T.C.

NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIEDSCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

INVESTIGATION OF PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR RESPONSES OF SOYBEAN CULTIVARS UNDER IRON DEFICIENCY

AMIR MAQBOOL

June 2018

MASTER THESIS
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Amir MAQBOOL tarafından **Dr. Öğr. Üyesi Emre AKSOY** danışmanlığında hazırlanan "**Investigation of Physiological, Biochemical and Molecular Responses of Soybean Cultivars Under Iron Deficiency**" adlı bu çalışma jürimiz tarafından Niğde Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü **Tarımsal Genetik Mühendisliği** Ana Bilim Dalı'nda Yüksek Lisans tezi olarak kabul edilmiştir.

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THESIS CERTIFICATION

I declare that all the information in the thesis is presented in accordance with the scientific and academic rules and that it is prepared in accordance with the thesis writing rules and that the reference to the source of any statements and information not belonging to me is fully cited in this work.

Amir

Amir MAQBOOL

ÖZET

SOYA ÇEŞİTLERİNİN DEMİR EKSİKLİĞİNE KARŞI GÖSTERMİŞ OLDUKLARI FİZYOLOJİK, BİYTOKİMYASAL VE MOLEKÜLER TEPKİLERİN İNCELENMESİ

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Demir (Fe), hem bitkiler hem de insanlar için temel mikro-besin maddelerinden bir tanesi olup, demir eksikliği en yaygın besinsel yetersizlikler arasında yer alır. Demir yetersizliği bitkilerde klorofil biyosentezinin azalmasına bağlı olarak gelişen demir eksikliği klorozuna (DEK) neden olur. Bu da doğrudan bitki verimini olumsuz yönde etkiler. Baklagiller içerisinde yer alan ve bir yağ bitkisi olan soya (*Glycine max.* L.), özellikle depoladığı demir miktarı bakımından tüm bitkiler arasında ikinci sırada yer aldığı halde, gelişimi esnasında karşılaşacağı demir eksikliği soya verimini büyük bir ölçüde azaltır. Bu yüksek lisans tezi kapsamında üç farklı olgunlaşma grubuna giren toplam 20 farklı soya çeşidinin demir eksikliğine karşı göstermiş oldukları fizyolojik, biyokimyasal ve moleküler tepkiler vejetatif ve generatif iki evrede belirlenmiştir. Bu kapsamda, demir eksikliğine maruz bırakılan bitkilerin klorofil indeksleri ve miktarları, fotosentez hızları, kök ve gövde yaş/kuru ağırlıkları, FRO enzim aktiviteleri ile yaprak, kök ve tohumdaki demir birikim miktarları belirlenmiştir. Ek olarak, stres uygulanan bitkilerin köklerindeki demir alımı ve taşınımından sorumlu *GmIRT1-like, GmFRO2-like, GmFERRITIN* and *AtNRAMP-like* [*GmDMT1;1]* genlerinin ifade seviyeleri belirlenmiştir. 20 soya çeşidinden III. olgunlaşma grubuna giren (orta-geççil) Atakişi ve Nova çeşitlerinin farklı hassasiyet tepkilerine yol açtıkları belirlenmiştir. Öte yandan,dayanıklı olarak belirlenen çeşitlerden Arısoy ve SA88'nın farklı dayanıklılık mekanizmalarını aktifleştirdikleri belirlenmiştir. Ayrıca, Ateom-7 bütün çeşitler arasında demir eksikliğine en dayanıklı çeşit olarak belirlenmiştir. Bu çalışma kapsamında dayanıklı olarak belirlenen çeşitler demir eksikliğinin görüldüğü İç Anadolu topraklarında yetiştirilmeye uygundur.

Anahtar Sözcükler: Demir eksikliği, soya, genotip taraması, dayanıklılık, *Glycine max*, fizyolojik tepkiler, biyokimyasal tepkiler, gen ifadesi

SUMMARY

INVESTIGATION OF PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR RESPONSES OF SOYBEAN CULTIVARS UNDER IRON DEFICIENCY

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Iron (Fe) is one of the essential micronutrients for both plants and humans, and Fe deficiency is among the most widespread nutritional deficiencies. Fe deficiency leads to Fe deficiency chlorosis (IDC) due to decreased chlorophyll biosynthesis, which, in turn, directly causes yield losses in plants. Soybean (*Glycine max.* L.) belongs to the legume family and is the top second plant species with the highest Fe content. However, soybean yields are negatively affected by Fe deficiency during growth in the field. In this master thesis, the physiological, biochemical and molecular responses of 20 different soybean varieties classified in three different maturation groups were determined against Fe deficiency in two developmental stages. In this context, chlorophyll indexes and amounts, photosynthesis rates, root length and shoot fresh/dry weights; FRO enzyme activities and iron accumulation in leaves, roots and seeds were determined from the plants exposed to Fe deficiency. In addition, expression levels of *GmIRT1-like*, *GmFRO2-like*, *GmFERRITIN* and *AtNRAMP-like* [*GmDMT1; 1*] genes responsible for Fe uptake and distribution were determined from the roots of stressed plants. Among 20 soybean varieties, two varieties (Atakişi and Nova) classified in third maturation group showed different sensitivities to Fe deficiency. On the other hand, two other varieties (Arısoy and SA88) were determined as tolerant, and they activated different tolerance mechanisms among other tolerant varieties. Moreover, especially Ataem7 showed the most tolerant phenotype among all tested varieties. The varieties determined to be IDC-tolerant are suitable for growing in Central Anatolian soils, where iron deficiency is highly observed.

Keywords: Iron deficiency, soybean, genotype screening, tolerance, *Glycine max*, physiological responses, biochemical responses, gene expression

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND ABBREVIATIONS

Symbols/Abbreviation	**Descriptions**
Fe	Iron
Fe^{2+}	Ferric Iron Ion
Fe^{3+}	Ferrous Iron Ion
Zn	Zinc
Mn	Manganese
Cd	Cadmium
Mg	Magnesium
CaO	Calcium oxide
FRO	*FERRIC CHELATE OXIDASE/REDUCTASE*
IRT1	*IRON REGULATOR TRANSPORTER 1*
FER	*FERRITIN*
NRAMP	*Natural Resistance-Associated Macrophage Protein*
EFL1B	*ELONGATION FACTOR 1-BETA*
CYP 2	*CYCLOPHILIN 2*
NIL	Near Isogenic Lines

CHAPTER I

INTRODUCTION

Iron (Fe) is one of the most essential micronutrients required by plants for a number of metabolic processes. Fe is considered as a king pin for plant growth and development, being required as a redox active metal involved in physiological and metabolic processes, including photosynthesis, respiration, nitrogen assimilation, sulfur metabolism, hormone biosynthesis, and production and scavenging of reactive oxygen species, osmoprotection, and pathogen defense (Hänsch and Mendel, 2009). Although iron is highly abundant in the soil than it is required by plants, it is mainly found in its oxidized (ferric) form, i.e. Fe^{3+}; therefore, it is often unavailable to the plants due to its low solubility (Kobayashi and Nishizawa, 2012). The low solubility is attributed towards high soil pH and high bicarbonate concentrations. Conclusively, it results in the limited uptake by plant roots because it cannot be readily absorbed by root cells (Lucena et al., 2006). Approximately 30 % of the world’s arable land faces this constraint. Incapability of the plant to acquire Fe from rhizosphere results in iron deficiency chlorosis or IDC visible in the interveinal tissues of young leaves. Fe deficiency results in developmental defects, at different plant growth stages, including chlorosis and growth retardation, leading to nutritional loss of the crop and the overall reduction of the yield (Briat et al., 2007). The first report of IDC was recorded in 1843 in grapes, where it is difficult to acquire ferrous Fe from soil particles due to high soil pH due to high carbonate and bicarbonate concentrations in the soil. Approximately, 30 % of the world’s soils are considered to be calcareous with low Fe availability (Takahashi, 2003).

Recent studies show that iron is also involved in the uptake of other nutrients as well. For example, sulfur, which is required by the plant as a macro-nutrient. If there is iron deficiency in the plant, sulfur uptake rate will also be reduced, resulting in lower yield and nutritional loss of the crop. The availability of iron has great effect on plant growth and yield (Zuchi et al., 2009; Astolfi et al., 2010, 2012).

Although iron is an essential nutrient for the plant, it becomes toxic for plants in the excess, causing the overproduction of reactive oxygen species (ROS), especially the hydroxyl radical (*OH), via the Fenton reaction. This causes the irreversible impairing of cellular structures and damages membranes, DNA and proteins (Kobayashi et al., 2012, Briat el., 2009). More ferrous iron (Fe^{2+}) absorption than the optimum amount by plants leads to the manifestation of typical leaf Fe toxicity symptoms called “bronzing”, brown spots starting from the leaf tips and distributed towards the leaf base. The roots of the plants affected by Fe toxicity become scanty, coarse, short, blunted and dark brown in color. Stunted root and shoot growth, limited yield (Becker et al., 2005; Dorlodot et al., 2005) and nutritional disorders (Ottow et al., 1983; Pereira et al., 2014) are also commonly reported. Thus, multifarious regulatory pathways have evolved to tightly balance Fe uptake, transport, metabolization, and storage for its supply and demand in different parts of the plant. Mitochondria and chloroplast pertains most of the iron in the cell. Majority of the proteins that are concerned with the electron transport chain contain iron as cofactor mainly conjugated with sulfur to form the Fe-S clusters. The biosynthesis of these clusters mainly requires concurrently reduced form of sulfur in the form of cysteine and of chelated Fe (Zuchi et al., 2009; Astolfi et al., 2010, 2012).

Besides plants, iron is also an important nutrient for humans and animals. Iron has three vital roles in the body of human beings: as it carries oxygen from the lungs to the rest of the body parts, maintained a healthy immune system and aiding energy production. Synthesis of many enzymes and proteins also depends upon the iron. This aspect is crucial during the recovery process from illness, wounds or following strenuous exercise and competing. The immunity system of a human body solely depends upon the concentration of iron in the body for its efficient functioning and physical and mental growth, particularly iron levels in body are more crucial during childhood and pregnancy, where the developing fetus solely depends upon the iron levels in the mother’s body. Lower iron content in a human body leads to slow the process of hemoglobin production, which means the transportation of oxygen is diminished resulting in muscle fatigue, abnormal dizziness and lower body immunity. Therefore, humans should consume sufficient amount of iron as part of their daily diet (Abbaspour et al., 2014). As a consequence, Fe deficiency is a major constraint for crop yield and quality, which eventually affects human health via food-chain, particularly to those people whose diets mainly relying on plant resources (Abadia et al., 2011). Increasing the content of iron in the major staple food crop species can be an important remedy to reduce the iron deficiency in human beings and animals (Graham et al., 1998; Graham and Welch, 2001; Cakmak, 2002).

Soybean (*Glycine max* L.) is an important nutritional crop belonging to the legume family, having high protein and oil content. Owing to the presence of high protein (40 %) and oil (20 %) content, it is regarded as a miracle crop. This miracle golden bean possesses high poly-unsaturated fats (85 %) and is also cholesterol free that enhances its adaptability for human health (Ambitsi et al., 2007; Dugje et al., 2009; Collombet, 2013). It is the highest produced legume crop, having the global production of about 230 million metric tons per year (Santos et al., 2016). Soybean is ranked second pertaining to the quantity of iron among all plant species after seaweed (USDA, 2016). In the past, soybean meal was also reevaluated for its dietary treatments of iron deficiency in some animals (Beard et al., 1996). Soybean is included in the list of those crops which are highly affected by iron deficiency due to high soil pH. IDC causes yield losses in soybean and other crop species as a result of the plant's inability to efficiently acquire iron from calcareous soils (Froechlich and Fehr, 1981). Fe deficiency in soybean leads to Fe-deficiency chlorosis (IDC) due to decreased chlorophyll biosynthesis, which, in turn, causes the yellowing of younger leaves, reduction in leaf area, shoot and root dry weight (Roriz et al., 2014). The loss in soybean yields due to IDC is predicted to be millions of tons every year (Naeve, 2006). Annual loss to IDC can be more than US$120 million in the United States (Hansen et al., 2003).

Traditional strategies to solve the problem of IDC-caused yield losses include soil amendments and foliar iron sprays (Schenkeveld et al., 2010), especially to correct mild chlorosis. However, they are not economically feasible. For this reason, the most commonly used strategy is still to select for IDC-tolerant crop genotypes. Soybean can be used as a model plant in order to understand the iron deficiency responses in plants, and to create bio-fortified crops having the high iron content in order to deal with the iron deficiency in humans (Aksoy et al., 2017). In many studies related with IDC tolerance in soybean, near isogenic lines (NIL) were used for easier comparisons. Although plants differ in their responses to iron deficiency and IDC tolerance has been studied in soybean, the effect of varietal differences in IDC is not well understood until now. Therefore, it is indispensable to analyze the physiological, biochemical and molecular responses of soybean cultivars under iron deficient conditions.

CHAPTER II

REVIEW OF LITERATURE

Soybean is an important legume crop, contributing towards the protein requirements of a large portion of world’s population. However, soybean production is curtailed by numerous factors, among which iron deficiency and high amounts of calcium salts are the critical ones. The effects of Fe-deficiency on soybean growth and possible options to cope with the problem have been extensively reviewed in this chapter.

2.1 Occurrence of Iron

Of the 87 elements in the earth’s crust, iron ranks fourth behind oxygen, silicon and aluminum. In soil, Fe is found 100 times more than calcium (Ca^{2+}), sodium (Na^{+}) and magnesium (Mg^{2+}), 1000 times more than zinc (Zn^{2+}), and 100,000 times more than iodine (I^{-}) (Turekian and Wedepohl, 1961). Iron is taken up by plant roots in two forms, as either Fe^{2+} (ferrous cation) or Fe^{3+} (ferric cation). Despite its abundance in the Earth’s crust, Fe is sparingly soluble under aerobic conditions, especially in high pH and calcareous soils, leading to significant yield losses (Mori, 1999).

2.2 Importance of Iron in the Plants

Iron is a fundamental element required for respiration, photosynthesis, and many other cellular functions, such as DNA synthesis, nitrogen fixation, sulfur metabolism and hormone production in plants (Vert et al., 2002). The redox properties of iron practically make it a vital element for all life forms. Iron is a component of cofactors that carry out electron transfer functions, or facilitate chemical transitions such as hydroxylations, radical-mediated rearrangements and (de)hydration reactions. Iron cofactors also function in oxygen transport, oxygen or iron sensing, or regulation of protein stability. The chloroplasts are particularly rich in iron–sulfur (Fe-S) proteins such as Photosystem I, ferredoxins and a range of metabolic enzymes. Mitochondria are another hotspot for iron enzymes, such as respiratory complexes containing multiple Fe-S clusters (complex I and II), a mix of Fe-S and haem (complex III) or haem and copper (complex IV). The peroxisomes and the endoplasmic reticulum contain haem proteins such as peroxidases and cytochrome P450s, whereas mono- and di-iron enzymes are found in all cell compartments (Brumbarova et al., 2015).

2.3 Iron Mobilization Strategies

The researchers are increasingly becoming interested in developing nutrient-rich plant foods, especially of Fe, through bio-fortification approach (Carvalho and Vasconcelos, 2013). It is evident from above section that soybean is a globally important crop and thus a potential candidate of bio-fortification. Unfortunately most of the bio-fortification strategies in soybean are concentrated on increasing sulfur amino acids (Dinkins et al., 2001) and vitamins, such as α-tocopherol (Dwiyanti et al., 2011), rather than increasing mineral concentrations. The research related with the development of crop plants rich in mineral nutrients must be focused on boosting the mobility and uptake of nutrients from soil, improvement/mobilization of these nutrients to edible portion of these crops and boost their storage in the edible tissues. To achieve this goal, a thorough understanding of the mineral transport system within the plants and its regulatory mechanisms is needed, which can be achieved through extensive experimentation.

Thus, to enhance Fe uptake and utilization in soybean, one of the possible strategies is to adapt a “bottom up” approach. The bottom up approach focuses on the mechanisms of Fe uptake with a hope that Fe addition will be taken up, mobilized and eventually stored in the edible parts of the plants. Apoplastic and/or symplastic pathways transport mineral elements in plants to the stele, from where these nutrients are supplied to xylem and transported to the shoots (White and Broadley, 2009).

Plant roots reduce Fe^{3+}-chelates and transport Fe^{2+} through the plasma membrane by a constitutive plasma membrane-bound ferric chelate reductase (Bienfait et al., 1985, 1989). However, Fe in calcareous soils is mostly found in the form of sparingly soluble Fe^{3+} compounds, which are not readily available for uptake by the roots. Thus, under such conditions, higher plants are forced to develop efficient strategies eventually making Fe soluble or converting it in readily available form to fulfill their Fe needs. In order to cope with the iron requirements higher plants have developed different types of strategies, commonly known as reduction strategy and chelation strategy. The main difference between these two strategies is the uptake of different oxidation states of iron. Fe^{2+} is taken up by reduction strategy plants whereas chelation strategy-based plants uptake iron in the form of Fe^{3+}-chelates. Moreover, recent studies also showed that many phenolic compounds, mainly coumarins, are involved in the iron uptake. All these strategies are explained briefly in this section.

i) Reduction-based Strategy (**Strategy I)**

ii) Chelation-based Strategy (**Strategy II)**

iii) Phenolics-based Strategy (**Strategy III)**

2.3.1 Strategy I

The non-grass monocots and dicot plants (e.g soybean) opt for strategy I for Fe uptake under Fe-deficient environments. Plants using reduction strategy acquire Fe in a three-step process initiated by the action of plasma membrane (PM) proteins present in the cells of the root epidermis. First, the rhizosphere acidification is stimulated by the proton excretion via a PM-localized H^+-ATPase (AHA) (Santi et al., 2009). Next step in the Fe uptake process is greatly facilitated by the reductase enzyme commonly known as FERRIC CHELATE REDUCTASE/OXIDASE (FRO), which converts insoluble Fe(III) to soluble Fe(II). The reduction of iron at this step has been proposed to be the rate-limiting for Fe-acquisition in reduction strategy-based plants (Kobayashi et al., 2012) Next, Fe (II) ions are transferred into the root epidermis cells by a divalent Fe transporter known as the IRON REGULATOR TRANSPORTER1 (IRT1). (Eide at al., 1996).

AHA family contains 12 members in Arabidopsis (Colangelo and Guerinot, 2004; Li et al., 2007). Among them, only *AHA2* and *AHA7* are upregulated under Fe deficiency, the former secretes protons to the rhizosphere, while the latter involves in development of root hairs (Santi and Schmidt 2009).

The role of iron reduction in the roots is well documented (Robinson et al.,1999; Connolly et al.,2003; Mukherjee et al.,2006); however, the role of the reductase in the leaf, fruit, and grain is still unclear. It is thought that iron reduction is necessary to reduce ferric iron in the aerial parts of the plant before being transported into the leaf cells (Larbi et al., 2001; Feng et al., 2006). FRO family consists of eight members (Jeong and Connolly 2009). It was found that AtFRO7 localizes to the chloroplast, and is required for efficient photosynthesis in young seedlings and for survival under iron-limiting conditions (Jeong et al., 2008). Iron reductase activity has been detected in leaves of different plant species, such as sunflower (de la Guardia and Alcántara, 1996), *Vigna unguiculata* (Brüggemann et al., 1993) and sugar beet (Gonzalez-Vallejo et al., 2000; Larbi et al., 2001). Members of the FRO family show various specificities of tissue expression. *FRO2* and *FRO5* are primarily expressed in roots while *FRO8* is primarily expressed in shoots (Mukherjee et al., 2006). *FRO6* and *FRO7* show high expression in all the green parts of the plant. *FRO3* is expressed at high levels in roots and shoots, and expression of *FRO3* is elevated in roots and shoots of iron-deficient plants. histochemical staining of *FRO3-GUS* plants revealed that *FRO3* is predominantly expressed in the vascular cylinder of roots. Interestingly, two other FROs (FRO3 and FRO8) localize to the mitochondria and might therefore contribute to mitochondrial iron homeostasis (Jeong and Connolly, 2009).

Expression of *FRO2* and *IRT1* is controlled by the basic helix-loop-helix (bHLH) domain-containing transcription factor known as Fe-DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) (Colangelo et al., 2004; Yuan et al, 2005). FIT forms heterodimers with 1b subgroup of bHLH transcription factors and is post-transcriptionally regulated by interacting with ETHYLENE-INSENSITIVE3 (EIN3), ETHYLENE-INSENSITIVE3-LIKE1 (EIL1) and the mediator subunit MED16 (Colangelo et al., 2004; Zhang et al., 2014). Soybean is a dicotyledonous plant and it has been found that it follows strategy-I for Fe uptake as shown in the Figure 2.1 (Römheld et al., 1987).

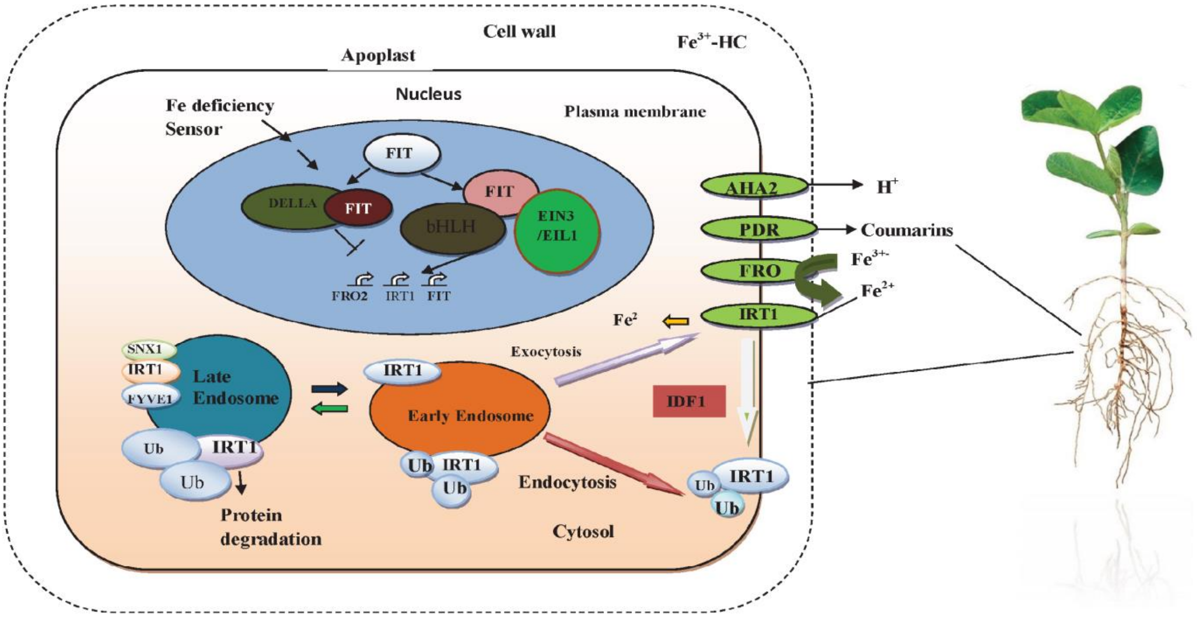

Figure 2. 1. Strategy I (Reduction strategy). Soybean and Arabidopsis follow this strategy for iron uptake from rhizosphere.

2.3.2 Strategy II

The Strategy II system involves the secretion of phytosiderophores (such as mugineic acids) and other derivatives such as 2’-deoxymugineicacid (DMA), epi-hydroxymugineic acid (epi-HMA) and 3-epihydroxy 2’-deoxymugineic acid (epi-HDMA) via roots (Connolly et al., 2000; Schaaf et al., 2004). Phytosiderophores are subsequently taken via YELLOW STRIPE TRANSPORTER1 (YS1) transporters which belong to OPT (OLIGOPEPTIDE TRANSPORTER) family. The transport of Fe^{3+}-chelate via YS1 transporter might be a proton-coupled transport (Schaaf et al., 2004; Nozoye et al., 2013). The phytosiderophores scavenge Fe, resulting in the formation of soluble Fe^{3+} complexes that can be taken up into the roots by active transport mechanisms (Waters et al., 2002; Connolly et al., 2003). This strategy is mainly seen in gramineous plants.

2.3.3. Strategy III

Strategy I plants were found to release an array of metabolites including phenolics, organic acids, flavins and flavonoids (Cesco et al., 2010). Previously phenolics were hypothesized to involve in the solubilization and reutilization of apoplastic Fe as reported in red clover (*Trifolium pretense*) (Jin et al., 2007). This feature was not well-thought-out to be part of the iron uptake mechanisms until coumarin-derived phenolics were reported in *Arabidopsis thaliana* grown at high pH conditions (Rodríguezet al., 2013; Schmid et al., 2014). The most important phenolics involved in the iron uptake are coumarins (Henriques et al., 2002). Iron deficient growth conditions lead to the synthesis and secretion of coumarins in roots of plants (Varotto et al., 2002). Coumarins have the ability to chelate and reduce Fe(III) (Yuan et al., 2005). Fraxetin, a coumarin was identified to mobilize Fe(III)-oxides by forming complexes with Fe(III) and reducing Fe(III) to Fe(II) (Vert et al., 2002). Nevertheless, phenolics were not able to complement the mutant phenotype of *ferric reduction oxidase2* (*fro2*) (Ivanov et al., 2012). Therefore, it is predicted that they are evolved in dicots as an alternative strategy to uptake Fe from the soil. Whether phenolics facilitate Fe uptake via reduction mechanism is still a controversy (Chen et al., 2017). Iron exists in the form of insoluble ferric chelates in soil, and ferric iron is also present in the apoplast bound to hemicellulose (HC). The solubilization of iron is carried out by AHA2 via acidification of the rhizosphere, and coumarin secretion through a transporter called ATP-BINDING CASSETTE G37 / PLEIOTROPIC DRUG RESISTANCE9 / POLAR AUXIN TRANSPORT INHIBITOR SENSITIVE1 (ABCG37/PDR9/PIS1) (Fourcroy et al., 2016; Ziegler et al., 2017). The transporter also regulates auxin distribution and homeostasis in roots by excluding IBA from the root apex but does not act directly in basipetal transport (Borghi et al., 2015).

2.4 Iron Translocation and Storage in Plants

Iron taken into the roots needs to be transported to the other aerial parts of the plants, where there is a critical need for iron dependent enzymes**.** IRT1 is mainly localized to the outward facing membrane of epidermal cells in plants (Barberon et al., 2014) signifying that is where iron first enters the symplastic pathway in which cells are connected by plasmodesmata. It is possible that efflux transporters localize to the inner membrane domain of root epidermal cells, but these has not been resolved yet (Dubeaux et al., 2015) NATURAL

RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN1 (NRAMP1) is proposed to collaborate with IRT1 for iron uptake, possible as a low-affinity uptake system (Castaings et al., 2016).

Nutrients can move through the apoplastic space constructed by the cell walls of epidermis and cortex cells to reach the endodermis. Here iron comes in contact with a barrier in the form of the Casparian strip, a layer composed of lignin, which forces all iron to pass into the symplast. Because of this reason the endodermis can be considered as the check point for the movement of iron in plants (Barberon et al., 2017). It is well reported that the amount of suberization of the endodermis changes in reaction to the environmental factors. Plants with the low iron contents showing a marked decrease in the suberization that results in an increase in permeability of the endodermis allowing more iron to enter in the vasculature (Barberon et al.,2014).

Due to its toxic nature and low solubility, Fe must be translocated in the form of complex chelators without causing damage to the redox reactions. Iron is translocated in the form of Fe^{2+}–nicotianamine (NA) complexes when it comes to the symplast. NA is a non-protein amino acid produced from *S*-adenosyl methionine by nicotianamine synthase (NAS), encoded by a small gene family in most plant species (Inoue et al., 2003: Bonneau et al. ,2016). Once iron passes through the endodermis, it can be loaded into the xylem for transport to the shoots. This process is operated by the pericycle, a complex layer of cells inside the endodermis of plants. A conduit is formed by the dead cells of xylem, therefore iron needs to be transferred from the symplastic space into the apoplast, probably by help of YELLOW STRIPE LIKE2 (YSL2) (DiDonato et al., 2004).and ferroportin (Morrissey et al., 2009), although biochemical evidence from transport studies is currently unavailable in this regard. In xylem, the prevailing form of iron is Fe^{3+}–citrate (Rellan-Alvarez et al., 2010) and accordingly Fe^{2+} must be oxidized to Fe^{3+}. Furthermore, citrate efflux is critical for iron translocation, and this is mediated by the efflux transporter FERRIC REDUCTASE DEFECTIVE3 / MANGANESE ACCUMULATOR1 (FRD3 / MAN1) in plants (Green et al., 2004) and its orthologue FRD3-LIKE1 (FRDL1) in some plants belonging to the Gramineae family, such as *Oryza sativa* (Yokosho et al , 2016).

Leaves are the vital tissues where iron is required for the process of photosynthesis. At this step iron re-enters in the symplast and FRO proteins cause the reduction of iron into Fe^{2+}. Then, it is again found to complex with NA to form Fe^{2+}–NA in leaf apoplast. A large proportion of transported iron is utilized by the plastids and mitochondria, where the transporters involved in each type of organelle have been described in recent reviews (Finazzi et al., 2015; Bashir et al, 2016). Iron is remobilized from leaf tissues and spreads to other sink organs via phloem. In Arabidopsis, the OLIGOPEPTIDE TRANSPORTER family protein, OPT3, was identified as a new transporter involved in the process of Fe translocation into the seeds. The studies revealed that *opt3* mutants had more iron accumulated in the leaves with less translocated to the other parts of the mutant plants (Zhai et al., 2014; Mendoza-Cózatl et al., 2014). Final destination of iron is considered to be the seed, where iron contents are important during germination before seedling develops a root to acquire nutrients from the soil. At this step, YSL transporters are involved in the seed loading (Jean et al., 2005). Two major mechanisms for the storage of iron have been identified in the plants: (i) restoration into the vacuoles and (ii) binding with the protein ferritin. The VACUOLAR IRON TRANSPORTER1 (VIT1) was first acknowledged in Arabidopsis as an orthologue of the yeast iron transporter CCC1. *vit1* mutant studies showed that the iron content of embryos was similar to the wild type, but the iron no longer accumulated in the vacuoles of the root endodermis and veins (Grillet et al., 2014; Roschzttardtz et al., 2009). NRAMP family of transporters release iron into the cytosol during germination process (Lanquar., 2005). Proteins from the VIT family are also known to be important for iron localization in rice grains and Brassica seeds (Zhang et al., 2012; Zhu et al., 2016).

Laulhere et al. (1993) reported that the ferritins are iron-storage proteins that accumulate in plastids during seed formation, and also in the leaves during senescence or iron overload. The amount of total iron stored in ferritin in the seed varies from specie to species (Zielińska-Dawidziak et al., 2015). Ferritins form a 24-subunit shell having the ability to store maximum of Fe^{3+} ions. Furthermore, the purified ferritins mainly in legumes are known to accumulate approximately 2500 ions (Theil et al. ,2011). The way in which iron is stored in seeds can affect its bioavailability when consumed, which is of great importance to the bio-fortification studies. Iron release from ferritins occurs during the growth of seedlings and greening of plastids. Depending on the concentration of the reducing agent ascorbate, either an overall iron release or uptake by ferritins from iron(III)-citrate may occur. Studies reveals that: (i) the chelated form of iron (but not ionic Fe^{3+}) is the substrate for iron reduction, which controls the subsequent uptake by ferritin; (ii) iron uptake by ferritins is faster at pH 8.4 than at pH 7 or 6 and is inhibited by an excess of strongly binding free ligands; and (iii) strongly binding free ligands are inhibitory during iron release by ascorbate. When reactions are allowed to proceed simultaneously, the iron chelating power is shown to be a key factor in the overall exchange.

Vert et al. (2002) reported that in response to iron deficiency the Arabidopsis roots induce the high expression of divalent cation transporter IRT1. Moreover, there is a genetic evidence that IRT1 is involved in the uptake of iron from the soil under iron deficient conditions. An Arabidopsis knockout mutant of *IRT1* showed chlorosis and severe growth defects in soil, leading to death. This defect is rescued by the exogenous application of iron. The mutant plants do not take up iron and fail to accumulate other divalent cations in low-iron conditions. IRT1–green fluorescent protein fusion, transiently expressed in culture cells, localized to the plasma membrane. Vert et al. (2002) also reported, through *promoter::glucuronidase* analysis and *in situ* hybridization, that IRT1 is localized in the external cell layers of the roots, specifically in response to iron starvation. These results clearly demonstrate that IRT1 is the major transporter responsible for high-affinity metal uptake under iron deficiency.

Thomine et al. (2003) reported that under Fe-starvation conditions, the GUS activity driven by the AtNRAMP3 promoter is upregulated without any changes in the expression pattern. Studies showed the impact of AtNRAMP3 disruption and overexpression on metal accumulation in plants. Under Fe-sufficient conditions, AtNRAMP3 overexpression or disruption does not lead to any changes in the plant metal content. Upon Fe starvation, AtNRAMP3 disruption leads to increased accumulation of manganese (Mn) and zinc (Zn) in the roots, whereas AtNRAMP3 overexpression decreases Mn accumulation. In addition, the overexpression of AtNRAMP3 down-regulates the expression of the primary Fe uptake transporter, IRT1, and FRO2. Expression of AtNRAMP3::GFP fusion protein in onion cells or Arabidopsis protoplasts showed that AtNRAMP3 protein localizes to the vacuolar membrane. To account for the results presented, studies revealed that AtNRAMP3 influences metal accumulation and IRT1 and FRO2 gene expression by mobilizing vacuolar metal pools to the cytosol.

Curie et al. (2000) reported that the sequence of five NRAMP proteins from *A. thaliana*. Sequence comparison suggests that there are two classes of NRAMP proteins in plants: *A. thaliana* (At) NRAMP1 and *Oriza sativa* (Os) NRAMP1 and 3 (two rice isologues) represent one class, and AtNRAMP2-5 and OsNRAMP2 represent the other class. ATNRAMP1 and OSNRAMP1 were able to complement the *fet3fet4* yeast mutant defective both in low- and high-affinity iron transports, whereas ATNRAMP2 and OSNRAMP2 failed to do so. In addition, *ATNRAMP1* transcript, but not *ATNRAMP2* transcript, accumulated in response to iron deficiency in the roots but not in the leaves. Finally, overexpression of *ATNRAMP1* in transgenic *A*rabidopsis plants lead to an increase in plant tolerance to toxic iron concentration. Interestingly, NRAMP1 was identified as Mn transporter. Iron was proposed as a primary high affinity substrate of NRAMP1 by noticing that iron deficiency played a central role for the growth defects of *nramp1irt1* double mutant, instead of Mn deficiency (Agorio et al., 2017). Taken together, these results demonstrate that ATNRAMP1 participates in the control of iron homoeostasis in plants.

Lanquar et al. (2004) reported that NRAMP gene family encodes integral membrane proteins mediating the transport of a broad range of transition metals in bacteria, fungi, plants, and animals. Studies were shown the regulation of ATNRAMP4 in Arabidopsis. In previous studies, AtNRAMP3 and AtNRAMP4 were found to transport Mn, Fe, and cadmium (Cd). Studies have exposed that, under Fe starvation, *AtNRAMP4* mRNA levels are up-regulated in Arabidopsis.

2.5 Transcriptional Control of Iron Uptake and Translocation

Different transcriptional factors control the uptake system of Fe in plants, and these transcriptional factors include bHLH proteins. Recently it has been suggested that the plants following strategy I for Fe uptake produce very specific Fe-deficiency compounds such as flavins and phenolics (Rodríguez-Celma et al., 2013) and scopoletins (Fourcroy et al., 2013). At present, numerous literature can be found related to Fe signaling in plants (Curie and Briat, 2003; Waters et., 2011; Hindt and Guerinot, 2012; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012).

Spatially organized networks control iron starvation responses in plants. *Arabidopsis thaliana* has evolved a special mechanism to cope with the deficiency of iron through the expression of *bHLH* transcription factor, *FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR* (*FIT*) (Ivanov et al., 2012). Since FIT belongs to the bHLH transcription factor family, it has the tendency to form heterodimers with other bHLH proteins (bHLH38 or bHLH39) (Wang et al., 2007). It is localized to toot epidermis cells (Bauer et al., 2007). The expression of *FRO2, AHA2* and *IRT1* are positively regulated by FIT. In an event of iron deficiency. It is worth mentioning that the expression of more than 40% of all iron deficiency-inducible genes is being regulated by *FIT*. Currently, the list of FIT regulated genes was extended from 73 to 448 genes (Mai et al., 2016). Therefore, *FIT* has distinctive significance and is indispensable for the normal growth and development in situations arising from iron deficiency (Long et al., 2010). FIT (a master regulator) is activated in response to low iron. The induction of iron acquisition genes, namely *FRO2, IRT1,* and *FIT* occurs by the interaction of FIT with other transcription factors, such as bHLHs and EIN3/EIL1 (Lingam et al., 2011).

Furthermore, Long et al. (2010) reported two genes which were induced by iron deficiency i.e., POPEYE (PYE) and BRUTUS (BTS). The former one encodes for a bHLH protein, while latter one is believed to be tightly co-regulated with PYE. PYE regulates the response to iron deficiency along with PYE-like (PYEL) transcription factors. The induction of *PYE* occurs in the pericycle under iron deficiency. The genes responsible for iron mobilization from roots to be translocated into the shoots is being negatively regulated by PYE protein. BTS is a E3 ligase protein with metal ion binding and DNA binding domains, which negatively regulates the response to iron deficiency. Its mRNA is cell-to-cell mobile (Selote et al., 2015; Thieme et al., 2015). Since, the expression of *PYE* is tightly co-regulated with *BTS*, BTS negatively regulates a response to iron deficiency (Long et al., 2010). Under low iron, BTS is stable and targets PYEL to fine tune the regulatory activities of PYEs. It is speculated that BTS may be involved in iron sensing. To this proclamation, it is interesting to know that BTS harbors an iron-binding hemery-thrin (HHE) domain and interacts with zinc and iron, when expressed in bacteria. The latest study advocates that HHE is crucial for the stability of BTS, but not for the E3 ligase activity, which is essential for the iron deficiency response (Matthiadis et al., 2016). The study further revealed that PYE along with BTS and other regulatory proteins, is responsible for the control of homeostasis of iron by regulating the gene expression in root stele, involved in iron homeostasis and many other biological processes.

The tight control of metal homeostasis in cells is governed by the trafficking of metal transporters between membranes of different compartments. Intracellular vesicle trafficking plays a major role in iron acquisition as well. Various multiple factors and secondary metabolites regulates the cycling of IRT1 between endosomes and the plasma membrane (Jeong et al., 2017). The localization of protein IRT1 between cell surface and trans-Golgi Network (Early endosomes) is being governed by ubiquitination and may be under the control of non-iron metals transported by IRT1. It ultimately provides another control layer for metal uptake.

Over-expression of *IRT1* or *FIT* does not necessarily lead to the accumulation of IRT1 or FIT proteins in Arabidopsis, proving a post-translational regulation of IRT1 and FIT accumulation under Fe deficiency. Several studies further showed that IRT1 is recycled between endosomal vesicles and the PM via ubiquitination-dependent pathway, where a RING-type E3 ubiquitin ligase, IRT1 degradation factor 1 (IDF1) was shown to be involved in ubiquitination of IRT1 (Shin et al., 2013). The posttranslational regulation of FIT is proposed to control the FIT activity by supplying fresh activators to its target promoter (Meiser et al. 2011). Moreover, it has been shown that 26S proteasome-mediated degradation of FIT is regulated by a burst in NO levels in the chloroplast (Arnaud et al. 2006).

Very recently, another level of posttranscriptional control for *FIT* was shown, where a protein family of repressors called DELLA bind to FIT and repress its downstream genes (Wild et al., 2016). In case of Fe deficiency, in one hand DELLA proteins accumulate in the root tip to repress the root growth, in the other, they are degraded by a proteasome-mediated pathway in the root elongation zone to release the repression on FIT-mediated Fe uptake mechanisms. Therefore, a tight regulation of Fe utilization-related genes at the transcriptional and post-transcriptional levels is essential to maintain rapid protein turnover to adapt to the environmental cues; however, control over several hundreds of genes that are expected to be involved in Fe-deficiency response according to microarray studies conducted with Fe-deficient plants is yet to be elucidated.

Another study conducted by Thomine and Vert (2013) is of the view that activation of genes at transcription level is being triggered by iron deficiency (FIT-triggered response). In addition to *IRT1* and *FRO2*, Metal transport encoding genes (*IRT2*, *MPT3*, *IREG2/ FPN2*) are co-regulated with *IRT1* to compartmentalize potentially toxic metals in the non-characterized intracellular vesicles (NCV) or vacuole in situation of high influx of metal.

In recent studies, other regulators of Fe signaling have been identified. In one of the studies, the bHLH transcription factor bHLH115 was identified as a positive regulator of the Fe-deficiency response (Liang et al., 2017). Loss-of-function of *bHLH115* causes strong Fe-deficiency symptoms and alleviates expression of genes responsive to Fe deficiency, whereas its overexpression causes the opposite effect. Chromatin immunoprecipitation assays confirmed that bHLH115 binds to the promoters of the Fe-deficiency-responsive genes *bHLH38/39/100/101* and *PYE*, which suggests redundant molecular functions with bHLH34, bHLH104, and bHLH105. Genetic analysis revealed that bHLH115 is negatively regulated by BTS after physical interaction with it. Thus, bHLH115 plays key roles in the maintenance of Fe homeostasis in *Arabidopsis thaliana*. In another study, the root-specific transcription factor MYB72 was identified as required for the onset of induced systemic resistance but is also associated with plant survival under conditions of iron deficiency (Zamioudis et al., 2014). Interestingly, *myb72* mutants showed altered responses to Fe deficiency and up-regulation of several genes involved in secondary metabolism and in the production of iron-mobilizing phenolic metabolites under conditions of iron deficiency.

Taken together, these studies suggest that there are several control layers and mechanisms to regulate Fe uptake and distribution in plants.

2.6 Iron Deficiency in Plants

Plants require iron for a large number of metabolic processes. Due to its low availability in high pH soils, and the impaired acquisition by roots, iron chlorosis is one of the most important limiting factors of plant development in many countries. Due to low availability of iron, the chlorophyll biosynthesis is hampered, which in turn, leads to low photosynthetic activity. This overall scenario directly means hampered crop yields leading to lower food production on the planet.

2.7 Soybean

Soybean (*Glycine max* L.) is a highly nutritious crop, containing higher amounts of protein (40 %) and oil (20 %) than traditional food sources such as meat, cheese and fish (Krishnan, 2005; Bolon et al., 2010). Soybean products are consumed by both humans and animals. The meal produced form soybean is a good source of proteins for animals, while humans consume soybean oil for their oil needs. Soybean oil is mostly used for food consumption and, more recently, for other uses such as biodiesel (Nwokolo, et al., 1996).

Global trade in soybeans and soybean products has risen rapidly since the early 1990s and, the global trade of soybeans crossed the total trade of wheat and total coarse grains during 2008-2009 (Lee et al., 2016). According to United States Department of Agriculture (USDA), global soybean trade is projected to increase by 22%, soybean meal by 20% and in soybean oil by 30% (Lee et al., 2016). The global production of soybean was approximately 230 million metric tons/annum in 2016 (Santos et al., 2016). The global production of soybeans is forecast to be 355.2 million tonnes in 2017–2018 (USDA, 2018). The top five countries; United States, Brazil, Argentina, China, and India, produce more 92% of the world’s soybeans (Masuda and Goldsmith, 2009).

2.8 Factors Affecting Soybean Yield

Several factors such as climatic conditions, soil fertility, pesticide use, balanced nutrition, stand establishment and agronomic practices influence the soybean yield. There are distinct factors at regional scales hampering the productivity of soybean. For example, soybean yield in Japan is attributed to disadvantageous climate characteristics for soybean cultivation, such as a rainy season at the time of sowing, drought stress after the rainy season, and typhoons (Fatichin et al., 2013; Matsuo et al., 2016). Excess soil moisture due to poor drainage in fields converted from paddy rice frequently inhibits the emergence and growth of soybean, which is a major constraint in Japanese soybean production because more than 80 % of the soybean crop is cultivated in converted paddy fields (Matsuo et al., 2013; Shimada et al., 2012).Similarly, water deficit is projected to be more frequent and intense, especially in the tropics and subtropics due to increased air temperature and altered rainfall patterns (Li et al., 2013; IPCC, 2015), which will require adaptation measures in agriculture.

Soybean is cultivated worldwide, and Fe deficiency is predominantly a limiting factor in the yield of soybean produced in calcareous soils (Rodríguez-Lucena et al., 2010). Most of the soybean cultivated lands in Turkey are calcareous, having higher pH levels, which adversely affects the uptake of micronutrients, leading to severe yield reduction (Maqbool et al., 2017). Similarly, the lower yield in the United States is attributed to higher soil pH (Naeve, 2006).

Among different nutrients, Fe deficiency-induced chlorosis (IDC) and subsequent lower Fe accumulation in the soybean seeds is a global problem. Substantial yield reduction is observed due to IDC in different regions of the world. As described above, higher pH levels create hindrance in the formation of Fe^{+2} ions readily available for uptake. Thus, this condition makes Fe biologically unavailable to the plants, and interveinal chlorosis is developed by the plants. Under such conditions, if Fe deficiency is not addressed, plants suffer from stunted growth, which ends with yield reduction (Froechlich and Fehr, 1981). The soybean yield is reduced to 20 % with each point increase in iron chlorosis score (Froehlich and Fehr, 1981). Keeping in view the current prices, these estimated losses sum up to $260 million annually in the USA as soybean prices have been increased since 2004 Naeve , 2006). The increased Fe-deficiency in arable lands, nutrient-efficient plants will play an important role in increasing the yield of crops in 21st century agriculture (Fageria et al., 2008).

2.9 Iron Deficiency Chlorosis (IDC) in Soybean

Iron chlorosis is a common agricultural concern, especially in calcareous soils where calcium carbonate increases the pH the soil solution to the range between 7.5 to 8.5 along with high concentration of bicarbonate (Lindsay and Schwab, 1982). It leads to the yellowing of leaves due to decreased amount of photosynthetic pigments, especially chlorophyll (Abadía and Abadía, 1993).

Several factors affect the bioavailability of iron in soils, including pH, redox potential, CEC (cation exchange capacity), and the presence of Fe complexing agents in the soil solution (Lindsay, 1979; Tabatabai and Sparks, 2005). Among these factors, pH is one of the predominant soil characteristics influencing Fe-solubility as a result of the pH-dependent dissolution of soil Fe(III)-hydroxides (Lindsay and Schwab, 1982; Schenkeveld et al., 2010). High pH and high pH-buffering capacity result in a low concentration of dissolved Fe in calcareous soils (Chaney, 1988; Schenkeveld, 2010). Higher pH of soil has an adverse effect on the ability of plans to reduce ferric iron. Ferrous iron is re-oxidized to ferric iron after absorption from roots and translocated to the leaves through xylem (Marschner, 1986). Although Fe is fairly abundant in soil, under such circumstances it becomes insoluble poorly available for absorption (Chaney, 1985, Marschner et al., 1996).

Further, it has also been reported that Fe-oxide phase and Mg^{2+} content of the solution significantly influences the bioavailability of Fe (Loeppert and Hallmark,1985). Nonetheless several researchers have reported that IDC in Minnesota, is associated with higher Mg^{2+} levels of soil and plant, higher soil Na^+ and Cl^-, higher Mg/Ca ratios, over-saturation of $CaCO_3$, higher plant P, higher soil moisture, lower soil temperature and higher bicarbonate levels (Inskeep and Bloom, 1984; Bloom and Inskeep, 1986; Inskeep and Bloom, 1986).

Salt concentration of the soil also significantly affect the IDC in soybean. A linear decrease in Fe bioavailability has been observed with increasing salt levels of the soils

(Dahiya and Singh, 1979). The exogenous application of Fe compounds is also unable to fix Fe-deficiency-induced IDC in the soils having higher levels of salts (Randall, 1981). Saturated EC paste significantly reduced the growth of plants in Looppert and Hallmark's (1985) study on chlorosis. The Mg/Ca ratio is also revealed to significantly affect IDC in soybean. These studies revealed that IDC is not only linked with the carbonate minerals but also with the presence of elevated salt levels in the soil. Therefore, it is well understood that both carbonates and elevated salt levels negatively impact the Fe availability, uptake and subsequent transport to the plant parts, which negatively affect growth and yield.

Numerous physiological processes, such as chlorophyll synthesis, respiration, nitrogen fixation, enzymatic activation and electron transfer, need Fe for their normal functioning (Taylor et al., 1982, Engels et al., 2012). The young leaves of the Fe-deficient plants show yellowing, suffer from reduced leaf areas and dry weight production of shoots; and roots are also severely decreased (Roriz et al., 2014). All these hampered growth traits decrease the soybean yield, and lead to substantial economic losses. The phenotype with chlorosis appears early in the growing season and disappears throughout the growing season as plants mature (Hansen et al., 2003). Often the symptoms of IDC appear early in the growth season in autumn. The long-term effects of iron deficiency stress were observed in the early autumn of the growing season (Tagliavini et al., 2001). These IDC-induced symptoms are overcome at the end of growing season by most of the soybean genotypes, however these genotypes are unable to resist to yield losses induced by IDC (Inskeep and Bloom, 1987). Hence the severity of these symptoms is linearly related to the yield reduction at the end of the growing season. Plants have developed several mechanisms to cope with the adverse effects of Fe-deficiency. These mechanisms help plant to efficiently uptake and mobilize Fe from the soil as well as within plant tissues and cells (Sperotto et al., 2012).

Several studies have been done on soybean in order to cope with the iron deficiency in recent few years. Niebur et al. (1981) have identified tolerant genotypes against IDC by estimating the agronomic characteristics of different cultivars. Breeding can be another tool to overcome this problem. For instance, cultivars with different IDC tolerance levels have been identified (Fehr et al., 1982). Genetic loci for iron deficiency have been identified (Lin et al., 1997; Wang et., 2008). Previous studies have recognized multiple quantitative trait locus (QTL) associated with IDC (Lin et al.,1997; Lin et al.,1998). In addition, the advent of microarray technology now allows for the identification of individual transcripts whose expression levels are affected by iron availability (O'Rourke et al., 2007). The availability of a whole-genome sequence assembly for the soybean genome has, for the first time, allowed us to genetically position differentially expressed genes induced by low iron availability. Moreover, recent advances in biotechnology has also enabled the scientists to suppress or over-express specific genes controlling specific mechanisms in plants (Jeong et al., 2009). Thus, these strategies need to be well-understood through experimental studies.

2.10 Molecular Characterization of IDC Tolerance Mechanisms in Soybean

Soybean homologs of *AtIRT1* and *AtFRO2* were characterized *in silico*, and an increase in their transcript abundances were confirmed in soybean roots under Fe-deficiency (Stribe, 2012). Similar to other abiotic stresses, plants activate a multitude of responses against Fe-deficiency, which are controlled by multiple genes (Li et al., 2016). Other genes (Rogers et al., 2009) and transcription factors (Peiffer et al., 2012) contributing to Fe uptake and translocation were recently identified in soybean.

Transcriptomic changes of soybean responses under Fe-deficiency were studied in Fe efficient line, Clark, Fe inefficient line T203 and Fe inefficient near isogenic line (NIL) of IsoClark, which was generated after a cross between Clark and T203. Generated in 1970’s (Bernard, 1975), genomes of Clark and IsoClark NILs are 98 % identical. IDC symptoms are generally observed in vegetative V3 developmental stage of soybeans. In an early microarray study to identify the differentially expressed genes (DEGs) in the roots of Clark and IsoClark under 14 days of IDC treatment, a total of 43 DEGs were found to be common in both lines (O’Rourke et al., 2007). Many of the identified genes were related to known stress-responsive pathways, while some of the DEGs were found to have a regulatory role in the cross-road of different signaling pathways. This indicates an activation of metabolic pathways, leading to the adjustment and conservation of cellular homeostasis under Fe-deficiency. The number of DEGs increase to 835 and 200 in the third trifoliate leaves of Clark and IsoClark, respectively, under same IDC conditions (O’Rourke et al., 2009). Only 18 transcripts were found to be common to both NILs. Myeloblastosis (Myb), helix-loop-helix (bHLH) and Elongation Factor 2 transcription factor binding sites were identified in the promotor regions of IDC-induced DEGs, indicating a complex transcriptional control of IDC responses in soybean. Some of these transcription factors regulate the downstream genes, such as *IRT1* and *FRO2,* in Arabidopsis (Weng and Guerinot, 2016). Similar to Arabidopsis, transcription factors belonging to the Ib sub-group of bHLH family were induced in the roots of both NILs, however the induction level was much higher in efficient Clark roots, suggesting a link between IDC tolerance and expression of bHLH transcription factors (Peiffer et al., 2012). Interestingly, the QTL region responsible from IDC tolerance in Clark was mapped to a location on Chromosome 3, where these specific bHLH transcription factors are clustered together, supporting the importance of gene duplications in abiotic stress tolerance (Courbot et al., 2007; Dong et al., 2016). One of the highest induced transcripts in efficient Clark line was *REPLICATION PROTEIN A SUBUNIT3* (*GmRPA3c*), which is one of the three subunits of RPA protein, a single-stranded DNA binding protein unique to Eukaryotes (Wold, 1997), and functions in maintaining genome integrity during DNA replication and cell-cycle checkpoint activation (Hass et al., 2012). *GmRPA3c* was induced in inefficient IsoClark leaves but suppressed in efficient Clark leaves (O’Rourke et al., 2009). Silencing of *GmRPA3c* in IsoClark decreased the general plant size while increasing the chlorophyll content (Zhang et al., 2010). When transgenic IsoClark plants were exposed to IDC, RNA metabolism and flavonoid biosynthesis pathways were induced, whereas photosynthesis pathways were suppressed, indicating the involvement of GmRPA in IDC tolerance as a negative regulator (Atwood et al., 2014). In a recent study, the DEGs were identified in the first trifoliate leaves of efficient Clark line under one and six hours of Fe deficiency (Lauter et al., 2014). Similar to previous reports, the expression levels of genes involved in hormonal signaling, DNA replication and Fe uptake were differentially altered under early Fe-deficiency treatment. The expression levels of transcription factors that had not been linked to IDC in previous studies were also found to be specifically altered in early Fe-deficiency treatment. DEGs identified in soybean transcriptomic studies under Fe-deficiency were grouped together to generate a list of common IDC-related transcripts (Aksoy et al., 2017). A total of 33 genes were identified as core IDC-related transcripts in soybean. Many of the core IDC-related transcripts in soybean were linked to Fe-deficiency responses or IDC tolerance in Arabidopsis; however, some are unique to soybean, such as Mini-chromosome maintenance proteins, *CYCLIN-DEPENDENT KINASED1;1*, and *CHROMATIN REMODELING8*. These genes are involved in the control of replication or transcription. Unexpectedly, some other soybean-specific genes linked to IDC are involved in lipid metabolism or protein folding, indicating a species-specific IDC response. Genes involved in phenylpropanoid synthesis pathway were induced specifically in IDC-treated soybean roots, suggesting a new type of IDC-tolerance mechanism specifically activated in IDC-tolerant soybean cultivars (Waters, 2016).

2.11 Possible Strategies to Ameliorate Iron Deficiency Chlorosis

2.11.1 The use of iron chelates

A limited success has been achieved to cure IDC-related subsequent yield losses in soybean through conventional approaches (Goos and Johnson, 2000). The conventional approaches include addition of Fe supplements and conventional breeding for development of IDC-tolerant genotypes. The Fe-supplementation is not a wise and cost-effective strategy as IDC evolves due to non-availability of Fe, rather than deficiency of Fe in the soils (Guerinot 2001). Amid these reasons, Fe fertilizers are still applied to ameliorate IDC in soybean in different regions of the world (Abadia et al., 2011). Different parameters based on ligands has been selected as criterion for the efficacy of fertilizer used in correcting Fe deficiency. These parameters include ability of ligands to maintain higher amounts of Fe in the solution, enabling the plants to use Fe, and when ligands are free they should uptake more Fe and supply to plants again (Lucena, 2003). This implies that the ligand should have affinity for Fe, high solubility in water and bioavailability to the plant.

The most commonly used Fe-chelators in agriculture for correcting Fe deficiency include ethylenediaminetetra acetic acid (EDTA) and ethylenediamine-N,N'-bis (2-hydroxyphenylacetic acid) (EDDHA) (Abadia et al., 2011). EDTA is unable to maintain the required Fe amounts under hydroponic conditions because of less available Fe in soil compared with EDDHA (Lucena, 2003). The compounds such as di ortho- (Hydroxy Benzyl Ethylenediamine Diacetic) acid (HBED) and N,N’-bis (2-hydroxy-5-methylbenzyl) ethylenediamine-N,N’-diacetic acid (HJB) are hexadentate ligands and have been tested for their ability to maintain Fe in soil solution (Lopez-Rayo et al., 2009). Both compounds have proven effective in maintaining the required amounts of Fe under calcareous soils (Nadal et al., 2012, 2013). From the chemical point of view, the previously mentioned chelating agents are hexadentate ligands of the polyaminocarboxylate family (Alvarez-Fernandez et al., 2005; Gomez- Gallego et al., 2005; Lopez-Rayo et al., 2009). Although it is recognized that the polyaminocarboxylate chelating agents are efficient in the treatment of IDC, the ligands are under investigation due to their persistence in the environment (Nowack, 2002, 2008). The limited amount of alternative Fe complexes calls for the identification of novel chelators which can be highly soluble, cost effective, highly bioavailable to the plant and environment-friendly.

For strategy I plants, the Fe chelates and complexes are the fertilizers that provide a fast recovery. Studies about the efficacy of Fe chelates and complexes should focus not only in economics but also in environmental costs. Traditionally, EDDHA/Fe^{3+} (ethylenediamine-N, N-bis (hydroxyphenylacetic) acid-based products have been used to control and solve this problem (Alvarez-Fernandez et al., 2005; Lucena, 2006). These chelates have a high stability in calcareous soils, being able to maintain Fe in the soil solution and transport it to the plant roots (Lopez-Rayo et al., 2009). However, there has been growing concern about the environmental risk of synthetic chelate application (Hyvonen et al., 2003); thus, other chelating agents, such as ethylene diamine disuccinic acid or IDHA (N-(1,2-dicarboxyethyl)-D,L-aspartic acid), have been tested as a biodegradable alternative to the use of recalcitrant products (Villen et al., 2007; Lucena et al., 2008; Rodríguez-Lucena et al., 2010). Both EDDS/Fe^{3+} and IDHA/Fe^{3+} are non-phenolic chelates structurally similar to EDTA/Fe^{3+} (ethylenediaminetetra acetic acid).

Several types of fertilizers are used with diverse success in correcting iron chlorosis. Inorganic salts, such as iron sulfate ($FeSO_4$) are normally of scarce effectivity. The ferrous phosphate Vivianite (Rosado et al., 2002) or the stabilized polyphosphates (Chandra et al., 2009) have a slow release behavior. Recently other nanoparticle materials such as the nonosponges are being studied (Vercelli et al., 2015).

2.11.2 Transgenics

Ferritin is known to store iron. By transferring the entire coding sequence of soybean ferritin gene in rice, the rice iron content was increased (Goto et al., 1999). The iron content of T1 seeds was as much as threefold greater than that of their untransformed counterparts. Recently transgenic rice plants were developed by over-expressing *OsIRT1* (Lee et al., 2009). This over-expression caused plant architecture to be altered. In addition, those plants were sensitive to excess Zn and Cd, signifying that OsIRT1 also transports those metals. As expected, iron and zinc contents were elevated in the shoots, roots and mature seeds of over-expressing plants. This demonstrates that *OsIRT1* can be used for enhancing micronutrient levels in grains.

Takahashi et al. (2001) reported that a barley genomic DNA fragment containing two *NAAT* genes, which encode for fundamental enzymes involved in the biosynthesis of phytosiderophores, were introduced into rice using Agrobacterium-mediated transformation. As described before in chelation strategy-based plants, phytosiderophores are natural iron chelators secreted from the roots to solubilize iron in the soil. The two transgenes were expressed in response to low iron nutritional status in both the shoots and roots of rice transformants. Transgenic rice expressing the two genes showed an increase in nicotianamine aminotransferase activity and secreted larger amounts of phytosiderophores than non-transgenics under low iron availability. Consequently, the transgenic rice showed an enhanced tolerance to low iron availability and had 4.1 times greater grain yields than that of the nontransformant rice in an alkaline soil.

Nicotianamine (NA) is one of the Fe chelator in plants intricating metal translocation into the seeds. Nozye et al. (2014) reported that soybean plants over- expressing the barley *NA SYNTHASE1* (*HvNAS1*) gene driven by the constitutive *CaMV 35S* promoter were produced using Agrobacterium-mediated transformation. The transgenic soybean plants were not affected by the iron deficiency as they followed normal growth pattern. Four fold increase in NA content was observed as compared to the non-transgenic plants. The level of *HvNAS1* expression was positively correlated with the amount of NA, and a high concentration of NA was maintained in the seeds in succeeding generations. Two-fold increase in seed iron content was observed in *NA SYNTHASE1* (*HvNAS1*) transgenic soybeans as compared to the non-transgenics. Furthermore, the transgenic soybeans showed tolerance to low Fe availability in calcareous soil. These results suggested that increasing the NA content in soybean seeds by the overexpression of *HvNAS1* offers potential benefits in agricultural productivity.

As described before, *FRO2* is the major gene involved in the reduction of Fe^{3+} to Fe^{2+}. Vasconcelos et al. (2006) reported that hetrologous overexpression of *FRO2* in transgenic soybean had showed less decrease in chlorophyll content and biomass production. Overexpression of *FRO2* can be used as an alternative way to reduce the yield losses incurred by low iron availability due to high pH.

The Arabidopsis gene *FRO6* (*AtFRO6*) encodes for a ferric chelate reductase, and it is highly expressed in green tisseus of plants. Ya li et al. (2010) reported that high level expression of the gene *AtFRO6* under the control of a *35S* promoter in transgenic tobacco plants. An increase in ferric reductase activity of 2.13 and 1.26 folds under iron- sufficient or iron-deficient conditions was observed compared to the non-transgenics. The enhanced ferric reductase activity resulted in increased concentrations of ferrous iron and chlorophyll, and reduced the chlorosis in the transgenic plants, compared to the non-transgenic plants. No significant difference was observed in ferric reductase activity and ferrous iron between transgenics and non-transgenics. These results suggest that FRO6 functions as a ferric chelate reductase for iron uptake by leaf cells, and overexpression of *AtFRO6* in transgenic plants can reduce iron deficiency chlorosis.

Unfortunately, the transformation and regeneration efficiencies of soybean are very low compared to other crops (Arias et al., 2017; Li et al., 2017; Raza et al., 2017). Therefore, the number of transgenic soybean studies to overcome IDC is limited.

2.11.3 Selection of tolerant genotypes

The use of Fe-efficient genotypes is the best option to overcome IDC in commercial plants like soybean. Plenty of researchers have focused on identifying molecular markers which could be used in crop improvement programs. There has been a considerable progress in soybean breeding for the development of Fe-efficient genotypes having increased IDC tolerance, and subsequent tracking of Fe-efficient phenotype selection. Unfortunately, these breeding efforts have been suffered from different environmental conditions affecting the observed/developed tolerance levels of IDC (Lin et al., 2000; Charlson et al., 2003).

Since soybean is highly sensitive to Fe-deficiency and subsequent IDC, several physiological and molecular mechanisms controlling uptake, transport and mobilization of Fe have been explored by different scientists (Vasconcelos et al., 2006; Roriz et al., 2014). A good number of genotypes having varying levels of sensitivity to Fe-deficiency are available, which makes soybean a good candidate to study above mentioned mechanisms of Fe-uptake and mobilization (Vasconcelos and Grusak, 2014). Conventional plant breeding is considered as one of the well-accepted measures to select tolerant genotypes; however, it suffers from a limited success until so far (Carvalho and Vasconcelos, 2013).

Selection of IDC-tolerant genotypes is inevitable to boost the soybean yield grown in the areas having dominant calcareous soils (Goos and Johnson, 2000). A unit increase in IDC score reduces 20 % yield of soybean (Froehlich and Fehr, 1981). Thus, IDC score could be used as a reliable predictor or criterion for the selection of Fe-efficient or IDC-tolerant soybean genotypes through simple selection trials under field conditions. Similarly, a linear reduction in the yield with IDC scores has also been observed in field trials (Naeve and Rehm, 2006). Although it seems that IDC score could be used as a criterion for selection of IDC-tolerant genotypes, yield must be taken into account when trials are conducted on calcareous soils or under Fe-deficient conditions. Moreover, several researches have suggested that soluble salts, calcium carbonate equivalent (CCE), soil pH, and soil Fe could not be used as reliable predictors for IDC-induced yield reduction in soybean (Franzen and Richardson, 2000; Hansen et al., 2003). Therefore, in addition to IDC score, more precise parameters should be identified to be used in soybean IDC tolerance breeding programs.

Two different genetic models have been explained to control IDC at molecular level

(Lin et al., 1997). These genetic models have also been shown by classical breeding approaches in earlier studies (Cianzio et al., 1980; Cianzio and Fehr, 1982). One model involves a major gene with modifiers (Cianzio et al., 1980) and associated quantitative trait locus (QTL) was located on chromosome 3, explaining more than 70 % of the phenotypic variations (Lin et al., 1997). The second model involves the identification of multiple genes, which represented a classical quantitative mode of inheritance (Cianzio and Fehr, 1982).

There are limited studies directly reporting the selection of IDC-tolerant soybean genotypes in greenhouse or field trials. The possible reasons for limited number of studies might be unavailability of sufficient germplasm at regional scales (Fairbanks 2000). The major hindrance in the way of breeding for IDC-tolerant cultivars has been the difficulties associated with screening for IDC tolerance using calcareous soils under field, greenhouse, and growth chamber conditions, where chlorosis symptoms cannot be constantly replicated among experiments. The reason for this lack of replication is probably due to the complex chemical and physical criteria in both the plant and soil that must be met in order for chlorosis to occur. The high variation in chlorosis symptoms due to non-genetic factors has reduced the effectiveness of selection for chlorosis tolerance.

In conclusion, physiological effects of IDC can be clearly observed in a larger soybean population in the field. Traditional strategies, such as soil remediation and foliar fertilizer applications, have been used to treat moderate soybean chlorosis (Schenkeveld and Temminghoff, 2011). Unfortunately, these strategies are not suitable for mass applications in the field because of the high cost. It is clear that increasing Fe deficiency will induce IDC and yield reduction in soybean, thus appropriate management options are directly needed to cope with the IDC and make soybean yields stable to fulfill the global requirements of soybean. Selection of iron efficient and tolerant cultivars is the most effective way to overcome IDC in plants. Therefore, the most appropriate strategy to avoid IDC is breeding for tolerant soybean lines. The aim of current study is to understand the physiological, biochemical and molecular responses of 20 different soybean cultivars under iron deficit conditions. Moreover, our primary objective is to evaluate efficient mobilizers for further breeding programs, because of genetic variation among the different genotypes.

The number of molecular studies that concentrate on soybean breeding for higher IDC tolerance has been increased in the literature in the last decade. SSR (Charlson et al. 2005; Charlson et al. 2003; Lin et al. 1997; Wang et al. 2008) and RFLP markers were developed (Lin et al. 1997; Lin et al. 2000) to be used in marker-assisted soybean breeding programs. Summarized in Table 1.1, several Quantitative Trait Loci (QTLs) linked to IDC tolerance in soybean were identified (Butenhoff 2015; Diers et al. 1992; Kassem et al. 2006; Mamidi et al. 2014; Peiffer et al. 2012; Ramamurthy et al. 2014). Several candidate genes that are shown to be related to Fe-deficiency in Arabidopsis were also found in close proximity of the identified QTLs (Table 1.1). These genes include orthologs of known Fe-deficiency response genes, such as *HMA5*, *NRAMP3*, *NAS3*, *FRO2*, *OPT1*, *YSL7*, *MTP8* and *IREG3*. Interestingly, some genes involved in photosystem protection, sugar metabolism and transcriptional control are also located in close proximity of QTLs linked to IDC tolerance in soybean, suggesting a species-specific IDC tolerance response.

Table 1.1. QTLs predicted to be associated with IDC tolerance in soybean

Gene model [a]	**Chromosome**	**Gene symbol**	**Gene annotation**	***Arabidopsis* AGI code**
Fe acquisition or transport				
Glyma01g187500	1	*NAPRT2*	NicotinatePhosphoribosyltransferase 2	AT2G23420
Glyma01g190700	1	*NRAMP3*	NaturalResistance-Associated-Macrophage-Protein 3	AT2G23150
Glyma01g200100	1	*NTR1;5*	Nitrate Transporter 1;5	AT1G32450
Glyma03g227500	3	*NTR1;2*	Nitrate Transporter 1;2	AT1G69850
Glyma03g231200	3	*NAS3*	Nicotianamine Synthase 3	AT1G09240
Glyma05g030700	5	*AHA11*	H(+)-ATPase 11	AT5G62670
Glyma07g067700	7	*FRO2*	Ferric Chelate Reductase 2	AT1G01580
Glyma08g101700	8	*MRP14*	Multidrug Resistance-Associated Protein 14	AT3G59140
Glyma08g102000	8	*MTP12*	Metal Tolerance Protein 12	AT2G04620
Glyma08g120500	8	*NTR1*	Oligopeptide Transporter-Related	AT1G59740
Glyma14g206600	14	*MTP8*	Vacuolar Manganese Transporter 8	AT3G58060
Glyma19g140000	19	*HMA5*	Heavy Metal ATPase 5	AT1G63440
Glyma19g32401	19	*OPT1*	Oligopeptide Transporter 1	AT5G55930
Glyma20g004200	20	*YSL7*	Yellow Stripe-Like 7	AT1G65730
Glyma20g096400	20	*IREG3*	Iron-Regulated Protein 3	AT5G26820
Transcription factors or transcription regulators				
Glyma01g189100	1	*WRKY15*	WRKY DNA-Binding Protein 15	AT2G23320
Glyma01g199200	1	*LSD1*	LSD1 Zinc Finger	AT1G32540
Glyma05g011300	5	*AGO10*	Stabilizer of Iron Transporter	AT5G43810
Glyma14g119000	14	*MYB56*	MYB Domain Protein 56	AT5G17800
Glyma14g181500	14	*BHLH030*	Basic Helix-Loop-Helix (bHLH) DNA-Binding Superfamily Protein	AT1G68810
Glyma14g200200	14	*WRKY33*	WRKY DNA-Binding Protein 33	AT2G38470
Glyma18g056600	18	*WRKY33*	WRKY DNA-Binding Protein 33	AT2G38470

* Adopted from Aksoy et al. (2017) with permission.

[a] According to Gene Call Version Glyma2.0.

CHAPTER III

MATERIALS AND METHODS

Study was conducted from the second week of May to the end of September in 2017. For the first one month of growth, the experiment was set up at greenhouse number 1 of Faculty of Agricultural Sciences and Technologies at Ömer Halisdemir University, Niğde, Turkey. After one month, the pots were carried out to the open netted greenhouse by the first one due to elevated temperatures. The experiment was designed according to complete randomized block design.

3.1 Plant Material

Twenty different cultivars of soybean (*Glycine max* L.) in 3 different development groups were used in this thesis study (**Table 3.1**). The seeds were obtained from Çukurova University and they were either originated from the USA or developed in a local soybean breeding program in Turkey (Kurt et al., 2010). Accordingly, the program was started in 1993 by crossing S4240 and Williams as parents, and it was intended to develop new varieties with superior whitefly tolerance. Cultivar S 4240 was developed by Sandoz Seed Co. in the USA whereas Williams was developed in the USA. S 4240 is a tall cultivar with brown pubescence, purple flower and indeterminate growth habit in maturity group of IV. It has high yield and whitefly resistance. On the other hand, Williams is a mid-maturing cultivar showing indeterminate growth habit with large leaves, brown pubescence, and white flower. It has high yield but whitefly sensitivity. Fe deficiency tolerance of these cultivars developed in the breeding program has never been tested before.

Table 3. 1. Soybean cultivars used in the study

Number	Soybean Cultivar	Origin	Development Group Number
1	Apollo	USA	II
2	Olympus	USA	II
3	Amcor	USA	II
4	Bravo	Turkey	III
5	Atakişi	Turkey	III
6	Sa 88	Turkey	III
7	Arısoy	Turkey	III
8	May-53-12	Turkey	III
9	Nova	Turkey	III
10	A-3127	Turkey	III
11	İlksoy	Turkey	III
12	Ataem 7	Turkey	III
13	Stressland	USA	IV
14	A-1621	Turkey	IV
15	KS-4694	USA	IV
16	Blaze	Turkey	IV
17	S-4240	USA	IV
18	Umut 2002	Turkey	IV
19	Adasoy	Turkey	IV
20	Türksoy	Turkey	IV

3.2 Growth Conditions and Stress Application

Seeds were grown *in vitro* in small tissue culture petri plates for five days on ¼ MS (quarter strength Murashige and Skoog) (Murashige and Skoog, 1962) media containing 0.8 % agar following surface sterilization by washing the seeds in 0.25 % sodium hypochlorite for 5 mins, followed by 3 times wash with distilled water. Seeds were germinated and grown in a growth chamber at 25°C and 60 % humidity. After five days, seedlings with similar root lengths (at similar growth stages) were transferred to the soil mixture. Before putting the seedlings into soil mixture, the roots were dipped into *Rhizobium japonicum* (USDA 110 strain) containing solution, which was prepared by overnight growth of 2 g of *R. japonicum* in 200 mL of Luria-Bertani (LB) liquid medium (McClure and Israel, 1979; Mullen et al., 1988). For each variety, fourteen pots were produced in the greenhouse. Six seedlings were transferred to each pot containing peat and perlite mixture at 2:1 ratio (pot diameter: 25 cm). Half of the pots were grown under Fe deficiency, where the soil pH was around 8-9 by addition of calcium carbonate (18 g/kg soil mixture), while the remaining pots were grown under normal conditions, i.e. without calcium carbonate, where the soil pH were 5-6. Plants were grown for 10 days at 25 ± 1°C (day) and 20 ± 1°C (night) and 50 % humidity under regional light cycle (14/10 h of day/night) under 580 ± 75 μmol PAR m^{-2} s^{-1}. After two weeks, three plants were thinned away from each pot, leaving only three plants per pot for better plant development. Plants were irrigated with well water early in the morning until they reached to R1 stage, then irrigation continued twice daily, once in the morning and in the evening. Remaining agronomic and pest management practices was maintained uniformly in all plants.

3.3 Physiological Measurements

All measurements were taken at two developmental stages: Vegetative growth stage of V2-V3 (25-30 days after germinating seedlings have been transferred to the soil) and regenerative growth stage of R4-R5 (90-100 days after germinating seedlings have been transferred to the soil). V2-V3 stage was chosen since this is the stage where Fe deficiency is the most prominent in soybean leaves, and R4-R5 stage was chosen since plants are the most sensitive to environmental stresses at this stage (Kaiser et al., 2014). In physiological measurements, each pot, including three plants, was taken as one biological replicate (total of 21 plants) from each of Fe deficient and sufficient conditions.

3.3.1 Determination of chlorosis score

When the soybean plants grown in normal and iron deficient conditions reached to the V2-V3 stage, they were scored according to a scale of one to six according to the chlorosis status. According to the chlorosis scale, group 1 is entirely green; group 2 is mild chlorosis; group 3 is completely yellow but the veins are green; group 4 is yellow and chlorosis in the veins; group 5 is full chlorosis (with necrosis); and group 6 is apical chlorosis (chlorosis of developing axillary buds) (Vasconcelos et al., 2006).

3.3.2 SPAD measurement

Following the scoring, SPAD-502 Plus chlorophyll meter (Konica Minolta) was used for the SPAD measurements at different stages of plant growth. SPAD measurements were taken from the top third trifoliate leaves of plants at V2-V3 and R4-R5 developmental stages. Three measurements were taken from each plant replicates (total of 21 plants) grown under Fe deficient and sufficient conditions.

3.3.3 Photosynthesis rate measurement

Photosynthesis rate was measured from the top trifoliate leaves at V2-V3 and R4-R5 stages of the plant by using Licor 6400 portable photosynthesis measurement device at noon. Three measurements were taken from each plant replicates (total of 21 plants) grown under Fe deficient and sufficient conditions.

3.3.4 Fresh and dry weight

The top trifoliate leaves of the plants were collected at V2-V3 and R4-R5 developmental stages. Fresh weight of the leaves was measured by analytical balance (Shimadzu), and recorded. Then, the leaves were dried in between papers at 65°C for 24 hours in an oven, and the dry weight of leaves were measured by analytical balance (Shimadzu), and recorded. Experiment was repeated with 21 biological plant samples grown under Fe deficient and sufficient conditions.

3.3.5 Root length measurement

At V2-V3 developmental stage, the roots are taken out of soil and washed with tap water to get rid of soil particles attached on the roots. Then, the length was measured with a ruler from the shoot attachment point all the way to the tip. Experiment was repeated with 9 biological plant samples grown under Fe deficient and sufficient conditions.

3.4 Biochemical Measurements

3.4.1 Total chlorophyll and carotenoid measurement

The top third trifoliate leaves were collected from the plants at V2-V3 and R4-R5 developmental stages for chlorophyll a, chlorophyll b and carotenoid measurements. Accurately weighted of fresh plant leaf sample was taken and homogenized in tissue homogenizer in 1 mL of 80 % (v/v) acetone (Sigma). Homogenized solution was kept at 4°C overnight for complete extraction. Homogenized sample mixture was centrifuged at 10,000 rpm for 5 minutes at 4°C. Absorption of the supernatant was measured by using a UV-Vis spectrophotometer (UV-1800, Shimadzu) at 470, 646,8 and 663,2 nm against 80 % (v/v) acetone as blank. Total chlorophyll content (chl a + chl b) was calculated according to the formula of (7.15 A663.2 + 18.71 A646.8)/1000/fresh leaf weight (Lichtenthaler, 1987). Measurements will be taken from 4 different plants. Experiment was repeated with four biological plant samples grown under Fe deficient and sufficient conditions.

3.4.2 FRO enzyme activity measurement

Root samples were collected at V2-V3 stage of the plants. FRO enzyme activity was analyzed from the roots by following the method of Aksoy et al. (2013). After the physiological measurements are completed, the roots of the three plants found in one of the normal and inadequate iron pots were removed from the soil. Then, the soil particles entangled in the roots were removed by shaking the roots in distilled water. Approximately 100 mg of five samples per cultivar (15 samples in total for each cultivar) was collected from the roots (close to the tip), washed three times in distilled water, and then the wet weights were weighed after damp drying the roots quickly with a tissue paper. The root samples were then placed in the solution used for FRO enzyme activity determination. The activity solution contains 0.1 mM Fe (III) -EDTA and 0.3 mM ferrous sulfate (both from Sigma), prepared in distilled water. The samples were incubated in the dark for 30-45 minutes at room temperature. The absorbance of the solution is then read at 562 nm against the blank solution. Measurements were taken from four biological plant samples grown under Fe deficient and sufficient conditions.

3.4.3 Metal content analysis

In order to measure the elemental accumulation levels, the top third trifoliate leaf and root samples were collected from the plants exposed to Fe deficiency and sufficiency at V2-V3 developmental stage. Then, the leaves or the roots pooled together after collected from three plants in one pot. In total, three pots were used, each representing one biological replicate. Then, the plants were grown until maturation, and they were left to dry. At the end of the generative period, the seeds were harvested from the plants grown in both Fe deficiency and sufficiency.

For determination of Fe levels, samples from leaves, roots and seeds were first shaken in a mixture containing 2 mM $CaSO_4$ and 10 mM EDTA for 10 minutes and then washed twice with distilled water to eliminate any metal particles attached on sample surface. The samples are then dried with a paper towel and divided into 3 technical repeats to be 100 milligrams (mg) and dried in an acid resistant borosilicate test tube for 48 hours in a 65°C oven (Aksoy et al., 2013). The dried samples were then weighed again and their dry weights were recorded. The samples in the test tubes were stored overnight at room temperature in 4 mL of 98.8% nitric acid (HNO_3) (re-distilled) (Sigma) and 1 mL of concentrated hydrochloric acid (HCl) (trace metal grade) (Sigma). The next day, samples were kept at 100°C for 1 hour, 150°C for 1 hour, 180°C for 1.5 hours, and lastly at 210°C until no liquid is left in the test tubes (Vasconcelos et al., 2006). Muffle furnace was used for acid digestion of samples. The dried specimens were re-dissolved with 10 mL of 2% HNO_3, and the Fe levels were determined in Inductively Coupled Plasma Mass

Spectrometry (ICP-MS) (Bruker Aurora M90) after the necessary dilutions (100 x for the seeds and 500 x for roots and leaves) have been performed using 2% HNO_3. Indium (EMD Millipore) as internal standard and appropriate standards (EMD Millipore) for calibration were used in accordance with the user's manual. Each sample was read five times in the pulse detector mode.

The reason for the use of ICP-MS in determining metal levels is that the amount of biological substance is sufficient for analysis, and the sensitivity of the assay is high due to the fact that the lower detection limit is lower than other similar detection systems. The data obtained was calculated by converting to a metal level of one millionth of a unit in dry weight (ppm / dry weight). The experiment was designed to involve three biological replicates per analysis.

3.5 Molecular Measurements

Based on the physiological and biochemical responses, two tolerant (Arısoy and Sa 88) and two sensitive (Atakişi and Nova) local soybean varieties were selected for molecular response analyses.

3.5.1 Total RNA extraction

For total RNA extraction, the top third trifoliate leaf and root samples were collected from the plants exposed to Fe deficiency and sufficiency at V2-V3 developmental stage, and they were immediately flash frozen with liquid nitrogen and stored at -86°C for RNA extraction. Total RNA was isolated using TRIzol reagent (Sigma) (Chomczynski and Sacchi, 1987). In short, 200 mg of tissues were lysed in cold mortar by help of liquid nitrogen. Then, the powder was transferred into 1.5 mL of Eppendorf tube, and 1 mL of TRIzol (Sigma) was added on top. Then, the samples were mixed in vortex and kept at room temperature for 10 minutes. Later, the tubes were centrifuged at 14.000 rpm at 4°C for 10 minutes, and the supernatant was taken and mixed with equal amount of chloroform after keeping at room temperature for 5 minutes. The tubes were centrifuged at 14.000 rpm at 4°C for 15 minutes. Then, the upper phase was transferred into a clean Eppendorf tube, and 500 µL of cold isopropanol was added. After a brief mixture of the solution in the tubes, they were kept at -20°C for 10 minutes. Then, they were centrifuged at 14.000

rpm at 4°C for 20 minutes. Finally, the predicated pellet was washed with 1 mL of 75 % of ethanol and was dried at room temperature for 10 minutes. Pellet was dissolved in 30 µL of DEPC-treated sterile distilled water. The concentration of total RNA was analyzed in a micro-spectrophotometer (nanodrop) and the quality of the RNA was visualized by 1 % agarose gel electrophoresis.

Total RNA samples were treated with DNase I (Thermo Fisher) to remove genomic DNA contamination according to the manufacturer's manual. Simply, 10 µg of total RNA was mixed with 10 µL of 10X reaction buffer, 10 U of DNase I and DEPC-treated water to make a final volume of 100 µL and incubated at 37°C for 30 minutes. Then, the reaction was stopped by addition of 10 µL of 50 mM EDTA and incubation at 65°C for 10 minutes After DNase I treatments, samples were precipitated by addition of 10 µL of 3M sodium acetate (pH: 5.2) and 330 µL of pre-cooled ethanol overnight at -20°C. Then, the samples were centrifuged at 14.000 rpm at 4°C for 30 minutes. Finally, the predicated pellet was washed with 1 mL of 75 % of ethanol and was dried at room temperature for 10 minutes. Pellet was dissolved in 20 µL of DEPC-treated sterile distilled water. The concentration of total RNA was analyzed in a micro-spectrophotometer (nanodrop) and the quality of the RNA was visualized by 1 % agarose gel electrophoresis.

3.5.2 RT qPCR

Total RNA samples (2 µg) were reverse-transcribed into cDNA using random hexamers and the Superscript III Reverse Transcriptase (Life Technology) in a total volume of 20 µl. One-twentieth of the reverse transcription products were analyzed using Qiagen Real Time PCR (Rotor-Gene Q) and SYBR Green I Master Mix (Life Technologies). In total, four target genes (*GmIRT1-like, GmFRO2-like, GmFERRITIN* and *AtNRAMP-like* [*GmDMT1;1*]) and one internal control gene (mentioned below) was amplified in RT-qPCR (Kaiser et.al., 2003; Santos et al., 2015). The primers for all genes were designed by using Primer3 software and given in **Table 3.2**. The amplification reaction and data analysis were performed as described in Salzman et al. (2005) by using $2^{\Delta\Delta Ct}$ method. Each reaction was run in technical triplicates and the melting curves were analyzed by Rotor-Gene Q 2.3.1.49 Software (Qiagene) to verify that only a single product is amplified.

In order to determine the best internal control gene that is going to be used for data normalization, amplification and melting curves of *ELONGATION FACTOR 1-BETA* (*EFL1B*), *CYCLOPHILIN2* (*CYP2*) and *18S rRNA* were analyzed (Jian et al., 2008). Accordingly, *EFL1B* was found to be the best internal control gene that can be used in iron deficiency studies in soybean. Therefore, it was used as an internal control for the normalization of data. The experiment was designed to involve three biological replicates per analysis.

Table 3. 2. Soybean genes and primers used in RT-qPCR

Gene Name	Accession Number/Homolog locus	Primer Sequences
ELFB1	AT5G60390	F 5’- GTTGAAAAGCCA GGGGACA-3’ R 5’- TCTTACCCCTTGA GCGTGG -3’
CYP2	AT2G21130	F 5’- CGGGACCAGTGTGCTTCTTCA-3’ R 5’- CCCCTCCACTACAAAGGCTCG-3’
18S rRNA	X75080.1	F 5’- TTAGGCCATGGAGGTTTGAG -3’ R 5’- GAGTTGATGACACGCGCTTA -3’
IRT1-like	XM_003520096.2	F 5’- GATTGCACCTGTGACACAAA -3’ R 5’- CAGCAAAGGCCTTAACCATA -3’
FERRITIN	U31648.1	F 5’- CCCCTTATGCCTCTTTCCTC -3’ R 5’- GCTTTTCAGCGTGCTCTCTT -3’
FRO2	XM_003528793.3	F 5’- AGGTGCTTATCTTCTGTTGTACACTTTCCCA-3’ R 5’- GCATTGCAGCTCTCTGTGTTGGA-3’
NRAMP (DMT1)	NM_001249798.2	F 5’- GCTTCCAAAGTCCTCACACC -3’ F 5’- TTGCTATGTCTGGTAATCCCAA -3’

3.6 Statistical Analyses

The data generated in the thesis was analyzed in MINITAB 18 software via variation analysis according to the randomized block design. The differences between the mean values were differentiated in Duncan's new multiple range test (MRT), where the statistical significance was set at 5 %. In some conditions, Fisher’s LSD test was performed following two-way ANOVA (analysis of variance) at significance level of 5 %.

CHAPTER IV

RESULTS

The current study was conducted to infer the morphological, physiological, biochemical and molecular responses of 20 different soybean genotypes under Fe-deficient and Fe-sufficient conditions in a pot experiment at the Department of Agricultural Genetic Engineering, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University. The results obtained during the whole course of the study are described in this chapter.

At the end of V2-V3 developmental stage, the genotypes grown under Fe-sufficient and deficient conditions were photographed and the chlorosis score was determined from the images according to Vasconcelos et al. (2006). The results are presented in Appendix A Accordingly, plants were separated into 4 chlorosis groups, from highest tolerance to the highest sensitivity. A2137, Olympus, Ateom-7, Arisoy, Bravo, Blaze, Ks4694 and Sa88 were found in the first group; Apollo, Turksoy, Stress Land and MAY5312 were found in the second group; Atakisi and Ilksoy were found in the third group; Nova, A1621, Adasoy, Amcor, S4240 and Umut2002 were found in the fourth group.

4.1 Fresh Weight of the top 3rd Trifoliate Leaf at V2-V3 Developmental Stage

The fresh weights of the top 3rd trifoliate leaves at V2-V3 developmental stage were significantly altered by Fe availability regimes, soybean genotypes and their interaction (Table 4.1). Overall, the genotypes grown under Fe-sufficient conditions had more fresh weight of the top 3rd trifoliate leaf at V2-V3 developmental stage than the ones grown under Fe-deficient conditions (Figure 4.1). In general, sensitive varieties showed higher decrease in leaf fresh weight compared to tolerant ones. The most significant decrease in fresh weight under iron deficit conditions was observed in ARISOY (32.30 %). On the other hand, APOLLO, BRAVO, STRESS LAND, MAY-5312, ILKSOY, A3127, OLYMPUS and BLAZE varieties showed decrease in leaf fresh weights; however, these changes were not significant (Figure 4.2). Interestingly, APOLLO showed one of the lowest leaf fresh weights among all genotypes under Fe-sufficient conditions.

Table 4.1. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interaction on fresh weight of the top 3rd trifoliate leaves at V2-V3 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	5.370	5.370	95.206	0.0001*
Genotypes (G)	19	4.709	0.235	4.174	0.0001*
Fe × G	19	1.847	0.097	1.724	0.035*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

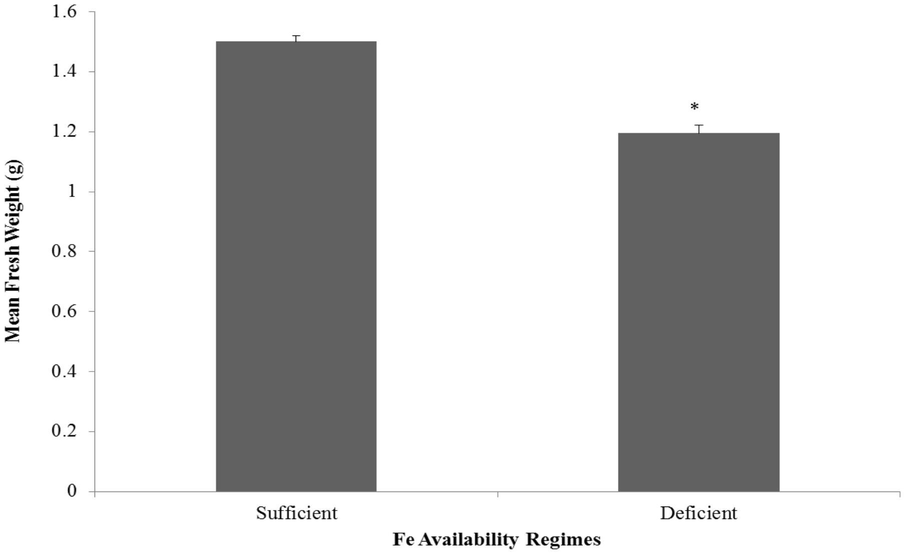

Figure 4.1. The effect of different Fe availability regimes on mean fresh weights of trifoliate leaves of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.2. Interactive effect of Fe availability regimes and soybean genotypes on fresh weight of the top 3rd trifoliate leaves at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient (S)	Fe-Deficient (D)	Percent Change (D / S)
Group 1	**A2137**	1.68 ± 0.09 a-c	1.44 ± 0.15 b-h	-14.28
	Olympus	1.68 ± 0.07 a-b	1.41 ± 0.13 c-i	-16.07
	Ataem-7	1.61 ± 0.11 b-d	1.09 ± 0.11 k-m	-32.30
	Arisoy	1.47 ± 0.12 b-f	0.99 ± 0.12 m	-32.65
	Bravo	1.44 ± 0.04 b-g	1.38 ± 0.07 d-j	-3.82
	Blaze	1.50 ± 0.06 b-e	1.19 ± 0.16 g-m	-20.66
	Ks4694	1.50 ± 0.09 b-e	1.13 ± 0.08 i-m	-24.67
	Sa88	1.61 ± 0.08 b-d	1.12±0.15 j-m	-30.43
Group 2	**Apollo**	1.21 ± 0.01 f-m	1.18± 0.03 h-m	-2.47
	Turksoy	1.94 ± 0.04 a	1.40 ± 0.10 b-f	-27.83
	Stress Land	1.43 ± 0.06 b-h	1.37 ± 0.08 d-j	-4.19
	MAY5312	1.38 ± 0.09 d-j	1.32 ± 0.07 e-k	-4.34
Group 3	**Atakisi**	1.37 ± 0.04 d-j	1.04 ± 0.08 l-m	-24.36
	Ilksoy	1.14 ± 0.06 i-m	1.07 ± 0.02k-m	-6.14
Group 4	**Nova**	1.40 ± 0.05 d-i	1.02 ± 0.08 l-m	-27.40
	A1621	1.51 ± 0.08 b-e	1.08 ± 0.08 k-m	-28.48
	Adasoy	1.29 ± 0.06 e-l	0.97 ± 0.061 m	-24.80
	Amcor	1.60 ± 0.10 b-d	1.15 ± 0.18 i-m	-28.12
	S4240	1.43 ± 0.08 b-h	1.02 ± 0.12 m	-28.67
	Umut2002	1.63 ± 0.06 b-d	1.21 ± 0.05 e-l	-25.77
	LSD value	**0.27375**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

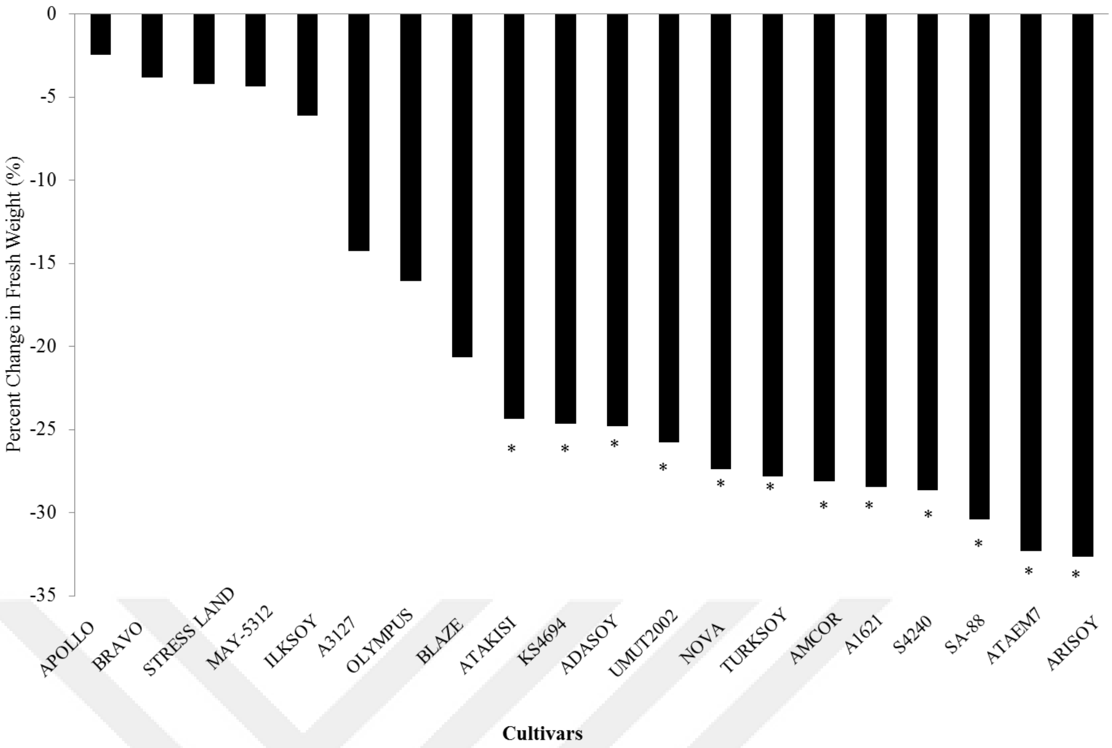

Figure 4. 2. Percent decrease in fresh weight of the top 3rd trifoliate leaves at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.2 Fresh Weight of the top 3rd Trifoliate Leaf at R4-R5 Developmental Stage

The fresh weight of the top 3rd trifoliate leaves at R4-R5 developmental stage was significantly varied by Fe availability regimes, soybean genotypes and their interaction (Table 4.3). Overall, the genotypes grown under Fe-sufficient conditions showed more fresh weight of the top 3rd trifoliate leaves at R4-R5 developmental stage than those found in Fe-deficient conditions (Figure 4.3).

Regarding the interactions among Fe availability regimes and soybean genotypes, the highest fresh weight was recorded for ADASOY under Fe sufficient regime, while the lowest fresh weight was observed for BLAZE under Fe-Deficient environment (Table 4.4). In general, sensitive varieties showed higher decrease in leaf fresh weight compared to tolerant ones.Moreover, significantly a higher decrease in fresh weight between R4-R5 developmental stage was observed in ARISOY (60.1 %), while a lower decrease was observed in APOLLO (1.91 %). These results indicate that the ability of genotypes to synthesize fresh biomass strongly varies according to the growth stage of the crop under Fe deficiency (Figure 4.4).

Table 4.3. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interaction on fresh weight of the top 3rd trifoliate leaves at R4-R5 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	8.630	8.630	193.965	0.0001*
Genotypes (G)	19	5.044	0.265	5.967	0.0001*
Fe × G	19	3.720	0.196	4.400	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

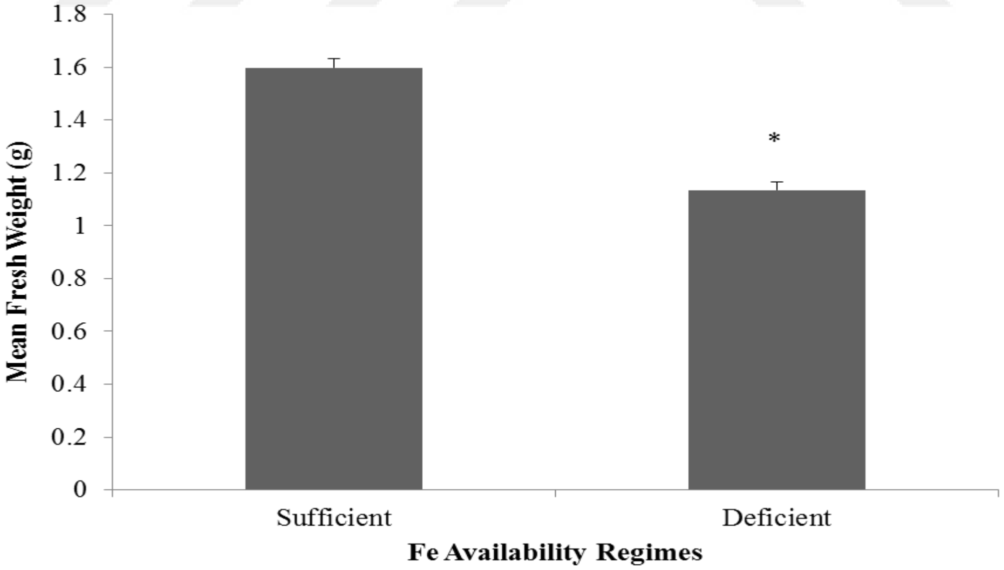

Figure 4.3. The effect of different Fe availability regimes on mean fresh weights of trifoliate leaves of soybean genotypes at R4-R5 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.4. Interactive effect of Fe availability regimes and soybean genotypes on fresh weight of the top 3rd trifoliate leaves at R4-R5 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A2137**	1.69 ± 0.15 c-f	0.98 ± 0.05 n-p	-42.01
	OLYMPUS	1.60 ± 0.13 d-h	1.28 ± 0.11 i-n	-20.00
	ATAEM-7	1.48 ± 0.07 e-k	1.22 ± 0.09 j-n	-17.56
	ARISOY	1.83 ± 0.19 a-d	0.73 ± 0.10 p-q	-60.10
	BRAVO	1.61 ± 0.13 d-h	1.14 ± 0.10 l-n	-29.19
	BLAZE	1.35 ± 0.09 g-m	0.68 ± 0.01 q	-49.63
	KS4694	1.65 ± 0.08 c-f	1.16 ± 0.04 l-n	-29.70
	SA88	2.01 ± 0.12 a-b	1.06 ± 0.08 m-o	-47.26
Group 2	**APOLLO**	1.20 ± 0.06 k-n	1.17 ± 0.07 l-n	-1.91
	TURKSOY	1.59 ± 0.11 d-h	0.83 ± 0.03 o-q	-47.80
	STRESS LAND	1.94 ± 0.03 a-c	1.18 ± 0.13 l-n	-39.18
	MAY5312	1.73 ± 0.10 b-e	1.19 ± 0.08 k-n	-31.21
Group 3	**ATAKISI**	1.21 ± 0.08 j-n	0.94 ± 0.10 n-q	-22.31
	ILKSOY	1.74 ± 0.01 b-e	1.08 ± 0.22 m-o	-37.93
Group 4	**NOVA**	1.34 ± 0.13 h-m	1.11 ± 0.12 m-o	-17.16
	A1621	1.49 ± 0.04 e-j	1.33 ± 0.19 h-m	-10.73
	ADASOY	2.04 ± 0.11 a	1.64 ± 0.11 d-g	-19.60
	AMCOR	1.66 ± 0.05 c-f	1.14 ± 0.12 l-n	-31.32
	S4240	1.54 ± 0.04 d-i	1.42 ± 0.06 f-l	-7.79
	UMUT2002	1.22 ± 0.02 j-n	1.12 ± 0.08 m-o	-8.20
	LSD value	**0.295**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

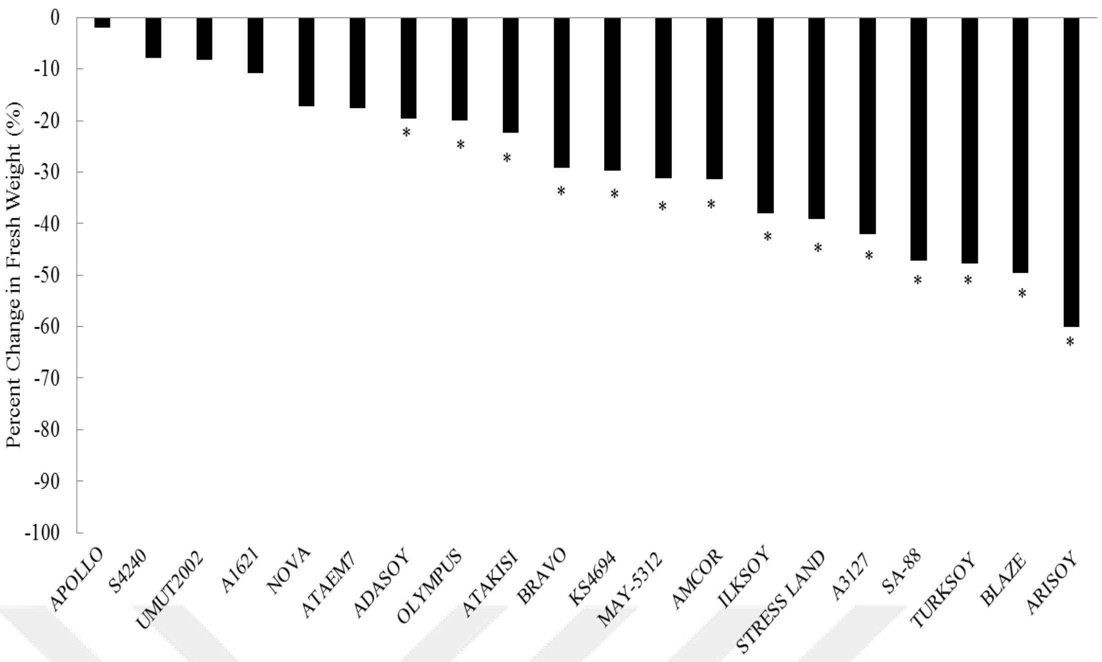

Figure 4.4. Percent decrease in fresh weight of the top 3rd trifoliate leaves at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.3 Dry Weight of the Top 3rd Trifoliate Leaf at V2-V3 Developmental Stage

The dry weight of the top 3rd trifoliate leaves at V2-V3 developmental stage was significantly influenced by Fe availability regimes, soybean genotypes and their interaction (Table 4.5). Overall, the genotypes grown under Fe-sufficient conditions showed higher amounts of dry weight of the top 3rd trifoliate leaves at V2-V3 developmental stage than they do under Fe-deficient conditions (Figure 4.5). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest dry weight was recorded for TURKSOY under Fe sufficient regime, while the lowest dry weight was observed for ARISOY under Fe-deficient environment (Table 4.6). Decrease in dry weight was observed for all the genotypes except for ATAEM 7 and ADASOY. Significantly largest decrease was obtained in BLAZE (48.97 %) while there was no change in BRAVO. Interestingly an increase in dry weight was observed in both ATAEM

7 and ADASOY when exposed to Fe deficiency (Figure 4.6). This suggest that these two varieties might accumulate more nutrients under Fe deficiency.

Table 4.5. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interaction on dry weight of the top 3rd trifoliate leaves at V2-V3 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes(Fe)	1	0.434	0.434	155.784	0.0001*
Genotypes (G)	19	0.461	0.024	8.695	0.0001*
Fe × G	19	0.176	0.009	3.325	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

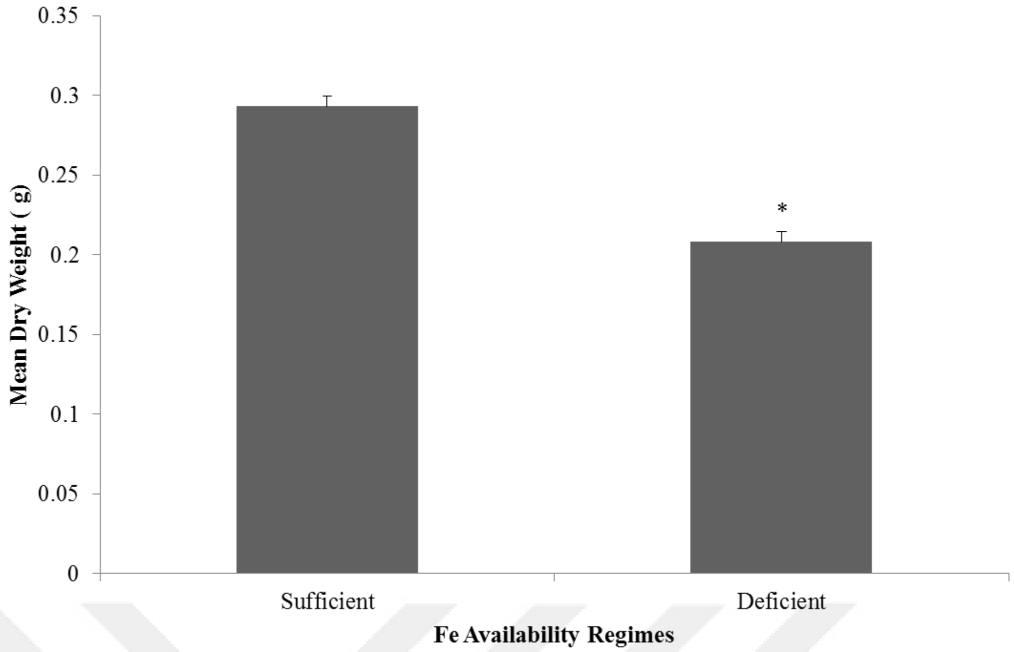

Figure 4. 5. The effect of different Fe availability regimes on mean dry weights of trifoliate leaves of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.6. Interactive effect of Fe availability regimes and soybean genotypes on dry weight of the top 3rd trifoliate leaves at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A2137**	0.24 ± 0.01 g-m	0.20 ± 0.01 l-q	-16.67
	OLYMPUS	0.35 ± 0.02 a-c	0.19 ± 0.02 l-q	-43.43
	ATAEM-7	0.19 ± 0.02 m-q	0.22 ± 0.02 i-o	+15.18
	ARISOY	0.28 ± 0.02 d-i	0.15 ± 0.01 q	-46.43
	BRAVO	0.31 ± 0.01 b-e	0.31 ± 0.04 b-f	0.00
	BLAZE	0.29 ± 0.01 c-h	0.14 ± 0.01 q	-48.97
	KS4694	0.35 ± 0.02 a-c	0.22 ± 0.01 j-o	-37.14
	SA88	0.30 ± 0.04 c-g	0.27 ± 0.02 e-j	-10.00
Group 2	**APOLLO**	0.23 ± 0.01 h-n	0.20 ± 0.01 l-q	-8.70
	TURKSOY	0.38 ± 0.01 a	0.24 ± 0.01 h-m	-36.58
	STRESS LAND	0.32 ± 0.01 b-e	0.27 ± 0.03 e-k	-15.63
	MAY5312	0.23 ± 0.01 h-n	0.21 ± 0.01 k-p	-8.70
Group 3	**ATAKISI**	0.32 ± 0.01 b-e	0.18 ± 0.01 m-q	-41.88
	ILKSOY	0.22 ± 0.01 i-o	0.19 ± 0.02 m-q	-13.64
Group 4	**NOVA**	0.25 ± 0.01 f-m	0.19 ± 0.01 m-q	-24.00
	A1621	0.32 ± 0.02 b-e	0.18 ± 0.01 n-q	-43.75
	ADASOY	0.14 ± 0.01 q	0.16 ± 0.01 o-q	+18.57
	AMCOR	0.27 ± 0.01 e-k	0.15 ± 0.01 p-q	-44.44
	S4240	0.37 ± 0.02 a-b	0.26 ± 0.03 e-k	-29.73
	UMUT2002	0.34 ± 0.01 a-d	0.17 ± 0.01 n-q	-47.65
	LSD	**0.061**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

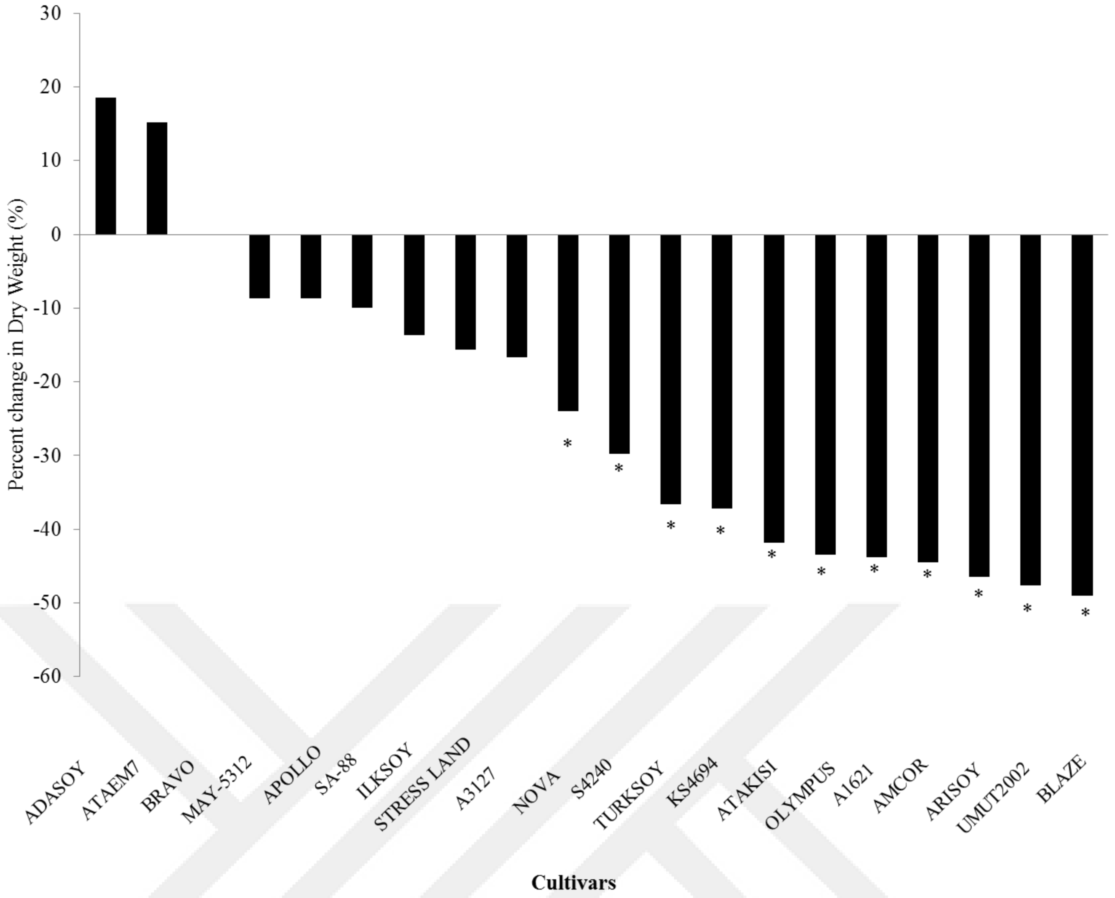

Figure 4. 6. Percent change in dry weight of the top 3rd trifoliate leaves at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.4 Dry Weight of the Top 3rd Trifoliate Leaf at R4-R5 Growth Stage

The dry weight of the top 3rd trifoliate leaves at R4-R5 developmental stage was significantly influenced by Fe availability regimes, soybean genotypes and their interaction (Table 4.7). Overall, the genotypes grown under Fe-sufficient conditions showed higher levels of dry weight of the top 3rd trifoliate leaves at R4-R5 developmental stage than they did under Fe-deficient conditions (Figure 4.7).

Regarding interactions among Fe availability regimes and soybean genotypes, the highest dry weight was recorded for BRAVO under Fe-sufficient regime, while the lowest dry weight was observed for ILKSOY under Fe-deficient environment (Table 4.8). Moreover, a decline in dry weight was observed for all the cultivars when exposed to Fe deficiency.

The highest significant decrease in dry weight was observed in BLAZE (54.45 %) and all others cultivars showed non-significant decrease in dry weight, the lowest decrease in dry weight was observed for STRESSLAND (non-signicant) (2.05 %) (Figure 4.8).

Table 4.7. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interaction on dry weight of the top 3rd trifoliate leaves at R4-R5 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	0.389	0.389	137.229	0.0001*
Genotypes (G)	19	0.452	0.024	8.392	0.0001*
Fe × G	19	0.236	0.012	4.389	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant (p<0.05)

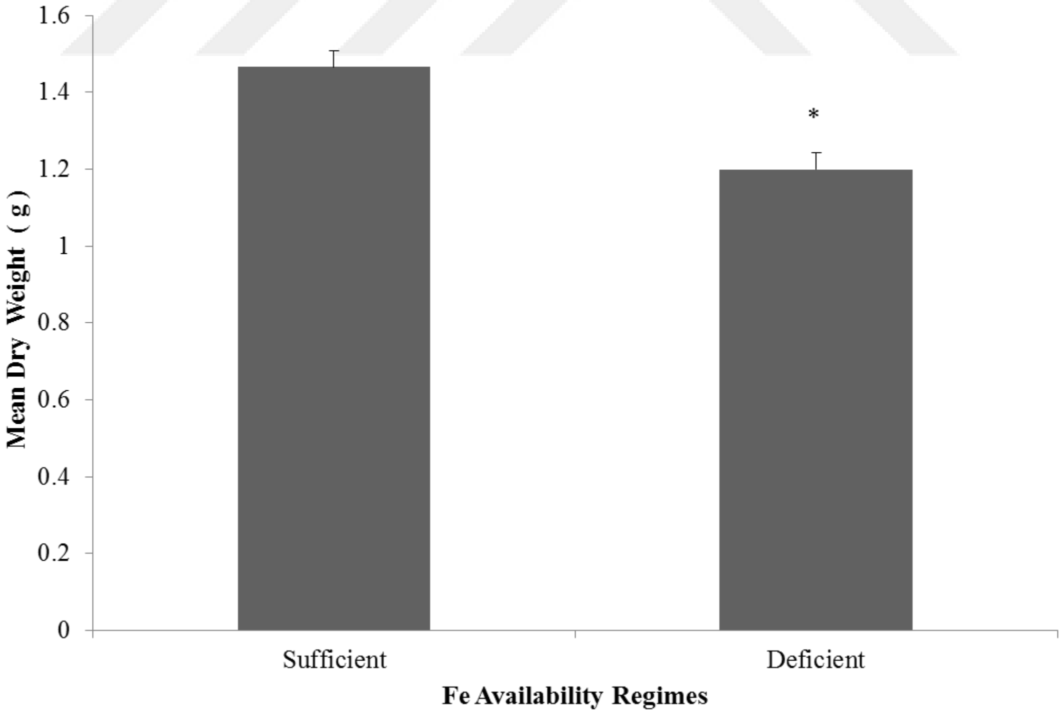

Figure 4. 7. The effect of different Fe availability regimes on mean dry weights of trifoliate leaves of soybean genotypes at R4-R5 developmental stage. * indicates

significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.8. Interactive effect of Fe availability regimes and soybean genotypes on dry weight of the top 3rd trifoliate leaves at R4-R5 developmental stage

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A2137**	1.11 ± 0.06 h-p	1.03 ± 0.23 k-p	-7.12
	OLYMPUS	1.66 ± 0.13 a-f	1.18 ± 0.11 g-p	-28.92
	ATAEM-7	1.26 ± 0.33 f-o	0.90 ± 0.05 m-p	-28.57
	ARISOY	1.33 ± 0.01 e-l	0.94 ± 0.23 l-p	-29.17
	BRAVO	2.03 ± 0.23 a	1.84 ± 0.02 a-d	-9.38
	BLAZE	1.91 ± 0.08 a-b	0.87 ± 0.09 o-p	-54.45
	KS4694	1.42 ± 0.10 c-k	1.08 ± 0.13 j-p	-24.37
	SA88	1.52 ± 0.21 b-h	1.28 ± 0.14 e-o	-15.79
Group 2	**APOLLO**	1.54 ± 0.11 b-g	1.26 ± 0.16 f-o	-18.18
	TURKSOY	1.42 ± 0.25 c-k	1.10 ± 0.13 i-p	-22.54
	STRESS LAND	1.46 ± 0.03 c-j	1.43 ± 0.10 c-k	-2.05
	MAY5312	1.38 ± 0.05 e-k	1.31 ± 0.12 e-m	-5.07
Group 3	**ATAKISI**	1.53 ± 0.03 b-g	1.41 ± 0.14 d-k	-7.84
	ILKSOY	0.89 ± 0.01 n-p	0.76 ± 0.05 p	-14.61
Group 4	**NOVA**	1.25 ± 0.04 f-o	1.07 ± 0.19 j-p	-14.40
	A1621	1.36 ± 0.08 e-l	1.12 ± 0.04 g-p	-17.65
	ADASOY	1.83 ± 0.14 a-c	1.69 ± 0.20 a-e	-7.65
	AMCOR	1.31 ± 0.09 e-m	1.11 ± 0.18 h-p	-15.27
	S4240	1.49 ± 0.08 b-i	1.29 ± 0.19 e-n	-13.42
	UMUT2002	1.53 ± 0.17 b-g	1.24 ± 0.16 g-o	-18.95
	LSD value	**0.417**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

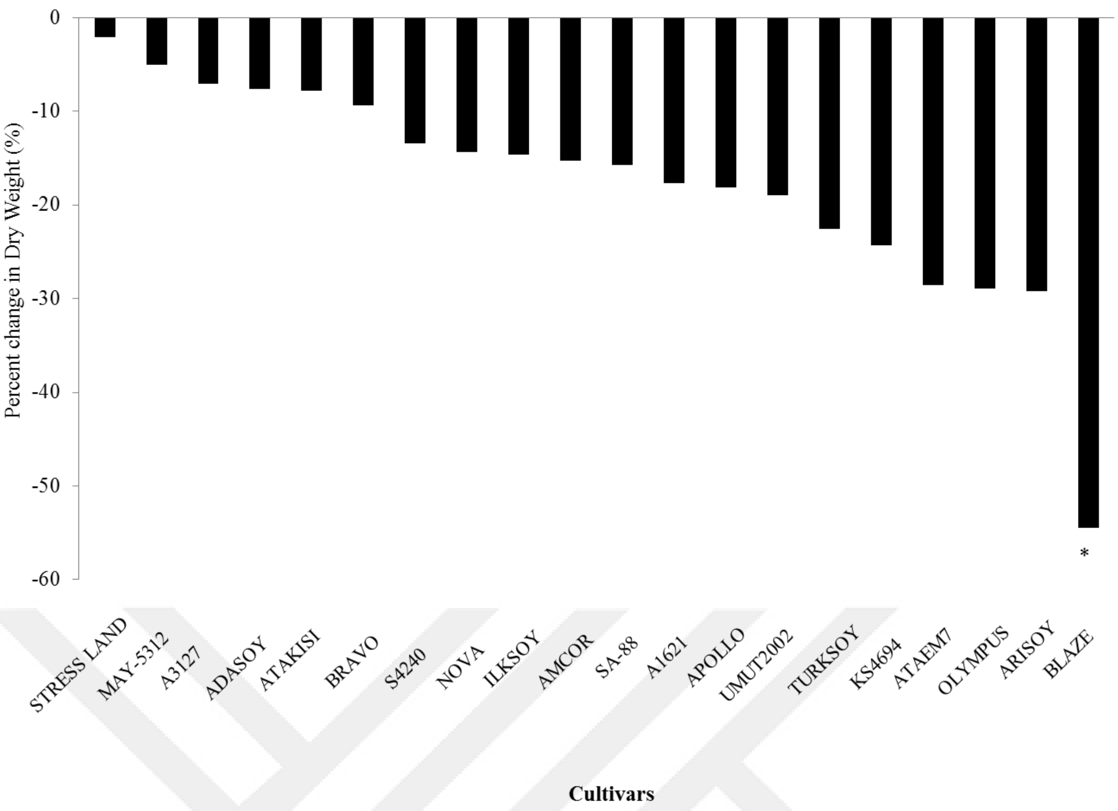

Figure 4. 8. Percent decrease in dry weight of the top 3rd trifoliate leaves at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.5 Root Length at R4-R5 Developmental Stage

The root lengths at V2-V3 developmental stage was significantly affected by Fe availability regimes, soybean genotypes and their interaction (Table 4.9). Overall, the genotypes grown under Fe-deficient conditions had longer root lengths at V2-V3 developmental stage than those grown under Fe-sufficient conditions (Figure 4.9; Appendix B). Regarding the interactions among Fe availability regimes and soybean genotypes, the longest root length was recorded for UMUT2002 under Fe-Deficient regime, while the lowest was observed for APOLLO under Fe-sufficient environment (Table 4.10). All genotypes showed a significant increase in root length under iron deficient conditions. ADASOY (183.62 %) showed significantly the highest increase in root length as compared to the rest of tested genotypes, while significantly lower increase was found in BRAVO (2.81 %) when compared to others under iron deficient conditions (Figure 4.10).

Table 4.9. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on root lengths at V2-V3 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	1	17451.506	17451.5	0.0001*
Genotypes (G)	19	19	15698.469	826.235	0.0001*
Fe × G	19	19	9184.369	483.388	0.000*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant (p<0.05)

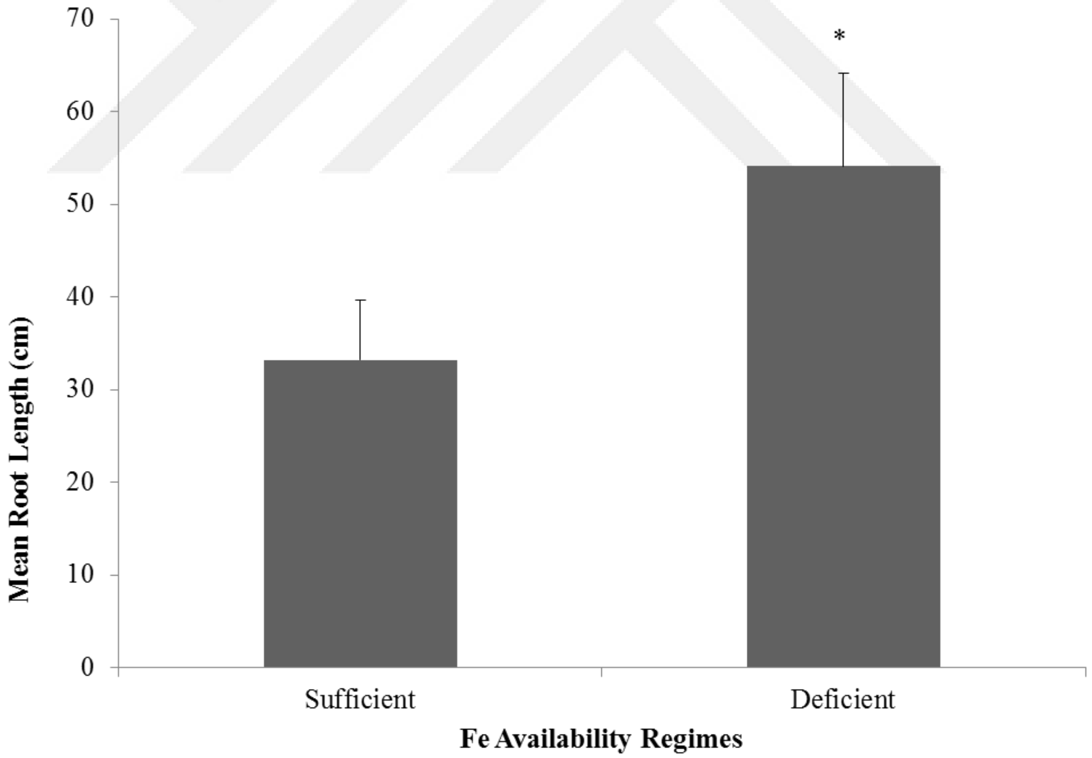

Figure 4. 9. The effect of different Fe availability regimes on mean root lengths of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes (p<0.05).

Table 4.10. Interactive effect of Fe availability regimes and soybean genotypes on root lengths at V2-V3 developmental stage

Chlrosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	A2137	25.25 ± 8.60 o-p	50.00 ± 3.52 f-k	+98.41
	OLYMPUS	27.00 ± 4.88 n-p	35.00 ± 5.75 i-p	+29.62
	ATAEM-7	33.25 ± 7.69 j-p	49.25 ± 5.43 f-k	+48.12
	ARISOY	43.00 ± 5.54 g-o	48.00 ± 8.13 f-k	+11.62
	BRAVO	53.25 ± 7.39 d-h	54.70 ± 8.04 e-j	+2.81
	BLAZE	32.75 ± 8.74 k-p	70.75 ± 4.30 b-d	+116.03
	KS4694	27.00 ± 4.81 n-p	45.00 ± 2.38 g-m	+66.66
	SA88	46.75 ± 14.7 f-l	63.00 ± 9.10 c-f	+34.75
Group 2	APOLLO	25.00 ± 6.31 o	45.00 ± 1.58 g-m	+44.44
	TURKSOY	25.75 ± 4.51 n-p	38.25 ± 1.79 h-p	+48.34
	STRESS LAND	39.50 ± 4.92 h-p	42.25 ± 7.10 g-p	+6.96
	MAY5312	33.75 ± 6.93 j-p	51.75 ± 8.91 e-i	+53.33
Group 3	ATAKISI	28.75 ± 2.71 m-p	42.75 ± 3.34 g-p	+48.69
	ILKSOY	26.75 ± 1.31 n-p	33.75 ± 1.25 j-p	+26.16
Group 4	NOVA	27.00 ± 6.51 n-p	73.75 ± 3.53 a-c	+173.14
	A1621	32.50 ± 3.52 k-p	43.50 ± 0.50 g-n	+33.846
	ADASOY	29.00 ± 3.41 l-p	82.25 ± 4.79 a-b	+183.62
	AMCOR	28.50 ± 5.29 m-p	57.50 ± 0.64 c-g	+101.75
	S4240	29.75 ± 2.04 l-m	68.00 ± 1.93 b-e	+128.571
	UMUT2002	49.75 ± 4.44 f-k	91.50 ± 10.16 a	+83.91
	LSD value	**17.82**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

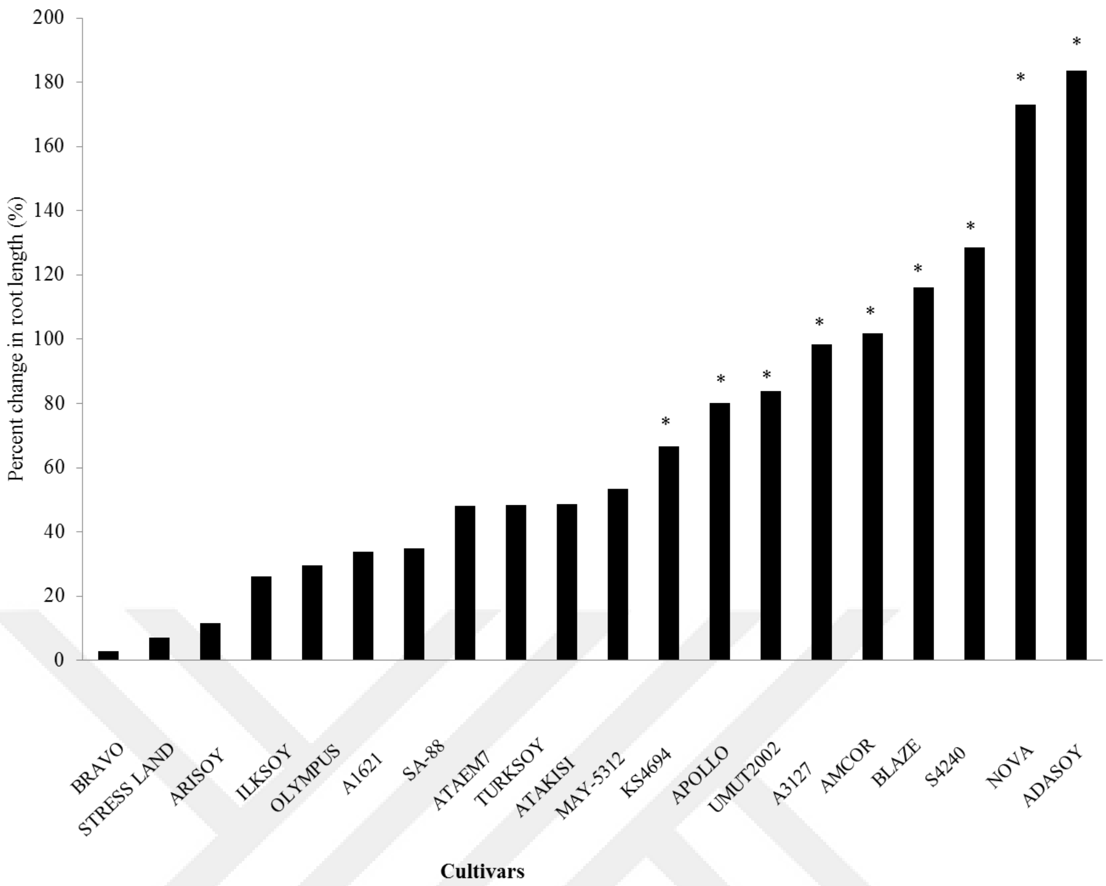

Figure 4.10. Percent change in root lengths at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.6 Chlorophyll Index (SPAD) Value at V2-V3 Developmental Stage

The SPAD value at V2-V3 developmental stage was significantly affected by Fe availability regimes, soybean genotypes and their interaction (Table 4.11). Overall, the genotypes grown under Fe-sufficient conditions recorded higher SPAD values at V2-V3 developmental stage than Fe-deficient conditions (Figure 4.11). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest SPAD value was recorded for ILKSOY under Fe-sufficient regime, while the lowest SPAD value was observed for AMCOR under Fe-deficient environment (Table 4.12). Moreover, significantly the highest decline in SPAD value was observed for AMCOR (33.21 %) while the lowest for BLAZE (2.12%) (non-significant) under iron deficient conditions (Figure 4.12).

Table 4.11. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interaction on SPAD value at V2-V3 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	2812.506	2812.5	341.527	0.0001*
Genotypes (G)	19	916.581	48.24	5.858	0.0001*
Fe × G	19	431.340	22.70	2.757	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$

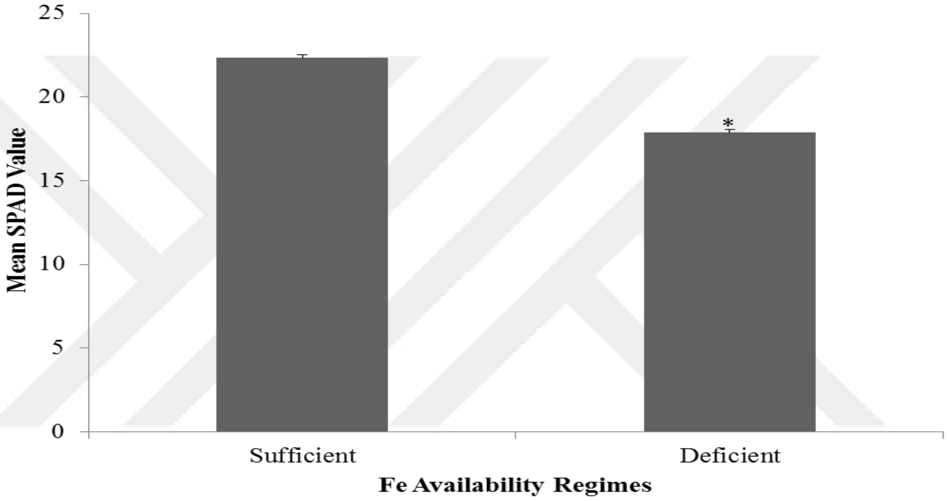

Figure 4.11.The effect of different Fe availability regimes on mean SPAD value of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.12. Interactive effect of Fe availability regimes and soybean genotypes on SPAD values at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change, (D / S)
Group 1	**A2137**	22.71 ± 1.09 b-d	18.71 ± 0.56 j-p	-17.61
	OLYMPUS	24.47 ± 0.83 a-b	17.81 ± 0.57 n-s	-27.22
	ATEAEM-7	21.98 ± 1.10 c-g	16.97 ± 0.46 p-s	-22.79
	ARISOY	21.23 ± 0.38 d-i	17.16 ± 0.48 o-s	-19.17
	BRAVO	24.08 ± 0.87 a-c	19.46 ± 0.87 h-n	-19.19
	BLAZE	20.78 ± 0.72 d-j	20.34 ± 0.57 f-l	-2.12
	KS4694	24.50 ± 1.48 a-b	17.97 ± 0.61 m-s	-26.65
	SA88	20.91 ± 0.48 d-i	16.86 ± 0.85 p-t	-19.37
Group 2	**APOLLO**	22.58 ± 0.77 b-e	20.54 ± 0.71 e-k	-9.03
	TURKSOY	21.54 ± 0.33 d-h	19.42 ± 0.69 h-n	-9.84
	STRESS LAND	25.23 ± 0.95 a	19.93 ± 0.82 g-n	-21.01
	MAY5312	22.68 ± 0.64 b-d	18.51 ± 1.19 k –q	-18.39
Group 3	**ATAKISI**	21.00 ± 0.69 d-i	16.19 ± 0.51 s-t	-22.90
	ILKSOY	25.07 ± 0.38 a	18.36 ± 0.39 l-r	-26.77
Group 4	**NOVA**	21.86 ± 0.25 d-g	15.87 ± 0.54 s-t	-27.40
	A1621	20.90 ± 0.61 d-i	16.29 ± 0.46 r-t	-22.06
	ADASOY	20.07 ± 0.73 f-m	16.48 ± 0.55 q-t	-17.89
	AMCOR	22.10 ± 0.68 c-f	14.76 ± 1.29 t	-33.21
	S4240	21.29 ± 0.68 d-i	19.21 ± 1.27 i-o	-9.77
	UMUT2002	21.93 ± 0.53 d-g	16.43 ± 0.82 q-t	-25.07
	LSD value	**2.130**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

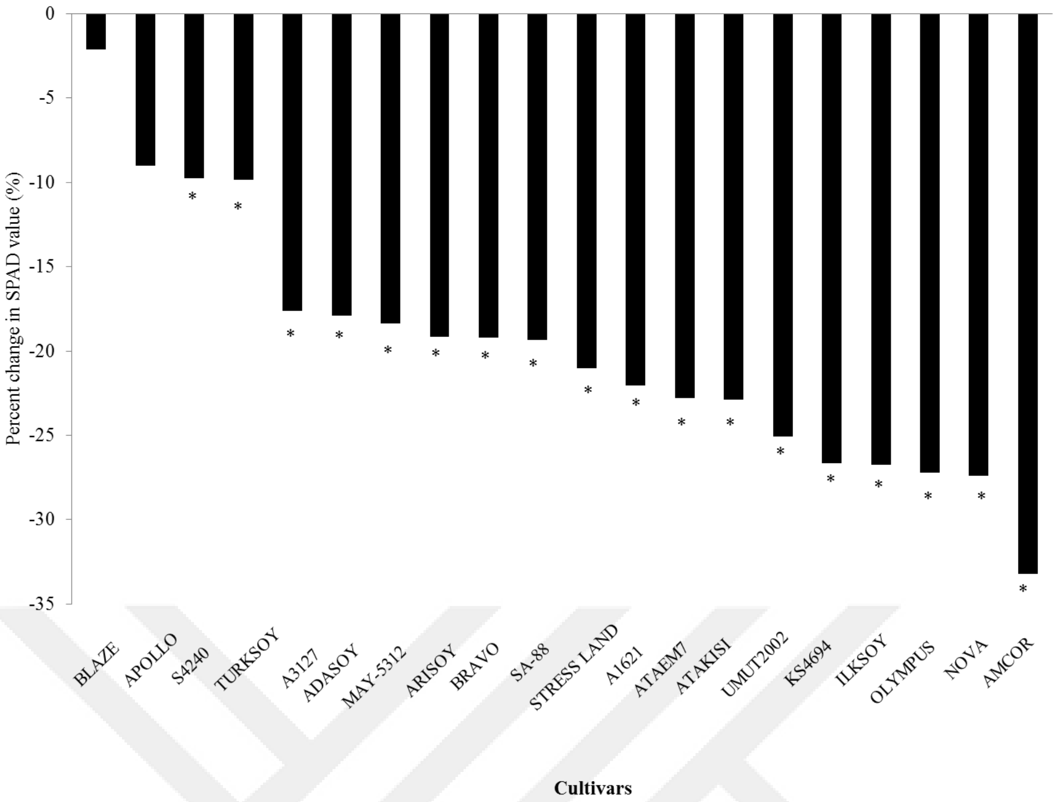

Figure 4. 12. Percent decrease in SPAD values at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.7 Chlorophyll Index (SPAD) Value at R4-R5 Developmental Stage

The SPAD value at R4-R5 developmental stage was significantly altered by Fe availability regimes, soybean genotypes and their interaction (Table 4.13). Overall, the genotypes grown under Fe-sufficient conditions had higher SPAD values at R4-R5 growth stage than Fe-deficient conditions (Figure 4.13). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest SPAD value was recorded for BLAZE under Fe-sufficient regime, while the lowest SPAD was observed for AMCOR under Fe-deficient environment (Table 4.14). Moreover, significantly the highest decrease in SPAD value was observed for ATAKISI (29.27%) and the lowest for STRESSLAND (2

Table 4.13. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on SPAD value at R4-R5 developmental stage

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	1649.236	1649.236	145.450	0.0001*
Genotypes (G)	19	2029.902	106.837	9.422	0.0001*
Fe × G	19	660.350	34.755	3.065	0.0001*

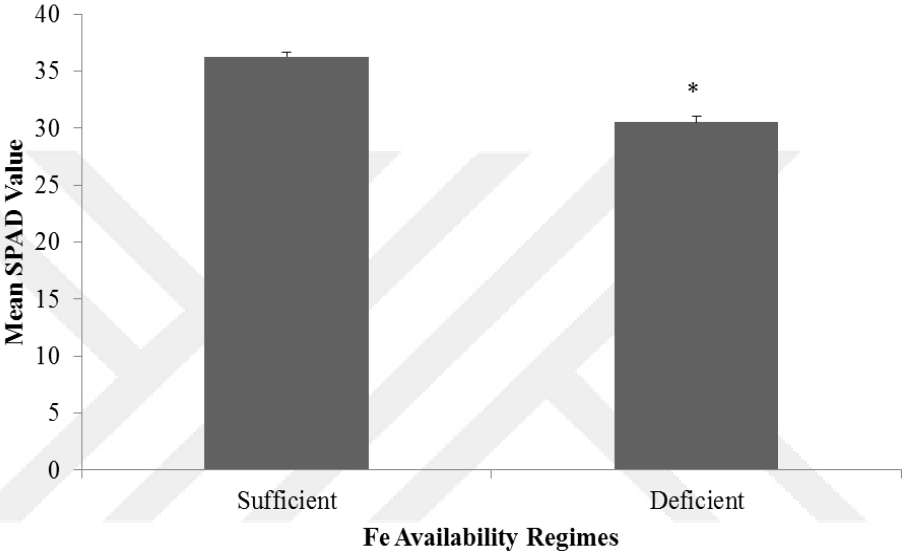

Figure 4. 13. The effect of different Fe availability regimes on SPAD value of soybean genotypes at R4-R5 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.14. Interactive effect of Fe availability regimes and soybean genotypes on SPAD values at R4-R5 developmental stage**.**

	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A2137**	38.82 ± 1.57 b-e	33.02 ± 0.56 g-m	-14.94
	OLYMPUS	39.46 ± 1.14 a-d	34.10 ± 2.24 g-k	-13.58
	ATEOM-7	34.59 ± 0.73 f-j	31.36 ± 0.57 i-o	-9.34
	ARISOY	35.61 ± 1.11 c-h	25.48 ± 2.87 q-s	-28.45
	BRAVO	39.30 ± 0.63 a-d	38.38 ± 3.39 b-f	-2.34
	BLAZE	43.66 ± 0.37 a	33.08 ± 2.54 g-m	-24.23
	KS4694	34.70 ± 1.32 e-j	30.02 ± 1.64 k-p	-13.49
	SA88	36.46 ± 0.70 b-g	29.35 ± 1.76 m-q	-19.50
Group 2	**APOLLO**	38.88 ± 1.16 b-e	34.11 ± 0.74 g-k	-12.27
	TURKSOY	35.75 ± 1.25 b-h	30.97 ± 2.38 j-o	-13.37
	STRESS LAND	34.51 ± 0.53 f-j	33.74 ± 1.60 g-l	-2.23
	MAY5312	39.80 ± 0.57 a-c	28.68 ± 2.28 n-r	-27.92
Group 3	**ATAKISI**	39.39 ± 0.92 b-d	27.86 ± 1.60 o-s	-29.27
	ILKSOY	39.84 ± 0.64 a-b	35.31 ± 0.74 d-i	-11.37
Group 4	**NOVA**	33.60 ± 1.12 g-l	24.59 ± 1.76 r-s	-26.82
	A1621	29.83 ± 0.62 l-p	26.26 ± 1.73 p-s	-11.97
	ADASOY	39.07 ± 1.33 b-d	34.40 ± 2.03 f-j	-11.95
	AMCOR	32.10 ± 0.85 h-n	23.79 ± 1.22 s	-25.89
	S4240	31.14 ± 0.72 i-o	29.83 ± 2.11 l-p	-4.21
	UMUT2002	32.97 ± 1.00 g-m	26.28 ± 1.30 p-s	-20.29
	LSD value	**4.205**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

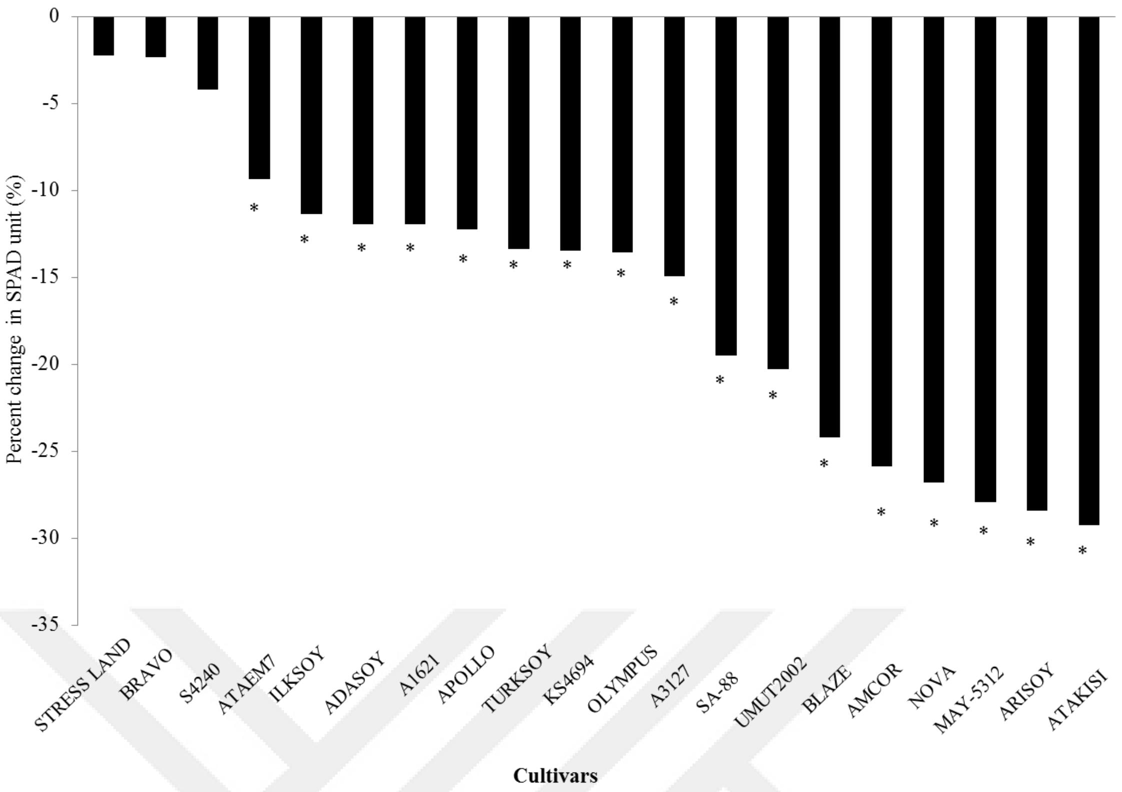

Figure 4. 14. Percent decrease in SPAD values at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.8 Total Chlorophyll Contents at V2-V3 Developmental Stage

The total chlorophyll contents at V2-V3 developmental stage was significantly affected by Fe availability regimes, soybean genotypes and their interaction (Table 4.15). Overall, the genotypes grown under Fe-sufficient conditions recorded higher amounts of total chlorophyll contents at V2-V3 developmental stage than those under Fe-deficient conditions (Figure 4.15). Regarding interactions among Fe availability regimes and soybean genotypes, the highest total chlorophyll content was recorded for ADASOY under Fe-sufficient regime, while the lowest chlorophyll content was observed for ATAKISI under Fe-deficient environment (Table 4.16). Moreover, decrease in chlorophyll contents was observed for all the genotypes except for ATAEM 7, which showed 21.82 % increase in chlorophyll content under Fe deficiency. Similarly, significantlly the highest decrease in chlorophyll content was observed for ATAKISI

(58.65 %) and the lowest decrease was observed for APOLLO (3.9 %) (non-significant) under iron deficient conditions (Figure 4.16).

Table 4.15. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on total chlorophyll content at V2-V3 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	1.963	1.963	423.160	0.0001*
Genotypes (G)	19	1.191	0.063	13.520	0.0001*
Fe × G	19	1.851	0.097	21.010	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

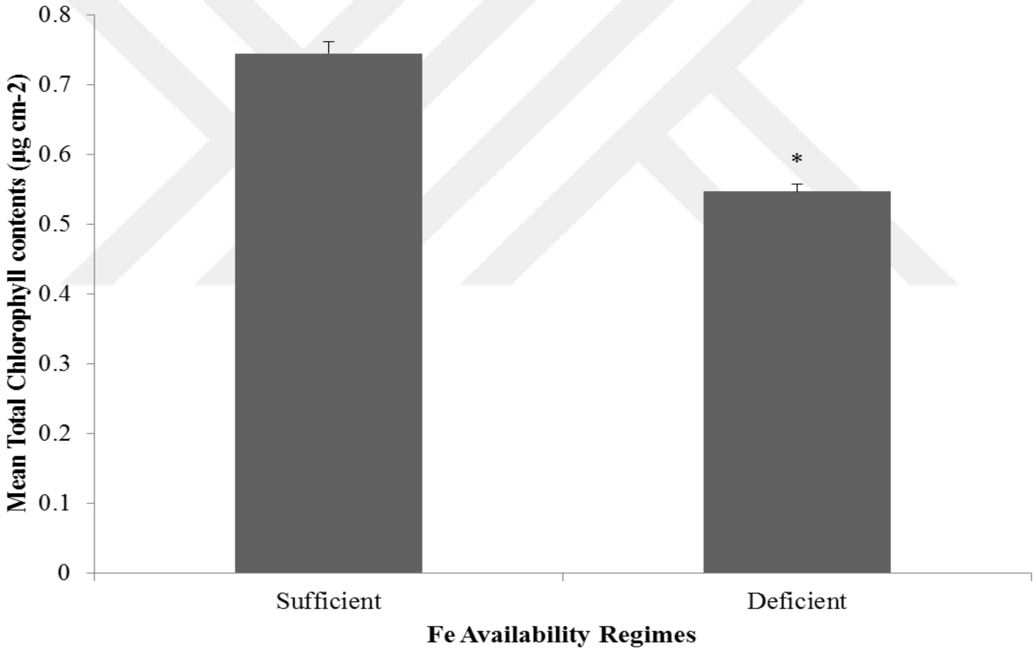

Figure 4.15. The effect of different Fe availability regimes on total chlorophyll content of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.16. Interactive effect of Fe availability regimes and soybean genotypes on total chlorophyll contents at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change
Group 1	**A2137**	0.68 ± 0.01 f-i	0.62 ± 0.01 h-k	-8.82
	OLYMPUS	0.69 ± 0.02 f-i	0.59 ± 0.04 j-n	-14.49
	ATAEM-7	0.55 ± 0.02 k-p	0.67 ± 0.01 f-j	+21.82
	ARISOY	0.60 ± 0.02 j-m	0.54 ± 0.02 l-p	-10.00
	BRAVO	0.71 ± 0.02 e-g	0.65 ± 0.02 e-j	-8.45
	BLAZE	0.61 ± 0.02 i-l	0.44 ± 0.01 r-s	-27.87
	KS4694	0.59 ± 0.01 j-n	0.45 ± 0.02 q-s	-23.73
	SA88	0.65 ± 0.01 g-j	0.51 ± 0.02 n-q	-21.54
Group 2	**APOLLO**	0.77 ± 0.05 c-e	0.74 ± 0.03 d-f	-3.90
	TURKSOY	0.62 ± 0.03 h-l	0.53 ± 0.02 m-q	-14.52
	STRESS LAND	0.88 ± 0.02 a	0.55 ± 0.01 k-p	-37.50
	MAY5312	0.82 ± 0.02 b-c	0.61 ± 0.01 i-l	-25.61
Group 3	**ATAKISI**	1.04 ± 0.02 a	0.43 ± 0.02 s	-58.65
	ILKSOY	0.81 ± 0.03 b-d	0.57 ± 0.02 k-o	-29.63
Group 4	**NOVA**	0.62 ± 0.01 h-l	0.52 ± 0.01 o -r	-16.13
	A1621	0.81 ± 0.03 b-d	0.53 ± 0.02 m-p	-34.57
	ADASOY	1.01 ± 0.03 a	0.45 ± 0.01 q-s	-55.00
	AMCOR	0.70 ± 0.01 e-h	0.49 ± 0.01 p-s	-30.00
	S4240	0.68 ± 0.02 f-i	0.56 ± 0.02 k-p	-17.65
	UMUT2002	0.85 ± 0.03 b-c	0.51 ± 0.02 o-r	-40.00
	LSD value	**0.0737**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

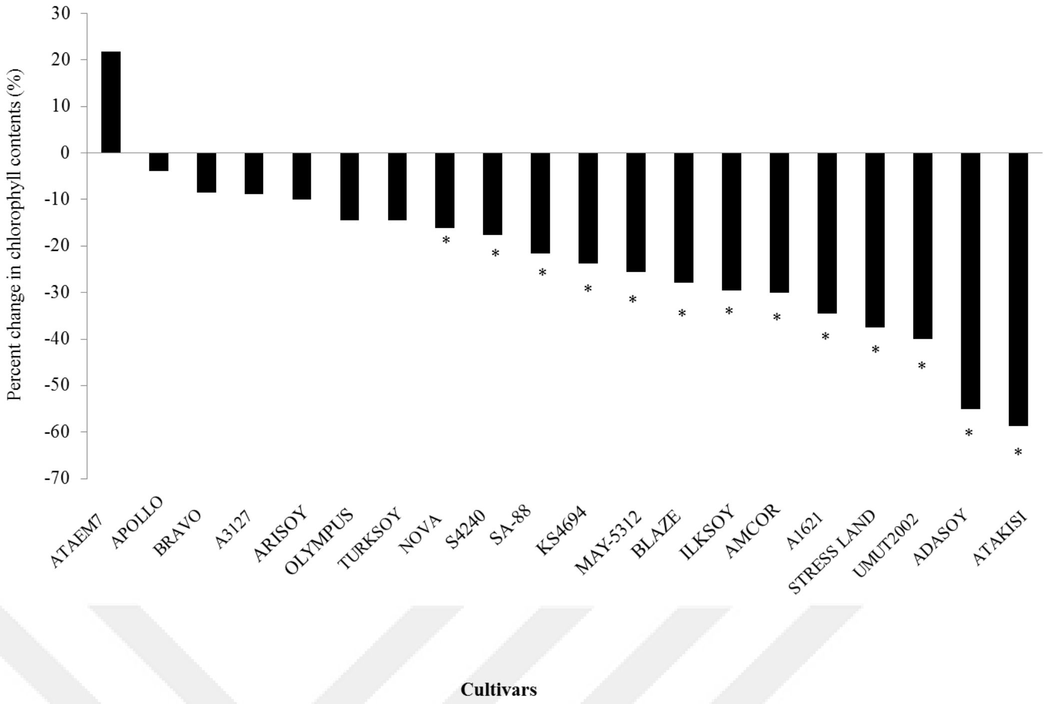

Figure 4. 16. Percent change in total chlorophyll contents at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) (p < 0.05).

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4.9 Total Chlorophyll Contents at R4-R5 Developmental Stage

The total chlorophyll contents at R4-R5 developmental stage was significantly altered by Fe availability regimes, soybean genotypes and their interaction (Table 4.17). Overall, the genotypes grown under Fe-sufficient conditions had higher total chlorophyll contents at R4-R5 developmental stage than those under Fe-deficient environment (Figure 4.17). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest total chlorophyll content was recorded for APOLLO under Fe-sufficient regime, while the lowest was observed for BRAVO under Fe-deficient environment (Table 4.18). Moreover, all genotypes showed decrease in total chlorophyll contents at R4-R5 developmental stage. Similarly, significantlly the highest decrease in chlorophyll content was observed for ARISOY (82.86 %) and the lowest decline was observed for Mays5312 (5.71 %) (non-significant) under iron deficient environment (Figure 4.18).

Table 4.17. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on total chlorophyll content at R4-R5 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	0.004	0.004	4.137	0.045*
Genotypes (G)	19	1.418	0.075	82.870	0.0001*
Fe × G	19	1.123	0.059	65.631	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

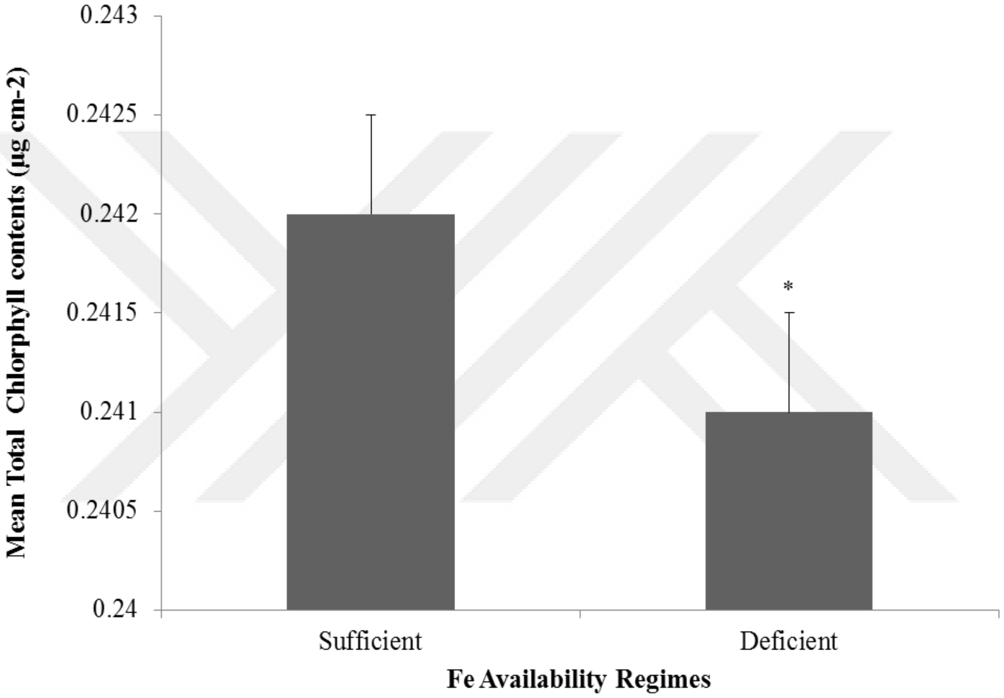

Figure 4. 17. The effect of different Fe availability regimes on total chlorophyll content of soybean genotypes at R4-R5 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.18 Interactive effect of Fe availability regimes and soybean genotypes on total chlorophyll contents at R4-R5 developmental stage**.**

Chlorosis Group	**Genotypes**	**Fe-Sufficient**	**Fe-Deficient**	**Percent Change (D / S)**
Group 1	**A3127**	0.44 ± 0.01 b-c	0.39 ± 0.01 c-d	-11.36
	OLYMPUS	0.44 ± 0.01 b-c	0.41 ± 0.02 b-c	-6.82
	ATAEM-7	0.29 ± 0.01 g-j	0.21 ± 0.02 m-q	-27.59
	ARISOY	0.35 ± 0.01 d-s	0.06 ± 0.02 t	-82.86
	BRAVO	0.28 ± 0.01 h-k	0.07 ± 0.03 t	-75.00
	BLAZE	0.19 ± 0.01 o-s	0.17 ± 0.02 q-s	-10.53
	KS4694	0.32 ± 0.01 e-h	0.15 ± 0.02 s	-53.13
	SA88	0.15 ± 0.01 s	0.07 ± 0.01 t	-53.33
Group 2	**APOLLO**	0.81 ± 0.01 a	0.22 ± 0.02 l-p	-72.84
	TURKSOY	0.26 ± 0.02 i-m	0.17 ± 0.01 p-s	-34.62
	STRESS LAND	0.23 ± 0.01 k-o	0.20 ± 0.01 n-r	-13.04
	MAY5312	0.35 ± 0.01 d-f	0.33 ± 0.01 e-g	-5.71
Group 3	**ATAKISI**	0.27 ± 0.01 i-l	0.18 ± 0.01 p-s	-33.33
	ILKSOY	0.46 ± 0.01 b	0.32 ± 0.02 e-h	-30.43
Group 4	**NOVA**	0.31 ± 0.01 f-i	0.24 ± 0.01 j-n	-22.58
	A1621	0.16 ± 0.01 r-s	0.14 ± 0.02 s	-12.50
	ADASOY	0.16 ± 0.01 r-s	0.05 ± 0.01 t	-68.75
	AMCOR	0.27 ± 0.01 h-k	0.23 ± 0.01 k-n	-14.81
	S4240	0.18 ± 0.01 o-s	0.14 ± 0.01 s	-22.22
	UMUT2002	0.44 ± 0.01 b-c	0.41 ± 0.01 b-c	-6.82
	LSD value	**0.048**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

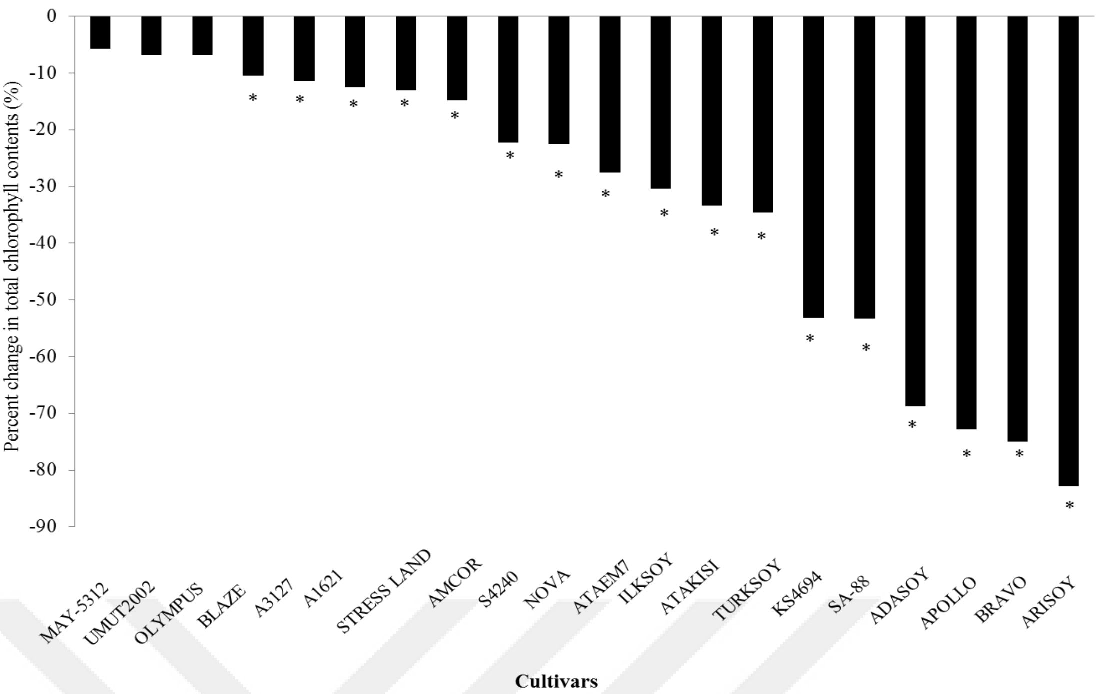

Figure 4.18. Percent change in total chlorophyll contents at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.10 FRO Enzyme Activity

The FRO enzyme activity was determined from the roots of 20 varieties grown at V2-V3 developmental stage under different Fe availabilities. The enzyme activity was significantly influenced by Fe availability regimes, soybean genotypes and their interaction (Table 4.19). Overall, the genotypes grown under Fe-deficient conditions showed significantly higher FRO enzyme activity than Fe-sufficient conditions (Figure 4.19). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest FRO enzyme activity was recorded for ADASOY under Fe-deficient regime, while the lowest FRO activity was observed for BLAZE under Fe-sufficient environment (Table 4.20). Similarly, an increase in FRO activity was observed in all genotypes except for ATAEM-7, which showed 32.92 % decrease in FRO enzyme activity under iron deficient condition (Figure 4.20). The highest significant increase in FRO enzyme activity was observed for BLAZE (1821.23 %) and the lowest increase was observed for ILKSOY (4.69 %) under iron deficient environment (Figure 4.20). FRO activity did not change in the roots of May5312 after Fe deficiency treatment (Table 4.20).

Table 4.19. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on FRO enzyme activity at V2-V3 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	15393.393	15393.393	580.325	0.0001*
Genotypes (G)	19	12169.133	640.481	24.146	0.0001*
Fe × G	19	8808.251	463.592	17.477	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

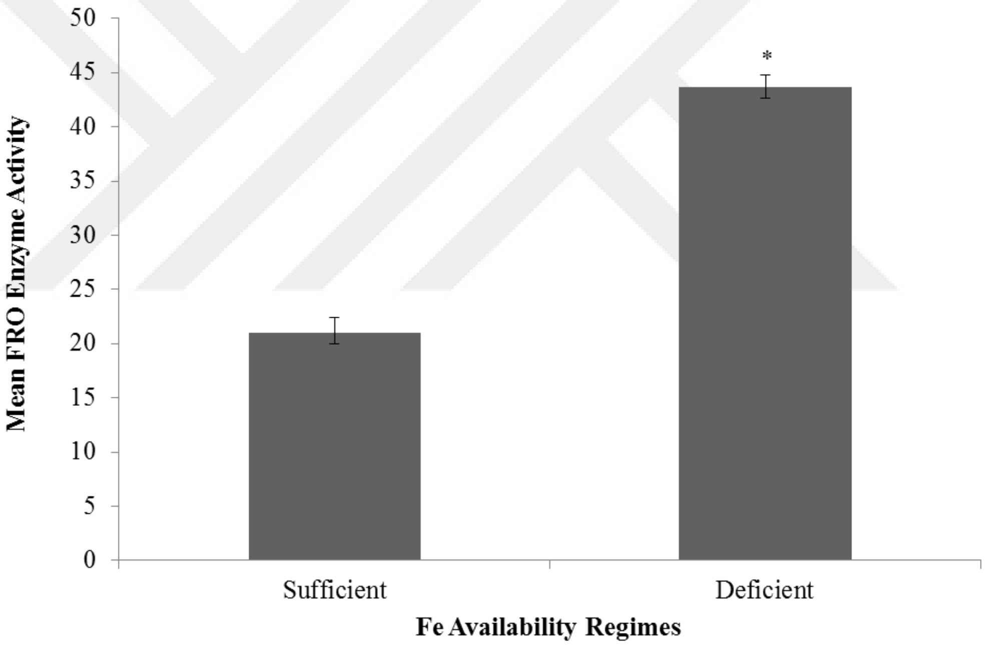

Figure 4. 19. The effect of different Fe availability regimes on FRO enzyme activities of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.20. Interactive effect of Fe availability regimes and soybean genotypes on FRO enzyme activities at V2-V3 developmental stage.

	Genotypes	**Fe-Sufficient**	**Fe-Deficient**	**Percent Change (D / S)**
Group 1	**A3127**	44.27 ± 4.42 d-h	46.97 ± 2.33 e-i	+6.10
	OLYMPUS	24.20 ± 2.76 l-n	45.34 ± 5.10 c-g	+87.38
	ATAEM-7	51.8 ± 3.42 b-d	34.75 ± 2.27 i-k	-32.92
	ARISOY	25.56 ± 4.23 l-m	49.25 ± 8.11 c-e	+92.68
	BRAVO	7.46 ± 1.03 r-s	21.42 ± 3.48 l-n	+187.13
	BLAZE	1.79 ± 0.16 s	34.39 ± 5.57 j-k	+1821.23
	KS4694	12.5 ± 1.91 o-r	58.04 ± 9.57 a-b	+361.73
	SA88	8.78 ± 1.29 q-s	37.68 ± 6.19 g -j	+328.72
Group 2	**APOLLO**	17.60 ± 1.10 m-p	36.77 ± 1.66 h-j	+108.92
	TURKSOY	23.53 ± 0.63 l-n	59.10 ± 3.03 a-b	+151.17
	STRESS LAND	3.57 ± 0.36 s	12.51 ± 1.90 o-r	+250.42
	MAY5312	46.88 ± 2.46 c-f	47.4 ± 3.73 c-f	+1.11
Group 3	**ATAKISI**	9.68 ± 1.58 p-s	51.75 ± 1.61 b-d	+434.61
	ILKSOY	40.9 ± 4.21 e-j	42.82 ± 4.54 f-j	+4.69
Group 4	**NOVA**	22.96 ± 1.94 l-n	40.39 ± 1.96 f-j	+75.91
	A1621	27.46 ± 4.42 k-l	46.30 ± 7.59 c-f	+68.61
	ADASOY	20.11 ± 2.85 l-o	61.41 ± 3.36 a	+205.37
	AMCOR	8.62 ± 1.28 q-s	44.43 ± 7.26 d-h	+415.43
	S4240	5.89 ± 0.82 r-s	53.03 ± 8.81 b-c	+800.34
	UMUT2002	16.88 ± 2.65 n-q	53.23 ± 8.84 a-c	+215.34
	LSD value	**0.048**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

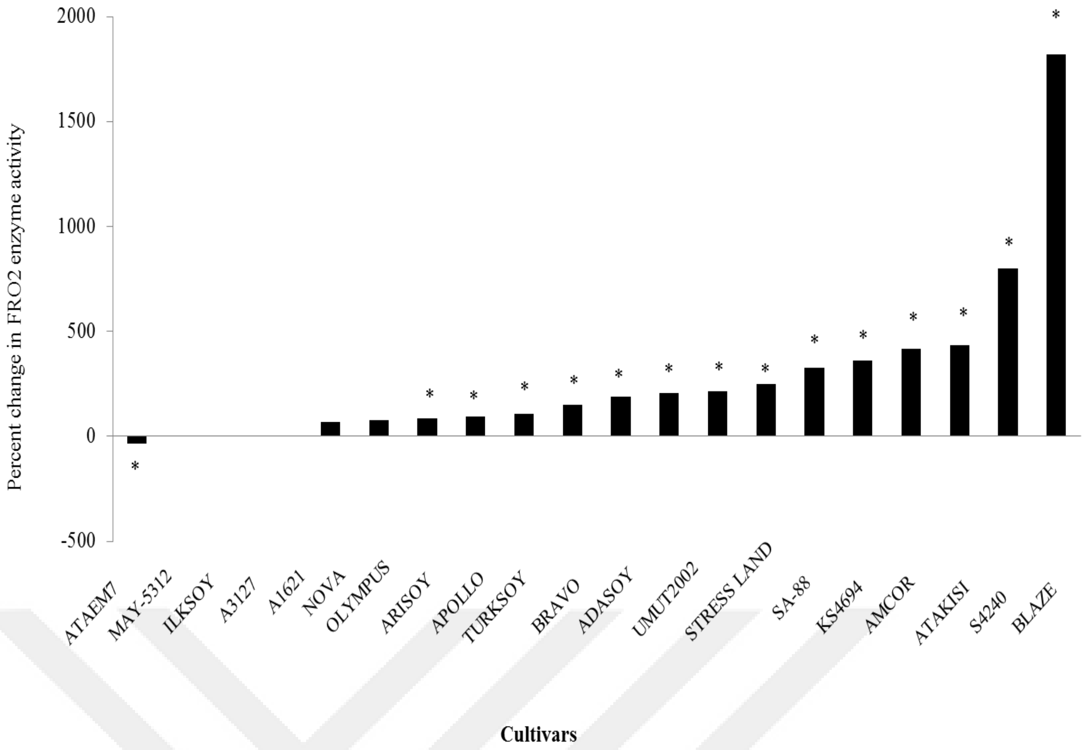

Figure 4. 20. Percent change in FRO enzyme activities at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

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4.11 Photosynthesis Rate at V2-V3 Developmental Stage

The photosynthesis rate at V2-V3 developmental stage was significantly affected by soybean genotypes and interaction among Fe availability regimes and soybean genotypes, while Fe availability regimes had no effect on photosynthesis rate at V2-V3 developmental stage (Table 4.21). This suggest that photosynthesis rate, by itself, is not enough to predict the IDC tolerance of soybean varieties.

The Fe-availability regimes had non-significant effect on photosynthesis rate at V2-V3 developmental stage although there was a decrease (Figure 4.21). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest photosynthesis rate was recorded for APOLLO under Fe-deficient regime, while the lowest was observed for STRESS LAND under Fe-sufficient condition (Table 4.22).

Unexpectedly, APOLLO, ATAEM7, BLAZE, BRAVO, OLYMPUS, SA88 and STRESS LAND showed increase in photosynthesis rates under Fe deficiency. Contrastingly in all other genotypes a decrease in photosynthesis rate was observed at V2-V3 developmental stage under iron deficient condition. Moreover, significantly the highest decrease in photosynthesis rate was observed in ATAKISI (55.9 %) and lower decrease was observed in A1621 (3.18 %) (non-significant) (Figure 4.22).

Table 4.21. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on photosynthesis rate at V2-V3 developmental stage**.**

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	16.718	16.718	2.924	0.088^{NS}
Genotypes (G)	19	2099.943	110.523	19.331	0.0001*
Fe × G	19	1254.285	66.015	11.546	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant (p<0.05)

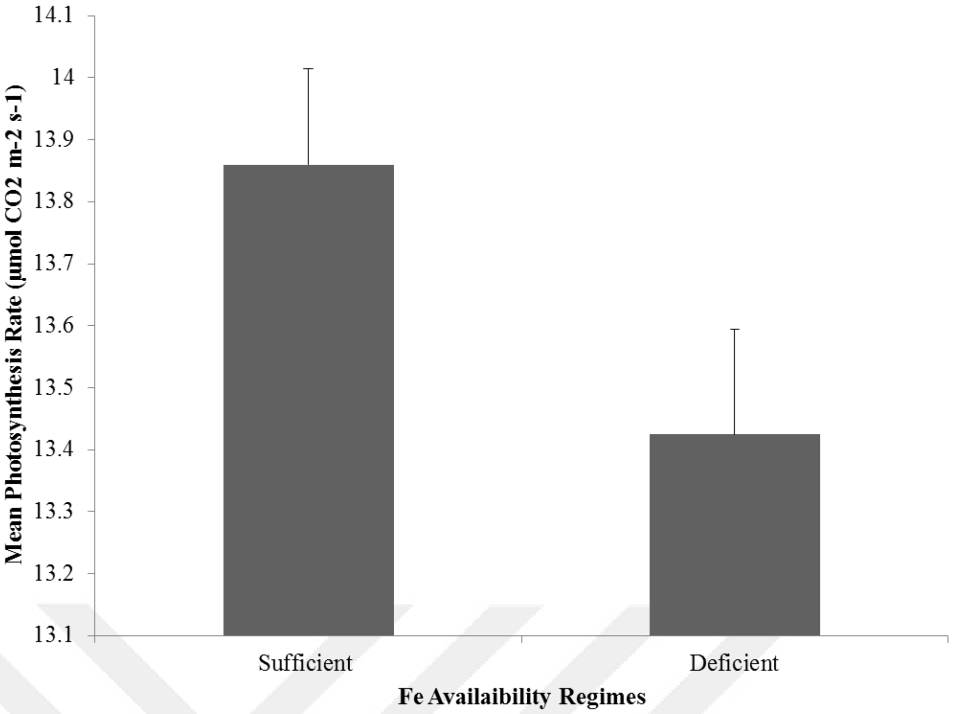

Figure 4.21. The effect of different Fe availability regimes on photosynthesis rates of soybean genotypes at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.22 Interactive effect of Fe availability regimes and soybean genotypes on photosynthesis rates at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	12.90 ± 1.03 k-s	8.54 ± 1.06 u-v	-33.80
	OLYMPUS	17.78 ± 0.98 a-d	18.28 ± 0.87 a-b	+2.81
	ATAEM-7	16.80 ± 1.12 a-f	17.72 ± 0.90 a-e	+5.48
	ARISOY	12.17 ± 0.89 n-s	9.40 ± 0.44 t-u	-22.76
	BRAVO	11.74 ± 2.09 o-s	15.30 ± 0.74 f-j	+30.32
	BLAZE	12.74 ± 1.14 l-s	16.25 ± 0.55 b-g	+27.55
	KS4694	14.83 ± 1.50 f-l	13.64 ± 0.58 h-p	-8.02
	SA88	12.99 ± 3.07 k-r	14.17 ± 0.86 g-n	+9.08
Group 2	**APOLLO**	11.58 ± 1.47 p-t	18.67 ± 0.73 a	+61.23
	TURKSOY	14.70 ± 1.92 f-m	11.58 ± 0.39 p-t	-21.22
	STRESS LAND	4.69 ± 1.85 w	12.46 ± 0.63 n-s	+165.67
	MAY5312	14.37 ± 1.60 g-n	13.68 ± 0.60 h-p	-4.80
Group 3	**ATAKISI**	15.51 ± 0.94 e-i	6.84 ± 0.72 v-w	-55.90
	ILKSOY	16.30 ± 1.23 b-g	12.49 ± 0.56 m-s	-23.37
Group 4	**NOVA**	17.58 ± 1.44 a-e	15.09 ± 0.65 f-k	-14.16
	A1621	13.54 ± 1.41 i-q	13.11 ± 0.73 j-q	-3.18
	ADASOY	18.02 ± 0.89 a-c	15.73 ± 1.05 d-i	-12.71
	AMCOR	11.38 ± 1.10 q-t	10.71 ± 0.42 s-u	-5.89
	S4240	11.60 ± 1.20 p-t	10.89 ± 0.50 r-t	-6.12
	UMUT2002	15.80 ± 1.51 c-h	13.84 ± 0.88 h-o	-12.41
	LSD value	**2.217**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

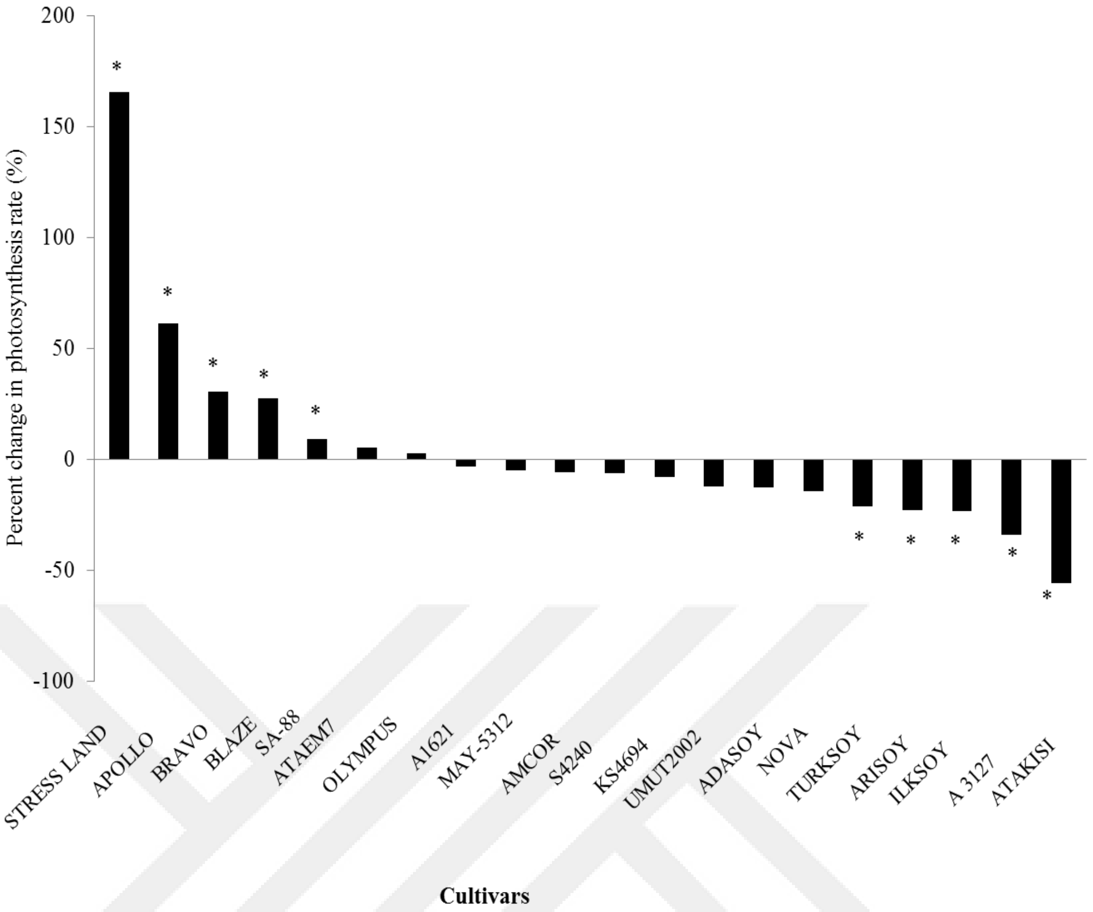

Figure 4.22. Percent change in photosynthesis rates at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

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4.12 Photosynthesis Rate at R4-R5 Developmental Stage

The Fe-availability regimes had significant effect on photosynthesis rate at R4-R5 developmental stage (Table 4.23). Overall, the genotypes grown under Fe-sufficient conditions had higher photosynthesis rates at R4-R5 developmental stage than Fe-deficient conditions (Figure 4.23). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest photosynthesis rate was recorded for NOVA under Fe-sufficient regime, while the lowest was observed for OLYMPUS, MAY5312 and ADASOY under Fe-deficient environment (Table 4.24). Unexpectedly genotypes STRESS LAND, ARISOY and UMUT 2002 showed increase in photosynthesis rates under Fe deficiency. Contrastingly in all other genotypes a decrease in photosynthesis rate was observed at R4-R5 developmental stage under iron deficient conditions (Figure 4.24).

Moreover, significantly the highest decrease in photosynthesis rate was observed in ADASOY (43.26 %) and the lowest decrease was observed in ATAKISI (2.86 %) (non-significant) (Figure 4.22).

Table 4.23. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on photosynthesis rate at R4-R5 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	257.719	257.719	26.945	0.0001*
Genotypes (G)	19	973.291	51.226	5.356	0.0001*
Fe × G	19	689.876	36.309	3.796	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

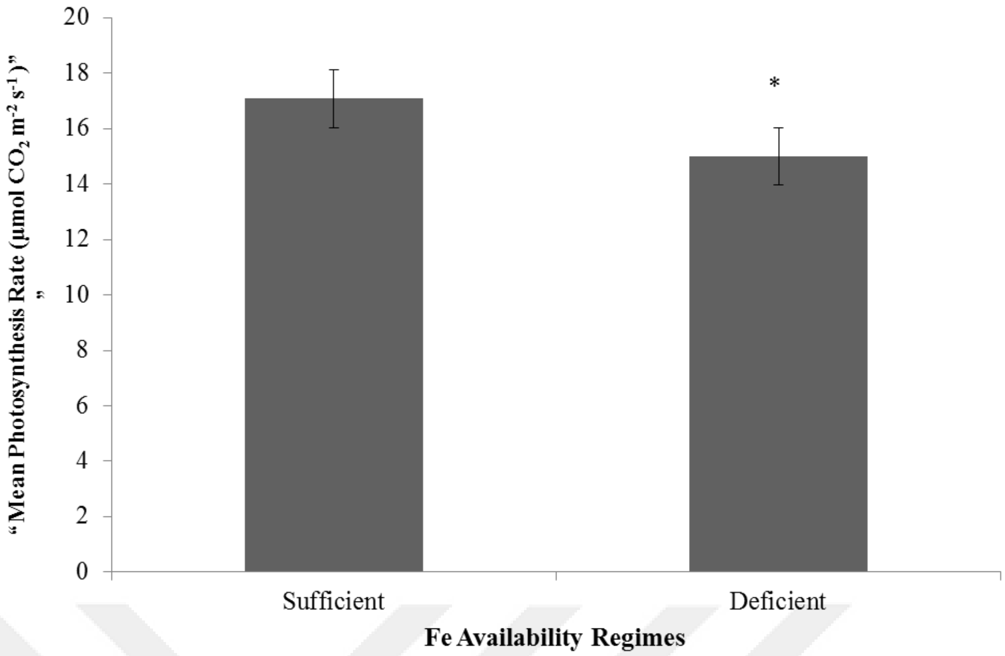

Figure 4. 23. The effect of different Fe availability regimes on photosynthesis rate of soybean genotypes at R4-R5 growth stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.24. Interactive effect of Fe availability regimes and soybean genotypes on photosynthesis rates at R4-R5 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	18.31 ± 0.90 b-e	10.95 ± 1.03 h-i	-40.20
	OLYMPUS	17.46 ± 0.94 c-f	10.43 ± 0.98 i	-40.26
	ATAEM-7	17.06 ± 0.87 c-f	11.16 ± 1.12 h-i	-34.58
	ARISOY	16.89 ± 0.83 c-f	19.44 ± 0.89 b-c	+15.10
	BRAVO	16.56 ± 1.05 c-f	15.39 ± 2.09 d-f	-7.07
	BLAZE	17.63 ± 0.57 c-f	14.73 ± 1.14 f-g	-16.45
	KS4694	15.00 ± 1.04 e-f	14.46 ± 1.50 f-h	-3.60
	SA88	17.89 ± 1.11 b-f	16.06 ± 3.07 c-f	-10.23
Group 2	**APOLLO**	18.55 ± 0.98 b-d	15.98 ± 1.47 c-f	-13.85
	TURKSOY	16.37 ± 1.26 c-f	15.80 ± 1.92 d-f	-3.48
	STRESS LAND	17.30 ± 1.14 c-f	17.73 ± 1.92 b-f	+2.49
	MAY5312	16.84 ± 0.71 c-f	10.77 ± 1.60 i	-36.05
Group 3	**ATAKISI**	15.86 ± 0.35 d-f	15.41 ± 0.94 d-f	-2.84
	ILKSOY	17.34 ± 0.91 c-f	16.73 ± 1.23 c-f	-3.52
Group 4	**NOVA**	21.15 ± 0.79 a-b	18.70 ± 1.44 b-d	-11.58
	A1621	15.36 ± 0.97 d-f	11.44 ± 1.41 g-i	-25.52
	ADASOY	15.65 ± 1.17 d-f	8.88 ± 4.81 i	-43.26
	AMCOR	17.25 ± 0.81 c-f	16.53 ± 7.36 c-f	-4.17
	S4240	16.72 ± 0.68 c-f	15.43 ± 1.20 d-f	-7.72
	UMUT2002	16.85 ± 1.28 c-f	23.47 ± 1.51 a	+39.29
	LSD value	**2.48**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

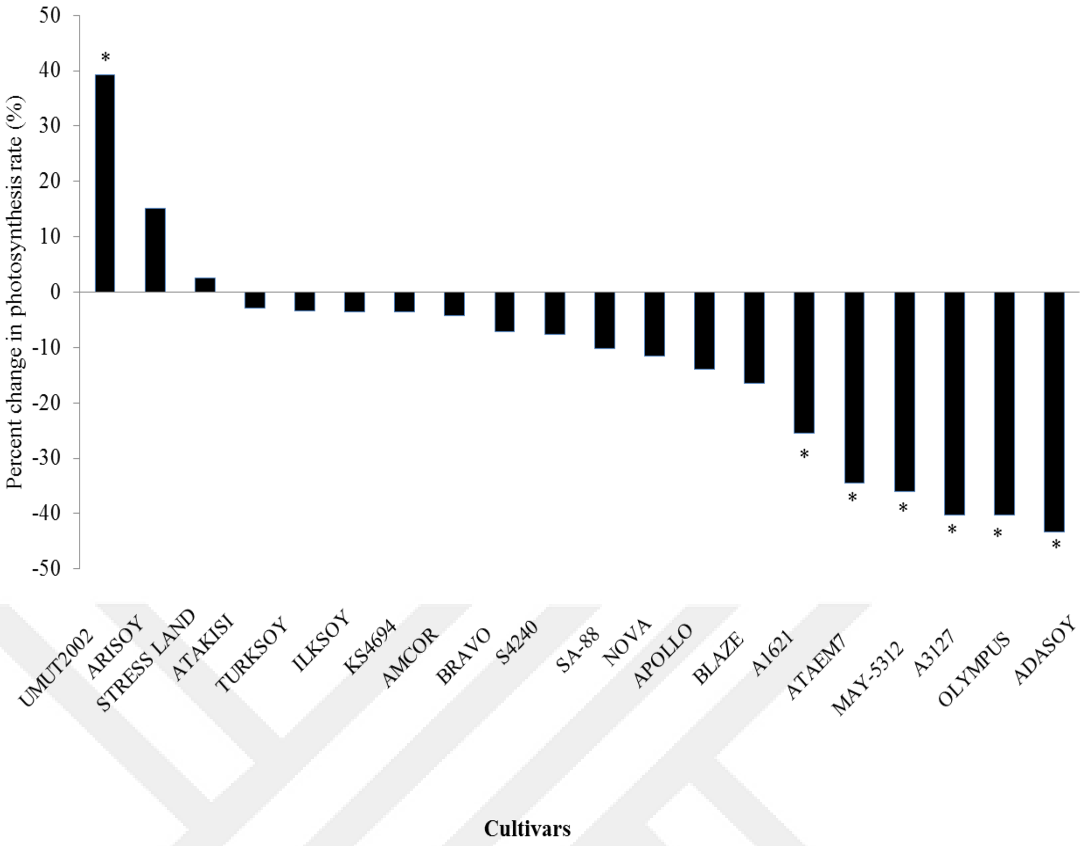

Figure 4. 24. Percent change in photosynthesis rates at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.13 Stomatal Conductance at V2-V3 Developmental Stage

The stomatal conductance at V2-V3 growth stage was significantly affected by soybean genotypes and interaction among Fe availability regimes and soybean genotypes, whereas Fe availability regimes had no effect in this regard (Table 4.25). Overall, Fe deficiency did not affect the mean stomatal conductance at V2-V3 growth stage while there was a non-significant increase under Fe deficiency (Figure 4.25). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest stomatal conductance was recorded for OLYMPUS and BLAZE under Fe-deficient regime, while the lowest was observed for STRESS LAND under Fe-sufficient environment (Table 4.26). Unexpectedly some cultivars showed decrease in stomatal conductance while other cultivars showed increase in stomatal conductance (Figure 4.26).

Table 4.25. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on stomatal conductance at V2-V3 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	0.006	0.006	1.810	0.179^{NS}
Genotypes (G)	19	1.433	0.075	22.528	0.0001*
Fe × G	19	1.012	0.053	15.911	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant ($p<0.05$)

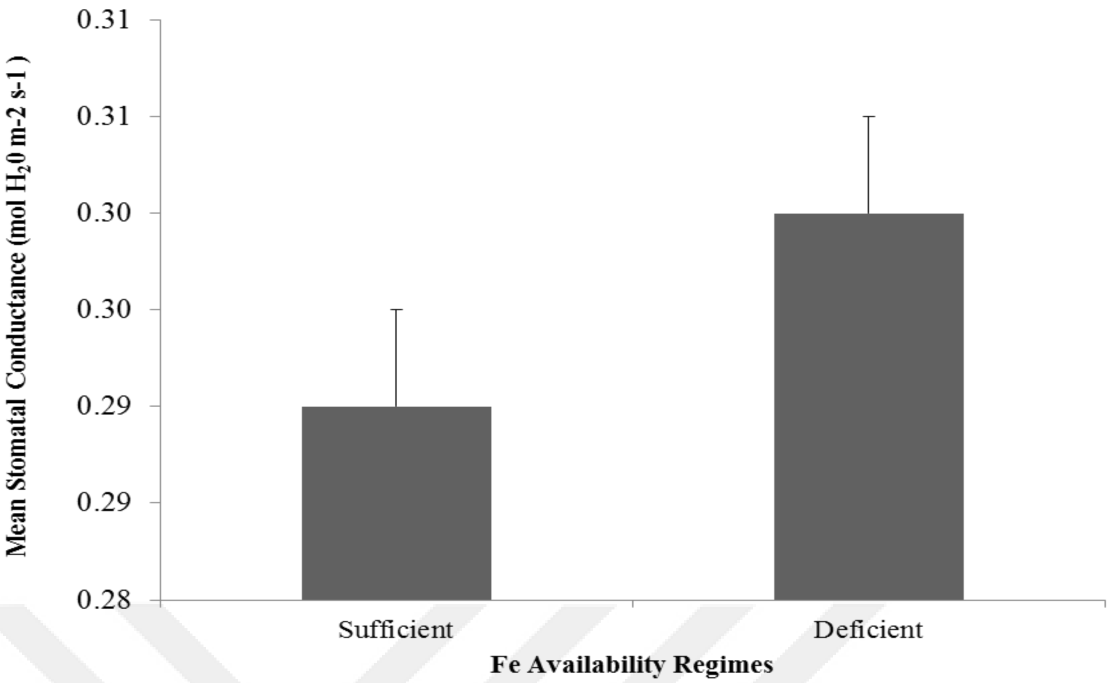

Figure 4. 25. The effect of different Fe availability regimes on stomatal conductance of soybean genotypes at V2-V3 growth stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.26. Interactive effect of Fe availability regimes and soybean genotypes on stomatal conductance at V2-V3 growth stage

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	0.3 8± 0.01 b-c	0.11 ± 0.01 r	-71.05
	OLYMPUS	0.46 ± 0.01 a	0.44 ± 0.01 a	-4.35
	ATAEM-7	0.30 ± 0.01 f-j	0.39 ± 0.02 b	+30.00
	ARISOY	0.20 ± 0.01 p-q	0.22 ± 0.01 n-q	+10.00
	BRAVO	0.28 ± 0.01 g-l	0.28 ± 0.01 h-m	0.00
	BLAZE	0.27 ± 0.01 h-n	0.47 ± 0.01 a	+74.07
	KS4694	0.25 ± 0.01 j-o	0.36 ± 0.01 b-e	+44.00
	SA88	0.27 ± 0.01 h-n	0.22 ± 0.01 n-q	-18.52
Group 2	**APOLLO**	0.27 ± 0.05 h-n	0.484 ± 0.02 a	+79.26
	TURKSOY	0.37 ± 0.01 b-d	0.29 ± 0.01 g-k	-21.62
	STRESS LAND	0.17 ± 0.01 q	0.25 ± 0.01 j-o	+47.06
	MAY5312	0.21 ± 0.01 o-q	0.24 ± 0.01 k-p	+14.29
Group 3	**ATAKISI**	0.32 ± 0.01 d-h	0.23 ± 0.01 m-q	-28.13
	ILKSOY	0.35 ± 0.01 b-f	0.21 ± 0.01 o-q	-40.00
Group 4	**NOVA**	0.29 ± 0.01 g-k	0.32 ± 0.01 d-h	+10.34
	A1621	0.21 ± 0.01 o-q	0.26 ± 0.01 i-o	+23.81
	ADASOY	0.32 ± 0.01 d-h	0.33 ± 0.01 c-g	+3.13
	AMCOR	0.21 ± 0.01 o-q	0.23 ± 0.01 l-p	+9.52
	S4240	0.25 ± 0.01 j-p	0.24 ± 0.01 k-p	-4.00
	UMUT2002	0.31 ± 0.05 e-i	0.36 ± 0.02 b-e	+16.13
	LSD value	**0.037**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

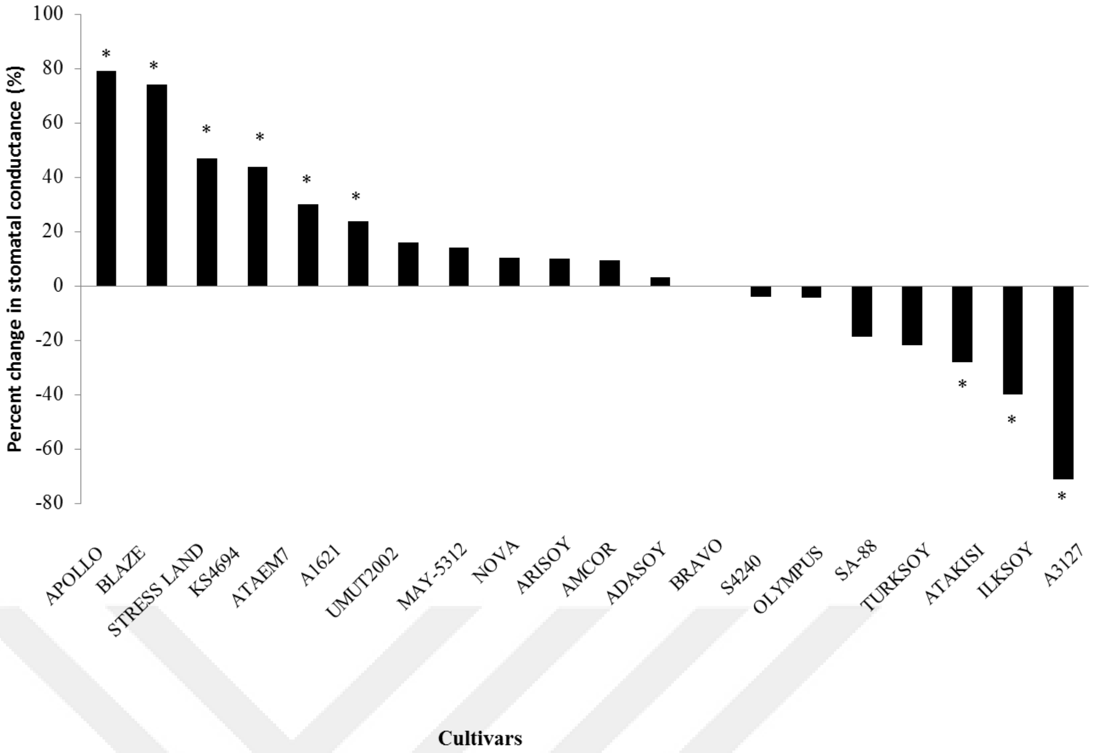

Figure 4. 26. Percent change in stomatal conductance at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) (p < 0.05)..

4.14 Stomatal Conductance at R4-R5 Developmental Stage

The stomatal conductance at R4-R5 growth stage was significantly altered by Fe availability regimes, soybean genotypes and their interaction (Table 4.27). Overall, the genotypes grown under Fe-sufficient conditions had higher stomatal conductance at R4-R5 growth stage than those under Fe-deficient conditions (Figure 4.27). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest stomatal conductance was recorded for NOVA under Fe-sufficient regime, while the lowest was observed for ADASOY under Fe-Deficient environment (Table 4.28). Similarly cultivars KS4649, UMUT2002, ILKSOY and TURKSOY showed increase in stomatal conductance while all other genotypes between R4-R5 growth stage under iron deficient conditions. Moreover, the highest decrease in stomatal conductance was observed in A-1621 (56.00 %) and the lowest in STRESS LAND (4.71 %) among all tested genotypes. However SA-88 and ATAKISI showed no change in stomatal conductance under different iron availability regimes (Figure 4.28).

Table 4.27. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on stomatal conductance at R4-R5 developmental stage.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	0.157	0.157	39.137	0.0001*
Genotypes (G)	19	0.884	0.047	11.613	0.0001*
Fe × G	19	0.235	0.012	3.088	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant (p<0.05)

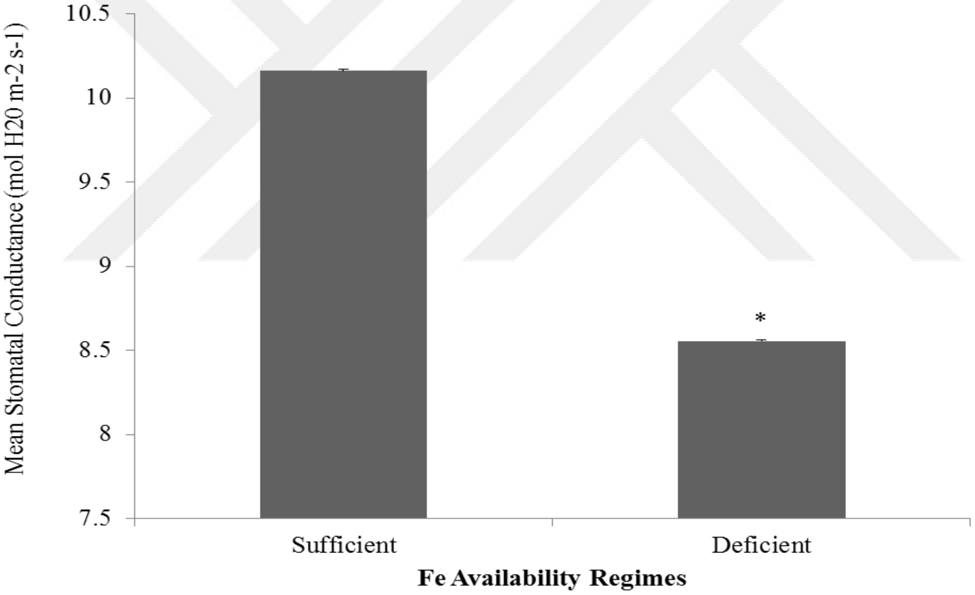

Figure 4. 27. The effect of different Fe availability regimes on stomatal conductance of soybean genotypes at R4-R5 growth stage. * indicates significant difference between the means under different Fe availability regimes (p<0.05).

Table 4.28. Interactive effect of Fe availability regimes and soybean genotypes on stomatal conductance at R4-R5 growth stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	0.30 ± 0.01 b-f	0.19 ± 0.01 k-o	-36.67
	OLYMPUS	0.31 ± 0.01 b-f	0.23 ± 0.01 g-m	-25.81
	ATAEM-7	0.25 ± 0.02 f-l	0.17 ± 0.01 m-p	-32.00
	ARISOY	0.34 ± 0.01 a-b	0.26 ± 0.01 e-k	-23.53
	BRAVO	0.31 ± 0.01 b-f	0.25 ± 0.01 f-l	-19.35
	BLAZE	0.28 ± 0.01 b-h	0.22 ± 0.01 g-m	-21.43
	KS4694	0.15 ± 0.01 n-p	0.17 ± 0.01 m-p	+13.33
	SA88	0.27 ± 0.01 d-i	0.27 ± 0.04 e-j	0.00
Group 2	**APOLLO**	0.34 ± 0.01 a-c	0.27± 0.01 c-h	-20.59
	TURKSOY	0.19 ± 0.01 j-o	0.28 ± 0.03 b-h	+47.37
	STRESS LAND	0.35 ± 0.01 a-b	0.33 ± 0.03 b-e	-5.71
	MAY5312	0.20 ± 0.01 i-o	0.13 ± 0.01 o-q	-35.00
Group 3	**ATAKISI**	0.17 ± 0.01 m-p	0.17 ± 0.01 m-p	0.00
	ILKSOY	0.21 ± 0.01 h-n	0.25 ± 0.01 f-l	+19.05
Group 4	**NOVA**	0.41 ± 0.01 a	0.27 ± 0.01 c-h	-34.15
	A1621	0.25 ± 0.01 f-l	0.11 ± 0.01 p-q	-56.00
	ADASOY	0.17 ± 0.01 m-p	0.07 ± 0.01 q	-54.12
	AMCOR	0.31 ± 0.01 b-f	0.18 ± 0.01 l-o	-41.94
	S4240	0.26 ± 0.01 e-j	0.18 ± 0.01 l-o	-30.77
	UMUT2002	0.29 ± 0.01 b-g	0.34 ± 0.05 a-d	+17.24
	LSD value	**0.072**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

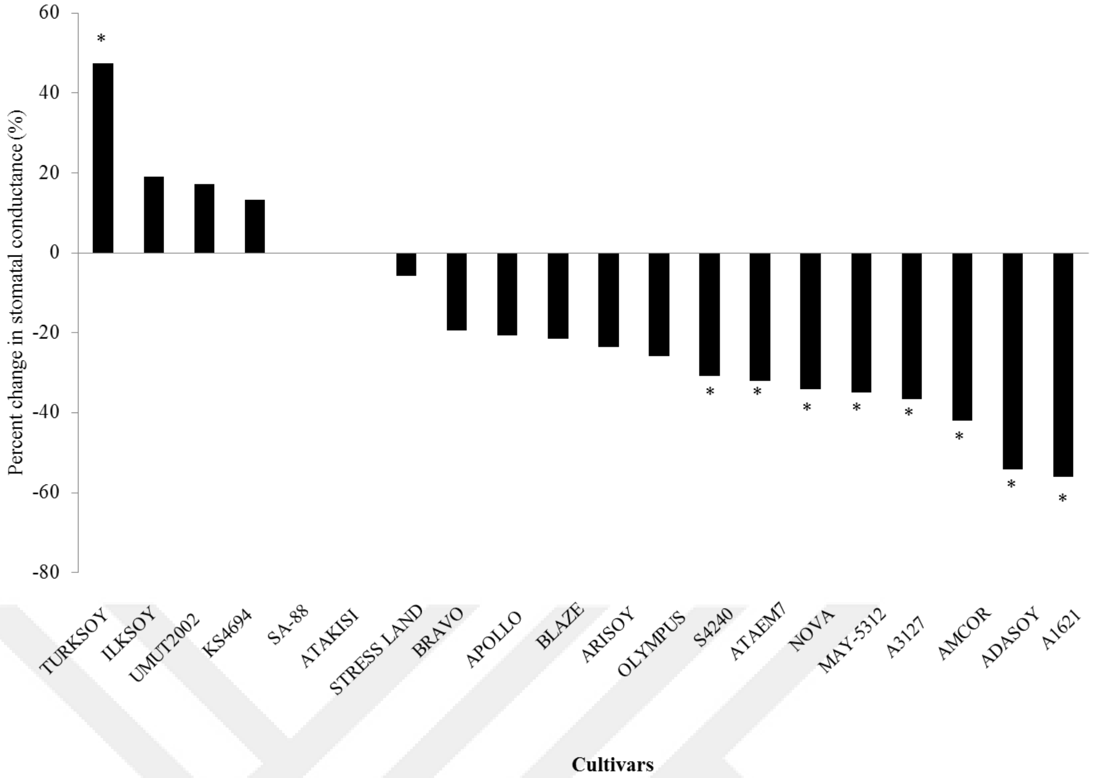

Figure 4. 28. Percent change in stomatal conductance at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.15 Transpiration Rate at V2-V3 Developmental Stage

The transpiration rate at V2-V3 developmental stage was significantly affected by Fe availability regimes, soybean genotypes and their interaction (Table 4.29). Overall, the genotypes grown under Fe-deficient conditions had higher transpiration rates at V2-V3 developmental stage than those under Fe-sufficient conditions (Figure 4.29). Regarding the interactions among Fe availability regimes and soybean genotypes, the highest transpiration rate was recorded for BLAZE under Fe-deficient regime, while the lowest was observed for ILKSOY under Fe-sufficient environment (Table 4.30). All genotypes showed a significant increase (153.28%) in transpiration rate under iron deficient conditions. ILKSOY had significantly the highest increase in transpiration rate compared to the rest of tested genotypes, while significantly the lowest increase (6.72%) in transpiration rate was computed in BRAVO (Figure 4.30).

Table 4.29. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on transpiration rate at V2-V3 developmental stage

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	1788.742	1788.742	481.287	0.0001*
Genotypes (G)	19	730.836	38.465	10.350	0.0001*
Fe × G	19	364.457	19.182	5.161	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant ($p<0.05$)

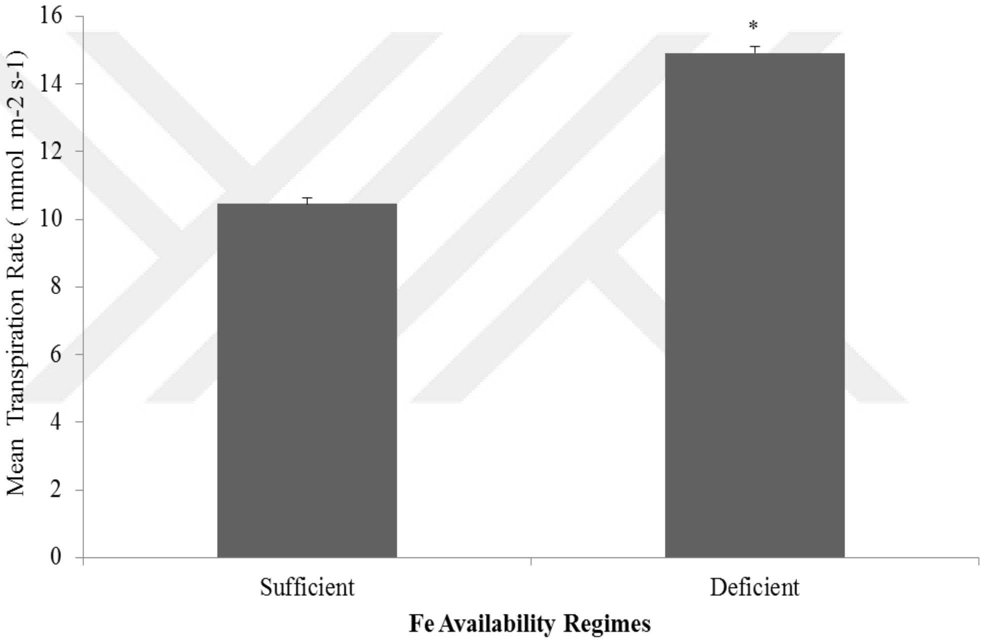

Figure 4. 29. The effect of different Fe availability regimes on transpiration rate of soybean genotypes at V2-V3 growth stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Table 4.30. Interactive effect of Fe availability regimes and soybean genotypes on transpiration rate at V2-V3 growth stage.

Chlorosis Group	**Genotypes**	**Fe-Sufficient**	**Fe-Deficient**	**Percent Change (D / S)**
Group 1	**A3127**	11.92 ± 0.89 l-p	13.54 ± 0.79 h-l	+13.59
	OLYMPUS	13.42 ± 0.66 i-l	15.72 ± 0.56 b-f	+17.14
	ATAEM-7	8.65 ± 0.64 s-t	16.91 ± 0.22 a-e	+95.49
	ARISOY	10.05 ± 0.52 q-s	14.03 ± 0.44 f-k	+39.60
	BRAVO	12.05 ± 0.43 l-o	12.86 ± 0.88 j-m	+6.72
	BLAZE	11.98 ± 0.89 l-o	17.56 ± 0.52 a	+46.58
	KS4694	11.17 ± 13.55 m-r	17.27 ± 0.45 a-c	+54.61
	SA88	11.53 ± 0.97 m-q	12.61 ± 1.22 k-n	+9.37
Group 2	**APOLLO**	10.55 ± 0.61 o-r	16.95 ± 0.53 a-d	+60.66
	TURKSOY	10.70 ± 0.93 o-r	14.95 ± 0.74 f-i	+39.72
	STRESS LAND	10.30 ± 0.60 o-s	13.93 ± 0.42 g-k	+35.24
	MAY5312	7.83 ± 0.42 t	14.26 ± 0.70 f-k	+82.12
Group 3	**ATAKISI**	10.14 ± 0.48 p-s	14.09 ± 0.88 f-k	+38.95
	ILKSOY	4.58 ± 0.79 u	11.60 ± 0.58 m-q	+153.28
Group 4	**NOVA**	9.63 ± 0.64 r-s	15.14 ± 0.61 e-i	+57.22
	A1621	10.18 ± 0.36 p-s	14.07 ± 0.80 f-k	+38.21
	ADASOY	10.46 ± 0.38 o-r	17.32 ± 0.90 a-b	+65.58
	AMCOR	10.28 ± 0.22 o-s	14.60 ± 0.68 f-j	+42.02
	S4240	11.01 ± 0.58 n-r	15.26 ± 0.92 d-h	+38.60
	UMUT2002	12.58 ± 0.79 k-n	15.48 ± 0.67 c-g	+23.05
	LSD value	**1.787**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

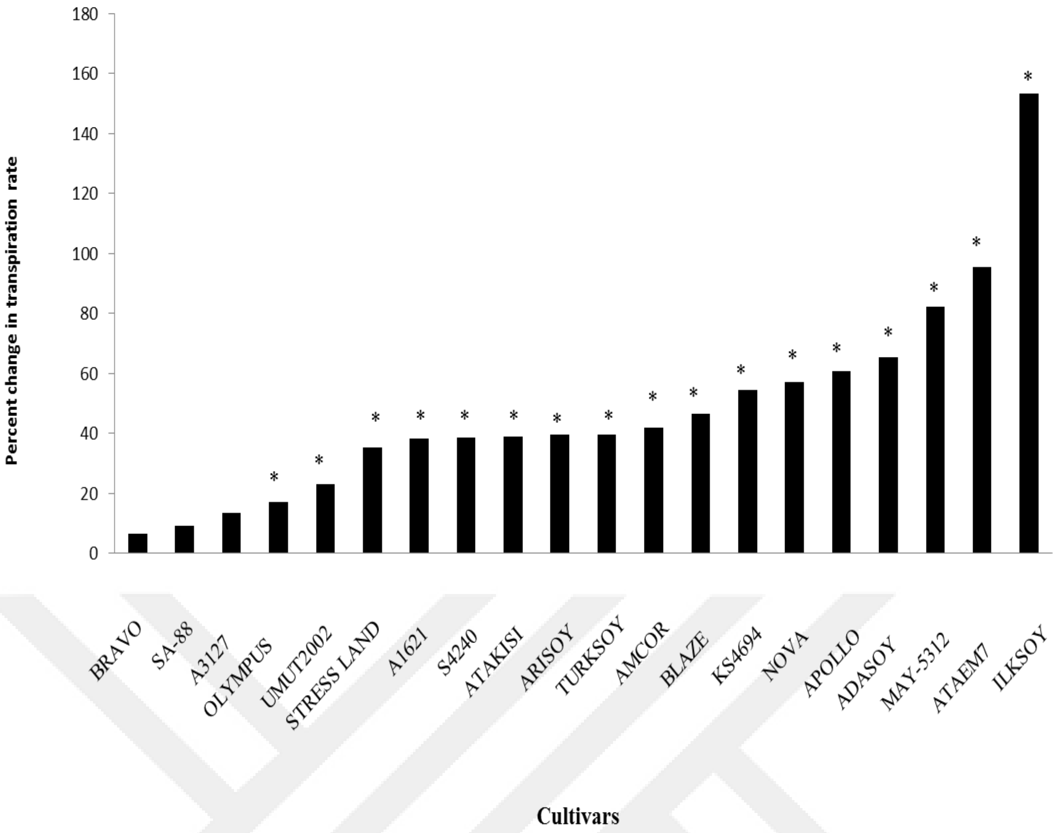

Figure 4. 30. Percent change in transpiration rate at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.16 Transpiration Rate at R4-R5 Developmental Stage

The transpiration rate at R4-R5 growth stage was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime were non-significant (Table 4.31). Overall, the genotypes grown under Fe-sufficient conditions had higher transpiration rates at R4-R5 growth stage than those grown under Fe-deficient conditions (Figure 4.31). The genotypes and interaction among genotypes and Fe-availability regimes had no effect in this regard (Tables 4.32). In fact, there was no statistical difference among many cultivars regarding the change in transpiration rate. However, in some of the cultivars there was significant change in transpiration rate as compared to the rest of the genotypes used for experiment. Significant increase in transpiration rate was observed for KS4649 while significantly lower increase in transpiration was seen in TURKSOY. Significantly more reduced (52.38%)

transpiration rate was seen in A1621, while there was lesser reduction (8.07%) in transpiration rate of SA88 (non-significant) as compared to the rest of tested genotypes. However, KS4694, ADASOY and TURKSOY showed increase in transpiration rates at R4-R5 growth stage in contratst to other tested genotypes (Figure 4.32).

Table 4.31. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on transpiration rate at R4-R5 developmental stage.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	154.569	154.569	5.170	0.024*
Genotypes (G)	19	767.136	40.376	1.351	0.156^{NS}
Fe × G	19	556.926	29.312	0.980	0.486^{NS}

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant (p<0.05)

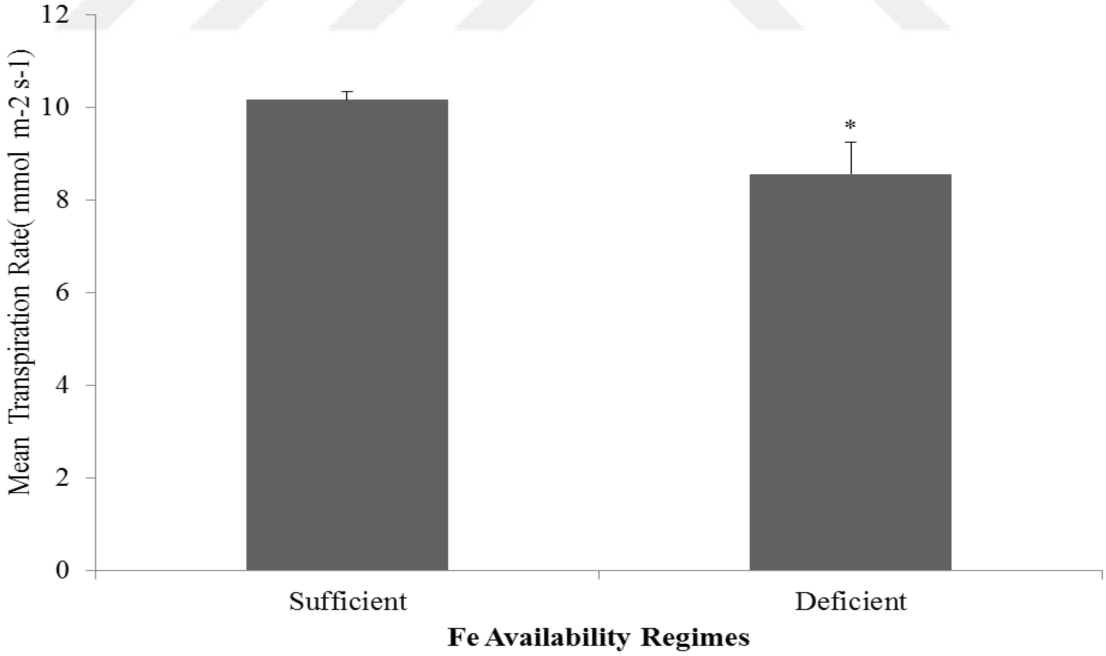

Figure 4. 31. The effect of different Fe availability regimes on transpiration rate of soybean genotypes at R4-R5 growth stage. * indicates significant difference between the means under different Fe availability regimes (p<0.05)

Table 4.32. Interactive effect of Fe availability regimes and soybean genotypes on transpiration rate at R4-R5 growth stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	10.44 ± 0.74 b-d	7.80 ± 0.89 b-e	-25.29
	OLYMPUS	11.08 ± 0.83 b-c	7.51 ± 0.66 b-e	-32.22
	ATAEM-7	8.70 ± 0.94 b-e	5.62 ± 0.64 c-e	-35.40
	ARISOY	11.44 ± 0.43 b-c	8.98 ± 0.52 b-e	-21.50
	BRAVO	11.45 ± 0.51 b-c	9.49 ± 0.43 b-e	-17.12
	BLAZE	11.51 ± 0.39 b-c	9.00 ± 0.89 b-e	-21.81
	KS4694	9.10 ± 0.69 b-e	18.98 ± 13.55 a	+108.57
	SA88	10.20 ± 0.53 b-d	10.07 ± 0.97 b-e	-1.27
Group 2	**APOLLO**	10.77 ± 0.34 b-c	9.18 ± 0.61 b-e	-14.76
	TURKSOY	7.98 ± 0.78 b-e	9.42 ± 0.93 b-e	+18.05
	STRESS LAND	11.92 ± 0.70 bs	10.64 ± 0.60 b-d	-10.74
	MAY5312	9.32 ± 0.47 b-e	5.60 ± 0.42 c-e	-39.94
Group 3	**ATAKISI**	7.68 ± 0.27 b-e	7.06 ± 0.48 b-e	-8.07
	ILKSOY	10.63 ± 0.46 b-d	9.48 ± 0.79 b-e	-10.82
Group 4	**NOVA**	12.39 ± 0.61 b	8.49 ± 0.64 b-e	-31.48
	A1621	9.45 ± 0.65 b-e	4.50 ± 0.36 d-e	-52.38
	ADASOY	3.91 ± 0.17 e	7.54 ± 0.38 b-e	+92.84
	AMCOR	11.29 ± 0.53 b-c	7.45 ± 0.22 b-e	-34.01
	S4240	9.86 ± 0.66 b-e	7.68 ± 0.58 b-e	-22.11
	UMUT2002	10.38 ± 0.66 b-d	10.14 ± 0.79 b-d	-2.31
	LSD value	**6.224**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

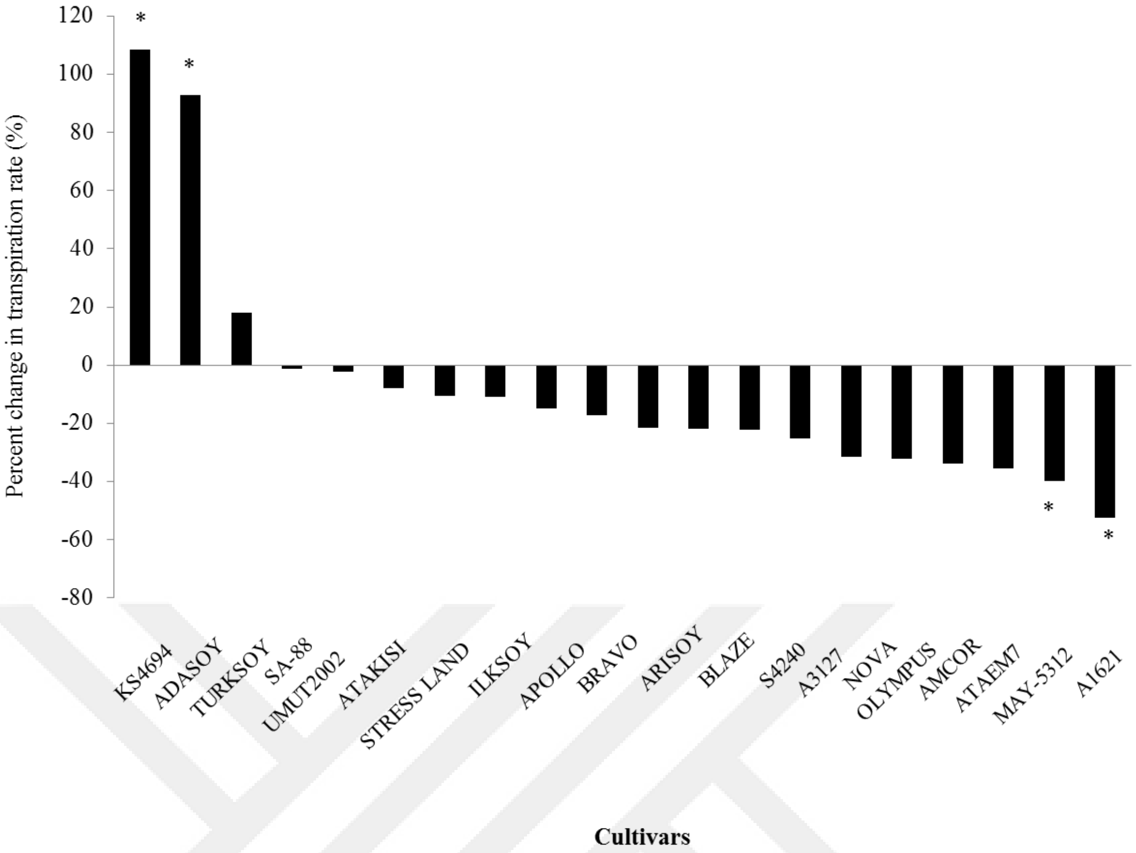

Figure 4.32. Percent change in transpiration rate at R4-R5 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

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4.17 Root Fe Accumulation at V2-V3 developmental stage

The root iron content level at V2-V3 growth stage was significantly altered by Fe availability regimes. Moreover, soybean genotypes and interaction among soybean genotypes and Fe availability are also significant (Table 4.33). Overall, the genotypes grown under Fe-deficient conditions had higher iron content at V2- V3 growth stage than those grown under Fe-sufficient conditions (Figure 4.33). However, the highest iron content was observed in A-1621 under iron deficient conditions while the lowest was observed in ATAEM-7 under iron sufficient conditions (Table 4.34). Interestingly some cultivars showed a significant increase in iron content levels and some of them showed a significant decrease in iron content under iron deficient conditions (Figure 4.34).

Table 4.33. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on root Fe accumulation levels at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$)

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	77962.116	77962.11	1455.636	0.0001*
Genotypes (G)	19	383170.81	20166.88	376.537	0.0001*
Fe × G	19	143692.43	7562.760	141.205	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant ($p<0.05$)

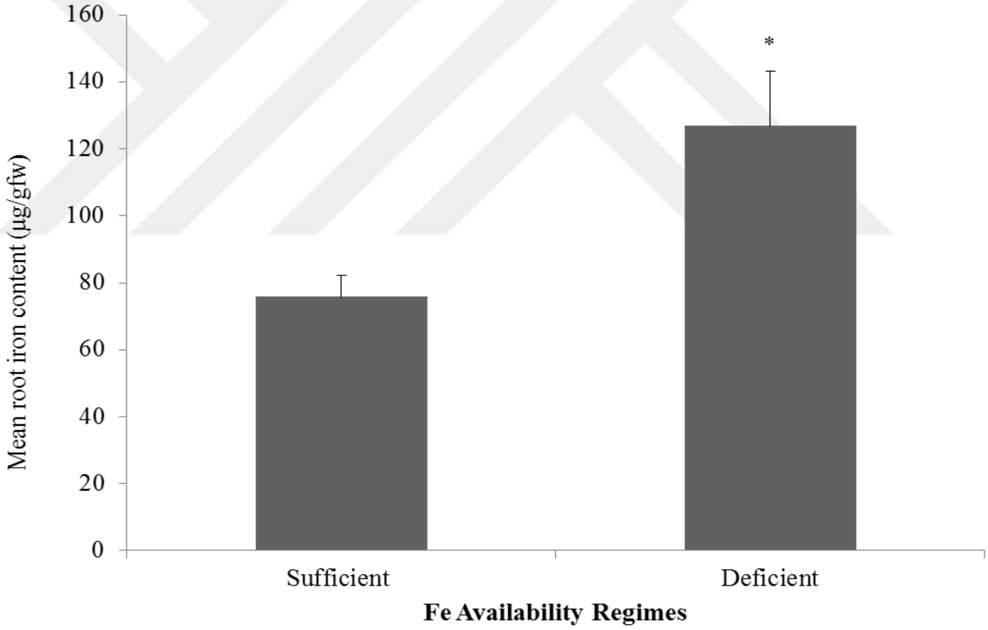

Figure 4. 33. The effect of different Fe availability regimes on root Fe accumulation levels of soybean genotypes at V2-V3 developmental stage.

Table 4.34. Interactive effect of Fe availability regimes and soybean genotypes on root Fe accumulation levels at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	29.56 ± 0.95 r-d	34.06 ± 1.04 r	+15.20
	OLYMPUS	32.33 ± 1.43 r	250.82 ± 6.04 b	+675.76
	ATAEM-7	17.43 ± 17.44 t	20.05 ± 0.62 s-t	+15.00
	ARISOY	34.02 ± 2.45 r	97.38 ± 9.03 k	+186.22
	BRAVO	82.58 ± 2.51 m	183.52± 8.16 d-e	+122.23
	BLAZE	77.77 ± 1.98 m	249.13 ± 3.30 b	+220.48
	KS4694	19.21 ± 0.90 s-t	48.57 ± 2.81 p	+152.84
	SA88	33.60 ± 0.98 r	153.49 ± 1.44 g	+356.77
Group 2	**APOLLO**	46.85 ± 1.98 p-q	100.93 ± 0.39 k	+115.49
	TURKSOY	89.64 ± 1.91 k-l	191.41 ± 5.69 d-e	+113.52
	STRESS LAND	91.32 ± 2.43 k-l	180.09 ± 4.05 e	+97.207
	MAY5312	28.10 ±1.91 r-s	35.98 ± 1.10 q-r	+28.038
Group 3	**ATAKISI**	140.67 ±11.86 h	55.08 ± 0.93 o-p	-60.84
	ILKSOY	50.74 ± 50.77 o-p	52.04 ± 0.58 o-p	+2.49
Group 4	**NOVA**	192.95 ± 192.96 d	220.10 ± 9.28 c	+14.01
	A1621	167.48 ± 5.36 f	274.74 ± 5.3 a	+64.05
	ADASOY	128.08 ± 2.72 i-j	137.90 ± 3.34 h-i	+7.67
	AMCOR	75.14 ± 4.36 m	61.61 ± 2.83 n-o	-18.00
	S4240	70.82 ± 2.27 m-n	70.86 ± 1.75 m-n	-14.54
	UMUT2002	118.74 ± 5.65 j	118.48 ± 1.93 j	-0.22
	LSD value	**11.892**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

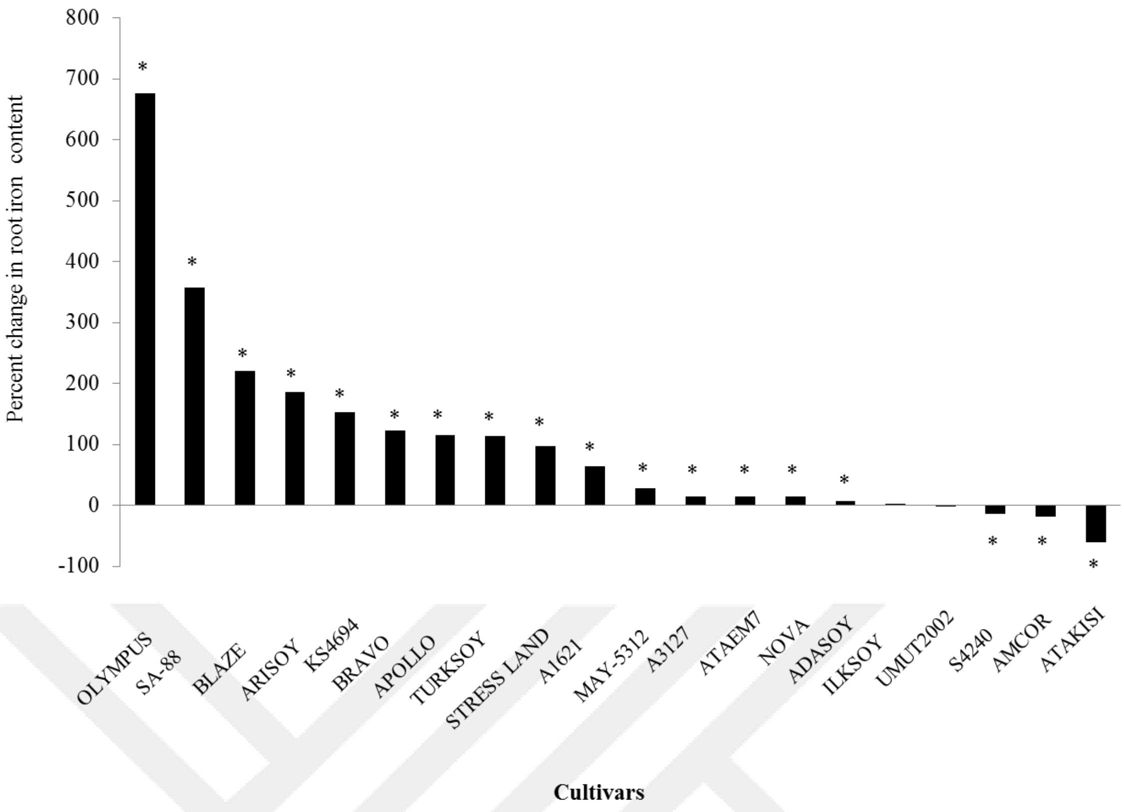

Figure 4.34. Percent change in root Fe accumulation level at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

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4.18 Leaf Fe Accumulation at V2-V3 Developmental Stage

The shoot iron content at V2-V3 developmental stage was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime are also significant (Table 4.35). Overall, the genotypes grown under Fe-sufficient conditions had higher iron content at V2-V3 developmental stage than Fe-deficient conditions (Figure 4.35). Moreover, the highest iron content was observed in S4240 under iron sufficient conditions while the lowest iron content was observed in ILKSOY under iron deficient conditions (Tables 4.36). Moreover the highest decrease in shoot iron content was observed in ATAKISI (50.53 %) while the lowest was observed in APOLLO (9.69 %) (non-significant) (Figure 4.36).

Table 4.35. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on shoot Fe accumulation levels at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	153949.36	153949.3	878.87	0.0001*
Genotypes (G)	19	700815.84	36885.04	210.57	0.0001*
Fe × G	19	130032.22	6843.80	39.070	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant ($p<0.05$)

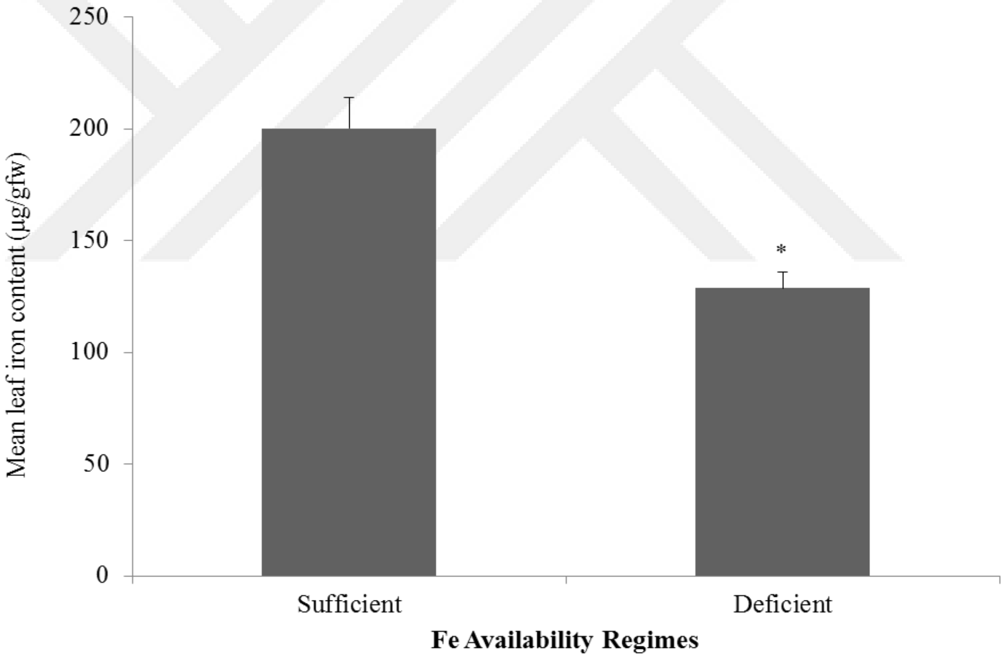


Figure 4. 35. The effect of different Fe availability regimes on shoot Fe accumulation levels of soybean genotypes at V2-V3 developmental stage.

Table 4.36. Interactive effect of Fe availability regimes and soybean genotypes on leaves Fe accumulation levels at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	51.906 ± 0.70q-s	38.241±1.25 r-s	-41.70
	OLYMPUS	142.839 ± 3.37k	96.566± 5.20m-n	-5.37
	ATAEM-7	63.185 ± 2.04 0-q	63.185±1.57 o-q	-31.07
	ARISOY	292.46 ± 10.90 b	173.86 ± 6.17 f-i	+11.51
	BRAVO	228.53 ± 3.94 d	200.94±1.57 e	-7.90
	BLAZE	163.59 ± 8.63 h-k	100.84±5.26 l-m	-43.29
	KS4694	71.17 ± 2.08 0-q	57.29± 3.21p-r	-37.95
	SA88	193.96 ± 13.51 e-f	153.72± 3.04i-k	+2.23
Group 2	**APOLLO**	149.88 ± 0.62 j-k	143.15± 3.29k	-9.69
	TURKSOY	235.87±6.661 c-d	179.87±5.07 e-h	-25.64
	STRESS LAND	234.65 ± 6.74 c-d	185.84± 7.83e-g	-54.78
	MAY5312	77.06 ± 2.44 n-p	61.11± 2.92o-q	-69.95
Group 3	**ATAKISI**	311.359 ± 10.61 b	119.71±7.56 l	-50.53
	ILKSOY	57.80± 3.20p-r	34.02±14.43 s	-52.72
Group 4	**NOVA**	251.67 ± 2.52 c	171.02±5.96 g-j	-50.05
	A1621	363.75 ± 15.28 a	148.77±5.33 k	-57.30
	ADASOY	238.24 ± c-d	186.09±3.04 e-g	-40.08
	AMCOR	155.01 ± 1.54 i-k	79.38±1.98 m-o	-50.96
	S4240	373.01 ± 19.61a	180.38±8.80 e-h	-8.15
	UMUT2002	358.93 ± 16.26 a	200.84±12.14 e	-79.81
	LSD value	**21.505**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

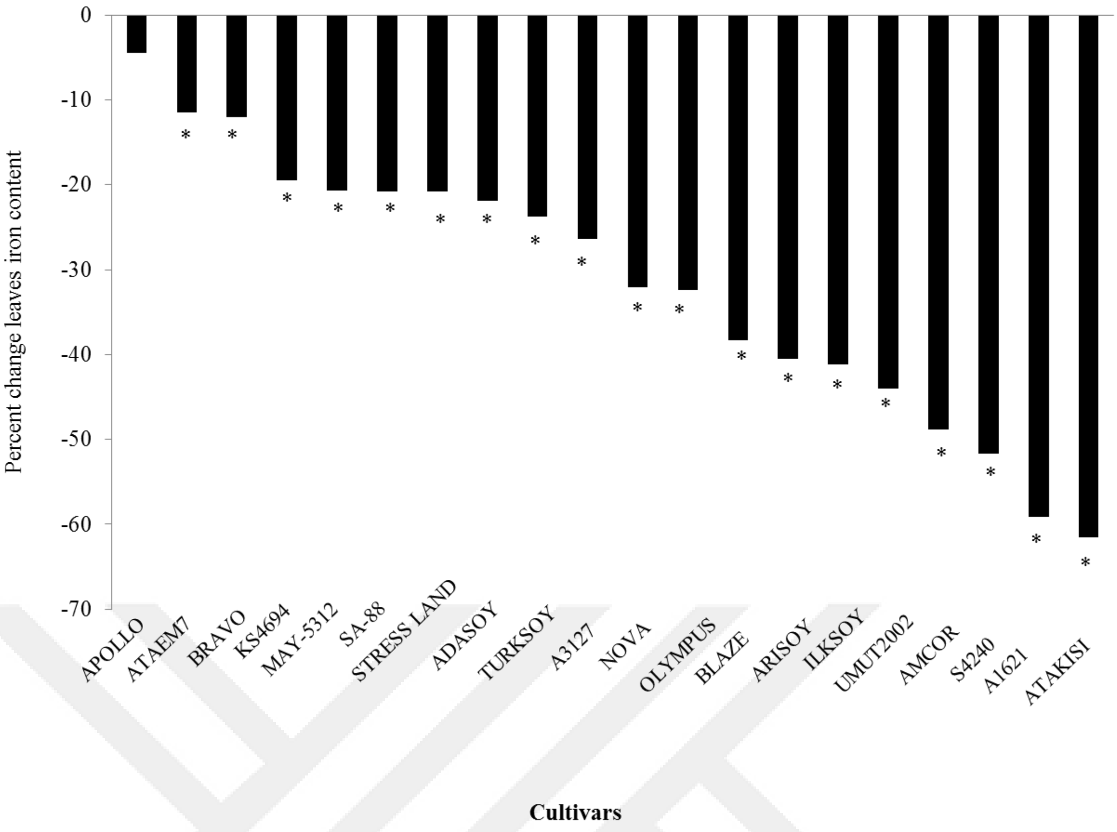


Figure 4. 36. Percent change in leaves Fe accumulation level at V2-V3 developmental stage under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.19 Seed Fe Accumulation

The seed iron content was significantly altered by Fe availability regimes, moreover soybean genotypes and interaction among soybean genotypes and Fe availability regime are also significant (Table 4.37). Overall, the genotypes grown under Fe-sufficient conditions had higher seed iron content than those under Fe-deficient conditions (Figure 4.37). The highest iron content was observed in A3127 under iron sufficient conditions while the lowest iron content was observed in A-1621 under iron deficient conditions (Tables 4.38). Interestingly increase in seed iron content was observed only in ARISOY and SA-88. Contrastingly all other tested genotypes showed a decrease in seed iron content under iron deficient conditions. Moreover, significantly the highest decrease in seed iron content was observed in UMUT 2002 (79.82 %) and the lowest was observed in OLPYMPUS (5.40 %) under iron deficit conditions (Figure 4.38).

Table 4.37. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on seed Fe accumulation levels at V2-V3 developmental stage. * indicates significant difference between the means under different Fe availability regimes ($p<0.05$).

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	16608.033	16608.03	1680.87	0.0001*
Genotypes (G)	19	137821.91	7253.785	734.146	0.0001*
Fe × G	19	20549.043	1081.529	109.460	0.0001*

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant ($p<0.05$)

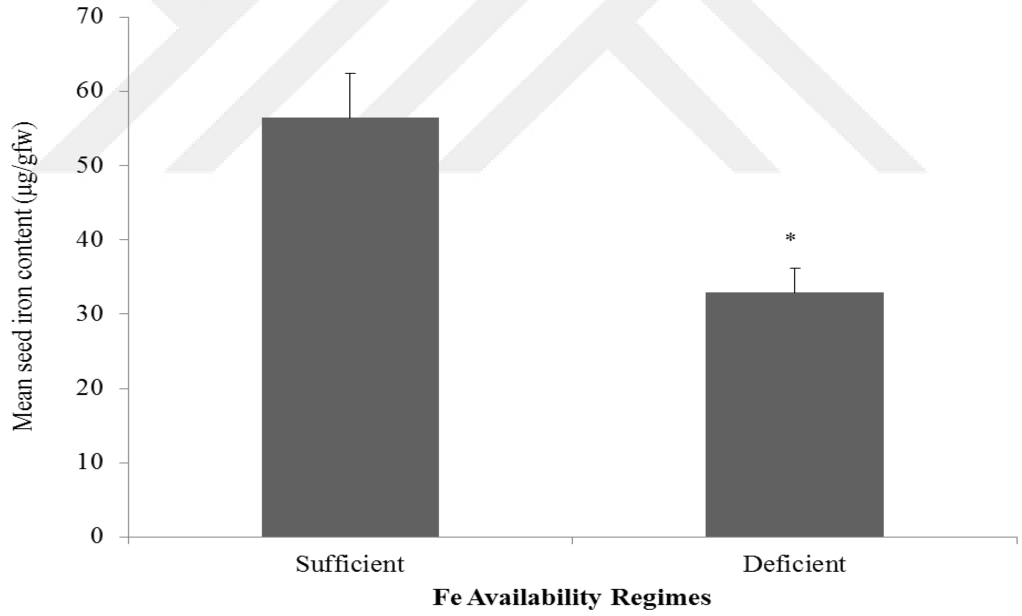


Figure 4. 37. The effect of different Fe availability regimes on seed Fe accumulation levels of soybean genotypes at V2-V3 developmental stage.

Table 4.38. Interactive effect of Fe availability regimes and soybean genotypes on seed Fe accumulation levels at V2-V3 developmental stage.

Chlorosis Group	Genotypes	Fe-Sufficient	Fe-Deficient	Percent Change (D / S)
Group 1	**A3127**	173.447 ±1.50 a	101.115 ± 3.03d	-63.61
	OLYMPUS	41.47 ±0.711 i-k	39.239 ± 0.87k-l	-5.40
	ATAEM-7	120.69±51.50 c	83.182 ± 3.03 e	-61.66
	ARISOY	30.286 ± 0.74m-n	33.773 ±0.59m	-49.47
	BRAVO	23.123 ±0.77 o-p	21.294 ± 0.70 p	-24.13
	BLAZE	59.440 ±0.17 h	33.703 ±0.41 m	-7.77
	KS4694	65.566 ±0.71 g	40.682 ± 1.23 j-k	-40.66
	SA88	5.151 ±0.66 t-v	5.266 ±0.81 s-v	-59.86
Group 2	**APOLLO**	34.199±0.62 l-m	30.884 ± 1.19m-n	12.54
	TURKSOY	74.247 ±1.83 f	55.134 ±0.91 h	-25.74
	STRESS LAND	46.102±0.55 i	20.845 ±0.67 p	-15.83
	MAY5312	148.258±5.19 b	44.542 ±0.64 i-j	-69.95
Group 3	**ATAKISI**	57.017 ±1.63 h	28.205±0.75 n-o	-17.73
	ILKSOY	72.6375.77 ± f	34.336 ±2.03 l-m	-52.73
Group 4	**NOVA**	84.614 ±1.04 e	42.262 ± I 5.47-k	-14.06
	A1621	4.253 ± 0.15u-v	1.816± 0.05 v	-57.27
	ADASOY	22.414 ±0.92 p	13.43 ±0.99 1 q	-31.72
	AMCOR	20.694 0.26 p	10.148 ±0.36 q-t	-50.99
	S4240	11.294 ± 0.31 q-r	10.373 ±0.35 q-s	-20.90
	UMUT2002	34.752 ±1.33 l-m	7.015 ± 0.12 r-u	-79.82
	LSD value	**5.108**		

Same letters within a column do not differ significantly ($p < 0.05$). * = Significant change (D / S) ($p < 0.05$).

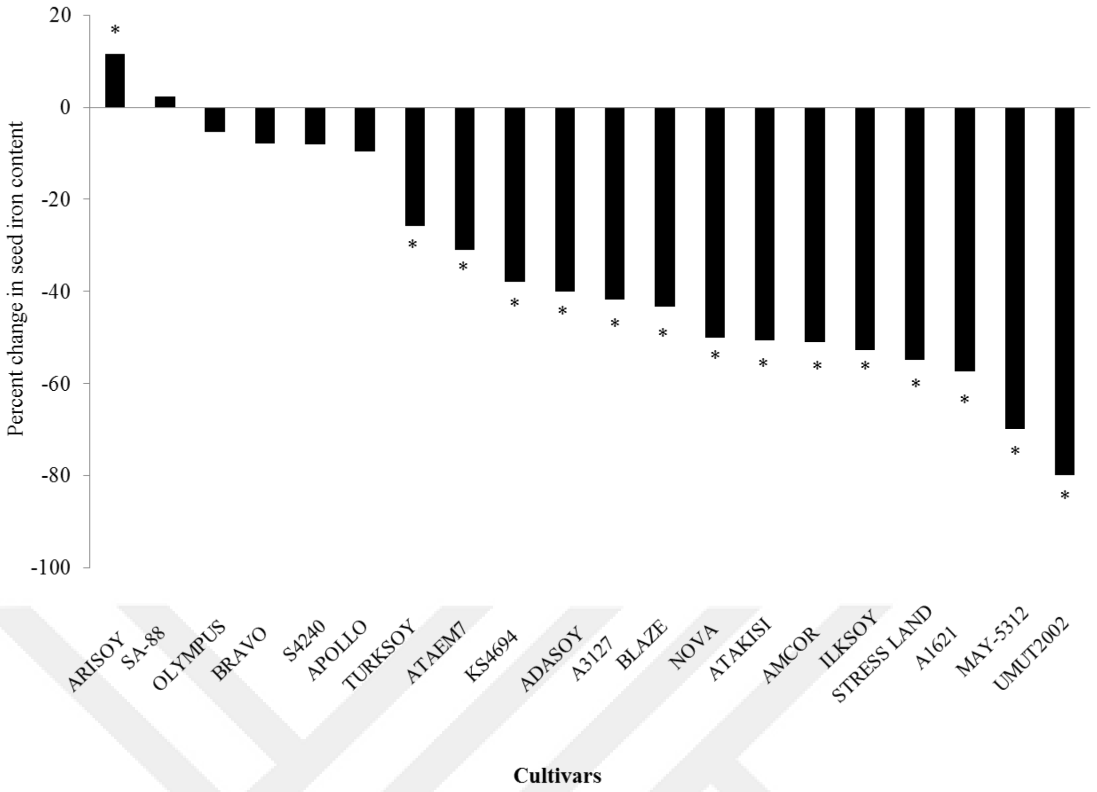


Figure 4. 38. Percent change in seed Fe accumulation level under iron deficient conditions. * = Significant change (D / S) ($p < 0.05$).

4.20 Relative Gene Expression Analyses

Depending on the results obtained from physiological and biochemical analyses, two tolerant (ARISOY and SA-88) and two sensitive (ATAKISI and NOVA) varieties in medium early maturity group (group III) were selected to determine the molecular effects of Fe deficiency at V3-V4 developmental stage. These varieties were all developed in the breeding program at Çukurova University in Turkey. RNA samples were isolated from the roots of plants grown in Fe-sufficient and deficient conditions. After following the DNase I treatment to remove the genomic DNA contamination, total RNA quality was determined by gel electrophoresis (Figure 4.39) and the quantity was determined by spectrophotometry (Appendix 3). Accordingly, 18S and 28S rRNA bands were observed sharply and the ratio of OD260/OD280 was in the range of 1.8 to 2.1, indicating that the quality of the RNA samples was good enough to proceed with 1st strand cDNA synthesis.

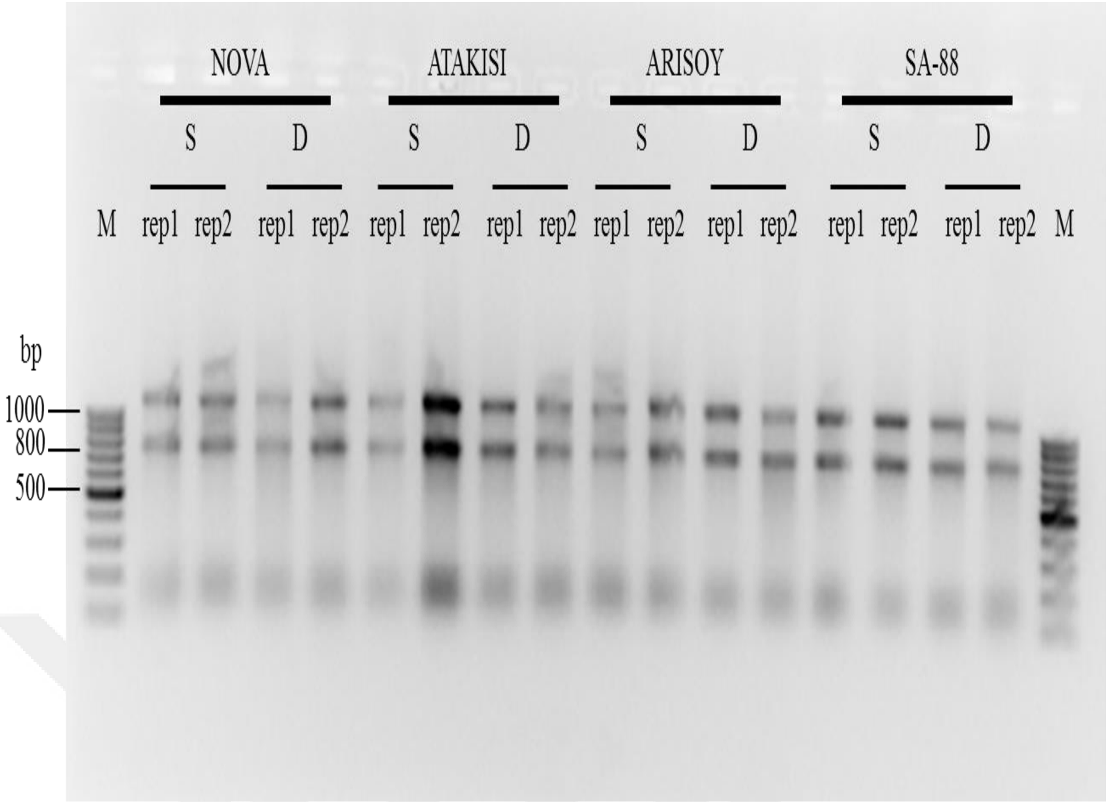


Figure 4. 39. Quality of total RNAs isolated from soybean genotypes. Two microliters of RNA samples were separated on 1.2 % agarose gel electrophoresis in 1 X TAE buffer for 1.5 hours. M represents for 1 kb DNA ladder (Thermo Scientific).

The transcript levels of *IRT1, FRO2, NRAMP and Ferritin* were assessed by Real Time quantitative PCR (RT-qPCR) in control (Fe-sufficient) and Fe-deficient roots of *Glycine max* in order to better understand the mechanism behind Fe uptake and translocation in plants. *FRO2* expression level was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime were non-significant (Table 4.39). There was no change in FRO2 expression in different genotypes under Fe-sufficient conditions (Appendix D supplementary figure 1). There was a significant increase in relative expression of *FRO2* under iron deficient conditions as compared to the iron sufficient conditions. Significantly the highest relative expression (28.91 folds) was recorded in ATAKISI among the four tested genotypes. Contrary to this, relatively the lowest expression (6.86 folds) was observed in ARISOY, while NOVA and SA-88 showed increase in *FRO2* expression levels of 24.87 and 24.92 folds, respectively, under iron deficient conditions (Figure 4.40).

Table 4.39. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on *FRO2* expression levels.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	2920.70	2920.70	25.24	0.001 *
Genotypes (G)	3	773.78	257.92	2.229	0.162
Fe × G	3	730.03	243.34	2.103	0.178

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant (p<0.05)

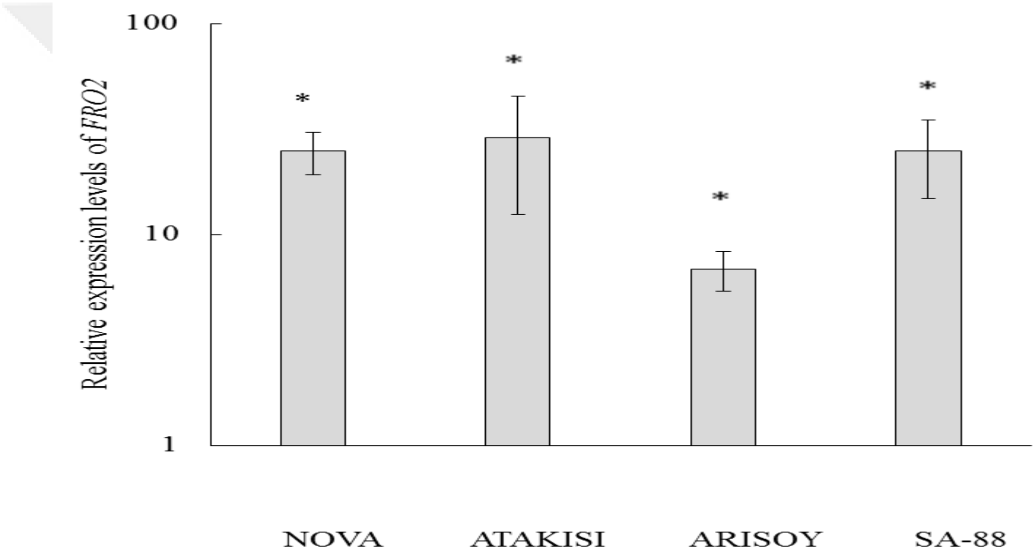


Figure 4. 40. RT-qPCR analysis of *FRO2* transcript levels in the roots of *Glycine max*. Expression levels relative to the Fe-sufficient conditions of each genotype.* indicates significant differences between Fe-deficient and sufficient conditions for each genotype (p<0.05).

IRT1 is the primary contributing factor of iron and heavy metal homeostasis in plants (Kobayashi and Nishizawa, 2012). Recent work shed light on the strong and rapid transcriptional induction of *IRT1* following iron deficiency. Therefore, we investigated the *IRT1* expression to confirm its transcriptomic behavior. *IRT1* expression level was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime were non-significant (Table 4.40). Under iron sufficient conditions, ARISOY revealed the highest expression (2.53 folds) of *IRT1* and the lowest was observed in SA-88 (1.92 folds) against Fe sufficient of NOVA, indicating that the genotype differences did not affect the *IRT1* expression under Fe sufficient conditions (Appendix D supplementary figure 2). Up-regulation of *IRT1* was observed in all tested genotypes under iron deficient conditions as compared to iron sufficient conditions. The highest fold increase in the expression levels of *IRT1* was observed in ATAKISI (11.43 folds) and the lowest was observed in SA-88 (7.18 folds). Moreover, NOVA and ARISOY showed 8.02 and 8.51 folds of increase, respectively, under iron deficiency (Figure 4.41).

Table 4.40. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on *IRT1* expression levels.

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	185.192	185.192	54.885	< 0.0001 *
Genotypes (G)	3	15.700	5.233	1.551	0.275
Fe × G	3	7.779	2.593	0.768	0.543

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant ($p<0.05$)

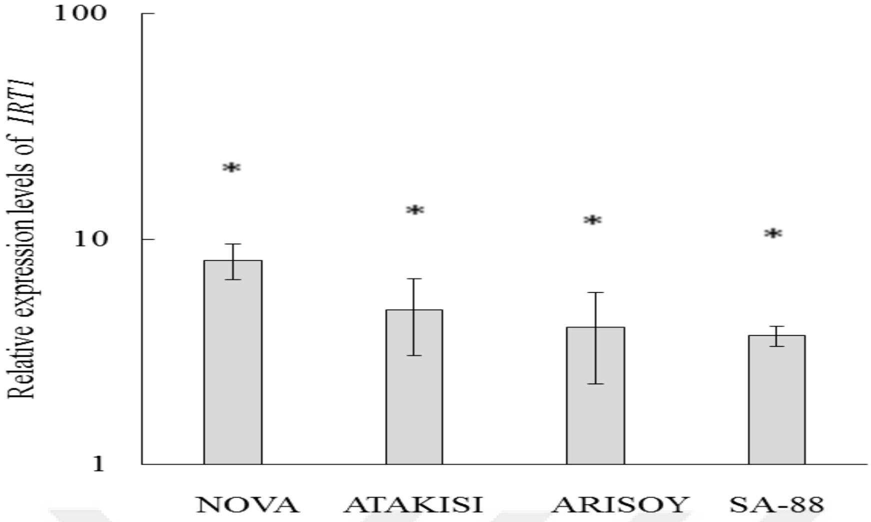


Figure 4. 41. RT-qPCR analysis of *IRT1* transcript levels in the roots of *Glycine max*. Expression levels relative to the Fe-sufficient conditions of each genotype.* indicates significant differences between Fe-deficient and sufficient conditions for each genotype ($p<0.05$).

A broad spectrum of transporters has been characterized, such as the Natural Resistance Associated Macrophage (NRAMP) proteins, involved in Fe import into the cytoplasm (Kim et al., 2006). The expression level of *NRAMP* (*DMT1*) was observed in order to better understand its behavior under iron deficient conditions. *NRAMP* expression level was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime were non-significant (Table 4.41). ARISOY and SA-88 revealed 3.98 and 2.63 folds of *NRAMP* expression levels under iron sufficient conditions (Appendix D supplementary figure 3). Significantly up regulation of *NRAMP* was observed in all tested genotypes under iron deficient conditions as compared to iron sufficient conditions. The highest expression of *NRAMP* was observed in ATAKISI (12.19 folds) and the lowest was observed in SA-88 (2.63 folds). Moreover, NOVA and ARISOY showed 3.91 and 3.98 folds of increase under iron deficiency (Figure 4.42). Interestingly down regulation of NRAMP was observed in ATAKISI under iron sufficient conditions.

Table 4.41. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on *NRAMP* expression levels.

Source	**DF**	**SS**	**MS**	**F value**	**P value**
Fe Availability Regimes (Fe)	1	79.109	79.109	6.070	0.039 *
Genotypes (G)	3	44.305	14.768	1.133	0.392
Fe × G	3	73.452	24.484	1.879	0.212

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant (p<0.05)

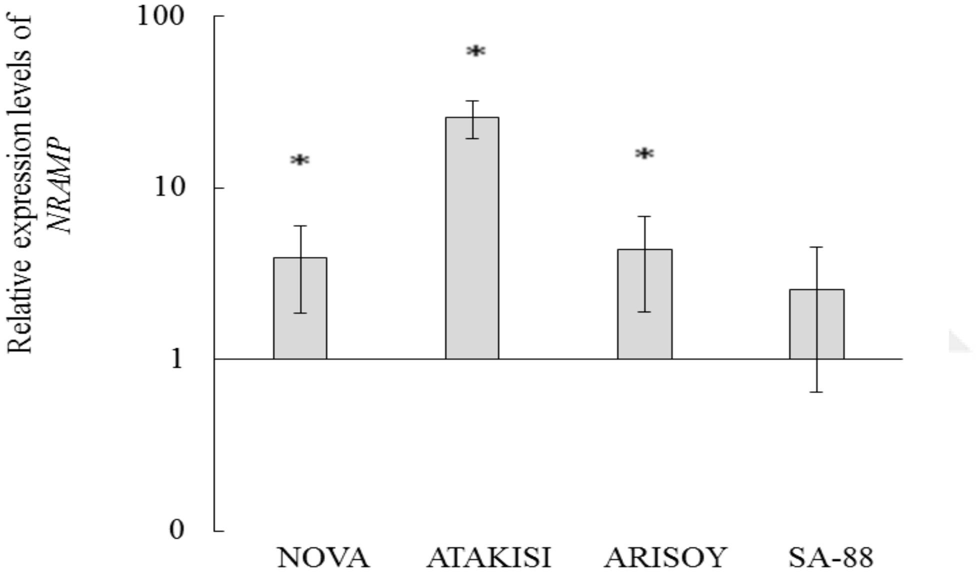


Figure 4. 42. RT-qPCR analysis of *NRAMP* transcript levels in the roots of *Glycine max*. Expression levels relative to the Fe-sufficient conditions of each genotype. * indicates significant differences between Fe-deficient and sufficient conditions for each genotype (p<0.05).

Fe homeostasis needs to be strictly controlled to avoid iron deficiency and toxicity (Liao et al., 2012). Therefore, storage proteins, such as Ferritin, play an important role in iron homeostasis (Briat et al., 2010). *FERRITIN* expression level was significantly altered by Fe availability regimes, while soybean genotypes and interaction among soybean genotypes and Fe availability regime were non-significant (Table 4.42). In the current study, expression of *FERRITIN* was observed in the roots of *Glycine max* under iron sufficient and deficient conditions. A decrease in *FERRTIN* expression was observed in all cultivars under iron deficit conditions as compared to iron sufficient conditions (Appendix D supplementary Figure 4). This is an expected result of this gene since its expression is repressed under Fe deficiency in Arabidopsis as well. Moreover, the highest decline in expression level was observed in ATAKISI (0.265folds) respectively lowest decrease was observed in NOVA (0.67 folds) under iron deficit conditions (Figure 4.43)

Table 4.42. Analysis of variance of Fe availability regimes, soybean genotypes and their mutual interactions on *FERRITIN* expression levels,

Source	DF	SS	MS	F value	P value
Fe Availability Regimes (Fe)	1	844.823	844.823	6.157	0.038*
Genotypes (G)	3	364.097	121.366	0.885	0.489
Fe × G	3	371.448	123.816	0.902	0.481

DF = degree of freedom, SS = sum of squares, MS = mean square, * = significant, ^{NS} = non-significant

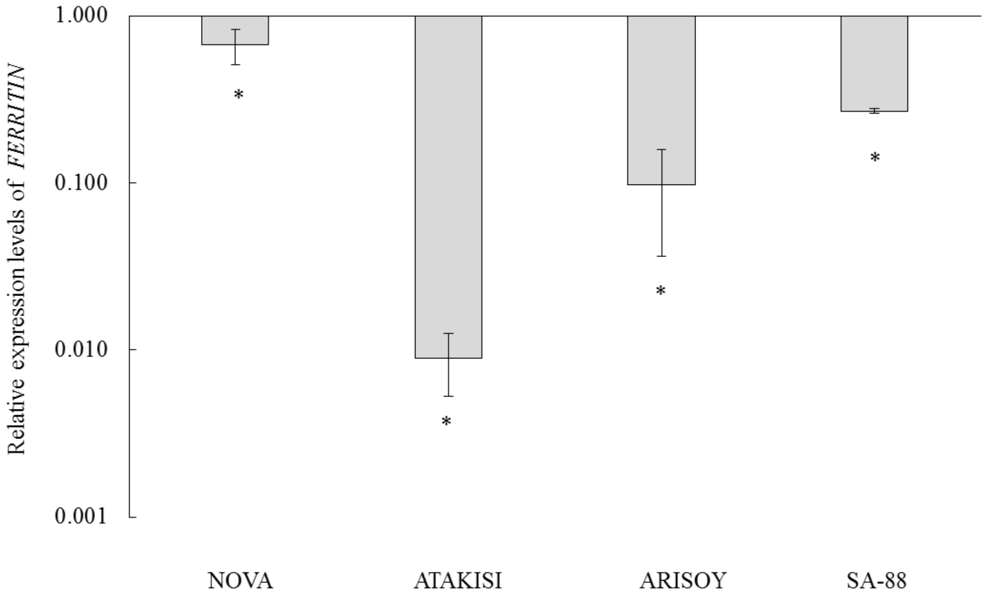


Figure 4. 43. RT-qPCR analysis of *FERRITIN* transcript levels in the roots of *Glycine max*. Expression levels relative to the Fe-sufficient conditions of each genotype.* indicates significant differences between Fe-deficient and sufficient conditions for each genotype (p<0.05).

CHAPTER V

DISCUSSION

Results of the study are briefly discussed in this chapter.

Iron deficiency chlorosis is known to reduce yield of soybeans even though no visual indication of iron chlorosis can be observed (O'Rourke et al., 2007). Selection of tolerant and efficient Fe mobilizers can be the best tool to overcome this problem. After 30 days under deficiency conditions, a clear effect on the leaf chlorophyll content and growth of the tested genotypes was recorded.

Gruber and Kosegarten (2002) showed that suppressed leaf formation and poor leaf growth are typical symptoms of IDC response, due to the high sensitivity of the meristematic apex to low iron availability in soybean. A similar observation has been reported in the current thesis study. Tolerant cultivars showed a minor decrease in leaf fresh weight whereas the sensitive cultivars showed a major decline in fresh weight of the leaves. These results suggest that the Fe availability influences the overall leaf fresh weight more drastically in sensitive cultivar.

Dry weight of leaves is the direct indication of minerals and nutrients in the leaves. Our major findings are comparable to the previous studies (Pestana et al., 2005, Jelali et al., 2010a, b; Msilini et al., 2012; Chebbi et al., 2013; Su et al,. 2014), as they showed a significant reduction in dry weight of plants exposed to direct Fe deficiency. It suggests that soybean is an Fe-efficient plant, which means iron has effective role in chlorophyll biosynthesis and uptake of other nutrients (Marsh et al., 1963; Miller et al., 1984). As a matter of fact, basic soil (with a pH around 9) was used in our experiment which decreases the uptake of iron and other nutrients; thus, decreased dry matter could be observed (Elkhouni et al., 2018). However, exceptionally genotype named "ATAEM-7" has showed increase in dry weight under iron deficit conditions in contrast to iron sufficient conditions. Similarly, a significant decrease in FRO2 enzyme activity was observed in ATAEM-7 under iron deficiency conditions as compared to all other tested genotypes, in which an increase in FRO2 enzyme activity was observed. This observation can be due to the natural high level of FRO2 enzyme activity in ATAEM-7, which in turn is correlated with higher levels of mineral uptake and accumulation and the increase in dry weight of plants.

SPAD is an indirect indicator of chlorophyll content as significant correlations have been established in earlier studies (Samdur et al., 2000; Boodi et al., 2015). In our study, a decline in SPAD value was observed under iron deficiency. These findings are comparable to the results of Chen et al. (2018) and Ceballos-Laita et al. (2018), owing to iron deficiency-induced chlorosis in the leaves, which causes the reduction in SPAD value. Chlorophyll index and chlorophyll content measurements goal to determine the damage level of chlorophylls in the leaves under iron deficiency. The negative effects of Fe deficiency on reduced chlorophyll contents have been well reported (Hansen et al., 2003; Lin et al., 2000). The Fe deficiency-induced chlorophyll changes/damages are associated with Fe-dependent enzymatic activities required to synthesize chlorophyll (Miller et al., 1995), and for proper functioning of carbon-fixation and electron transport in photosystems (Andaluz et al., 2006). The reduction in chlorophyll contents and chlorophyll index of sensitive genotypes can be correlated with the hampered activities of Fe-dependent enzyme activities in the chloroplasts. On the other hand, the tolerant genotypes managed to perform the normal activities of Fe-dependent enzymes for chlorophyll synthesis, which resulted in lesser changes in chlorophyll index and chlorophyll contents (Gruber and Kosegarten, 2001).

It is well reported that 80 percent of the iron is found in the photosynthetic cells of the plants, where it is the key element for the biosynthesis of chlorophyll, many heme molecules, biosynthesis of cytochromes and electron transport chain due to its pivotal role in the redox reactions (Maathuis, 2009). A significant decrease in the photosynthetic rates of chlorotic leaves under Fe deficiency was observed in the study as compared to the green leaves Fe efficient conditions. Previously iron deficiency has been reported to cause deleterious effects on many photosynthetic parameters, including: (i) decreases in leaf photosynthetic pigment concentrations; (ii) decreases in photosynthetic rates; (iii) decreases in photosynthetic efficiency and electron transport through PS II at steady-state photosynthesis estimated by Chl fluorescence, together with increases in thermal energy dissipation within the PS II antenna, and (iv) a high relative proportion of the de-epoxidated forms A and Z in the V + A + Z pigment pool (larbi et al., 2004). Our findings are comparable to the previous studies (Ren et al., 2018; Ucar et al., 2017), because all the photosynthesis-related parameters demonstrated strong responses to decreasing Fe supply, as has also been observed for the plants (Willson et al., 2017). Additionally, chlorophyll deficiency is directly proportional to iron deficiency and chlorophyll deficiency is correlated with the photosynthesis rate in the plant (Pal et al., 2015).

When exposed to light, iron-deficient plants show considerably lower efficiency of excitation energy capture by open PSII reaction centers (Fv'/Fm'), quantum yield of PSII electron transport (PhiPSII), and photochemical quenching coefficient (qP), but markedly higher non-photochemical quenching (NPQ) (Jiang et al., 2007). These results suggest that both the donor and the acceptor sides of PSII complex are damaged by iron deficiency; cyclic electron transport around PSI in iron-deficient soybean plants might play an important role in inducing the excitation energy dissipation and meeting the demand for extra ATP as a compensation for the loss of phosphorylation capability. Therefore, we can conclude that the soybean cultivars that show higher chlorosis are prone to accumulate less Fe in their leaves, which in turn decreases the level of chlorophyll biosynthesis, photosynthesis and overall fresh and dry weight of the leaves. On the other hand, the cultivars that can tolerate Fe deficiency well show the opposite reactions since they can accumulate higher levels of Fe in their leaves. Interestingly, in a study of major Fe transporter in root epidermis membranes, IRT1, in Arabidopsis it was found that *irt1* mutants showed increased levels of photosensitivity dues to degradation of photosynthetic apparatus, which in turn decreased the chlorophyll fluorescence (Varotto et al., 2002). Therefore, similar to the results presented here, a single mutation in *IRT1* gene drastically alters the photosystem mainly due to lower levels of Fe uptake from rhizosphere into the roots.

At the shoot level, the absence of Fe is known to inhibit chlorophyll and chloroplast, leading to the development of chlorosis, especially in younger leaves (Henriques et al., 2002). Higher levels of Fe deficiency could even change the ultrastructure of thylakoid membranes and chloroplasts (Bashir et al., 2015). Similar observations were recorded in the current study which strengthen our argument that sensitive cultivars were not able to continue normal growth and development, which ended with lower chlorophyll contents in the leaves. The inherent genetic makeup of the cultivars might be the other reason for significant differences among sensitive and tolerant cultivars (Vasconcelos and Grusak, 2014).

When compared to the literature, the findings of this thesis suggest that the chloroplast ultrastructure, chlorophyll biosynthesis, and photosystem and electron transport chain proteins could be protected well in tolerant soybean cultivars since they accumulate required levels of Fe in the shoots. Moreover, changes in Fe levels should be highly regulated in the cells since low levels of Fe can inhibit the photosynthesis due to the reasons mentioned above, but it becomes toxic when accumulates in excess. In this case, Fe^{2+} interacts with hydrogen peroxide (H_2O_2) to form hydroxyl radicals (OH•) via the Fenton reaction in chloroplasts and mitochondria (Mittler, 2017). Since these free radicals can attack the proteins of photosystem and electron transport chain, their accumulation drastically inhibits photosynthesis and eventually causes chlorosis and tissue damage (Nowaczyk and Plumeré, 2016). Taken together, change in Fe homeostasis in chloroplasts inhibits the photosynthetic electron transport, therefore increases the chance of free radical production. Many photosynthetic organisms have evolved antioxidant systems around the proteins of photosystem and electron transport chain in chloroplasts to protect them against free radical attacks (Gupta et al., 2018). Similar to cyanobacterial systems (Lax et al., 2007; Kranzler et al., 2013), tolerant soybean cultivars might have developed some new strategies in the chloroplasts to cope with Fe shortages. Antioxidant enzyme systems should be worked in these tolerant cultivars (compared to sensitive ones) in the future to further clarify the potential involvement of free radical-scavenging mechanisms in soybean under Fe deficiency.

To acquire iron, almost all plant species reduce soil Fe (III) to Fe (II) by Ferric-chelate reductases (*FRO2*) present in the plasma membrane of root epidermal cells, according to the recent literature (Aksoy et al., 2018). The reduced Fe product is then taken up by Fe (II) transporter proteins. These activities are induced under Fe deficiency (Nikolic and Pavlovic, 2018). Ferric chelate reductase (*FRO2*) expression increased Fe (III)-chelate reductase activity under iron deficient conditions (Waters et al., 2002). Similar results were observed in this thesis. Although FRO enzyme activity increases in sensitive vanities, but plants unable to uptake more iron from soil under stress conditions. It can be correlated with poor plant growth, low SPAD and total chlorophyll contents in the leaves. Moreover, the cultivars having the higher FRO enzyme activity under normal conditions have showed less increase in the enzyme activity under iron deficient conditions, indicating that these cultivars already had higher basal levels of FRO activity and therefore could tolerate Fe deficiency better than the sensitive ones with lower levels of basal FRO activity. Similarly, higher fresh weight, dry weight, chlorophyll contents and SPAD values were observed in the cultivars having higher natural FRO enzyme activity. Interestingly ATAEM-7 showed the highest FRO enzyme activity under normal conditions. These results give the indication that natural FRO enzyme activity is the rate limiting step for the uptake and translocation of iron from soil to the other aerial parts of the plants. This observation was supported by the findings of Satbhai et al. (2017). Accordingly, they showed a correlation between FRO activity, *FRO2* transcript abundance and root length in *Arabidopsis thaliana* accessions under iron deficiency. Moreover, they also identified *FRO2* locus as the major determinant of genetic variations under Fe deficiency.

Our results are comparable to the previous findings (She et al., 2018; Moreira et al., 2018). The increased Ci, together with non-significant correlation, suggest that non-stomatal limitation may contribute to the decrease of Fe (Farquhar and Sharkey, 1982). These results are similar to that of Fernandez et al. (2008) viz. iron sufficient plants showed lower transpiration rates and iron deficit plants showed increase in transpiration rates at V2-V3 developmental stages. However, contrasting results were found at R3-R4 developmental stages as some iron sufficient cultivars have more transpiration rates and transpiration rate of the Fe deficient leaves were kept at low levels due to the lower degree of stomatal opening as compared to green leaves, in agreement with Larbi et al. (2006). These results indicate that developmental stage of the plant is critical to Fe homeostasis, and plant modifies its transcriptional control according to the environment in order to uptake iron from soil and translocate it between roots to shoots.

It has been hypothesized that, for some genotypes e.g ATAEM7, Fe is necessary for appropriate functioning of the reductase enzyme itself (Blair et al., 2010). Although most studies imply that Fe reduction is induced under Fe deficiency (Wang et al., 2013; Zha et al., 2014), it has already been described that this is not always true (Vasconcelos and Grusak., 2006; Santos et al., 2015). In order to understand how Fe deficiency affects the mineral composition in *G. Max* organs, root and shoot tissues at V2-V3 developmental stage and seeds (after harvest) were analyzed by ICP-MS. When soybean cultivars were exposed to low iron availability, they appeared to accumulate more Fe in the roots; however, shoot iron content decreased up to many folds under iron deficient conditions. It has been reported before that in response to shortage in mineral nutrition, plants usually allocate more resources to the roots (Hermans et al., 2006; Santos et al., 2015). Our results are comparable to the previous findings (Santos et al. 2016), where soybean roots showed increase in iron contents, while shoots showed a major decline in iron content. Moreover, decrease in seed iron contents was observed for all the cultivars except for APOLLO. Unexpectedly APOLLO showed an increase in seed iron content under iron deficient conditions. Therefore, this variety may be used in future biofortification breeding programs to enhance the seed iron content. As previously reported, plants exhibit tight homeostatic control to prevent accumulation of iron where it is not needed, and this may limit iron redistribution to edible tissues such as seeds (Connorton et al., 2017).

To further understand the mechanisms triggered by Fe unavailability, it is critical to understand the key conserved molecular players involved in nutrient uptake (e.g. FRO2 and IRT1), transport (e.g. NRAMP,) and storage (e.g. ferritin).

IRT1 is the essential iron transporter for the uptake of iron from the soil (Vert et al., 2002). It is the main Fe importer in dicotyledonous plants, including soybean **(**Korshunova et al., 1999: Potocki et al., 2013). Ferric chelate reductase expression of *FRO2* increased the Fe(III)-chelate reductase activity under iron deficient conditions (Waters et al., 2002). FRO2 and IRT1 are subject to tight regulation at the transcriptional and post-transcriptional levels. As reported by previous studies, under low availability of iron, the activity of the FIT regulatory system triggers to increased *IRT1 and FRO2* gene expression. (Sivitz et al., 2012). Similarly, current study revealed the up regulation of *IRT1* under iron deficit conditions in all tested genotypes, comparable to previous findings in Arabidopsis (Jeong et al., 2009). Two varieties (NOVA and ATAKISI) assumed as sensitive on the basis of physiological and biochemical results. They showed higher expression of *FRO2* and *IRT1* whereas the tolerant cultivars showed lower expression as compared to the sensitive ones. It was shown that the expression of *FRO2* significantly increases whereas *IRT1* expression do not change in the roots of soybean Williams 82 exposed to low levels of Fe deficiency for 14 days in hydroponics (Santos et al., 2016). These differences between the results of this thesis study and Santos et al. (2016) findings might be due to the experimental setup and the length of the stress application. It is already known that

IRT1 and *FRO2* expression is affected from the length of the stress application (Aksoy et al., 2013). Moreover, it was already established that V3-V4 stage is the best developmental stage to observe the effects of Fe deficiency in soybean (Vasconcelos and Grusak, 2014). Moreover, the sensitive cultivars showed more FRO2 enzyme activity under iron deficit conditions as compared to the tolerant one, but the dry weight and total chlorophyll results showed that the sensitive cultivars have failed to translocate iron from roots to the upper aerial parts of plants.

A broad spectrum of transporters has been characterized, such as the Natural Resistance Associated Macrophage (NRAMP) proteins, involved in Fe import into the cytoplasm (Kim et al., 2006). *NRAMP* gene expression is increased upon Fe starvation suggests that these proteins are involved in plant Fe nutrition (Kim et al., 2006). Accordingly, under Fe deficiency, as plants need more remobilization of Fe to respond to their needs, *NRAMP-like* expression increases in (Santos et al., 2016). Similarly, a significant increase in the *NRAMP-like* transcript levels was observed in the roots of all tested genotypes under iron limited conditions in the current study.

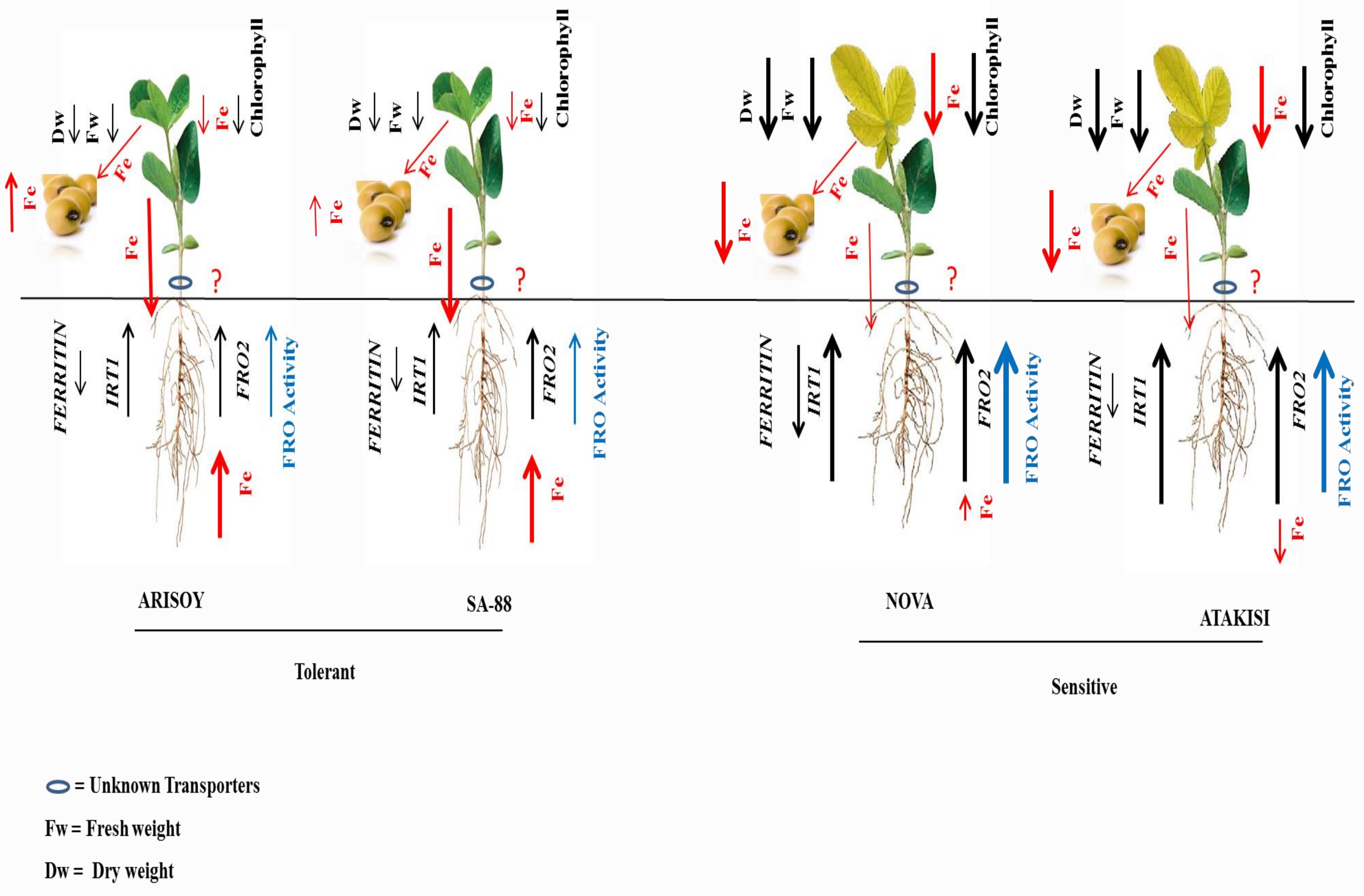
Fe homeostasis needs to be strictly controlled to avoid iron deficiency and toxicity (Liao et al., 2012). Therefore, storage proteins, such as ferritin, play an important role in iron homeostasis (Briat et al., 2010). Similar to Santos et al. (2016), a significant decrease in *FERRITIN* transcript level was observed under iron deficit conditions. Qu et al. (2005) has reported similar down-regulation of *FERRTIN* gene due to low iron scarcity. Findings of Goto et al (1998) are also in coincidence with our results, i.e. a significant decrease in *FERRITIN* transcript level was observed in iron-limited conditions owing to the fact that free and ferritin-bound irons in plants exist in equilibrium, so over-expression of ferritin may disturb this equilibrium, resulting in the sequestration of essential free iron (Briat and Lobreaux, 1997; Gueriont and Yi, 1994). Additionally, the demand for iron uptake is increased by *FERRITIN* over-expression and the accumulation of undesirable metals may be restricted under normal culture conditions (Qu et al., 2005).

Overall the combination of the results from physiological, biochemical and molecular analyses indicate that Arısoy and SA88 are the tolerant varieties with different tolerance mechanisms. As shown in Figure 5.1, chlorophyll index and total chlorophyll content of Arısoy decreases, which directly influences a decrease in photosynthesis rate.

Moreover, the fresh and dry trifoliate leaf weight decreases together with a much lower level of Fe accumulation in the leaves. Meanwhile, more Fe accumulates in the roots of Arısoy under Fe deficiency, which does not enhance the expression of FRO2 and FRO activity that much; therefore, plant cannot uptake enough Fe from the rhizosphere. Although it cannot uptake Fe from the soil, since it translocates more Fe from shoots to the roots, there will be a higher Fe pool in the roots, which inhibits the activation of Fe deficiency signaling in this organ. Interestingly, the same plan cannot translocate Fe from roots back to the shoots; therefore, there will be a local Fe deficiency in the leaves. However, this effect is not very high as the basal level of Fe in the leaves (under normal Fe conditions) is high enough to compensate for the decrease in photosynthesis and other parameters related with it. In contrast to Arısoy, the other tolerat variety, SA88, behaves in a different way to tolerate Fe deficiency. First of all, chlorophyll index and total chlorophyll content of SA88 decreases to a similar level when compared to Arısoy; however, its trifoliate fresh and dry weights along with the photosynthesis rate was not affected as much as Arısoy. The main reason for thes observations is that the Fe accumulation in the leaves of SA88 was less low than that found in Arısoy. Therefore, it does not show Fe deficieny symptoms in the green organs as much as Arısoy does. Next, it accumulates more and more Fe in its roots compared to Arısoy since it can activate FRO2 expression and FRO activity more than Arısoy. FER expression was also less suppressed in the roots of SA88 than it did in the roots of Arısoy due to high level of Fe. Overall, these observations indicate that SA88 still continues to operate (or even enhance) Fe uptake mechanisms in the root epidermis cells. Moreover, it translocates iron from shoots to the roots; but, cannot bring it back to the shoots. Therefore, there is a higher Fe pool in the roots, and plant does not sense the Fe deficiency. Interestingly, it was suggested that higher Fe pools in the roots of the Arabidopsis plants block the activation of FRO (Garcia et al., 2013). But, in the case of SA88, FRO activity and root Fe uptake might have been enhanced due to an unknown signal generated locally in Fe deficienct leaves, and this signal might activate the FRO and IRT1 even if the local root Fe levels are high.

When the sensitive varieties were compared, Atakişi accumulated lower levels of Fe in trifoliate leaves compared to Nova; therefore, it showed much lower levels of chlorophyll index, total chlorophyll content, leaf fresh and dry weights (Figure 5.1). Moreover, FRO activity was much lower in the roots of Atakişi than it did in the roots of Nova. One reason for this is that Atakişi did not translocated more Fe from leaves to the roots in contrast to Nova. Meanwhile, both varieties did not uptake enough Fe from the rhizosphere. Hence, the expression of FERRITIN was the lowest in the roots of Atakişi. Overall, sensitive vareties were sensitive to Fe deficiency since they cannot uptake enough Fe from the soil into the roots and some varieties could not translocate iron from roots to the shoots .

Figure 5.1. Summary of the iron deficiency responses of tolerant and sensitive soybean varieties.



CHAPTER VI

CONCLUSION

The current study concludes that different soybean cultivars used in Turkey significantly differ in their ability to uptake and mobilize Fe under Fe-Deficient or unavailable conditions. The main conclusions from the obtained results of the current study are enlisted as under;

- Iron deficiency significantly affected the biomass production (fresh and dry biomass), physiology (photosynthesis rate and chlorophyll content), enzyme activity (FRO) and gene expression (*FRO2*).
- The soybean genotypes included in the study differed in morphophysiological attributes, biomass production and gene expressions under Fe deficiency.
- Under strict criteria, we can conclude four varieties as tolerant (ARISOY, SA-88, ATAEM-7 and APOLLO) since they did not show a major decline in fresh weighs, chlrophyll contents and iron contents in major aerial parts of plants. Moreover, three varieties were designated as the most sensitive ones (NOVA, ATAKISI and BLAZE) as they showed increase in FRO enzyme activity, *FRO2* gene expression levels and decrease in fresh weight, dry weight, chlorophyll contents and shoot and seed iron contents.
- The genotypes were unable to translocate iron into the shoots and seeds under Fe deficiency.
- The cultivar ATAEM-7 showed a unique behavior under iron deficit conditions. All cultivars showed increase in FRO enzyme activity under iron deficient conditions, while ATAEM-7 showed a decrease in FRO enzyme activity and increase in shoot iron content and dry weight. This behavior had never been reported before in any soybean genotypes. However, in some cases iron is required by the proper functioning of FRO by itself, and this may be the reason. More research in this regard is needed.
- The cultivars having natural higher FRO enzyme activity were found to be IDC-tolerant, showing less decrease in fresh weight, dry weight, chlorophyll content, iron content in leaves and seeds under iron deficient conditions.

- It can be concluded that natural FRO enzyme activity is the rate limiting step for iron uptake in plants.
- The cultivars designated as ‘tolerant’ need further studies to increase iron mobilization towards shoots and seeds through breeding or biotechnological tools.
- Careful selection of efficient mobilizers could help in reducing the yield losses incurred by calcareous soil, which cover as about 30 percent of the world arable land. The identified cultivars in the current study could be potential candidates of cultivation on calcareous soils in the country. These tolerant varities can be used for further breeding programs for the development of new iron deficiency tolerant varieties or iron efficient varieties.

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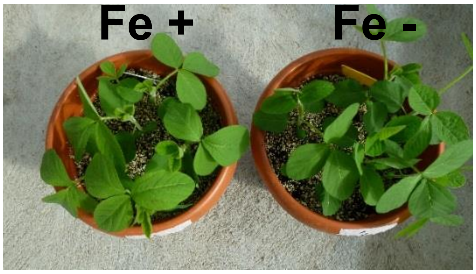
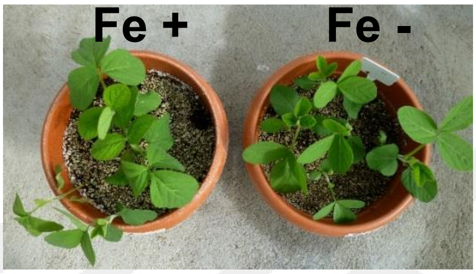
APPENDIX

APPENDIX-A

IDC SCORING

Cultivars	Image	Chlorosis Group
Olympus		**1**
A-3127		**1**
Ataem-7		**1**
Arısoy		**1**

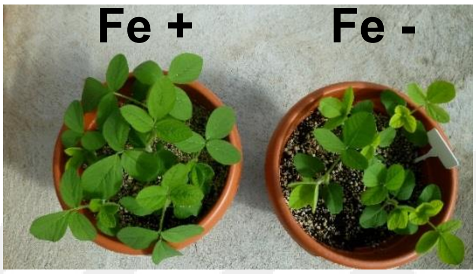
APPENDIX A (Continue)

Bravo		**1**
Blaze		**1**
KS-4694		**1**
SA88		**1**

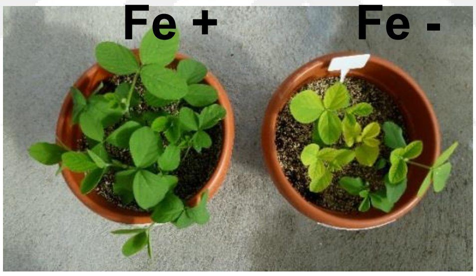
APPENDIX A (Continue)

Apollo		**2**
Türksoy		**2**
MAY-5312		**2**
Stress Land		**2**

APPENDIX A (Continue)

Atakişi		**3**
İlksoy		**3**
Nova		**4**
Adasoy		**4**

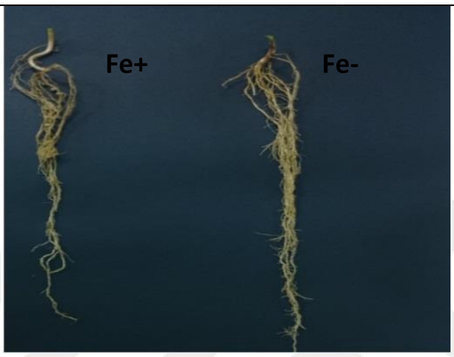
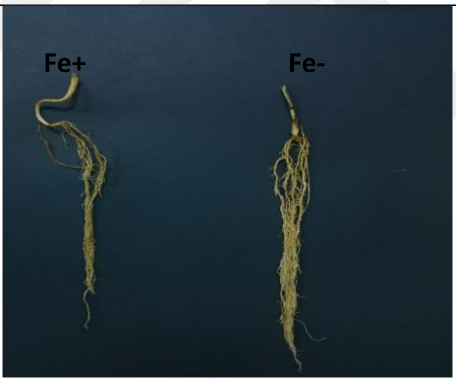
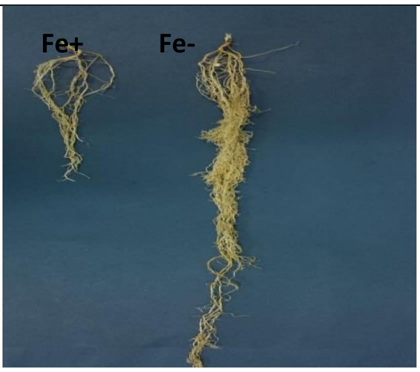
APPENDIX A (Continue)

Umut2002		**4**
A1621		**4**
Amcor		**4**
S-4240		**4**

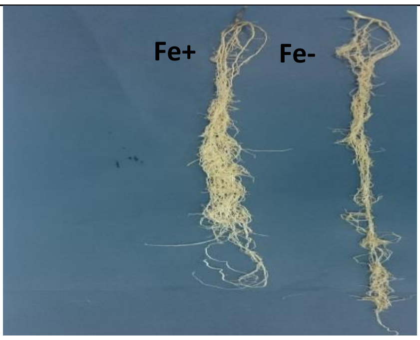
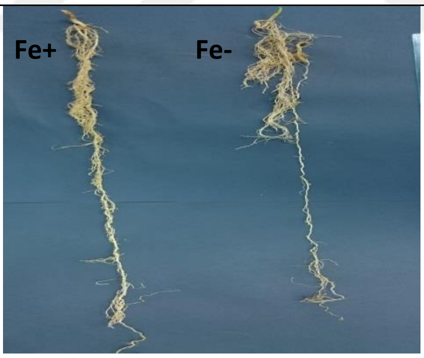
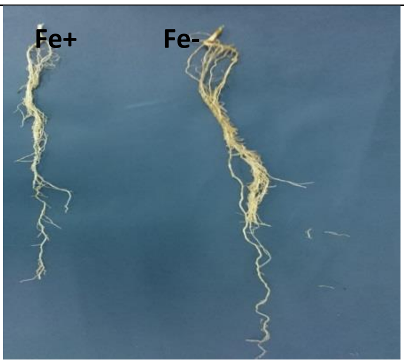
APPENDIX B

ROOT LENGTHS

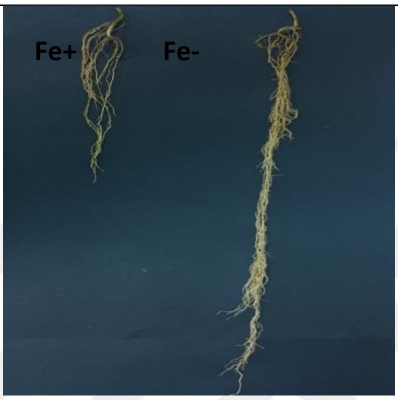
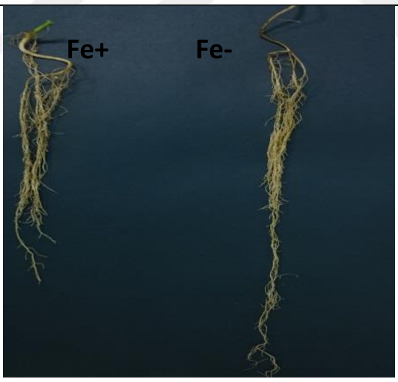
Roots of *Glycine Max* under iron deficient (high pH) conditions.

Cultivars	Image	Chlorosis Group
Olympus		**1**
A-3127		**1**
Ataem-7		**1**

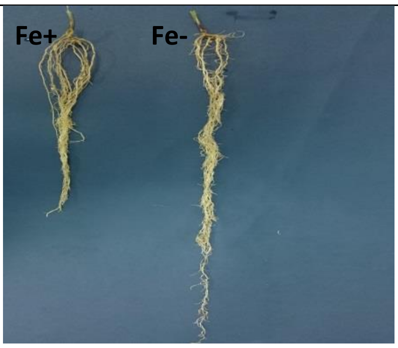
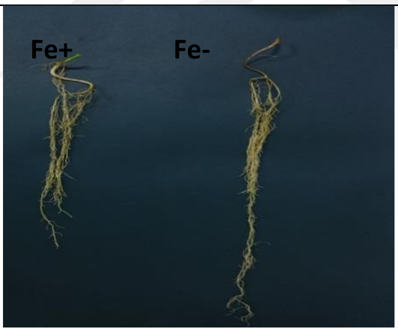
APPENDIX B (Continue)

Arısoy		**1**
Bravo		**1**
Blaze		**1**
KS-4694		**1**

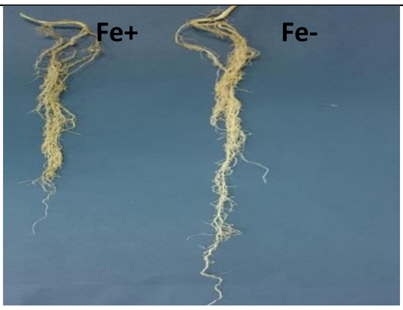
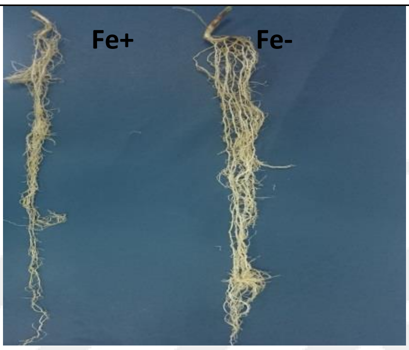
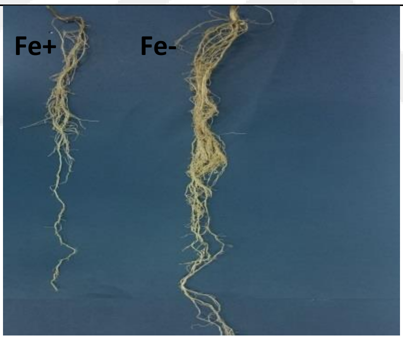
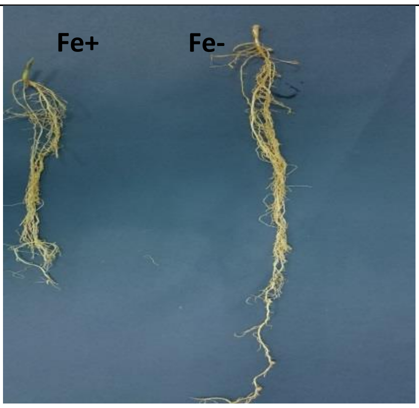
APPENDIX B (Continue)

SA88		**1**
Apollo		**2**
Türksoy		**2**
MAY5312		**2**

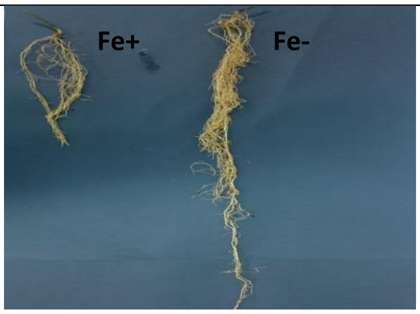
APPENDIX B (Continue)

Stress Land		**2**
Atakişi		**3**
İlksoy		**3**
Nova		**4**

APPENDIX B (Continue)

Adasoy		**4**
Umut2002		**4**
A-1621		**4**
Amcor		**4**

APPENDIX B (Continue)

S-4240		**4**

APPENDIX C

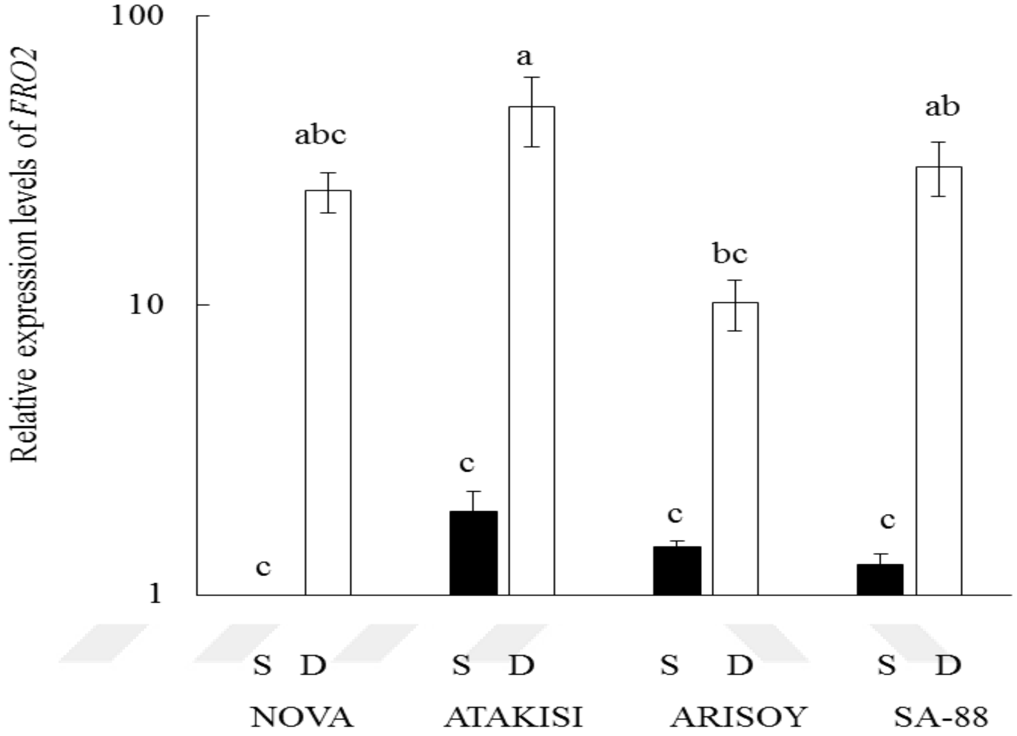
QUANTIFICATION OF RNA SAMPLES

RNA quantification by nano-photospectrometer.

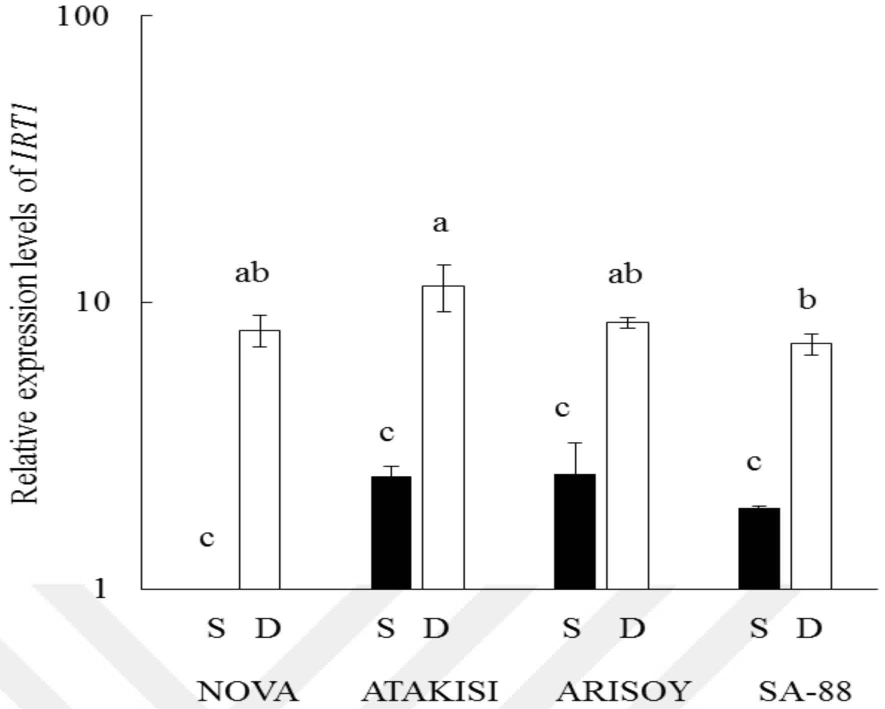
Sample	RNA Concentration (ng/µL)	OD 260/280	Sample	RNA Concentration (ng/µL)	OD 260/280
NOVA-SUF	732.9	2.06	ARISOY-SUF	607.32	2.00
NOVA-SUF	416.99	1.99	ARISOY-SUF	682.33	2.05
NOVA-DEF	1008.62	2.01	ARISOY-DEF	352.69	2.06
NOVA-DEF	765.09	1.99	ARISOY-DEF	412.24	2.01
ATAKISI-SUF	626.02	2.01	SA-88-SUF	688.2	1.96
ATAKISI-SUF	970.81	2.00	SA-88-SUF	688.2	2.09
ATAKISI-DEF	899.7	2.03	SA-88-DEF	1151.19	1.89
ATAKISI-DEF	536.75	1.98	SA-88-DEF	431.87	2.11

APPENDIX D

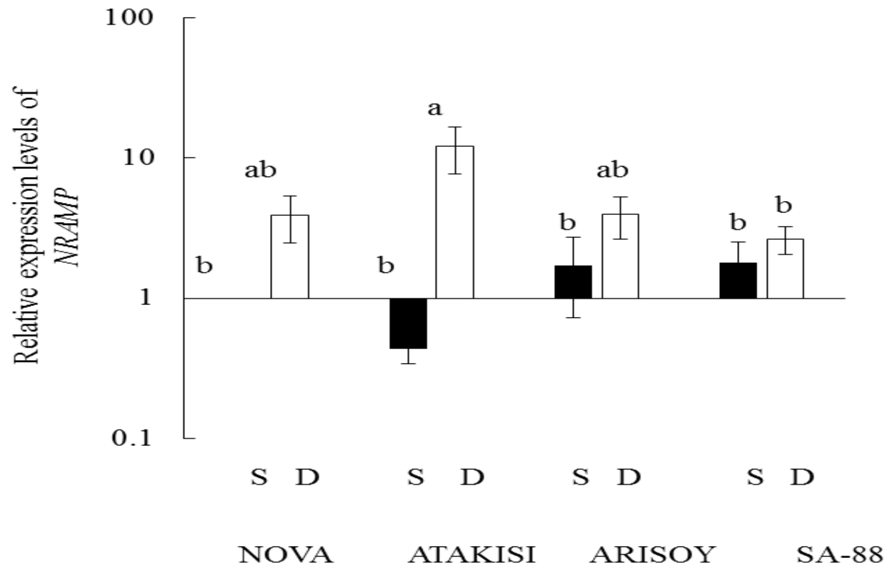
Results of Relative Gene Expression Analyses



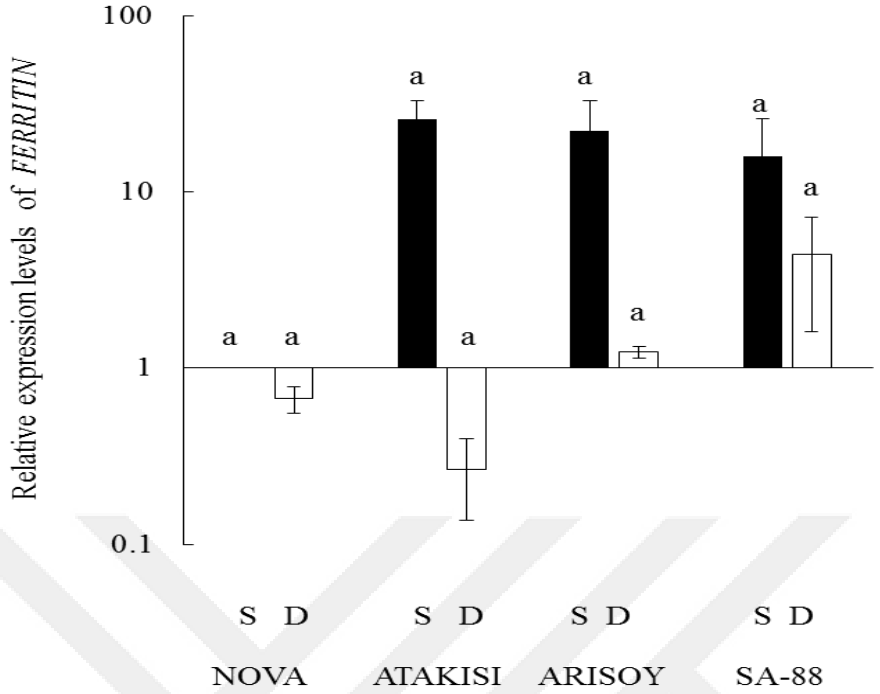
Supplementary Figure 1. qRT-qPCR analysis of *FRO2* transcript levels in the roots of *Glycine max*. under iron sufficient and deficient conditions against sufficient of NOVA. Different letters indicate significant differences among different Fe treatments on genotypes against Nova Fe-sufficient condition ($p<0.05$).



Supplementary Figure 2. qRT-qPCR analysis of *IRT1* transcript levels in the roots of *Glycine max*. under iron sufficient and deficient conditions against sufficient of NOVA. Different letters indicate significant differences among different Fe treatments on genotypes against Nova Fe-sufficient condition ($p<0.05$).



Supplementary Figure 3. qRT-qPCR analysis of *NRAMP* transcript levels in the roots of *Glycine max*. under iron sufficient and deficient conditions against sufficient of NOVA. Different letters indicate significant differences among different Fe treatments on genotypes against Nova Fe-sufficient condition ($p<0.05$).



Supplementary Figure 4. qRT-qPCR analysis of *FERRITIN* transcript levels in the roots of *Glycine max*. under iron sufficient and deficient conditions against sufficient of NOVA. Different letters indicate significant differences among different Fe treatments on genotypes against Nova Fe-sufficient condition (p<0.05)

CURRICULUM VITAE

Amir Maqbool was born on October 15, 1992 in Multan, Pakistan. He completed his higher secondary education from MPHSS College Multan, Pakistan in 2011. Afterwards he joined Bahauddin Zakariya University, Multan Pakistan in 2011 for his undergraduate studies. He completed his B.Sc (Hons) in Agriculture from Department of Plant Breeding And Genetics, Faculty of Agricultural Sciences and Technologies in September 2015. Afterwards he enrolled in Graduate School of Natural and Applied Sciences, Department of Agricultural Genetic Engineering at Niğde Ömer Halisdemir University, Niğde, Turkey to pursue his master education under the Supervision of Assistant Professor Dr. Emre Aksoy. During his master thesis research, he worked on ‘Physiological Biochemical and Molecular Response of Different Soybean Cultivars Under Iron Deficiency’. He knows English, Urdu, Turkish and Punjabi languages.

Publications Produced from Thesis Work

A total of 7 publications have been made from this master thesis. These publications are listed below:

SCI publication:

1. Aksoy, E., **Maqbool, A**., Tindas, İ. and Caliskan, S., 2017. Soybean: A new frontier in understanding the iron deficiency tolerance mechanisms in plants. Plant and Soil, 418(1-2), pp.37-44.

Invited Speaker:

1. Sevgi Çalışkan, Emre Aksoy, Amir Maqbool, Mehmet Emin Çalışkan "Correlation Analysis of Some Responses of Soybean Cultivars Under Iron Deficiency" 3. International Plant Breeding Congress, October 15 - 19, 2017, Kyrenia, TRNC.

Conference Presentations:

1. Emre Aksoy, Ahmet Şahin, Amir Maqbool, Sevgi Çalışkan "In silico Identification and Characterization of Potential Molecular Markers Related with Iron Deficiency Tolerance in Soybean" International Agricultural Science Congress, May 9-12, 2018, Van, Turkey (Oral presentation).

2. Emre Aksoy, Amir Maqbool, Sevgi Çalışkan "Soybean Breeding Program Concentrating on Seed Iron Content" International Agricultural Science Congress, May 9-12, 2018, Van, Turkey (Oral presentation).

3. Amir Maqbool, Emre Aksoy, Sevgi Çalışkan "Analyses of Some Molecular Responses of Some Local Soybean Varieties under Iron Deficiency" 8th Agriculture Student Congress with International Participation, April 27-29, 2018, Niğde, Turkey.

4. Amir Maqbool, Emre Aksoy “Ferric-chelate Reductase: Could It Be Used as an Alternative Method to Understand Iron Sensitivity in Soybean?” International Green Biotechnology Congress, September 11-13th, 2017, Istanbul, Turkey.

5. Amir Maqbool, Emre Aksoy “Analyses of Some Physiological Responses of Soybean Cultivars Under Iron Deficiency” International Green Biotechnology Congress, September 11-13th, 2017, Istanbul, Turkey.