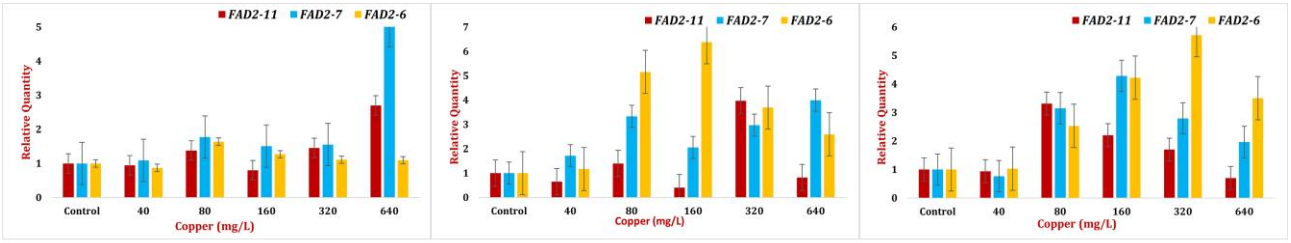

Figure 7. Changes in the expression levels of concentration-dependent *FAD2* genes in the leaf, root and cotyledon samples of ASOL cultivar under copper stress.

4. Discussion and Conclusion

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 copper heavy metal stress.

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 [2,5,22].

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 copper. 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 study support that *FAD2* genes give similar responses to 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 [2]. 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 [23,24].

In the studies in the literature; it has been stated that *FAD* genes play a role in defense by increasing their expression levels in adverse environmental conditions [25]. Overexpression of the *FAD3* gene in tomato plant increased the tolerance of tomato seedlings to salt stress [26] while overexpression of *FAD3*, *FAD8* and *FAD7* genes increased the tolerance of the tobacco plant to drought [24] and cold stress [27]. It has been determined that *FAD2* genes are involved in the defense mechanism against salt stress in sunflower [28].

In addition, it was reported that the expression levels of *FAD2* and *FAD6* genes increased against salt stress in *Arabidopsis* seedlings [29]. 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 [30].

In the literature, it has been stated that the *FAD2* gene in *Arabidopsis* plant [31,32] and soybean seed [25] provides adaptation to temperature change through the production of polyunsaturated fatty acids. It was determined that *FAD2* gene expression increased in response to cold stress in maize and flaxseed [33].

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 [34]. Similarly, overexpression of *FAD8* also reduces the damage of cold stress [35]. In the study on the *FAD2*-3 gene in soybean, an increase in the unsaturated fatty acid composition and mRNA expression levels of the *FAD2*-3 and *FAD2*-4 genes were determined under cold stress [36].

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}) [34]. 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 [4].

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 [4,23,24,30].

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. As a result, short-term exposure of the safflower plant to low Cu concentrations caused an increase in GSH synthesis to maintain normal plant growth. Long-term exposure of the safflower plant to high Cu and Pb stresses negatively affected the GSH metabolic chain and created serious toxicity for the safflower plant. The change in the expression level of *GSH* detected in this study is similar to the change in the expression level of *FAD2* genes obtained in this study [37].

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.

As a result, when the data obtained from this study were evaluated as a whole, it was determined that the expression levels of *FAD2* genes increased at low concentrations in safflower cultivars subjected to copper stress. It is thought that this result, which is similar to the results of the studies in the literature, is due to the use of copper as a micronutrient by plants. Gautam et al. (2016) emphasized that although copper is used as a micronutrient by plants, its toxic effect depends on time and dose. The decrease in the expression of *FAD2* genes at increasing copper 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 [38].

In addition, some studies in the literature have reported that the expression profiles of various genes are tissue specific [2,5,39,40]. 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 copper, lead and cadmium heavy metal stresses. 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 [20] 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.

In conclusion, when evaluated in general, *FAD* 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. Further study of the *FAD2* gene will help improve oil quality and stress resistance in plants. The data obtained from this study; it has been shown that *FAD2* genes play a critical role in defense against heavy metal stress in safflower cultivars. With this study, it has been shown that the *FAD2* gene is a good candidate gene for future studies in the safflower plant.

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Conflicts of interest

Authors have no any financial or personal relationships with other individuals or organizations that might inappropriately influence this work during the submission process.

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

Copper (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**	-	-	-	-	-	-
	40	9,52709	3,25869	9,78642	1,22671	0,61054	3,12453	0,70824	0,35249	1,80395
	80	7,25209	8,64408	14,34087	1,13818	1,72735	2,08053	0,65713	0,99729	1,20119
	160	14,36040	4,26039	0,26121	2,11419	0,15802	0,05815	1,22063	0,09123	0,03357
	320	18,31624	10,45389	2,46048	4,52803	1,53114	0,37356	2,61426	0,88400	0,21568
	640	15,64965	10,22171	2,39775	5,05895	1,68295	0,42246	2,92079	0,97165	0,24391
Root	C	**1**	**1**	**1**	-	-	-	-	-	-
	40	10,21834	4,23551	17,75621	1,44590	0,24112	1,75852	0,83479	0,13921	1,01528
	80	9,19766	19,27982	16,32068	1,28658	1,33311	0,93925	0,74281	0,76967	0,54228
	160	16,41403	22,97337	3,08384	1,51342	5,65527	0,96597	0,87377	3,26507	0,55771
	320	24,00828	6,27329	3,86109	4,94038	0,92114	0,46993	2,85233	0,53182	0,27131
	640	13,63664	17,65678	18,59443	1,25539	2,12837	1,51181	0,72480	1,22882	0,87285
Cotyledon	C	**1**	**1**	**1**	-	-	-	-	-	-
	40	17,76059	26,60507	68,94573	7,51593	2,07409	1,96295	4,33933	1,19748	1,13331
	80	37,97217	62,40647	2,52341	0,78094	1,53874	0,68661	0,45087	0,88839	0,39641
	160	45,55923	12,12712	2,24263	2,06927	0,87661	0,89004	1,19469	0,50611	0,51386
	320	25,49977	21,60077	9,80563	1,39320	1,37903	5,03193	0,80436	0,79619	2,90519
	640	25,25063	52,05280	1,07139	3,66284	4,82043	0,33793	2,11474	2,78308	0,19511

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

Copper (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**	**-**	**-**	**-**	**-**	**-**	**-**
	40	0,05922	0,01776	3,44860	0,00641	0,00264	0,60798	0,00370	0,00152	0,35102
	80	1,15899	0,34140	4,69802	0,16282	0,08823	1,05221	0,09400	0,05094	0,60749
	160	3,94107	1,98152	0,41915	0,46491	0,25692	0,11314	0,26842	0,14833	0,06532
	320	0,05169	0,03562	0,50805	0,00165	0,00297	0,13908	0,00095	0,00172	0,08030
	640	0,20602	0,90926	0,70442	0,02916	0,02739	0,18699	0,01684	0,01581	0,10796
Root	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	0,26578	0,69412	0,12194	0,03492	0,26990	0,02906	0,02016	0,15583	0,01678
	80	0,06304	0,32170	0,50149	0,01676	0,01936	0,09590	0,00968	0,01118	0,05537
	160	1,06525	0,80694	2,55612	0,51007	0,26483	0,62659	0,29449	0,15290	0,36176
	320	0,32814	1,56911	0,02966	0,22424	0,58741	0,01384	0,12947	0,33914	0,00799
	640	0,55033	1,56057	2,57069	0,07128	0,45185	0,66367	0,04115	0,26088	0,38317
Cotyledon	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	3,27540	3,14683	2,68838	0,79194	0,18088	0,38939	0,45722	0,10443	0,22482
	80	2,15452	4,28460	4,55956	0,77066	1,22941	0,79458	0,44494	0,70980	0,45875
	160	4,88732	2,79960	6,11191	0,58131	0,20280	0,21916	0,33562	0,11709	0,12653
	320	5,53135	1,96931	3,77943	0,43000	0,18402	0,74524	0,24826	0,10624	0,43026
	640	1,83898	0,76723	1,13290	0,44195	0,04145	0,52609	0,25516	0,02393	0,30374

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

Copper (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**	**-**	**-**	**-**	**-**	**-**	**-**
	40	0,71418	0,92850	0,85593	0,01962	0,11416	0,09517	0,01133	0,06591	0,05495
	80	1,21438	2,27720	1,97064	0,07615	0,19837	0,69643	0,04397	0,11453	0,40208
	160	7,18868	7,68719	5,49709	0,33927	0,12300	0,79313	0,19588	0,07101	0,45791
	320	5,97464	11,04703	2,44064	0,81481	2,66538	0,45699	0,47043	1,53886	0,26384
	640	8,39685	9,71704	7,75369	0,41647	1,43460	1,23540	0,24045	0,82827	0,71326
Root	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	0,96684	15,99135	15,31854	0,38203	7,29866	7,75784	0,22057	4,21388	4,47899
	80	1,06279	8,10906	16,47391	0,22857	0,90857	7,72605	0,13196	0,52456	4,46064
	160	6,80145	34,98766	26,90692	2,47309	11,65494	3,49816	1,42784	6,72898	2,01966
	320	3,35926	13,84117	21,29567	0,31249	5,81812	4,10248	0,18041	3,35909	2,36857
	640	21,72537	24,98494	9,65203	4,88303	11,92643	2,81343	2,81922	6,88573	1,62434
Cotyledon	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	47,31467	22,55803	17,67609	28,05617	4,29628	1,22927	16,19824	2,48046	0,70972
	80	14,46213	29,21194	29,95519	2,03249	2,23540	6,75816	1,17346	1,29061	3,90183
	160	16,57346	23,90170	37,78259	4,79114	2,31777	9,95296	2,76617	1,33816	5,74635
	320	4,38973	15,61766	27,47067	2,36646	3,45049	4,94963	1,36628	1,99214	2,85767
	640	58,49239	23,45923	30,16695	21,16872	4,62733	7,48355	12,22177	2,67159	4,32063

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

Copper (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**	**-**	**-**	**-**	**-**	**-**	**-**
	40	1,38013	1,77597	1,63842	0,18419	0,06263	0,05936	0,10634	0,03616	0,03427
	80	0,79980	1,50553	1,26841	0,07320	0,22608	0,34370	0,04226	0,13053	0,19843
	160	1,45178	1,55396	1,10833	0,07061	0,09066	0,14029	0,04077	0,05234	0,08099
	320	2,70180	5,02902	1,09384	0,55771	1,55321	0,21495	0,32199	0,89675	0,12410
	640	0,94471	1,09166	0,86755	0,03336	0,14549	0,08108	0,01926	0,08400	0,04681
Root	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	10,21834	4,23551	17,75621	1,44590	0,24112	1,75852	0,83479	0,13921	1,01528
	80	9,19766	19,27982	16,32068	1,28658	1,33311	0,93925	0,74281	0,76967	0,54228
	160	16,41403	22,97337	3,08384	1,51342	5,65527	0,96597	0,87377	3,26507	0,55771
	320	24,00828	6,27329	3,86109	4,94038	0,92114	0,46993	2,85233	0,53182	0,27131
	640	13,63664	17,65678	18,59443	1,25539	2,12837	1,51181	0,72480	1,22882	0,87285
Cotyledon	C	**1**	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**
	40	17,76059	26,60507	68,94573	7,51593	2,07409	1,96295	4,33933	1,19748	1,13331
	80	37,97217	62,40647	2,52341	0,78094	1,53874	0,68661	0,45087	0,88839	0,39641
	160	45,55923	12,12712	2,24263	2,06927	0,87661	0,89004	1,19469	0,50611	0,51386
	320	25,49977	21,60077	9,80563	1,39320	1,37903	5,03193	0,80436	0,79619	2,90519
	640	25,25063	52,05280	1,07139	3,66284	4,82043	0,33793	2,11474	2,78308	0,19511