

EFFECT OF BORON AND IRON STRESS ON PHYTOSIDEROPHORE
PRODUCTION IN BARLEY

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY
YETKİN ÇAKA İNCE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

FEBRUARY 2015

Approval of the Thesis

**EFFECT OF BORON AND IRON STRESS ON PHYTOSIDEROPHORE
PRODUCTION IN BARLEY**

submitted by **YETKİN ÇAKA İNCE** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural ÜNVER
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan ADALI
Head of Department, **Biology**

Prof. Dr. Meral YÜCEL
Supervisor, **Biology Dept., METU**

Examining Committee Members:

Prof. Dr. Füsün EYİDOĞAN
Faculty of Education, Başkent University

Prof. Dr. Meral YÜCEL
Biology Dept., METU

Assoc. Prof. Dr. Çağdaş SON
Biology Dept., METU

Assoc. Prof. Dr. Remziye YILMAZ
Food Engineering Dept., Hacettepe University

Asst. Prof. Dr. Mehmet Cengiz BALOĞLU
Genetics and Bioengineering Dept., Kastamonu University

Date: 05.02.2015

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Yetkin aka İNCE

Signature:

ABSTRACT

EFFECT OF BORON AND IRON STRESS ON PHYTOSIDEROPHORE PRODUCTION IN BARLEY

İnce, Yetkin Çaka

M.S., Department of Biology

Supervisor: Prof. Dr. Meral Yücel

February 2015, 109 pages

Boron and iron are essential nutrients for growth and development of higher plants. Deficiency and toxicity of them cause significant changes in physiological, cellular and molecular make-up of plants. Iron uptake in barley is mainly achieved by production and release of phytosiderophores to the environment. On the other hand, production of phytosiderophores are affected by concentrations of boron and iron. Four enzymes which function in phytosiderophore hydroxymugineic acid (HMA) production namely NAS, NAAT, IDS2, and IDS3 showed differential expression profiles when plants were treated with different concentrations of iron and boron. In this study, we investigated the potential link between iron, boron, and phytosiderophore production in barley. For this purpose the barley seedlings were treated with deficiency or toxicity of boron and iron as well as combinations of these and expression levels of aforementioned genes were examined. In addition to gene expression analysis, physiological parameters, total boron, iron, and zinc content and photosynthetic performance of seedlings were considered.

Keywords: barley, boron, iron, zinc, stress, phytosiderophore

ÖZ

ARPADA BOR VE DEMİR STRESİNİN FİTOSİDEROFOR ÜRETİMİNE ETKİSİ

İnce, Yetkin Çaka

Yüksek Lisans, Biyoloji Bölümü

Tez Yöneticisi: Prof. Dr. Meral Yücel

Şubat 2015, 109 sayfa

Bor ve demir yüksek bitkilerin büyümesi ve gelişimi için elzem besin maddeleridir. Bu maddelerin eksikliği ve aşırı fazlalığı bitkilerin fizyolojik, hücresel ve moleküler yapılarında kayda değer değişikliklere yol açar. Arpada demir alımı başlıca fitosideroforların üretimi ve çevreye salınımı ile olur. Diğer yandan fitosiderofor üretimi bor ve demir konsantrasyonlarından da etkilenir. Bitkiler farklı demir ve bor konsantrasyonlarında büyütüldüklerinde, fitosiderofor hidroksimugineik asit (HMA) üretiminden sorumlu dört enzim (NAS, NAAT, IDS2 ve IDS3) farklı ekspresyon profilleri göstermektedir. Bu çalışmada arpada demir, bor ve fitosiderofor üretimi arasındaki olası bağlantıyı araştırılmıştır. Bu amaçla, arpa fideleri bor ve demir eksikliği, fazlalığı ve bu durumların kombinasyonlarıyla muamele edilmiş ardından daha önce adı geçen genlerin ekspresyon seviyeleri incelenmiştir. Bunun yanı sıra, bitkilerin fizyolojik parametreleri, toplam bor, demir ve çinko içerikleri ve fotosentetik aktiviteleri göz önünde bulundurulmuştur.

Anahtar Kelimeler: arpa, bor, demir, çinko, stress, fitosiderofor

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Prof. Dr. Meral Yücel for her guidance, teaching, and advices throughout this thesis. I would also like to thank Prof. Dr. Hüseyin Avni Öktem for the opportunities they have provided me as a member of their research group.

I wish to express my gratitude Dr. M. Tufan Öz who helped me like an unofficial co-supervisor. His advices, comments, and encouragements were valuable for me throughout this study and previous studies.

I also want to thank the members of thesis examining committee; Prof. Dr. Füsün Eyidoğan, Assoc. Prof. Dr. Remziye Yılmaz, Assoc. Prof. Dr. Çağdaş Son, Asst. Prof. Dr. Cengiz Baloğlu for evaluating this thesis; and their suggestions and comments to make the final version of this thesis better.

I would like to thank Prof. Dr. Yasemin Ekmekçi, Şeküre Çulha Erdal and Yasemin Öztürk from Hacettepe University for their help in element analyses and photosynthetic measurements. I would also like to thank Prof. Dr. Vasıf Hasırcı, and his assistants Damla Arslantunalı and Gökhan Bahçecioğlu for helping me to prepare samples for element analyses.

I would like to thank METU Central Laboratory, Molecular Biology and Biotechnology R&D Center for usage of plant growth chamber.

I wish to thank all of my laboratory friends, Dr. Çağla Sönmez, Dr. Oya Akça, Hamit Battal, Dilek Çam, Melih Onay, Ayten Eroğlu, Dr. Farhad Taghipour, Işkın Köse, Görkem Baysal, Evrim Elçin, Ceren Tuğrul, Onur Bulut, Dilan Akın, Evrim Aksu, and Kaan Arıcı for their helps, collaboration and suggestions. I would like to thank especially Dr. Ceyhun Kayıhan for his advices, comments, and suggestions throughout the thesis.

I would like to thank all of the members of Prof. Dr. Gülay Özcengiz Laboratory starting from İsmail Cem Yılmaz. They sincerely helped me when I needed any instruments or chemicals. I am also thankful to Aktan Alpsoy and Emre Evin for their help and suggestions in qPCR.

I am also thankful to my friends Soner Yıldız, Ahmet Yanbak, Bülent Ateş, Sinem Çelebiöven, Gökçe Aköz, Eren Şengül, Ender Aykanlı, Cevahir Karagöz, and Mine Özsoy for letting me bore them with my scientific problems.

I would also like to thank The Scientific and Technological Research Council of Turkey (TÜBİTAK) for their financial support to my education.

Last but not the least; I would like to express my thanks to my family; my mother A. Füsün İnce, my father H. Hüseyin İnce, my brothers Bilsev and Kutalmış İnce, and my sister Özey Kara for believing and encouraging me throughout all my life and my nephew Gökalp, nieces Gökçe and Ece for their presence. I would also like to thank Deniz Kara, Miray Akyunus, and Lütfiye Derya İnce for their friendships and supports.

This study was supported by Research Fund of METU Grant No: BAP-07-02-2014-007-045.

TABLE OF CONTENTS

ABSTRACT	v
ÖZ	vi
ACKNOWLEDGEMENTS	vii
TABLE OF CONTENTS	ix
LIST OF FIGURES	xii
LIST OF TABLES	xiv
LIST OF ABBREVIATIONS	xv
INTRODUCTION	1
1.1 Barley	1
1.1.1 Stress Conditions Affecting Growth of Barley	3
1.2 General Stress Tolerance Mechanisms of Plants	4
1.3 Boron	7
1.3.1 Functions of Boron on plants	8
1.3.2 Boron uptake in plants	9
1.3.3 Boron Deficiency and Toxicity	9
1.3.4 Molecular Aspects of B Stress	11
1.4 Iron	11
1.4.1 Functions of Iron on plants	12
1.4.2 Iron uptake in plants	12
1.4.3 Biosynthesis of phytosiderophores	14
1.4.4 Iron Deficiency and Toxicity	15
1.4.5 Molecular Aspects of Fe Stress	17
1.5 Zinc	18
1.6 Fe-B-Zn-PS relations in plants	18
1.7 Aim of the study	20
MATERIALS & METHODS	23

2.1	Materials	23
2.1.1	Plant Material	23
2.1.2	Growth Media.....	23
2.1.3	Culture conditions.....	23
2.1.4	Chemicals, Reagents and Kits	23
2.1.5	Primers.....	24
2.1.6	Instruments	24
2.2	Methods.....	25
2.2.1	Seed Surface Sterilization, Germination and Hydroponic Culture.....	25
2.2.2	Stress application.....	25
2.2.3	Collection of Plant Material	26
2.2.4	Physiologic Parameters.....	26
2.2.4.1	Fresh and Dry Weight Analysis.....	26
2.2.4.2	Relative Water Content.....	26
2.2.4.3	Length of Shoots and Roots.....	26
2.2.5	RNA Isolation and Characterization.....	27
2.2.5.1	Decontamination	27
2.2.5.2	Total RNA Extraction	27
2.2.5.3	RNA Clean-up and DNA Removal.....	28
2.2.5.4	Determination of RNA Quality and Quantity	28
2.2.6	cDNA Synthesis	28
2.2.7	Polymerase Chain Reaction (PCR).....	29
2.2.8	Determination of Boron, Iron, and Zinc Content in Shoots	33
2.2.9	Chlorophyll (Chl) <i>a</i> fluorescence measurements	33
2.2.10	Statistical Analysis	34
RESULTS & DISCUSSION		35
3.1	Physiologic Parameters	35
3.1.1	External appearance of cultivars.....	35
3.1.2	Length of Shoots and Roots.....	35

3.1.3	Fresh Weight of Shoots and Roots	37
3.1.4	Dry Weight of Shoots and Roots.....	39
3.1.5	Relative Water Content of Shoots	40
3.2	Element Analysis	42
3.2.1	Shoot B-content	42
3.2.2	Shoot Fe and Zn-content:	43
3.3	Chlorophyll Fluorescence and Photosynthetic Performance	46
3.4	Gene Expression Analysis	60
3.4.1	RNA Integrity	61
3.4.2	Conventional PCR.....	61
3.4.3	Q-PCR	62
3.4.3.1	Standard Curve Construction.....	63
3.4.3.2	Calculation and Comparison of Amplification Efficiencies.....	68
3.4.3.3	IDS3 and NAS Expression Level Changes.....	69
3.4.3.3.1	Fold changes of IDS3and NAS in Day-1	71
3.4.3.3.2	Fold changes of IDS3 and NAS in Day-5.....	75
	CONCLUSION	77
	REFERENCES.....	80
	APPENDICES	103
A.	COMPOSITION OF HOAGLAND’S SOLUTION	103
B.	ALIGNMENT RESULTS OF AFFYMETRIX BARLEY1 PROBES AND CORRESPONDING MRNA SEQUENCES	104
C.	PHOTOSYNTHETIC PARAMETERS.....	107
D.	Q-PCR SUPPLEMENTARY DATA.....	108

LIST OF FIGURES

FIGURES

Figure 1.1 The cereal production in hectares and metric tons globally	2
Figure 1.2 The top barley producing countries in 2012	3
Figure 1.3 Structure of mugineic acid and its complex with Fe^{3+} , ferric mugineate	13
Figure 1.4 Biosynthesis of mugineic acids.....	15
Figure 3.1 Leaves of Tarm-92 seedlings after 5 days.	36
Figure 3.2 The length of shoots and roots of Tarm-92.....	38
Figure 3.3 The fresh weight of shoots and roots of Tarm-92.....	39
Figure 3.4 The dry weight of shoots and roots of Tarm-92	40
Figure 3.5 The relative water content (%) of shoots of Tarm-92.....	41
Figure 3.6 The B content of shoot tissues of Tarm-92.....	43
Figure 3.7 The Fe content of shoot tissues of Tarm-92	45
Figure 3.8 The Zn content of shoot tissues of Tarm-92	46
Figure 3.9 The radar-plot presentation of selected JIP test parameters	49
Figure 3.10 The normalized F_0 values shoot tissues of Tarm-92.....	50
Figure 3.11 A scheme of the energy cascade from PSII light absorption to electron transport.....	51
Figure 3.12 The normalized TR_0/ABS values shoot tissues of Tarm-92	52
Figure 3.13 The normalized ET_0/TR_0 values shoot tissues of Tarm-92.....	59
Figure 3.20 The normalized $\text{PI}(\text{total})$ values shoot tissues of Tarm-92	60
Figure 3.21 Electrophoretic separation of representative total RNA samples	61
Figure 3.22 Conventional PCR results.....	62
Figure 3.23 Agarose gel of PCR products.....	63
Figure 3.24 Optimization of primer concentrations in Q-PCR.	65
Figure 3.25 Standard curve of GAPDH(a), IDS3 (b), and NAS(c)	67
Figure 3.26 Melt curve of standards; GAPDH (a), IDS3 (b), and NAS(c);	68

Figure 3.27 Efficiency comparison GAPDH vs. IDS3 and GAPDH vs. NAS	69
Figure 3.28 Average fold changes of NAS & IDS3(calibrators D-0 & D-1)	74
Figure 3.29 Average fold changes of NAS & IDS3 (calibrator D-5)	75

LIST OF TABLES

TABLES

Table 1.1 Down regulated genes related with phyto siderophore production in root tissues of Tarm-92 under B deficiency	20
Table 2.1 Primer sequences and product sizes	24
Table 2.2 Reaction conditions for conventional PCR	30
Table 2.3 Cycling conditions for conventional PCR.....	30
Table 2.4 Reaction conditions for Real Time PCR.....	31
Table 2.5 Cycling conditions for real time PCR	31
Table 3.1 The Ct values of standards	66
Table 3.2 Correlation Statistics of Standard Curves	66
Table 3.3 Average fold changes (AFC) of NAS and IDS3 according to calibrators Day-0, Day-1, and Day-5	73
Table A.1 Composition of ½ Hoagland’s Solution	103
Table A.2 Iron and Boron compositions of stress conditions	103
Table B.1 Alignment results of AB011269.1 and Contig10741_at sequences.....	104
Table B.2 Alignment results of AF136941.1 and Contig10741_at sequences	105
Table B.3 Alignment results of D37796.1 and Contig12916_s_at sequences	106
Table C.1 Summary of the formula and terms of JIP test parameters and their description	107
Table D.1 Fold changes of IDS3 expression according to Day 0, Day 1, and Day 5 controls	108
Table D.2 Fold changes of NAS expression according to Day 0, Day 1, and Day 5 controls	109

LIST OF ABBREVIATIONS

ABA	abscisic acid
ABS	photon flux absorbed
AHA	<i>Arabidopsis</i> H ⁺ -ATPases
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BaMMV	bymovirus
bp	Base pairs
BSMV	barley stripe mosaic hordeivirus
Chl	Chlorophyll
DEPC	Diethylpyrocarbonate
DMA	2'-deoxymugineic acid
DNA	Deoxyribonucleic acid
DRR	DNase Removal Reagent
DW	dry weight
epi-HDMA	3-epihydroxy 2'-deoxymugineic acid
epi-HMA	3-epihydroxymugineic acid
ET	electron transport
FRO2	Fe(III) chelate reductase
FW	fresh weight
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMA	hydroxymugineic acid
ICP-AES	inductively coupled plasma-atomic emission spectroscopy
IDS2-3	iron deficiency sensitive 2-3
IRT1	Fe(II) transporter
LHCI	light harvesting complex I
LHCII	light harvesting complex II

MA	Mugineic acid
MIP	major intrinsic protein
NA	nicotianamine
NAAT	nicotianamine aminotransferase
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAS	nicotianamine synthase
NIP	nodulin 26-like intrinsic proteins
OEC	oxygen-evolving complex
PCR	polymerase chain reaction
pH	a measure of the acidity or basicity of an aqueous solution
PIP1	plasma membrane intrinsic protein
pKa	acid dissociation constant at logarithmic scale
ppm	Parts per million
PS	Phytosiderophore
PSI	photosystem I
PSII	photosystem II
QA	Quinine A
QB	Quinine B
Q-PCR	Real-time (Quantitative) polymerase chain reaction
RC	Reaction center
RG-II	rhamnogalacturonan II
RNA	Ribonucleic acid
RWC	Relative water content
SAM	S-Adenosyl methionine
TR	trapping flux

CHAPTER 1

INTRODUCTION

1.1 Barley

Barley is a member of *Triticeae* tribe of *Poaceae* family. The species is self-pollinating and diploid having $2n = 14$. Archaeological remains of barley grains indicates that it was domesticated 10,000 years ago in the Fertile Crescent [1]. Cultivated barley (*Hordeum vulgare* spp. *vulgare* L.) is one of the eight founder crops of agriculture [2]. Barley ranks fourth in terms of production quantity and cultivation area among other cereal crops [3] (Figure 1.1).

Barley is grown for a variety of purposes but used mainly as fodder for animals, carnivorous fish feed, malt for a large list of alcoholic beverages including beer and whiskey, component of various foods, bread, and nonalcoholic beverages [4]. Barley grain is composed of mainly starch (60 – 64%), arabinoxylans (4.4 – 7.8%), β -glucans (3.6 – 6.1%), cellulose (1.4 – 5.1%), simple carbohydrates (0.6 – 4.6%), proteins (8 – 15%), and lipids (2 – 3%) in dry weight [5]. Generally, barley cultivars with high protein content are used for food and feeding whereas, starchy cultivars are used for malting.

Among other cereal crops, barley is one of the most widely adapted species. It is grown in diverse environments like northern Scandinavia, Himalayan Mountains, and monsoon paddies because of its genetic variation.

Average world production of barley between 2002 and 2004 is approximately 144 million metric tons (MT) annually (Figure 1.1). The top barley producer in the world is Russian Federation. Turkey is ranked as the sixth country globally (Figure 1.2 [6]).

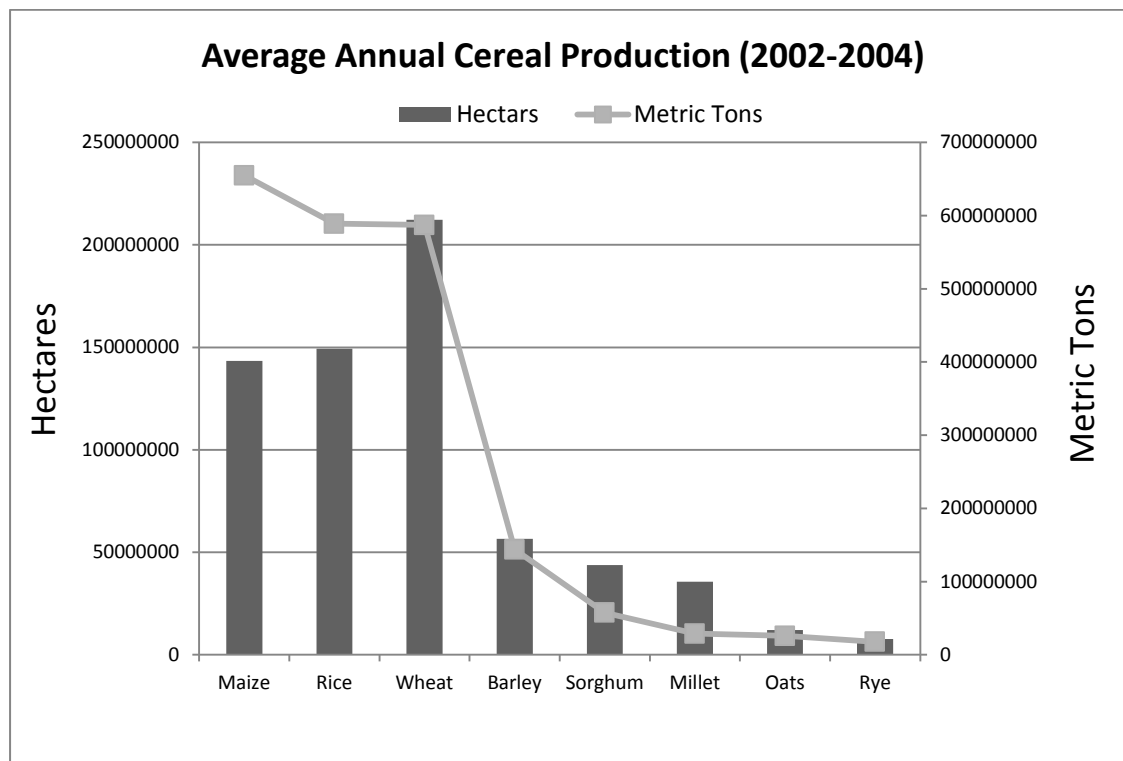


Figure 1.1 The cereal production in hectares and metric tons globally [3]

Barley is a model species for physiological and genetic research on *Triticeae* [7], because of its wide diversity in genetics, morphology, and physiology and less complex genome sequenced in 2012 [8]. Seven pairs of nuclear chromosomes, one mitochondrial and one chloroplastic chromosome (total of 5000 Mbp) compose the genome [9].

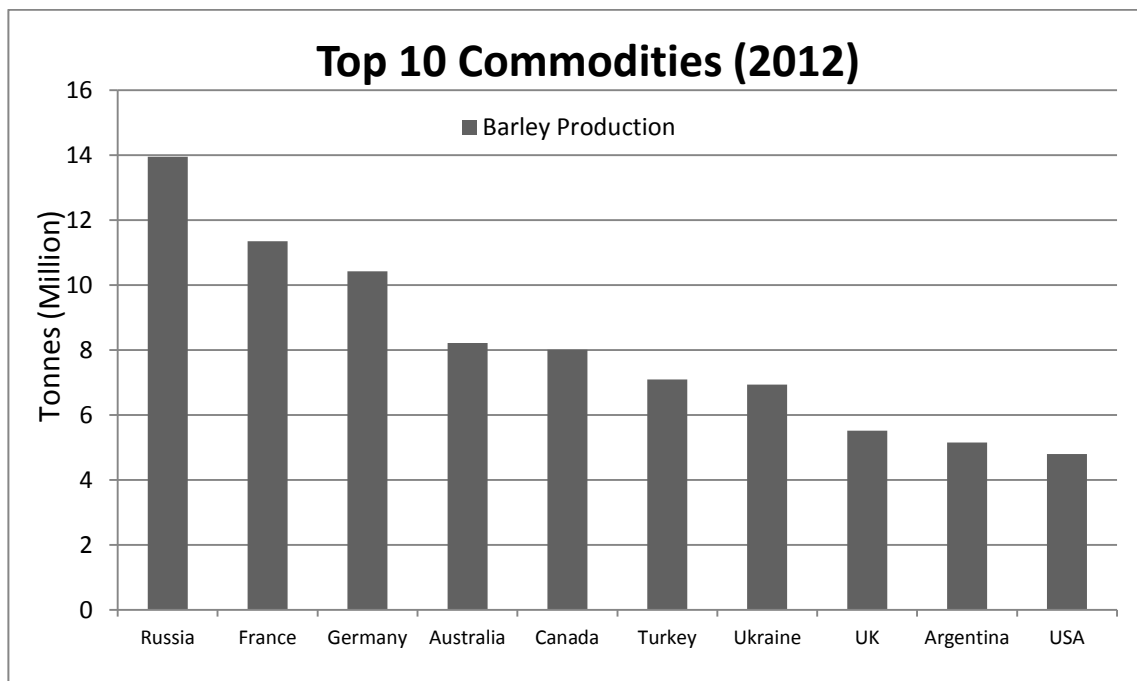


Figure 1.2 The top barley producing countries in 2012 [6]

1.1.1 Stress Conditions Affecting Growth of Barley

Its adaptable nature to many diverse environments makes geographic and ecological range of barley quite wide compared to other cereals. However, there are biotic and abiotic factors affecting the yield and growth of barley.

Biotic factors include viral, bacterial or fungal diseases. *Blumeria graminis* f.sp. *hordei* causing powdery mildew [10], *Rhynchosporium secalis* causing leaf scald [11], *Puccinia hordei* causing barley rust[12], certain species of *Fusarium* causing head [13], and *Cochliobolus sativus* causing various diseases [14] are some of fungi species responsible for biotic stress. In addition to fungal diseases, barley is susceptible to bacterial blight caused by *Xanthomonas campestris* pv.*translucens*. There are also several viruses like barley stripe mosaic hordeivirus (BSMV) and barleymild mosaic bymovirus (BaMMV) [15]causing diseases on barley.

Abiotic stress, the negative impact of non-living factors such as drought, salinity, toxins, pesticides etc. on the living organisms, is considered as the most harmful factor

affecting crop growth and productivity worldwide [16]. Abiotic stresses affect barley during stages of development including early stages of reproductive development [17].

Excess amounts of some elements such as Al, Mn, Cu, and B cause mineral stress resulting in large crop yield losses. According to Clark, mineral stress has negative effects on about 25% of agricultural land [18]. Therefore, it is accepted as one of the main reasons limiting the plant growth over the majority of the land [19].

Boron and iron stress are two important abiotic stresses for barley. It is reported that cereals have low sensitivity to B deficiency [20]. However, B deficiency can affect barley adversely. Male fertility is depressed in barley under B deficiency, which is resulted in grain set failure [21]. In addition, vegetative growth of barley can be either enhanced [21] or depressed [22] under B deficient conditions. Mengel and Kirkby (2001) regarded barley as moderately tolerant to B toxicity [23]. However, two Turkish barley cultivars (Anadolu and Tarm-92) have been reported as B tolerant [24]. High soil alkalinity, the common problem in semi-arid and arid soils is related to many types of mineral stress including B toxicity and Fe deficiency. In Fe deficient conditions, Gramineous plants exhibit morphological and physiological changes including alterations in root morphology [25] and stimulation of phytosiderophore release from roots [26].

1.2 General Stress Tolerance Mechanisms of Plants

The responses of plants to various abiotic and biotic stresses are complex and involve numerous adaptations in different manner. After stress exposure, activation of ion channels, kinase cascades [27], and reactive oxygen species (ROS) [28]; and accumulation of several different phytohormones [29] lead plant to reprogramme its genetic machinery to minimize stress induced damage [30].

After sensing stress, reactive oxygen species (ROS) [31], [32]; Mitogen-Activated Protein Kinase (MAPK) cascades [33], [34]; several phytohormones such as abscisic acid, salicylic acid, jasmonic acid, and ethylene; and certain transcription factors are activated to function as signaling molecules.

Low amounts of ROS regulate stress responses, whereas high levels cause serious damage leading to even death of the cells on plants [35], [36]. The role of ROS in cell communication [37], and as a secondary messenger functioning in defense gene activation [38], [39] have been shown. Since the excess amount of ROS is harmful to plants, they need to be degraded after their function, and the increase of molecules responsible for degradation of ROS is accepted as an indicator of stressed plants. These antioxidant molecules responsible for ROS degradation are enzymes including glutathione peroxidase (GPX), superoxide dismutase (SOD), mono dehydro ascorbate reductase (MDHAR), glutathione reductase (GR), de hydro ascorbate reductase (DHAR), catalase (CAT), ascorbate peroxidase (APX), glutathione S-transferase (GST) and water-soluble compounds such as glutathione and ascorbate [40], [41].

The stress tolerance mechanisms of plants vary depending on the type of the stress. However, the main tolerance mechanisms against excess mineral uptake are shared among plants. Four mechanisms are defined; (i) restriction of uptake, (ii) active efflux, (iii) compartmentation, and (iv) complexing the excess mineral with other molecules.

i. Exclusion of excess mineral from the plant

Generally, accumulation of lower concentrations of an excess mineral in tolerant cultivars compared to sensitive ones suggests exclusion is one of the main strategies to cope with excess amounts of minerals. The ways that plants use to prevent excess mineral uptake include precipitating or complexing of minerals around the roots.

Precipitation of metals can be achieved by increasing pH values of rhizosphere or by anion excretion. It has been shown that tolerant *Z. mays* cultivar excrete P anions against Al toxicity compared to sensitive one showing toxicity symptoms [42]. Organic acids have also been accepted as possible chelates. There are several studies showing increased amount of organic acids like malic acid, citric acid, oxalic acid more in tolerant cultivars against Al toxicity [42]–[46]. However, other examples of organic acid chelate function inside of the cells rather than the outside.

Exclusion of B from roots is also proposed as one of the main tolerance mechanism against B toxicity. The comparison of tolerant and sensitive barley cultivars under B toxicity showed that tolerant cultivars significantly accumulate less B [47].

ii. *Active efflux*

Although active efflux is accepted as a possible mechanism of tolerance to mineral stress, there is very limited research on it. However, partially active efflux of B from roots has been shown [47]. In addition, AtBOR4, a B transporter in Arabidopsis, has been demonstrated as having B efflux activity in yeast cells [48]. Another possible example of active efflux is loading metals to older leaves and shedding them to get rid of excess amount of the metal [49].

iii. *Compartmentation within vacuoles*

Mineral tolerance could be achieved by compartmentation of them into places in which they will not be able to react with other cellular substances. The most probable sites for this kind of places are the vacuoles. In literature, several studies have demonstrated the use of vacuoles to get rid of excess minerals. Under excess Zn content, increase of vacuolar in barley leaves hold responsible for increase in total Zn content of leaves [50]. Similar mechanism for salt tolerance, compartmentation of Na in vacuoles, was also proposed [51]. Other species under different mineral stress conditions have acted similarly, such as *Z. mays* under Al toxicity [52], *D. caespitose* under Zn toxicity [53], *N. tabacum* under Cd toxicity [54]. In addition, compartmentation of B in vacuoles have been proposed in wheat cultivars against B toxicity [55].

iv. *Complexing*

Making mineral unable to interact with other cellular substances can be achieved by complexing them with substances specialized to bind them. Metallothioneins, phytochelatins, organic acids, inorganic and organic ligands like phytate are used to bind metals under toxic conditions to prevent damage.

In several different plant species, studies showed that the role of members of metallothioneins, which are cysteine rich, low molecular weight proteins [56] in metal tolerance of plants. Cu-binding protein in *A. gigantean* [57], metallothionein production in *A. thaliana* for Cu tolerance [58], increased protein production to bind excess Cu in tolerant *S. oleracea* [59] are some examples.

Like metallothioneins, phytochelatins are also cysteine-rich but they are non-protein metal binding peptides [60]. Phytochelatins binding to excess Cu and Cd in *Z. mays* [61], production of phytochelatin under excess Zn, Cu, and Cd in *Rubia tinctorum* roots [62] and after exposure to Cd in *N. tabacum* [54] were observed.

Organic acids are also able to form complexes with metals inside or outside of cells. There are several studies showing increased amount of organic acids like malic acid, citric acid, oxalic acid more in tolerant cultivars against Al toxicity [42]–[46]. Furthermore, great increases in production of citric acid and malic acid and relatively lower amounts of increases in production of succinate and fumarate were observed in Cu and Zn tolerant *N. plumbaginifolia* cultivars under Zn or Cu toxicity [63].

Another possible metal complexing agent is phytate. Zn-phytate complexes were detected in tolerant *D. caespitosa* roots more than in sensitive ones [53]. In addition, several plants including Al tolerant *Z. mays*, have metal deposit associated phytate in their vacuoles [52].

1.3 Boron

Boron (B) has intermediate properties between metals and non-metals[64]. Lithosphere and hydrosphere are quite rich with their B concentration 5-10 mg/kg in rocks[65], 3-30 µg/kg in rivers[66] and ~4.5 mg/L in ocean[67]. B in soil is found mostly combined in borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), colemanite ($\text{CaB}_3\text{O}_4(\text{OH})_3 \cdot \text{H}_2\text{O}$), ulexite ($\text{NaCaB}_5\text{O}_6(\text{OH})_6 \cdot 5(\text{H}_2\text{O})$), tourmaline (crystalline boron silicate mineral compounded with elements such as Al, Fe, Mg, Na, Li, K) and borate minerals (boracite- $\text{Mg}_3\text{B}_7\text{O}_{13}\text{Cl}$), which are inaccessible to plants. The only form of soil B that is accessible to plants is water soluble boric acid (H_3BO_3). It is a weak monobasic acid (pKa 9.24) and un-dissociated under most soil pH conditions. Besides, boron is non-mobile in

plants, which requires supplementation of B continuously from soil or planting media in all plant meristems. These properties of B make it unique among other essential nutrients.

1.3.1 Functions of Boron on plants

Boron is essential for normal growth of higher plants [68]. However, the requirements of B within the plant kingdom differ markedly, but generally dicots have a greater requirement for B than monocots [69]. B in plant functions directly or indirectly in cell wall synthesis and structure, membrane structural maintenance, sugar transport, lignification, carbohydrate metabolism, respiration, nitrogen fixation, RNA metabolism, phenol metabolism, and ascorbate metabolism [70].

The role of B in cell wall structure and function has been demonstrated in radish root cell walls by isolation and characterization of a B-polysaccharide complex [71]. The capacity of B to form borate esters with apiose residues of rhamnogalacturonan II (RG-II) [72] makes it essential for cell wall structure and function [73].

Another role of B is related on maintenance of integrity of plasma membrane [74]. In B deficient conditions, it was shown that membrane bound H^+ pumping ATPases have lower activities [75]. ATPase activity was restored after B re-supply, indicating effects of B on ion fluxes occur directly or indirectly by membrane bound ATPases. Rather than direct effects on ATPase activity, possible binding of B to hydroxyl groups of glycoproteins and glycolipids in plasma membranes may be the main reason for alteration of membrane permeability [74]. B complexes in glycoproteins and glycolipids help stabilization of membrane, providing an optimum conformation for enzymes and channels [74], [76].

In addition to structural functions of B in plants, B complexes with NAD^+ can cause disruption of metabolism [77] and other ribose containing molecules [78], [79], and functional roles in regulatory or signaling processes as being a part of signal molecules [80]. Moreover, roles in plant reproduction [81], abortion of flower initials [82], premature flower and fruit drop [83], male sterility [84], impairment of male gametogenesis [85], and pistil sterility [86] are reported.

1.3.2 Boron uptake in plants

The most common form of boron at soil pH values 5.5-7.5 is the soluble boric acid (H_3BO_3) which is the available boron source for plants. Three different mechanisms for boric acid uptake by plants are suggested depending on B availability in soil:

I. Passive diffusion of B across the lipid bilayer under adequate/excessive conditions of B availability [87]. Theoretical considerations of lipid permeability coefficient for boric acid suggested that plants achieve boron by passive diffusion [88], which is supported experimentally [89], [90].

II. Diffusion of B is facilitated by the major intrinsic protein (MIP) channels, channels that transport neutral molecules [91]. A member of MIP superfamily, plasma membrane intrinsic protein 1 (PIP1) expression resulted in 30% increase in B absorption in *Xenopus laevis* oocytes [92]. In addition, molecular studies in *Arabidopsis thaliana* revealed a boric acid channel (*AtNIP5;1*), a member of the MIPs family [93]. Expression of *AtNIP5;1* is upregulated in B deficient conditions [93]. Having a close homolog sequence to *AtNIP5;1*, *OsNIP3;1* in rice has also been identified as a boric acid channel and is required for normal growth under B deficient conditions [94]. Another study demonstrated that *H. vulgare* PIP1;3 and PIP1;4 transformed cells of yeast are more sensitive to boron [95].

III. A high affinity transport system depending energy is used under low B conditions; it has been shown that active B uptake by roots occurs [90], [96]. The expression of a BOR transporter in rice (*OsBOR1*) in root elongation zone of rice under B deficiency suggests the active and efficient B uptake into root cells [97].

1.3.3 Boron Deficiency and Toxicity

Availability of B to plants is affected by many diverse physical factors including rainfall, pH, soil texture and moisture, temperature, and clay mineralogy [23], [98]. Under high rainfall conditions, boric acid, the available B form to plants, can be easily leached [65]. This leads to B deficiencies in many regions such as Japan, China, USA, and Brazil. In contrast, B cannot be leached enough under low rainfall conditions causing accumulation to toxic levels [99]. Therefore, arid and semiarid regions are

found to be B toxic [100] like Central Anatolia (Konya, Eskişehir, Aksaray) and South-eastern regions of Turkey [101] South Australia, California and Chile [102], [103].

Although the requirements of B within the plant kingdom differ markedly [69], The requirement of Gramineous monocots is 4 – 10 $\mu\text{g B/g}$ dry weight and it is 20 – 55 $\mu\text{g B/g}$ dry weight for dicots [23], [104].

The symptoms of B deficiency appear firstly at growing points. However, plant species owing higher B in young leaves and growing parts are exceptions. Stunted appearance, discolored leaves, poor pollination, hollow stems and fruit and brittle, and loss of fruiting bodies are characteristics of B deficiency [23]. Most of the symptoms are associated with requirement of B in the function and structure of cell wall [72]. Moreover, impairment of fruit formation under B deficiency has been reported in grapes because of its requirement in pollen tube growth, pollen germination, and pollen viability [105]. Since, reproductive growth has higher requirement for B than vegetative growth [81], impairments in crop production under B deficiency have also been reported. Other B deficiency symptoms include inhibition of root and shoot elongation, loss of cell wall plasticity, and inhibition of cell division.

High concentration of soil B is mostly associated with lands showing volcanic activity recently and receiving low rainfall [66]. B toxicity is frequently associated with soils lacking of sufficient drainage [98]. B toxicity in plants can be diagnosed using visible symptoms. Reduction of growth, and easily visible chlorosis followed by necrosis on older leaves are typical symptoms of B toxicity [77], [103], [106], [107]. In addition, symptoms of toxicity include fruit disorders, bark necrosis and stem dieback rather than leaf burn and necrosis [103] leaf cupping resulting from disturbance of cell wall cross-linking and expansion [108].

A study on *Dittrichia viscosa* revealed that B deficiency resulted in a significantly decrease in growth. However, it has no negative effect on photosynthetic parameters [109]. On the contrary, studies in citrus showed that chlorophyll content decreased more under B deficiency than B toxicity [110]. Another study evaluating photosynthetic performance wheat cultivars under B toxicity by JIP test reported that B-tolerant cultivar

exhibited a better photosynthetic performance compared to the sensitive cultivar, concluding photosynthetic performance of cultivars can be used to determine their resistance to B [111].

1.3.4 Molecular Aspects of B Stress

Although direct information on the signal transfer from B deficiency to nuclei is not present, deficiency changes the expression level of genes involving in nitrogen metabolism [112], B uptake [113], cell wall formation [114], and oxidative stress [115].

Three hypotheses were suggested to explain the signal transport mechanism to nuclei: (i) the imbalance in cellular redox caused by B deficiency may induce a gene by transfer of signal from cell wall to cytoplasm [115]; (ii) a mechanical cascade of signals may be induced as a response to fluctuations in B concentrations [116]; (iii) B may interact with transcription factors directly as a cellular signal [117]. The second hypothesis is supported by changes in cytoskeletal proteins polymerization pattern [118], [119] and the endocytic pathway inhibition for internalization of B-cross-linked RG-I pectins in brefeldin A-induced compartments [120] under B deficiency.

B toxicity induces changes in gene expression levels in *Arabidopsis* [121]. One of identified genes is a zinc finger family transcription factor (*At1g03770*). It could regulate the expression level of genes involved in physiological response to toxicity of B [121]. The putative role of B, interactions with transcription factors as a cellular signal, might be supported by this result. Another important late response was found as the induction of jasmonic acid related genes under B toxicity [122].

1.4 Iron

Iron (Fe) is the most abundant element in the Earth by mass. Although Fe exists in a wide range of oxidation states (−2 to +6), two most common ones are +2 (ferrous) and +3 (ferric). Fe reacts with oxygen and forms various oxide and hydroxide compounds. Iron (II,III) oxide (Fe_3O_4), and iron (III) oxide (Fe_2O_3) are the most common ones.

1.4.1 Functions of Iron on plants

Iron is another essential micronutrient for many plant functions. Some of the important functions of iron in plants include chlorophyll development and function, energy transfer, constituent of certain enzymes and proteins, plant respiration and metabolism, and nitrogen fixation.

Nearly 90% of the Fe is stored in the chloroplasts [123] where photosynthesis, heme biosynthesis, and Fe-S cluster assembly take place. Chlorosis, the characteristic symptom of Fe deficiency in plants (see section 1.3.4) is indication of decrease in chlorophyll content. Fe is a crucial element in photosynthetic pigment metabolism and ultrastructure of chloroplasts [124].

Mitochondria also employ Fe in respiration, heme biosynthesis and Fe-S cluster synthesis [125]. For mitochondria function, proper Fe homeostasis is essential, allowing transporters and Fe sequestering proteins to work. In rice pollen, mitochondria are deformed under Fe deficiency, which reduced seed yield [126], because development of flower is dependent on energy [127]. Many Fe related genes are highly expressed in pollen containing anthers [128]–[131].

1.4.2 Iron uptake in plants

Fe is one of the most common elements in the earth; however, its availability for plants is very low due to low solubility of Fe in soil [132]. In soil that are well aerated and/or at physiological pH, ferrous (Fe^{2+}) is readily oxidized to water insoluble ferric (Fe^{3+}). Plants need to solubilize Fe to take up it from the soil. Two different strategies have been evolved by plants to take up Fe from soil.

Strategy I (Reduction-based strategy)

It is used by non-grasses. The first step is proton release under Fe-deficient conditions. The pH of the soil is lowered by proton extrude in rhizosphere by epidermis expressed *Arabidopsis* H^+ -ATPases (AHA) [133] leading increased Fe^{3+} solubility. It was reported that Fe^{3+} becomes 100-fold more soluble for every one unit drop in pH [134]. Under deficiency three different AHA genes were upregulated in root epidermis

[135]. However, AHA2 has the highest expression level, it has been accepted as the primary root AHA in response to deficiency of Fe [136].

Fe(III) chelate reduction is second method that non-graminaceous species has evolved in response to Fe deficiency. Fe^{3+} is reduced by ferric chelate reductases to the more soluble Fe^{2+} . *Arabidopsis* mutants having the defective copy of the gene (*frd1*) developed severe chlorosis under Fe deficiency [137]. *FRO2*, being mapped to the same location as *frd*, was expressed in epidermal cells of roots in Fe-depleted samples, supporting that it is the main Fe(III) chelate reductase in *Arabidopsis* [138]. Identification of Fe(III) chelate reductases have also been achieved in tomato and pea [139], [140].

Finally, after reduction, the divalent metal transporter *AtIRT1* can transport Fe (II) into the root epidermal cells [141]. Transportation of other elements including Zn, Mn, Cd, Co, and Ni are also done by *AtIRT1* [142]–[144]. IRT1 orthologs have also been found in tomato [128], in pea [145] and in a strategy II plant, rice [146].

Strategy II (Chelation-based Strategy)

Siderophores are microbial originated Fe containing chelates and are found widely bacteria and fungi [147]. Under Fe deficiency, grasses release another form of this microbial originated chelates, phytosiderophores [148]. However, this type of molecules cannot be found in dicots [149]. Phytosiderophores (PS) are non-protein amino acids that have the ability to solubilize Fe^{3+} ions and form Fe-PS complexes.

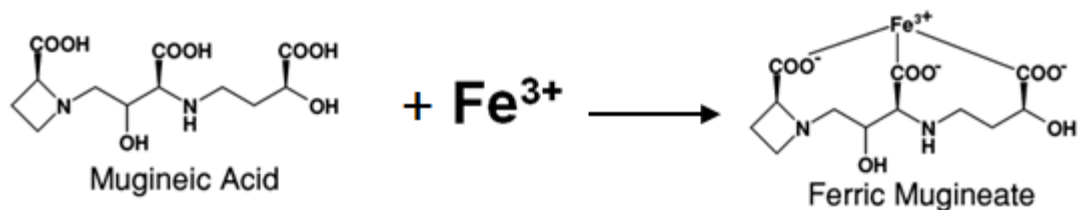


Figure 1.3 Structure of mugineic acid and its complex with Fe^{3+} , ferric mugineate

Grasses use the chelation based strategy to uptake Fe. They release soluble siderophores having high affinity for Fe^{3+} . Mugineic acid family (MA family) phytsiderophores bind Fe^{3+} in rhizosphere efficiently, and then complexes of Fe^{+3} -PS (Figure 1.3) are transported into root epidermis through a high affinity uptake system [26]. It has been discovered that grasses survive under more drastic Fe-deficient conditions, because chelation strategy is more efficient than reduction [150].

1.4.3 Biosynthesis of phytosiderophores

2'-deoxymugineic acid (DMA), 3-epihydroxymugineic acid (epi-HMA), and 3-epihydroxy 2'-deoxymugineic acid (epi-HDMA) are members of MA family phytosiderophores. MA production and secretion in each grass differs but always increases in response to Fe-deficient conditions confirming that tolerance to Fe-deficiency is related with PS secretion [64]. Barley, being more tolerant to Fe-deficiency, has MA, HMA, and epi-HMA; while other grasses including wheat, rice, and corn use only relatively low amounts of DMA [151]. MAs are synthesized from S-Adenosyl methionine (SAM) through several steps beginning with synthesis of the key intermediate, nicotianamine (NA) by nicotianamine synthase (NAS) (Figure 1.4). NA has the ability to bind Fe^{2+} and Fe^{3+} and other metals both in grasses and non-grasses. However, NA is not secreted, which suggests intra and intercellular role rather than the extracellular role in both strategies. In grasses nicotianamine aminotransferase (NAAT) converts NA to 3'-keto DMA [152] which produces the common precursor of all MAs, DMA [151]. Two barley genes, iron deficiency sensitive 2 and 3 (IDS2 and IDS3) whose expression is upregulated under Fe-deficiency supporting the catalyze activity to form epi-HMA and epi-HDMA [153].

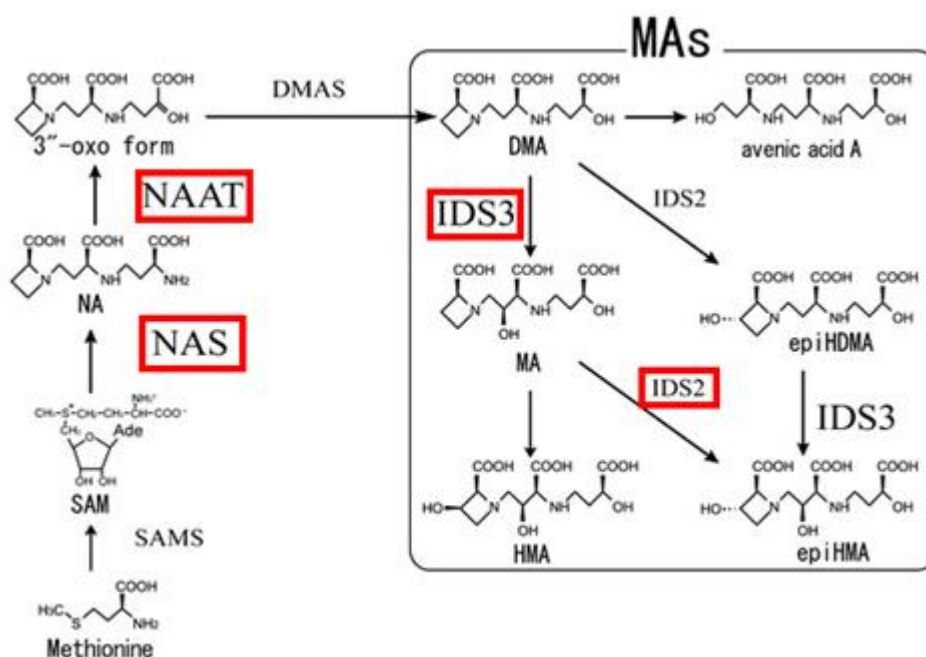


Figure 1.4 Biosynthesis of mugineic acids [154]

1.4.4 Iron Deficiency and Toxicity

Iron stress is one of the important yield limiting factors in crop production. Deficiency or toxicity occurs in a variety of soils.

Fe deficient soils are usually seen the areas where the pH value is higher than 6 [155]. Above pH 4, each unit increase in pH decreases Fe^{3+} solubility by about 1000 [156]. High levels of lime, heavy metals, nitrate, and organic matter content are generally associated with Fe deficiency. In addition, poor aeration, unbalanced cation ratios, root infections by nematodes, and high temperature differences are the signs of Fe deficiency. It is a problem in calcareous soils[157] which is about 5.2 million hectares of the world land surface [158].

Fe toxicity, which is not as common as deficiency, is seen in acid soils. On acid soils, Fe^{2+} form is favorable in such level that is toxic to plants. Poor drainage, highly reducing conditions, and high sulphide concent, which are also characteristics of rice

soils, are often associated with Fe toxicity [159]. Therefore, Fe toxicity is a serious problem for rice production.

Both deficiency and toxicity of Fe can be easily identified by visual symptoms. In the case of deficiency, first symptoms are seen in younger leaves as a chlorosis developing interveinally in the new leaves. As the level of deficiency increases, the area between the veins turns yellow to bleached-white. Unless the deficiency is not extremely severe, the veins remain green [160]. In the early stage of growth, plants are more susceptible to Fe deficiency.

Unlike deficiency symptoms, old leaves present first symptoms as a response to Fe toxicity. Brown spots starting from tips of lower leaves in rice are the signs of Fe toxicity in rice [161]. Fe toxicity may affect plants in two ways; (i) direct effect as death of the plant when lethal doses, (ii) indirect effect causing nutritional imbalance when longer but relatively lower doses are applied to plants. Excess Fe may inhibit uptake, transport and utilization of other nutrients like P, K and Zn [162].

As mentioned earlier, the first visible symptom of Fe-deficiency is chlorosis of leaves, which is associated with loss of chlorophyll content even though chlorophyll chelates Mg rather than Fe. Once Fe is uptaken into plant, it is sequestered by ferritin located predominantly in plastids [163]. It was reported that Fe deficiency significantly decreases the number of thylakoid membranes per chloroplast whereas the structures of other Fe-rich organelles remains unchanged [164]. Fe deficiency also changes the active Rubisco levels [165]. Furthermore, loss of photosystem I (PSI) activities under Fe deficiency were reported [166], [167]. The light harvesting complex I (LHCI) was drastically reduced whereas LHCII was not affected from Fe deficiency. The loss of PSI is associated with reduce in LHCI. In addition, PSI contains Fe-S proteins functioning in electron transfer to NADP^+ . Ferredoxin, one of the most important Fe containing proteins in photosynthesis, is considered as the direct recipient of electrons from the pathway [168]. Therefore, negative effects of Fe deficiency on PSI can be explained by decrease in number of Fe containing proteins.

1.4.5 Molecular Aspects of Fe Stress

Under Fe deficient conditions a response called strategy I (described in section 1.3.2) is induced in all plants except grasses [132]. Increase in expression of genes involved in this response such as Fe(III) chelate reductase (FRO2) and the Fe(II) transporter (IRT1) are observed in *Arabidopsis* [169], [170]. IRT1 is the major protein transporting Fe from soil [171]. The increase in expression of various metabolic enzymes under Fe deficiency suggests dramatic changes in carbon metabolism [172], [173]. Another study showed that roots and shoots of *Arabidopsis* respond differently to Fe deficiency [174]. Moreover, same study reported a correlation between repression of photosynthetic enzymes and increasing time to exposure to Fe deficiency. Other Fe-deficiency induced genes in *Arabidopsis* include genes encoding enzymes involved in glycolysis, the citric acid cycle, and the oxidative pentose phosphate pathway; products involved in mobilization and export of carbon in the form of sugars and starch.

Grasses like barley and wheat use strategy II (described in section 1.3.2) as Fe deficiency response. Microarray results in barley roots grown in Fe deficiency revealed that expression of genes involved in methionine synthesis (first product of MAs- see section 1.3.3) [175]. Another study comparing abundances of proteins in barley under Fe deficiency showed that three enzymes involved in PS production named iron deficiency sensitive2 (IDS2), IDS3, and a methylthio-ribose kinase were increased [176]. In addition, promoter analysis of IDS3 in *Arabidopsis* and tobacco showed that IDS3 promoter is Fe deficiency inducible and its expression is root specific [177]

As a response to Fe toxicity, four *AtFer* genes that encode ferritin, are differentially expressed in both roots and shoots [178]. The same transcripts accumulated in plants in response to H₂O₂ treatment. H₂O₂ and Fe(II) can react to form hydroxyl radicals. Thus, cells can be protected from damage of oxidative stress by storing iron using ferritin. The protection of plant cells from damage of oxidative stress caused by Fe toxicity can be achieved by increase in expression of *AtFer* genes. In addition, Fe toxicity and production of abscisic acid (ABA) is correlated [179]. The

reduction of ferritin levels in a maize ABA-deficient mutant [180] also indicates the ferritin-Fe toxicity interaction [145].

1.5 Zinc

Zinc is another important micronutrient for plants. It is a component of enzymes functioning protein synthesis and energy production. It is also responsible for maintenance of structural integrity of biomembranes and has an important role in seed development. Zn enzymes are involved in regulation of main mechanisms of cells like DNA-transcription, RNA-processing, and translation. A well-known motif for these processes, zinc-finger motif composes up to 4% of the predicted proteins in *Arabidopsis* [181]. In addition, all types of DNA-polymerases and RNA-polymerases, histone deacetylases, splicing factors, and enzymes involved in RNA-editing in mitochondria and chloroplasts are Zn dependent [182]. It has several other important functions in chloroplasts and mitochondria [183]–[186]. Furthermore, the cytoplasm, lysosome, and the apoplastic space are compartments with Zn-dependent hydrolytic activities [187]–[190].

The importance of Zn in the present study comes from its availability conditions in nature and relations with the given stress conditions. As in Fe and unlike B, the availability of Zn to plants decreases in high pH soils. Thus, co-occurrence of Fe deficiency, Zn deficiency, and B toxicity is possible, as indicated previously [191]–[193]. In addition, studies have revealed that there is competition between Cu, Mn, Fe, and Zn uptake [194]–[197]. There are reports confirming Fe deficiency increases Zn uptake and vice versa [198]–[201]. This relation between Fe and Zn is not limited to graminaceous plants, Fe deficiency causes excess Zn uptake also in tobacco and *Arabidopsis* [199], [202].

1.6 Fe-B-Zn-PS relations in plants

As mentioned in section 1.4, co-occurrence of Fe deficiency, Zn deficiency, and B toxicity is possible [191]–[193]. Thus, it is important to understand the relation between these three elements and the molecules responsible for uptake of two.

To begin with, Fe and Zn interactions have been studied over years. It has been reported that competition between Zn and Fe for the transporters *HvIRT1* and *HvIRT2* [203] and *ZmYS1* [204] is present. In addition, uptake of Zn increased 15-fold and accumulation of Zn increased 16-fold in *Zea mays* under Fe deficiency [201]. This result is expected since two pathways for the uptake of Zn from Zn-phytosiderophores in grasses was proposed, one via the transport of the free Zn cation and the other via the uptake of nondissociated Zn-phytosiderophores [205]. However, the relation between Fe and Zn is not limited to graminaceous plants, Fe deficiency also causes increased Zn uptake also in tobacco and *Arabidopsis* [199], [202]. The role of *AtIRT1* and *AtIRT 2* in transportation of both Zn and Fe was detected [141], [143].

In the case of Fe and B, Lukaszewski and Blevins showed that's quash and alfalfa root tips contained high concentrations of Fe accumulated in root cell wall under B deficiency [206]. In addition, low levels of ascorbate were also detected, which might prevent the reduction of iron to the ferrous form that plants can use. Precipitation of phosphorus by ferric and formation of a type of Fe plaque inhibiting root uptake efficiencies was suggested [105].

Gene expression analyses in barley roots by microarray in response to Zn and Fe deficiency revealed that NAS and NAAT were upregulated, which was also confirmed by Northern Blot analysis [154].

In 2007, Patterson et al. investigated protein abundances from B-tolerant (Sahara) and B-intolerant (Clipper) barleys grown under B non-toxic conditions by using iTRAQ peptide tagging (iTRAQ) [176]. Among the proteins that they investigated, IDS2 and IDS3 showed an increased abundance in tolerant plants. Sahara seedlings accumulated significantly more B under Fe deficiency, but Clipper accumulated similar amounts of B under both Fe-deplete and Fe-replete conditions. When Fe concentrations were compared, it was found that Sahara accumulated slightly more Fe than Clipper in both Fe-replete and Fe-depleted conditions.

Öz (2012) found that NAS, IDS3, and NAAT were down-regulated according to microarray results in B-tolerant Tarm-92 after 5 d of B deficiency [207] (Table 1.1).The

conclusion was that transcriptome modulation might occur under B deficiency to decrease or stop the uptake of other micronutrients such as iron. A possible competition between B and other micronutrients for uptake might explain this.

Table 1.1 Down regulated genes related with phytosiderophore production in root tissues of Tarm-92 under B deficiency[207]

Fold Change	Putative function	Probe Set ID
33.53 ↓	IDS3	Contig12916_s_at
32.17 ↓	IDS3	D37796_at
30.85 ↓	NAS 1	Contig10741_at
15.99 ↓	NAAT A	Contig7288_at
5.82 ↓	IDS3	AB024007_at

Zn-B relation has been also studied on different species. It was reported that B toxicity was more severe and appeared first in Zn deficient barley plants in comparison to Zn sufficient ones [208]. Zn-fertilization studies in wheat cultivars revealed large differences in shoot B concentration under Zn deficiency and fertilization [209]. It was proposed that Zn can inhibit the negative effect on excess B uptake on plants [209]. Another study in tomato about effect of Zn on the alleviation of excess B uptake was confirmed the relation between Zn and B [210]. Zn application revealed the symptoms of B toxicity partially. In addition, microarray analysis of barley cultivar Tarm-92 revealed a slight increase of zinc transporter under B deficiency [207]

1.7 Aim of the study

Elevated B levels in soil constrain agricultural production in arid and semi-arid regions in the world including Turkey, California, Chile, and South Australia. Having

more than 70% of B reserves of world, agricultural production in Turkey suffers from B toxicity symptoms including decreases in yield of cereals such as wheat and barley

Studies are needed to understand B resistance mechanisms and take necessary actions to reduce the damage caused by B toxicity on agriculture. Recent studies revealed that there might be a relationship between B content of barley and phytosiderophores responsible for Fe uptake in grasses both in mRNA and protein levels. The relation between B and Fe might lead us not only understanding the B tolerance mechanisms of grasses, but also potential use of B fertilizers in abnormal Fe conditions in soil. Therefore, this study aims to understand potential link between iron, boron, and phytosiderophore production by investigating gene expression levels of NAS and IDS3, two of four enzymes responsible for phytosiderophore mugineic acid, in B tolerant barley Tarm-92 under different concentrations of B and Fe. In addition to gene expression analysis, physiological parameters, total B, Fe and Zn content and photosynthetic performance of seedlings were considered.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1 Plant Material

The seeds of Turkish cultivar Tarm-92 were obtained from Central Research Institute for Field Crops (Ankara, Turkey) and were used as plant material.

2.1.2 Growth Media

Half-strength Hoagland's nutrient solution [211] was used for seedling growth. Boric acid (H_3BO_3) solution was used for application of B stress. The compositions of growth solution and stress application solutions are given in Appendix A (Table A.1). Sterilized distilled water by autoclaving 20 min at 121°C was used to prepare growth media and all of its components. Adjustment of pH to 5.7 – 5.8 was done after sterilization of the media.

2.1.3 Culture conditions

Hydroponic cultures of plants were maintained in growth chambers at $22\pm 2^\circ\text{C}$ with 16 h light ($300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) and 8 h dark photo-cycle and with 70% relative humidity.

2.1.4 Chemicals, Reagents and Kits

The chemicals and reagents were purchased from AppliChem GmbH (Darmstadt, Germany), Life Technologies (Carlsbad, CA, US), Thermo Scientific (Ontario, Canada), Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, US), and Ambion (Austin, TX, US). Distilled or ultrapure water were used to prepare all of the media and solutions.

2.1.5 Primers

Primers (Table 2.1) were designed using a Primer3 Input v.0.4.0 program (<http://bioinfo.ut.ee/primer3-0.4.0/>) and OligoAnalyzer 3.1 (Integrated DNA Technologies -<https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) using regions of the corresponding sequences available at NCBI (<http://www.ncbi.nlm.nih.gov>) aligning Affymetrix Barley1 available at NetAffx™ Analysis Center (<http://www.affymetrix.com/analysis/index.affx>). All primers were ordered from Alpha DNA (Montreal, Canada). Detailed information on sequences and probes are given in Appendix B.

Table 2.1 Primer sequences and product sizes

Name	Sequence (5'-3')	Product Size (bp)
HvGAPDH/Sense	GTGAGGCTGGTGCTGATTACG	198
HvGAPDH/Antisense	TGGTGACAGCTAGCATTTGAGAC	
HvNAS/Sense	GTGTGGTCAGCATCCATACG	151
HvNAS/Antisense	ACTTGGCACACTACCCTCGT	
HvIDS3/Sense	CACCGTGCAATGACAACTC	195
HvIDS3/Antisense	TGTGAGATTGAGCGATGACC	

2.1.6 Instruments

The growth of plants was performed in SGC1700 plant growth chamber manufactured by Weiss Gallenkamp Ltd (Loughborough, UK).

In conventional PCR experiments thermal cycler manufactured by Bio-Rad Laboratories Inc (Hercules, CA, US) was used. Real-time PCR was performed using Corbett Rotor-Gene™ 6000 (Qiagen, Valencia, CA, USA). Centrifugations were done using 3-16PK and 3K30 centrifuges (Sigma GmbH; Osterode, Germany). Spectroscopic measurements of nucleic acids were performed using Nanodrop 2000 (Thermo Scientific, Ontario, Canada). Gel electrophoresis was performed using systems manufactured by

Bio-Rad Laboratories Inc (Hercules, CA, US). Gel documentation was done using GelDoc-It Imaging System (UVP Ltd; Cambridge, UK).

B, Fe, and Zn content in tissues was quantified using IRIS Intrepid inductively coupled plasma-atomic emission spectroscopy (Thermo Elemental, USA). The polyphasic, OJIP fluorescence transient measurements were performed with a Handy PEA fluorimeter (Hansatech Instruments Ltd., Norfolk, UK).

2.2 Methods

2.2.1 Seed Surface Sterilization, Germination and Hydroponic Culture

Barley seeds were surface sterilized for 20 min with gentle continuous mixing in 3% (w/v) sodium hypochlorite with 0.05% (v/v) Tween-20 at room temperature. The seeds were rinsed using sterile distilled water for 4-5 times.

Sterile filter papers were used to dry surface sterilized seeds. Then the seeds were placed in plastic petri dishes containing sterile filter papers (60-70 seeds per dish) and covered with a single layer of sterile filter paper. Then filter papers were moisturized with 3-5 mL of $\frac{1}{2}$ Hoagland's nutrient solution and petri dishes were maintained in growth chambers at dark for germination for 2 days. After 2 days, germinated seeds in petri dishes were placed in plastic pipette tip containers filled with half-strength Hoagland's solution supplemented with 10 μM H_3BO_3 and 50 μM NaFe(III)EDTA which is sufficient for barley, and grown for 7 days in growth chamber at given conditions. The transparent lids of the containers were kept closed for 2 days to protect seedlings from drying and then opened. The solution was refreshed regularly. Stress applications took place at the end of 9 days (2+7) after sowing.

2.2.2 Stress application

Nutrient solutions of 9-day-old seedlings were replaced with half-strength Hoagland's solutions (Appendix A) prepared for stress conditions (Fe-deficient, B-deficient, B-toxic(5 mM), B-toxic(25 mM), Fe-deficient+B-deficient, Fe-deficient+B-toxic(5 mM), Fe-deficient+ B-toxic(25 mM)), as listed in Table A.2 in Appendix A.

Control groups were maintained in fresh solutions containing 50 μM NaFe(III)EDTA and 10 μM H_3BO_3 .

2.2.3 Collection of Plant Material

Root tissues of seedlings were used as material for RNA isolation. The tissues were blotted dry, harvested by using scissors cleaned with 70% (v/v) ethanol, frozen immediately in liquid nitrogen and stored at -80°C for further use. Collection of samples was done at the 0th, 1st, and 5th days of stress application. Tissues of 10 to 12 seedlings were pooled in order to minimize variation between individuals.

Shoot tissues of barley seedlings were used in determination of B, Fe, and Zn content. The tissues were harvested by using scissors and let to dry in an oven at 50°C for a week before measuring weights.

2.2.4 Physiologic Parameters

2.2.4.1 Fresh and Dry Weight Analysis

At the 14th day of growth the weight of shoot and root tissues from control and stressed groups were recorded as fresh weight (FW). Then shoots tissues were floated on distilled water for 24 hours at room temperature. The weights of hydrated shoot tissues were recorded as turgid weights (TW). After three days of the drying in an oven at 80°C , dry weights (DW) were taken.

2.2.4.2 Relative Water Content

The shoot samples which were collected at the 5th day of stress treatment were used to determine relative water content (RWC) according to Smart and Bingham [212];
$$\text{RWC (\%)} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) * 100$$

2.2.4.3 Length of Shoots and Roots

At the 14th day of growth the length of shoot and root tissues from control and stressed groups were recorded by using a ruler.

2.2.5 RNA Isolation and Characterization

2.2.5.1 Decontamination

All solutions and equipments contacting with RNA samples were treated by overnight incubation in 0.1% (v/v) Di ethyl pyro carbonate (DEPC) in a vertical laminar flow and autoclaved at 121°C for 20 min to get rid of RNase contamination. Heat-labile equipments, benches, and gloves were cleaned using RNaseZap (Ambion).

2.2.5.2 Total RNA Extraction

A single-step method of RNA isolation from barley root tissues was performed according to previous description[213] using TRIzol reagent (Life Technologies). The tissues weighting 100 – 150 mg were powdered in pre-cooled mortars by pestles and liquid nitrogen. Powdered samples were immediately transferred to pre-cooled 2 mL-tubes and suspended in 1 mL TRIzol reagent then vortexed vigorously for 15 min at room temperature in order to dissociate nucleoprotein complexes completely. Samples were then centrifuged at 21,000*g* for 5 min at room temperature to precipitate insoluble material. 900 µL of the supernatant were transferred to clean 1.5 mL-tubes and 200 µL of chloroform were added. The mixtures were vortexed vigorously for 15 sec and incubated at room temperature for 3 min. Samples were then centrifuged at 21,000*g* for 15 min at 4°C for phase separation. Then 450 – 500 µL of the upper aqueous phase were transferred to clean 1.5 mL-tubes and 200 µL of chloroform was added once more. The mixtures were vortexed vigorously for 15 sec and incubated at room temperature for 3 min. Samples were then centrifuged at 21,000*g* for 5 min at 4°C.

After phase separation, 375-400 µL of the upper phase were transferred to clean 1.5 mL-tubes. Then 0.3 M NaOAc (pH 5.2) was added into transferred solutions. One-volume of pre-chilled isopropanol was added on the tubes and mixed gently for RNA precipitation. Samples were incubated at -20°C for 30 min and then centrifuged at 21,000*g* for 10 min at 4 °C. RNA is often visible as a white pellet after centrifuge. The supernatant was discarded carefully and RNA pellet was washed with 1 mL pre-chilled 75% (v/v) ethanol. Samples were mixed gently and briefly and centrifuged at 21,000*g* for 5 min. The supernatant was discarded and ethanol washing step was repeated. After

washing the pellet with pre-chilled 75% (v/v) ethanol twice, leftover ethanol was totally removed by brief air drying for 5 – 10 min. The pellet was not dried completely as this greatly decreased RNA solubility. Finally the RNA pellet was dissolved in DEPC-treated ultrapure water.

2.2.5.3 RNA Clean-up and DNA Removal

DNA contamination in RNA samples was removed using RapidOut DNA Removal Kit (Thermo Scientific) according to instructions of the manufacturer. In a total reaction volume of 20 μ L, approximately 2 μ g of total RNA was incubated at 37°C for 30 min in the presence of 1 U DNase I and 1X DNase buffer with $MgCl_2$ (supplied by manufacturer). DNase Removal Reagent (DRR) was used to remove DNase from the mixture. After vortexing vigorously, 2 μ L of DRR for each unit of DNase I added and the mixture was incubated at room temperature for 2 min by gently mixing 2-3 times to resuspend the DRR without splashing. Then the tube was centrifuged at ≥ 800 g for 0.5 – 1 min to pellet the DRR. The supernatant, containing DNA-free and DNase-free RNA was transferred into a new tube.

2.2.5.4 Determination of RNA Quality and Quantity

The concentrations of DNA-free and DNase-free RNAs were determined by recording the absorbance at 260 nm using Nanodrop 2000 (Thermo Scientific, Ontario, Canada).

The ratio of OD at 260 and 280 nm (OD_{260}/OD_{280}) was used to determine the RNA purity. RNA preparations having ratio values of ~ 2.0 for OD_{260}/OD_{280} were accepted as pure. Agarose gel electrophoresis was used to check quality and integrity of isolated total RNA by using major rRNA species as references.

2.2.6 cDNA Synthesis

cDNA synthesis from total RNA samples was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the instructions of the manufacturer. 0.5 μ g of total RNA and 1.2 μ L of primer mix composed of random

hexamer and oligo (dT)₁₈ primers (1:5 molar ratio) was mixture in a total volume of 12 µL using nuclease free water and incubated at 65°C for 5 min. After chilling on ice, 4 µL of 5X reaction buffer (supplied by manufacturer), 1 µL of RiboLock™ Ribonuclease Inhibitor (20 U/µL), 2 µL of dNTP mix (10 mM), and 1 µL of M-MuLV RT (200 U/µL) was added making a 20 µL-mixture. The reaction mixture was incubated in a thermal cycler at 25°C for 5 min followed by 42°C for 60 min. The reaction was terminated by incubation at 70°C for 5 min. The mixture was stored at -20°C if it will be used in a week (-80°C was used for longer storage).

2.2.7 Polymerase Chain Reaction (PCR)

Conventional PCR was performed by using MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, US) to check primers-samples and to find optimal annealing temperature. *Taq* DNA Polymerase (Thermo Scientific) buffered with 10X *Taq* Buffer with (NH₄)₂SO₄ was used in all PCR. Reaction and cycling conditions of conventional PCR are given in Table 2.2 and Table 2.3 respectively.

Gene expression level changes under aforementioned stress conditions were measured on Corbett Rotor-Gene™ 6000 using two-step Q-PCR method (Qiagen, Valencia, CA, USA). All Q-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific). Reaction and cycling conditions of Real Time PCR are given in Table 2.2 and Table 2.3 respectively.

Table 2.2 Reaction conditions for conventional PCR

Reagent	Final Concentration
Primer I	0.3 μ M
Primer II	0.3 μ M
dNTPs	0.2 mM
10X <i>Taq</i> Buffer with (NH ₄) ₂ SO ₄	1 X
MgCl ₂	1.5 mM
<i>Taq</i> DNA Polymerase	0.05 units/ μ l
DMSO	3 %
cDNA	2.5 ng/ μ l

Table 2.3 Cycling conditions for conventional PCR

Cycle Conditions	
95 °C	3 min
95 °C	20 sec
55* °C	30 sec
72 °C	1 min
30 X cycle to step 2	
72 °C	5 min
4 °C	∞

*55 °C was accepted as optimal for all three of the genes.

Table 2.4 Reaction conditions for Real Time PCR

Reagent	Final Concentration
Primer I	0.15 μ M
Primer II	0.15 μ M
Maxima SYBR Green/ROX qPCR Master Mix (2X)*	1 X
cDNA	2.5 ng/ μ l

**Provides a final concentration of 2.5 mM MgCl₂*

Table 2.5 Cycling conditions for real time PCR

Cycle Conditions	
95 °C	10 min
95 °C	15 sec
55 °C	15 sec
72 °C	30 sec
35X cycle to step 2	

Relative quantification, target gene (NAS and IDS3) - the control gene (GAPDH) amount ratio, was performed to analyze gene expression level changes. This ratio is then compared between control and stress induced samples. The genes are separately amplified using same sample. Equal amounts (0.5 ug) from all RNA samples were pooled after DNA removal in a single tube. Then this pooled cDNA was used to determine amplification efficiencies of genes of interest. A tenfold dilution series from the cDNA pool were prepared. Then each dilution series was amplified in real-time Q-PCR. Standard curves were constructed for all three genes by using CT values obtained. The amplification efficiencies (E) for each target were calculated by:

$$E=10^{(-1/S)}-1$$

equation in which “S” equals to slope of the standard curve. Second, efficiency comparison was done by plotting the difference in C_T values against log template amount. The difference in PCR efficiency was determined by calculating slope of the line. If the slope of the resulting straight line is <0.1 , amplification efficiencies are comparable.

After efficiency comparison, two different analyses were used:

- I. It was used for IDS3 which showed different amplification efficiency since the slope of the resulting straight line is not <0.1 . In this method;
 - GAPDH was chosen as an appropriate endogenous reference whose expression level does not change under the experimental conditions or between different tissues
 - A dilution series of tenfold dilutions from pool cDNA was prepared to construct standard curves for IDS3 and GAPDH
 - Real-time PCR/Q-PCR was performed
 - The C_t values for the standards and the samples of interest were determined
 - Standard curves for both IDS3 and GAPDH by plotting C_T values (Y-axis) against the log of template amount or dilution (X-axis) were constructed
 - The amount of target and reference in the samples of interest were calculated using their C_t values and the corresponding standard curve
 - To calculate the normalized amount of target, the amount of target was divided by the amount of reference (because replicate reactions were performed, the average value was used)
 - Three calibrator samples were defined (Control in Day 0, Day 1, and Day 5) and the relative expression level of the target gene in the samples of interest was compared by dividing the normalized target amounts by the value of the calibrator
 - Fold changes according to calibrator samples were calculated and showed in graphs

- II. For NAS, which showed comparable amplification efficiency since the slope of the resulting straight line is <0.1 , $\Delta\Delta Ct$ method was used. In this method;
- The ΔCt value was determined by subtracting GAPDH Ct value from NAS Ct value for each sample
 - Three calibrator samples were defined (Control in Day 0, Day 1, and Day 5) and the $\Delta\Delta Ct$ value was determined by subtracting the calibrator ΔCt value from the ΔCt value of each sample
 - The normalized level of target expression relative to the calibrator was calculated by using the formula $2^{-\Delta\Delta Ct}$

2.2.8 Determination of Boron, Iron, and Zinc Content in Shoots

After 9, 10 and 14 day of growth (0, 1 and 5 day of stress treatments), the shoot tissues were collected and dried at 50°C for a week. The dried tissues were weighted and cut into small pieces to fit in crucibles using scissor. About 0.2-0.3 g of dried samples were ashed at 550 °C for 6 hours, and dissolved in 65% HNO₃. After incubation 15 minutes in room temperature, solutions were diluted with distilled water to 25 mL. Then the solutions were filtered by using filter papers into new containers after another incubation step for 30 minutes. The filtered samples were stored at 4 °C for further use. The B, Fe, and Zn content (ppm) in tissues was quantified using inductively coupled plasma-atomic emission spectroscopy (*ICP-AES, IRIS Intrepid, Thermo Elemental, USA*) analysis in Department of Mining Engineering, Hacettepe University (Ankara, Turkey). A tenfold dilution series from the standards of each element were prepared in ppm values. Then, the standard curves for each element were prepared. After the measurement of elements, each of the samples was quantified by using the standard curves and comparing the standard values.

2.2.9 Chlorophyll (Chl) *a* fluorescence measurements

The polyphasic, OJIP fluorescence transient measurements were performed with a Handy PEA (Hansa tech Instruments Ltd., Norfolk, UK) fluorimeter on selected leaves of the cultivars at room temperature. Following a 30-min dark adaptation, samples were illuminated with continuous light (650 nm peak wavelength; 3,000 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$

¹maximum light intensity for 1 s) provided by three LEDs, and the Chl*a* fluorescence signals were recorded according to Strasser and Strasser [214]. The OJIP transient was analyzed by the JIP test based on the energy fluxes theory for biomembranes in a photosynthetic sample, which leads to the equations and calculations for the efficiencies for the whole energy cascade from absorption to the reduction of the end electron acceptors at the PSI acceptor side and the performance indexes [215]. The fluorescence parameters (Appendix C, Table C.1) were calculated by Microsoft Excel and also according to the JIP test [215].

2.2.10 Statistical Analysis

The experiments, arranged in a completely randomized design, were repeated three times. Differences among treatments were tested using *MINITAB* statistical program. Statistical variance analysis (*ANOVA*) of the data was performed and data were compared using *Tukey's* test at the 5% level.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Physiologic Parameters

3.1.1 External appearance of cultivars

The effects of given stress conditions to barley cultivar Tarm-92 on some parameters of growth and physiology were investigated after 5 days of stress. Necrotic and yellowish leaves were observed in seedlings treated with stress solutions containing toxic dosage of boric acid after 5 days (Figure 3.1) as previously described in literature [106], [216]. In addition, lateral root growth was inhibited as boric acid concentration increases. Effect of deficiency conditions on leaves or roots is not visually distinguishable compared to control. When Fe-deficiency was applied accompanied with B-toxicity (either 5 mM or 25 mM), still there are no clear visual effects on leaves or roots.

3.1.2 Length of Shoots and Roots

As a primary phenotypic effect of induced stresses, significantly reduced shoot growth in both of 25 mM boric acid treatments, and reduced root growth in conditions with 5 mM and 25 mM boric acid treatments was observed (Figure 3.2). Reduced photosynthetic rate and water use efficiency have been suggested as the cause of the restriction of growth [217]. It is reasonable to expect reduced photosynthetic activity in necrotic leaves, which is also confirmed by JIP-test results (see section 3.3). Fe-deficiency did not seem to have a significant effect on the shoot and root growth when applied with other stress conditions, as in comparison to optimal growth conditions. Although reduced shoot growth in response to other stress treatments was present, even



Figure 3.1 Leaves of Tarm-92 seedlings in control (a), B-deficiency (b), 5 mM B-toxicity (c), 25 mM B-toxicity (d) solutions after 5 days.

for conditions including 5 mM boric acid treatment, they are not significantly different from control or others. However, significantly reduced root growth in 5 mM boric acid treatment with or without Fe-deficiency was also present. Root growth reduction in response to B toxicity has previously been observed in barley [106], [55], wheat[218], and tomato [219].

3.1.3 Fresh Weight of Shoots and Roots

B-toxicity (5 mM and 25 mM) with or without Fe-deficiency caused a significant reduce in fresh weight (FW) of shoot tissues (Figure 3.3). In addition, 25 mM treatments were different from 5 mM treatments. As in length results, Fe-deficiency did not affect fresh weight values of shoots when applied with other stress conditions. However, fresh weight of roots showed a little different response than the shoots. Root fresh weight results of all stress conditions were significantly different from control treatment. Since, roots are the first region of plants that faced with stress and forced to adopt several structural and functional modifications[220], it is expected to see such a significant decrease in root fresh weights rather than shoots. Furthermore, 25 mM boric acid treatments were significantly different from other stress conditions as well, indicating severity of stress among others. Although accompany of Fe-deficiency to other stress conditions did not have a significant effect on root fresh weight, slight decreases in Fe-deficient root fresh weights compared to corresponding stress conditions were observed. The similar influence of B toxicity on growth parameters of barley is also shown in previous studies[106], [55].

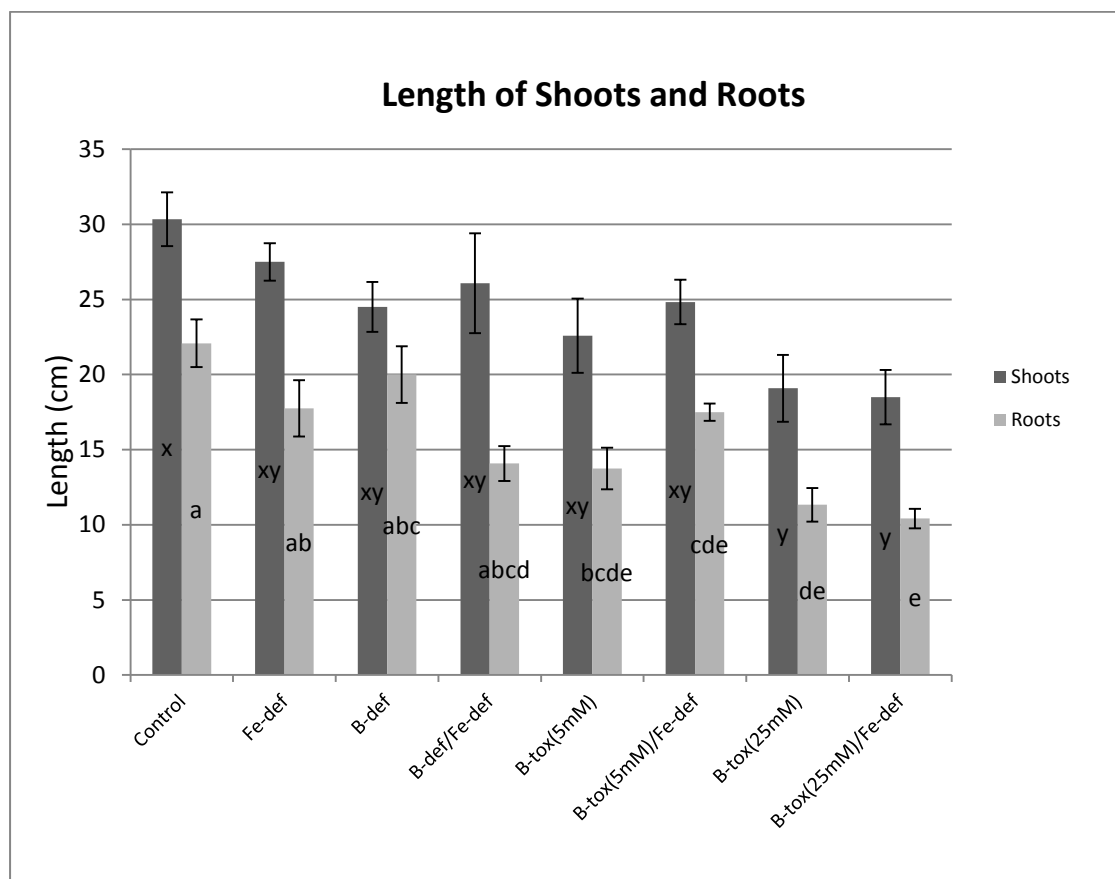


Figure 3.2 The length of shoots and roots of Tarm-92 exposed to given stress conditions after 5 day (14th day of growth). Each value represents the mean $\pm$ SEM of six independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

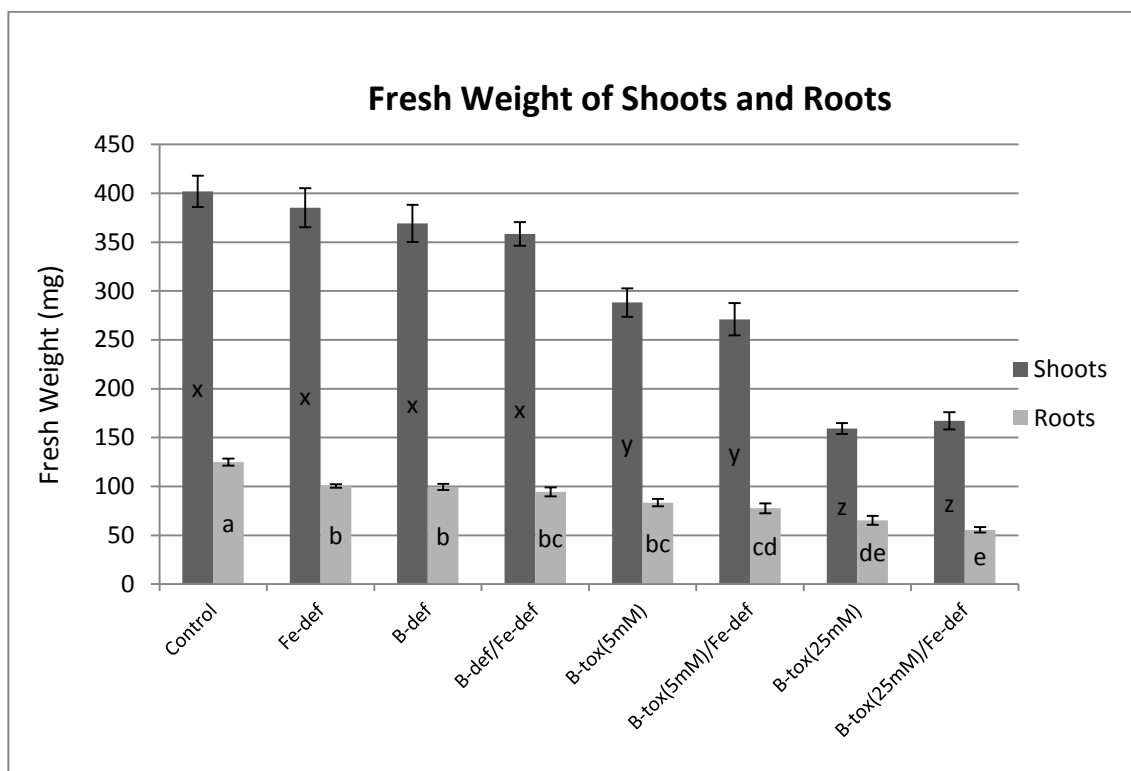


Figure 3.3 The fresh weight of shoots and roots of Tarm-92 exposed to given stress conditions after 5 day (14th day of growth). Each value represents the mean $\pm$ SEM of six independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with *ANOVA*

3.1.4 Dry Weight of Shoots and Roots

Similar to fresh weight results, B-toxicity (5 mM and 25 mM) with or without Fe-deficiency caused a significant reduces in dry weight (DW) of shoot tissues (Figure 3.4). 25 mM treatments were also significantly different from 5 mM treatments. Effect of Fe-deficiency was not significant for both shoot and root dry weight compared to other conditions and control group. Unlike the fresh weight results and effect of 25 mM treatments, 5 mM boric acid treatments did not reduced root dry weight. Tarm-92 is a B-tolerant barley cultivar [24], so insignificant reduction in dry weight in response to 5 mM B treatment is expected.

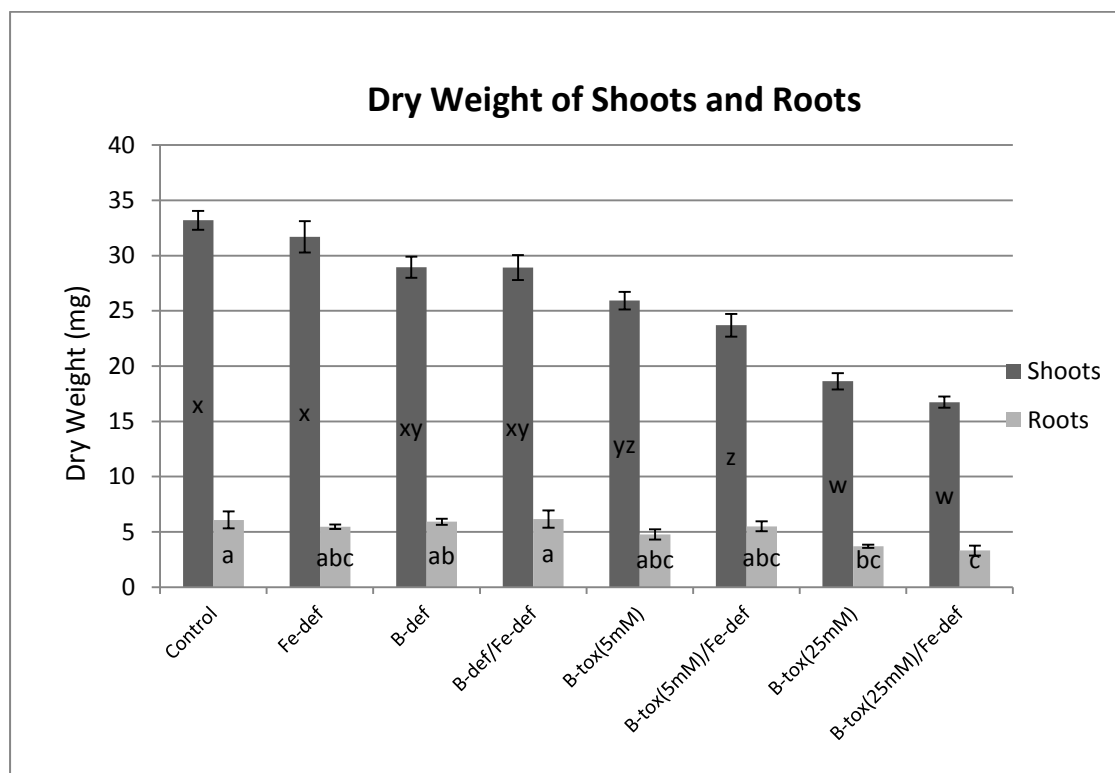


Figure 3.4 The dry weight of shoots and roots of Tarm-92 exposed to given stress conditions after 5 day (14th day of growth). Each value represents the mean $\pm$ SEM of six independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with *ANOVA*

3.1.5 Relative Water Content of Shoots

Relative water content(RWC) has long been in use as an appropriate estimate of plant water status [221]. Both leaf water potential and osmotic adjustment can be estimated by this simple method. Depending on species, RWC normally range between 98% in fully turgid transpiring leaves to about 30-40% in severely desiccated and/or dying leaves. At around initial wilting, the typical RWC of the most crop species is about 60% to 70%, with exceptions. In the case of barley cultivar Tarm-92 with given stress conditions, the values range between about 90% and 60% depending on the severity of the stress. RWC values of treatments B-toxicity (25 mM) and B-toxicity (25 mM) accompanied with Fe-deficiency were significantly different from the control and the rest of the stress treatments (Figure3.5). However, B-toxicity (5 mM) treatment alone

and accompanied with Fe-deficiency was not significantly different from control, confirming the cultivar is B-tolerant. As in FW and DW results, effect of Fe-deficiency was not significant both for other conditions and control group.

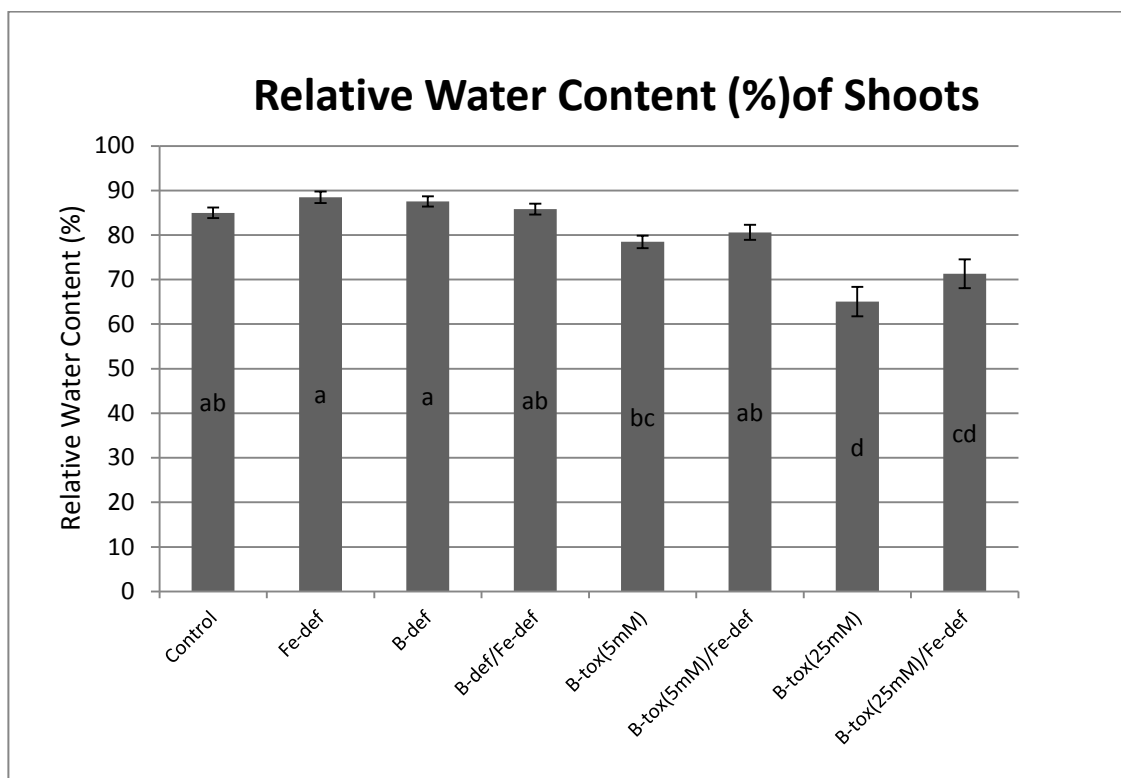


Figure3.5 The relative water content (%) of shoots of Tarm-92 exposed to given stress conditions after 5 day (14th day of growth). Each value represents the mean $\pm$ SEM of six independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with *ANOVA*

Overall, changes in physiological parameters were detected in all of the given stress conditions. Especially, the conditions with 25 mM boric acid treatment disrupted the growth of seedling in every aspect, where Fe-deficiency did not significantly affect the growth when applied with other stress conditions. In addition, as a B-tolerant barley cultivar [24], Tarm-92 showed fairly a good growth performance under 5 mM B

treatment. Further, B deficiency did not result in significant decrease in any of the growth parameters, except root fresh weight.

3.2 Element Analysis

Element analysis of seedlings under mineral stress is important to determine the effectiveness of stress response mechanisms of plants against induced stress conditions. Determination of shoot element contents for the corresponding mineral and other minerals which are considered as related to stress response mechanism against that particular stress or general can provide meaningful data to understand the response mechanism in a great extent. From the data, the role of the transporters, enzymes, and other metabolites (phytosiderophores in our case) involving in the stress response mechanism can be predicted. Therefore, B-Fe-Zn content of shoots were determined using ICP-AES in the 1st and 5th day of the stress treatment to help understanding of the overall stress response mechanism under aforementioned stress conditions.

3.2.1 Shoot B-content

B accumulates in a pattern from leaf base to tip. The older the leaf, the more B accumulation occurs. This leads marginal or tip chlorosis and necrosis [216] as observed in the present study (see section 3.1.1). As expected, toxic levels of B resulted in significant increase in B-uptake for both 1 day and 5 day after stress induction (Figure 3.6). A previous study on wheat cultivars under different toxic B stress conditions showed similar results to ours [111]. However, other stress conditions did not affect the B status of plants. A study investigating Fe status and B uptake on barley cultivars showed that B-tolerant barley accumulated more B in Fe-deficiency significantly after 110h, which was not observed for B-intolerant cultivar [176]. However, Fe-deficiency did not result in significant increase of B accumulation after 120 h of stress application according to our data. In addition, it was not affected B-content when applied with B-toxicity. The difference between two studies can be explained by the difference in cultivars, or there is no relation between Fe-status and B accumulation of B-tolerant barley cultivars. However, it should be noted that Patterson and colleagues (2007) used

oldest leaves for determination of B content where entire shoots were used as material in the present study.

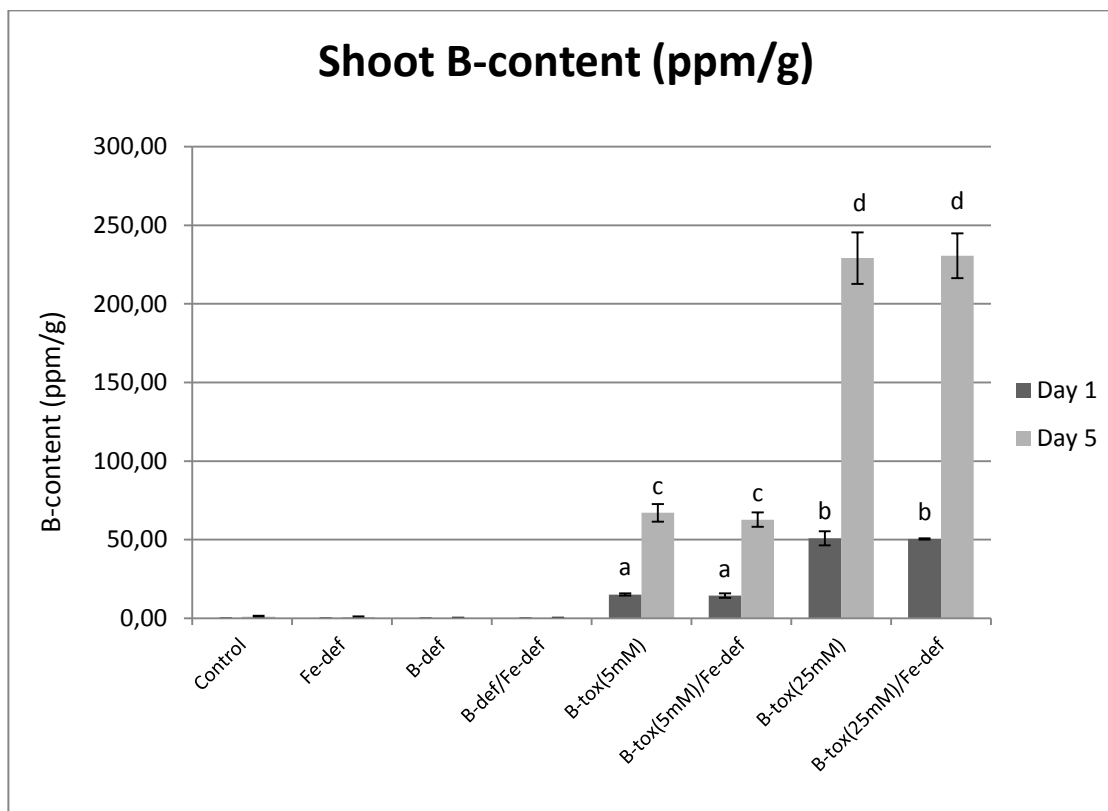


Figure 3.6 The B content of shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 0, 1 and 5 day. Each value represents the mean $\pm$ SEM of three independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with *ANOVA*

3.2.2 Shoot Fe and Zn-content:

The relation between plant's B, Fe and Zn status was studied over the years [201], [205], [209], [210]. In addition, it was reported that alkaline soils contain low concentrations of Zn and Fe and high concentrations of B [191]–[193]. uptake of Zn increased 15-fold and accumulation of Zn increased 16-fold in *Zea mays* under Fe deficiency [201]. Zn-fertilization studies in wheat cultivars revealed large differences in shoot B concentration when Zn depleted or repleted [209]. Another study in tomato

about effect of Zn on the alleviation of excess B uptake was confirmed the relation between Zn and B [210]. Zn application revealed the symptoms of B toxicity partially. Significant decrease in Fe and increase in Zn content of B-tolerant and B-intolerant barley cultivars under Fe-deficiency was also observed starting the first day of the stress application [176]. However, our results did not show any significant difference for both Zn and Fe content in any of the induced stress conditions (Figure 3.7 and Figure 3.8).

First, no change in Fe content at least in Fe deficiency is an interesting result. However, it can be explained by the fact that usage of entire shoots for element analysis. The youngest leaves are used generally for Fe content determination[176], because Fe is a microelement and plants Fe status before the application of stress conditions is already adequate for survival of the seedlings. The stored Fe in old leaves before the stress application can be used by younger leaves, keeping total Fe status constant. In addition, after the first mechanisms detected Fe deficiency, seedlings may take necessary cautions to collect all of the remaining Fe in the media, e.g. increasing the biosynthesis of phytosiderophores. In nature, the element composition of the surroundings of the plants does not change in days. Therefore, the overall responses to element stress conditions should be long termed, because if limited Fe is available around the plant, it would not change until the roots grow enough to find a Fe-rich area. The first step of a long time response might be collecting all of the present Fe in surroundings, so plant will not need Fe for relatively long time. The constant Fe content in 5th day of stress may be explained by this. The number of phytosiderophores increased in a great amount to collect the remaining of Fe from previous media and stored for further use. Thus, Fe content remained the same in comparison to control.

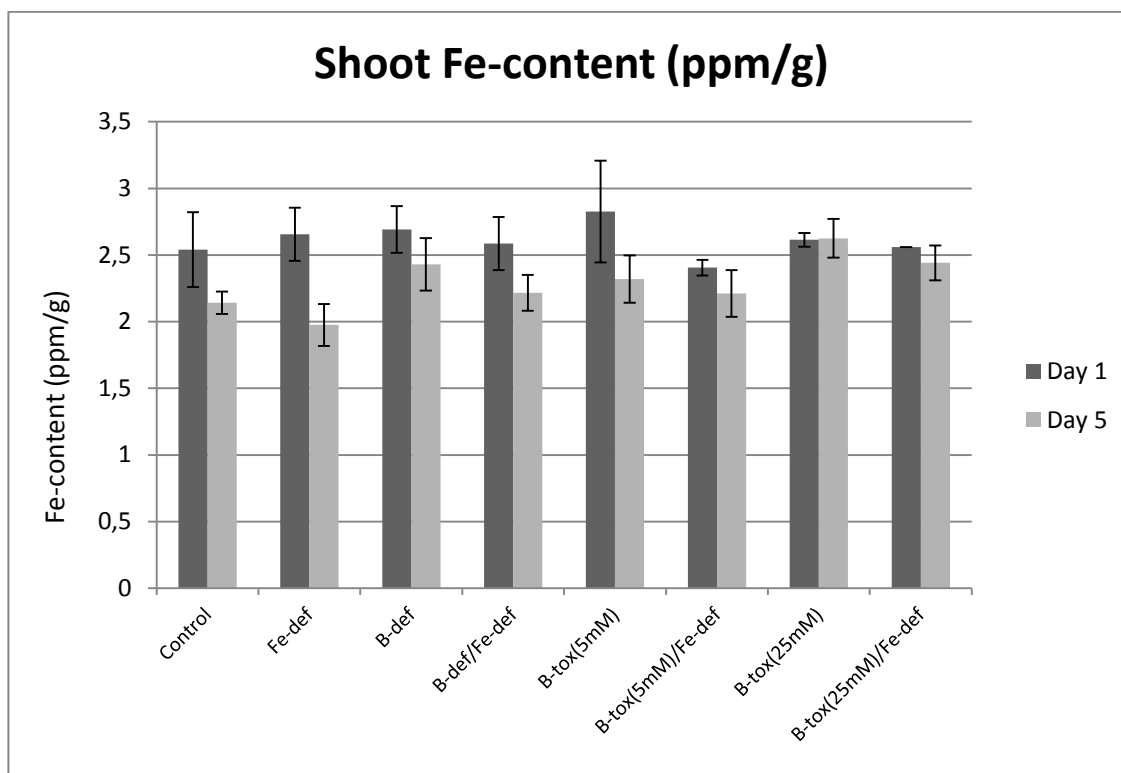


Figure 3.7 The Fe content of shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 1 and 5 day. Each value represents the mean $\pm$ SEM of three independent replicates. No values are significantly ($P < 0.05$) different from others according to the *Tukey's* test in conjunction with *ANOVA*

Zn content was determined to be used as a supporting data of response mechanism of seedlings. However, we failed to observe the leading data, change in Fe content, in the present study. The increase in Zn content under Fe deficiency is detected in several studies [176], [201], suggesting the role of phytosiderophores in Zn uptake [205]. The steady Zn content under Fe deficiency in the present study can be explained by proposing that the number of phytosiderophores did not increase. However, the expression levels of mRNAs of phytosiderophore producing enzymes (see section 3.4) did not confirm this statement. Further investigation is needed to explain the situation.

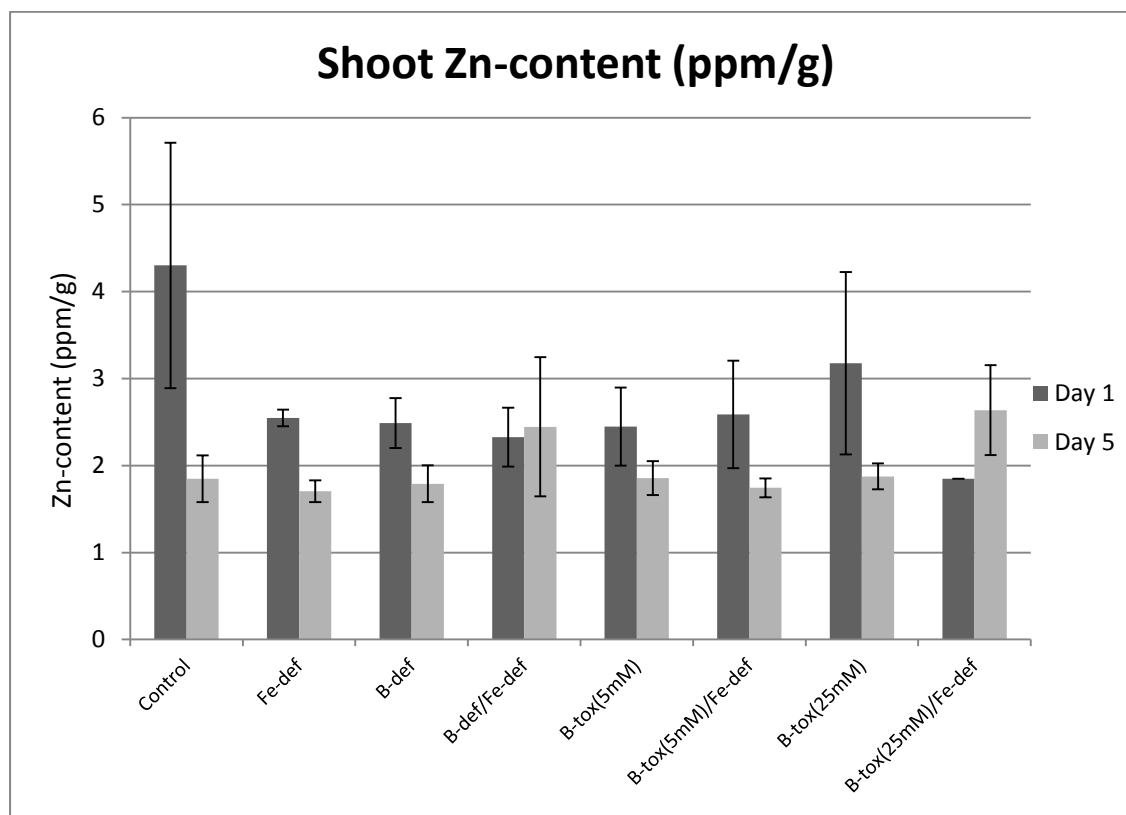


Figure 3.8 The Zn content of shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 1 and 5 day. Each value represents the mean $\pm$ SEM of three independent replicates. No values are significantly ($P < 0.05$) different from others according to the Tukey's test in conjunction with ANOVA

3.3 Chlorophyll Fluorescence and Photosynthetic Performance

The reduced growth of seedling under stress is related to decrease in photosynthetic activity [217]. Chlorosis and necrosis in leaves, which are also observed in the present study, are associated with the loss of chlorophyll content. In addition, B-toxicity results in severe effects on biochemical processes including reduction in chlorophyll (Chl) contents and photosynthetic rates [103], [111], [222], [47]. The changes in the photosynthetic processes can be determined by the help of chlorophyll fluorescence which is defined as a noninvasive and quantitative tool [223]. Especially, the effects of B toxicity that induces chlorosis and/or necrosis in leaves can be investigated by studying Chl a fluorescence which is sensitive to a variety of abiotic

stresses [224]. The JIP-Test is a method for analysis of fast OJIP fluorescence induction kinetics [214]. The test quantifies *in vivo* energy fluxes passing through reaction centres (RCs) and photosystems (PS), evaluating photosynthetic performance of plants [214], [225]. Information about the structure, conformation and function of the photosynthetic apparatus in any physiological state can be provided by JIP test [215]. Different steps and phases of the transient with the redox states of PSII is linked through the test allowing correlation of phases with the efficiencies of electron transfer (ET) in the intersystem chain between PSII and PSI and to the end electron acceptors at the PSI acceptor side [215].

Photosynthetic activity of plants is affected negatively by various abiotic stresses, which eventually cause reduced growth [217]. B stress induced damage in various plants, as an abiotic stress, has been studied by evaluation of Chl *a* fluorescence [226], [227]. Recently, photosynthetic response of two wheat cultivars under B toxicity has been investigated [111]. However, photosynthetic response data on B stress induced barley cultivars is not available.

In addition to B-stress, Fe-deficiency is another important condition when considering photosynthetic activity because important steps in photosynthetic pigment metabolism, and ultrastructure of chloroplast, are directly dependent on iron [228]. Studies on the structure and function of the photosynthetic apparatus of the green unicellular algae *Chlamydomonas reinhardtii* under Fe-deficiency revealed that the antennaprotein complexes are affected differently, where light harvesting complex I (LHCI) is drastically reduced and LHCII abundance remains fairly constant [229]. The other photosynthesis related effects of Fe deficiency includes decreased number of thylakoid membranes [164], changes in active Rubisco levels [165], loss of photosystem I (PSI) activities [166], [167]. PSI contains Fe-S proteins functioning in electron transfer to NADP^+ . Ferredoxin, one of the most important Fe containing proteins in photosynthesis, is considered as the direct recipient of electrons from the pathway [168]. Therefore, negative effects of Fe deficiency on PSI can be explained by decrease in number of Fe containing proteins.

The analysis of changes in Chl a fluorescence kinetics in B-tolerant barley cultivar Tarm-92 [24] can provide valuable information on structure and activity of PSII under various B-treatments and response of a B tolerant barley cultivar against different toxic concentrations of B. As expected, Chl a fluorescence kinetics measurements showed alterations in the photosynthetic apparatus' efficiency of stress induced plants in the present study. The results of selected JIP test parameters (Appendix C) are showed in Figure 3.9as a radar-plot. In addition to radar-plot presentation, the results of each parameter are given in details containing the results of *Tukey's* test in conjunction with *ANOVA* (Figure 3.10 to Figure 3.20). The value of each parameter was normalized by dividing the value of control.

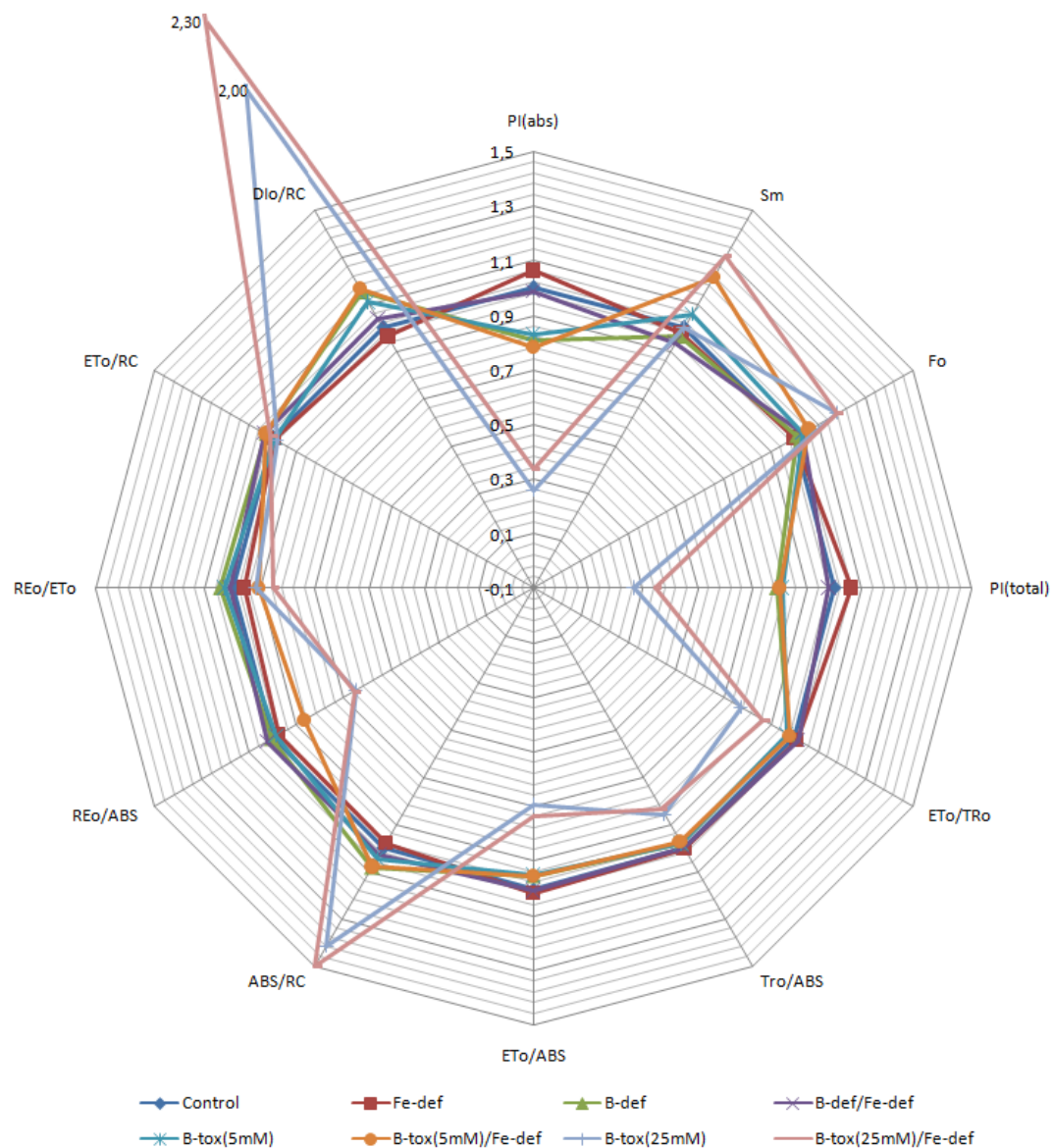


Figure 3.9 The radar-plot presentation of selected JIP test parameters quantifying photosynthetic efficiencies of dark-adapted leaves of Tarm-92 exposed to given stress conditions after 5 day. Mean values of parameters were plotted relative to their respective control values ($n = 8$)

OJIP fluorescence transient measurements* were performed after 30-min dark adaptation, because in the dark adapted state of the photosynthetic apparatus, QA is accepted as fully oxidized [215].

Krause and Weis were defined initial fluorescence intensity (F_0) as the representation of the fluorescence emission by the excited antenna Chl a molecules before the migration of excitation to the reaction centres (RCs) [230]. F_0 is measured when all RCs of PSII are open. F_0 values increased significantly in B-toxicity (25 mM) with or without Fe-deficiency (Figure 3.10). Other treatments did not result in significant change.

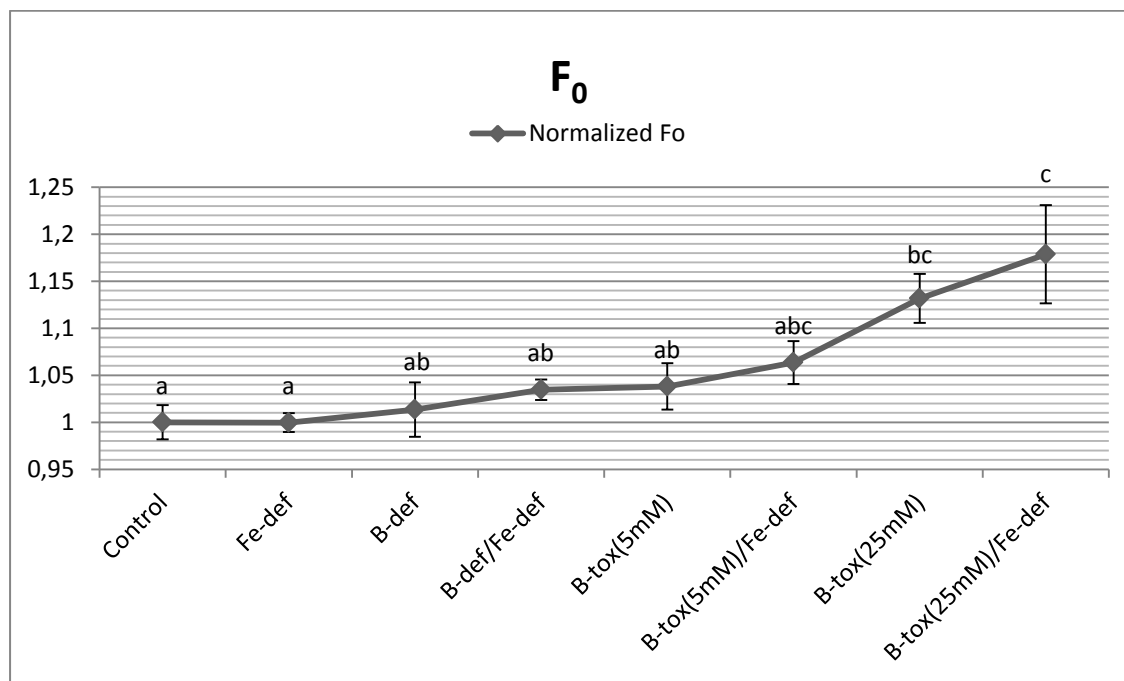


Figure 3.10 The normalized F_0 values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

* Letters in OJIP describes the time steps where the parameters were calculated; O step- at 0 s; J step- at 2 ms; I step- at 30 ms; P step- time to reach peak or maximum value

Increase in F_0 can be the sign for an irreversible damage to PSII caused by the physical separation of LHCII from the PSII core complexes [231]. Increase in F_0 value occurs when the number of functional Chls not connected to the RCs of PSII increases. In other words, the lower the value of F_0 means the higher number of active and open RCs [232]. The results showed that Tarm-92 is tolerant to B-toxicity even for high B concentrations (5 mM). However, deadly concentrations of B (25 mM) disrupt the photoprotective capacity resulting in a higher degree of damage than other treatments. The fairly small and regular increases in treatments involving Fe-deficiency are expected, but insignificant to cause serious damage.

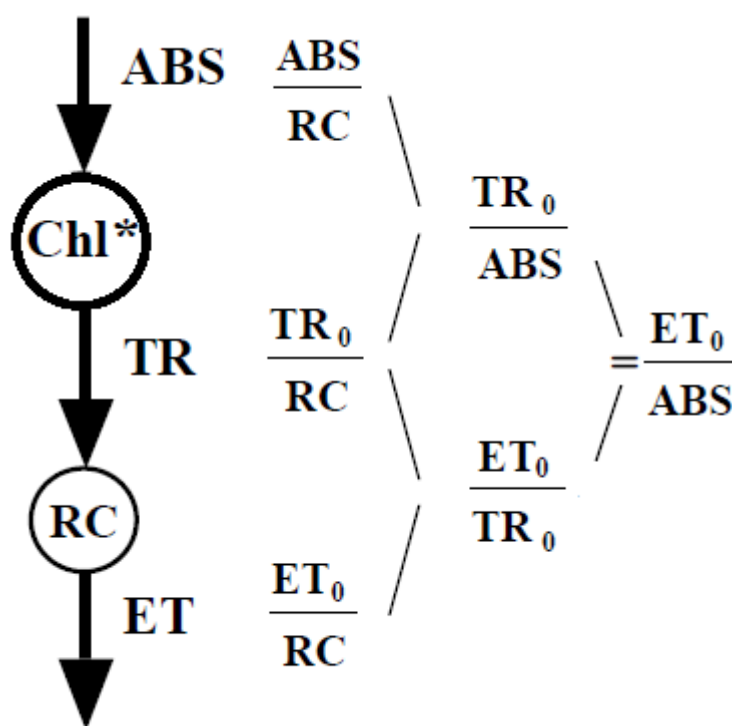


Figure 3.11 A scheme of the energy cascade from PSII light absorption to electron transport [214]

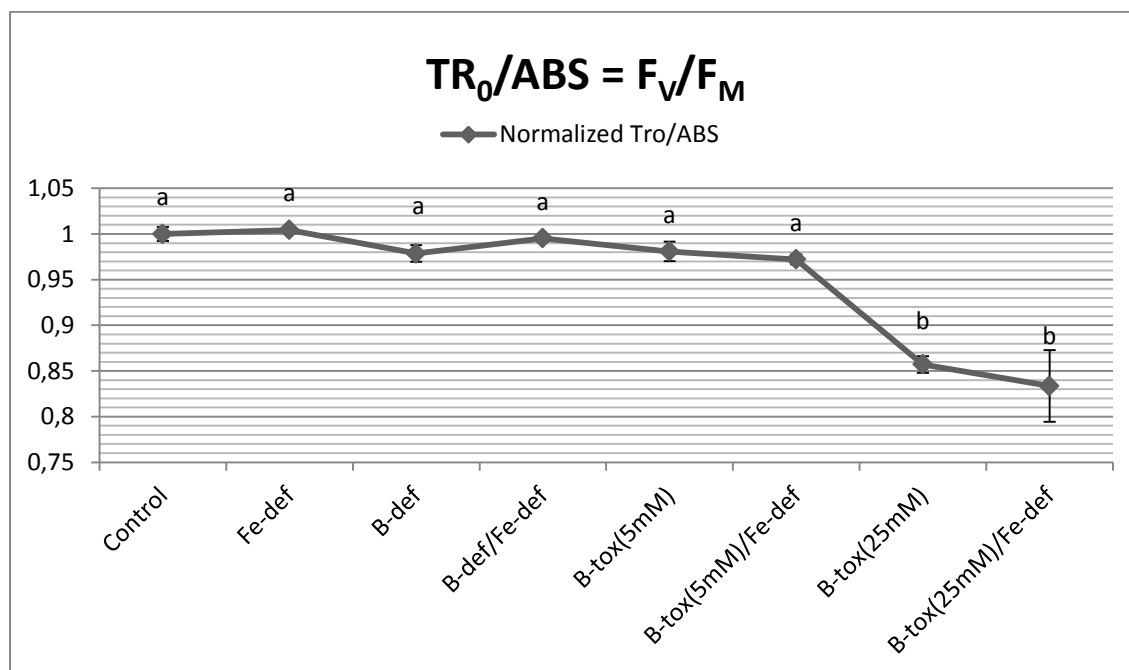


Figure 3.12 The normalized TR_0/ABS values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

A simplified scheme is given in the Figure 3.11 where ABS is photon flux absorbed by the antenna pigments and creating excited Chl; TR is channeled excitation energy as trapping flux to RC to be converted to redox energy by reducing QA to QA⁻; and ET is the electron transport further than QA⁻ leading ultimately CO₂ fixation [215]. The ratios of these values give valuable information on PSII activity of the seedlings.

The ratio of TR_0/ABS ($[F_M - F_0]/F_M = F_V/F_M$) describes the potential yield of the primary photochemical reaction of PSII [233]. It was defined as the most widely used PSII efficiency indicator [234]. Biotic and abiotic stresses lead significant decreases in F_V/F_M [230]. F_M value describes the maximum fluorescence yield, where the reduction of all QA is achieved. The TR_0/ABS value in B-toxicity treatments containing 25 mM boric acid decreased significantly compared to control (Figure 3.12). Change in other treatments is not significant. A severe decrease in 25 mM B containing treatments

indicates that effect on the donor side is relatively higher than that of the acceptor side indicating the cessation of PSII activity in those treatments.

The probability that a trapped exciton moves an electron into the electron transport chain further than QA^- , $ET_0/TR_0 = 1 - V_J$. Only the values for 25 mM boric acid containing treatments significantly decreased (Figure 3.13). Although it was not significantly affected, decrease in Fe deficiency is expected for this parameter because electron transfer from QA^- to QB is achieved by a reaction involving Fe [230].

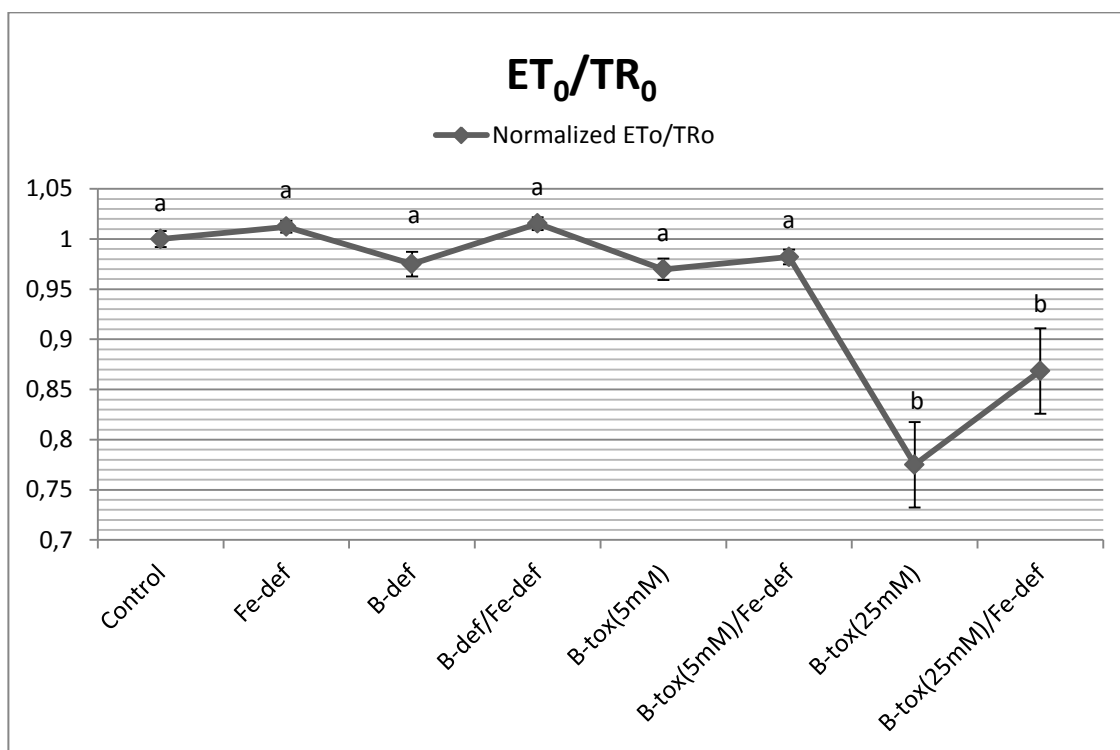


Figure 3.13 The normalized ET_0/TR_0 values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the Tukey's test in conjunction with ANOVA

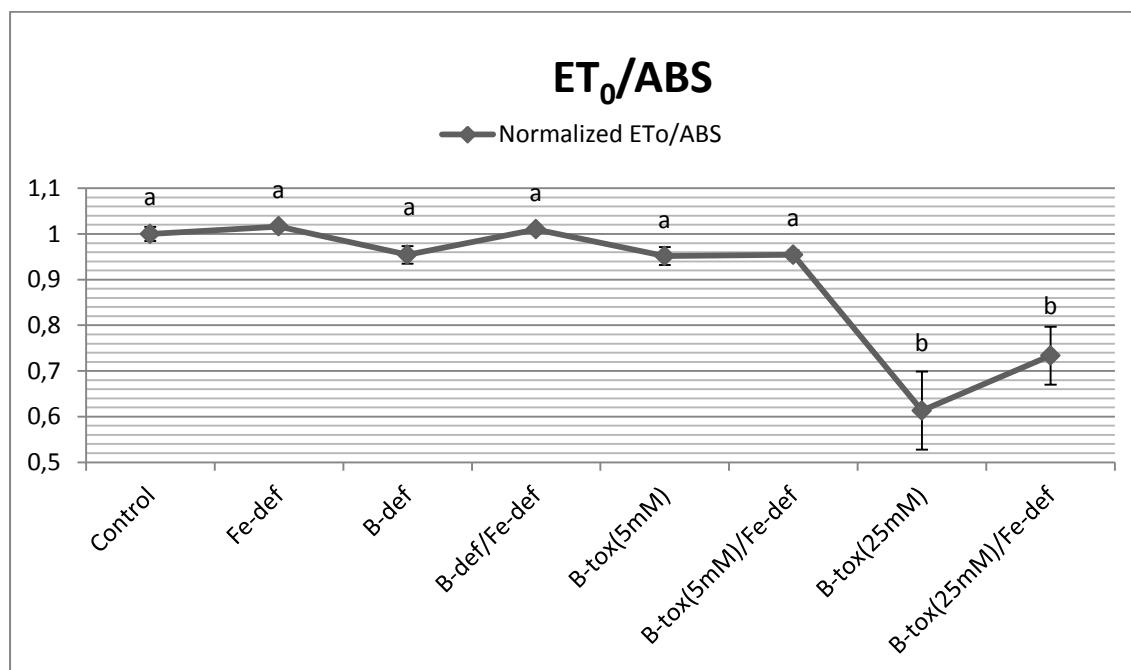


Figure 3.14 The normalized ET_0/ABS values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with *ANOVA*

The ratio of $ET_0/ABS = (TR_0/ABS).(ET_0/TR_0)$ describes the quantum yield for electron transport at $t=0$, and the overall expression of the PSII activity. The values in seedlings decreased significantly again only after 25 mM B treatment with or without Fe-deficiency (Figure 3.14). Therefore, it can be said that PSII activity of barley cultivar Tarm-92 is diminished only by conditions with 25 mM B treatment.

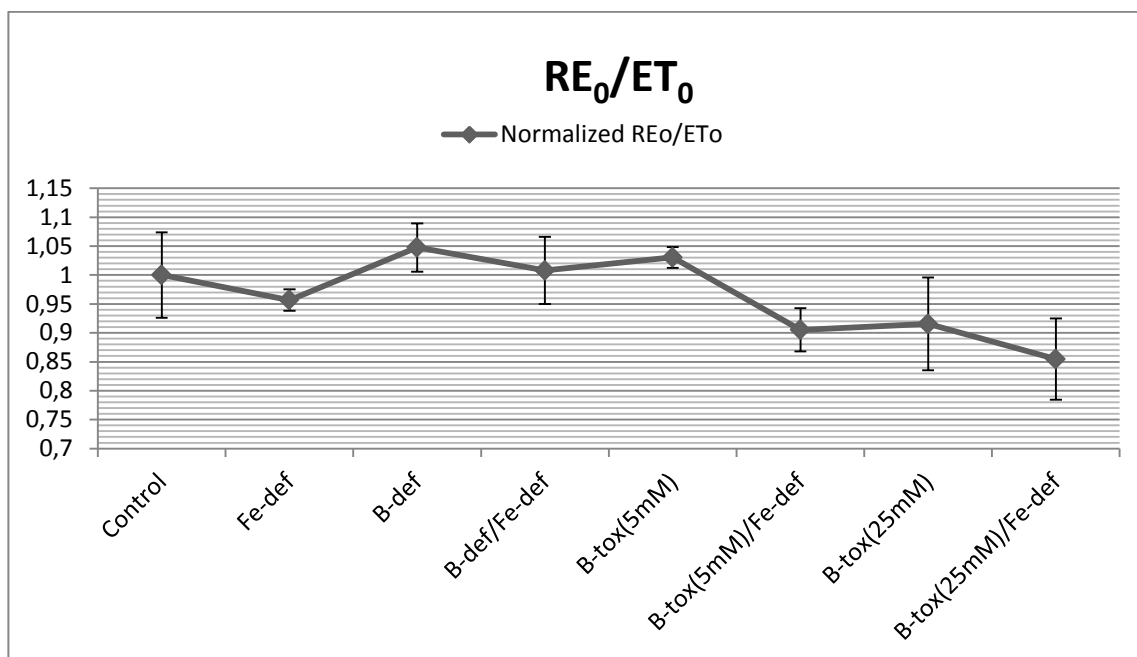


Figure 3.15 The normalized RE_0/ET_0 values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

RE_0/ET_0 , $(1-V_J)/(1-V_I)$, is the efficiency of an electron movement from intersystem electron acceptors to PSI final electron acceptors [225]. Since Fe deficiency results in loss of PSI activities [166], [167], and Fe containing compounds involves in several steps of PSI, the effect of Fe deficiency should be present at this parameter. As seen in Figure 3.15, there is no significant difference detected among the stress conditions. However, slight decreases in all of Fe deficient conditions are present compared to corresponding values.

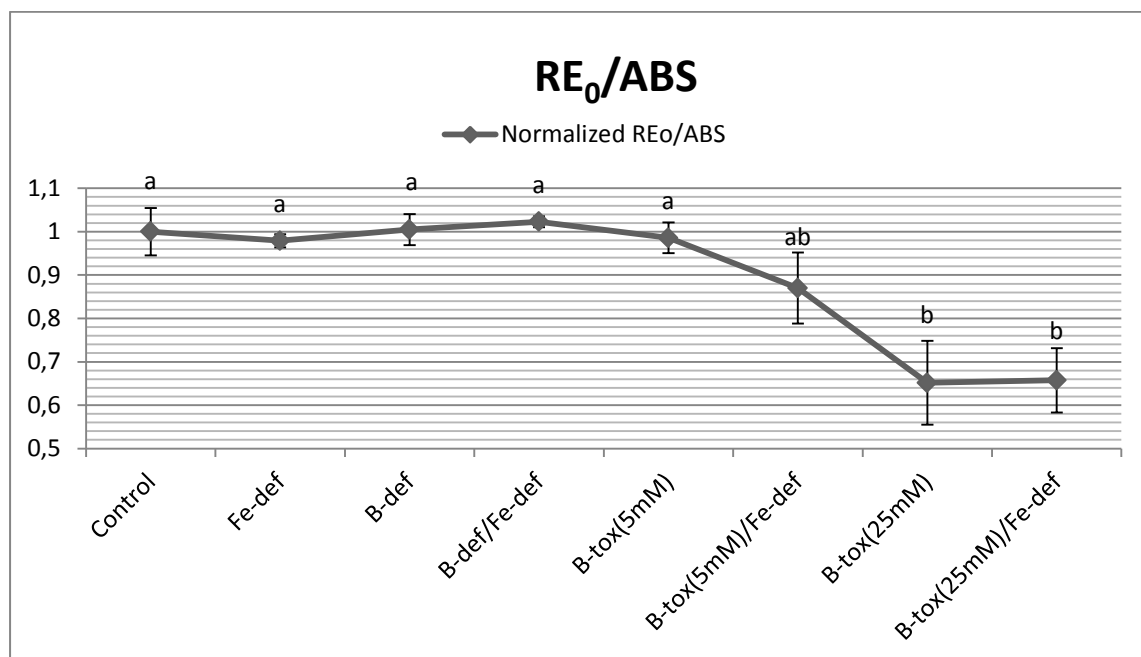


Figure 3.16 The normalized RE₀/ABS values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

RE₀/ABS is the quantum yield of electron transport from QA- to PSI final electron acceptors [225]. Unlike the RE₀/ET₀, it gives valuable information about the activity of the system in a wider range starting from PSII to the end of PSI. Significant decrease was present again in only 25 mM B treatments (Figure 3.16). The similar effect of Fe deficiency may be expected since the value also includes information about PSI activity, but there is not. It may indicate that the damage on PSII activity is more serious than PSI's.

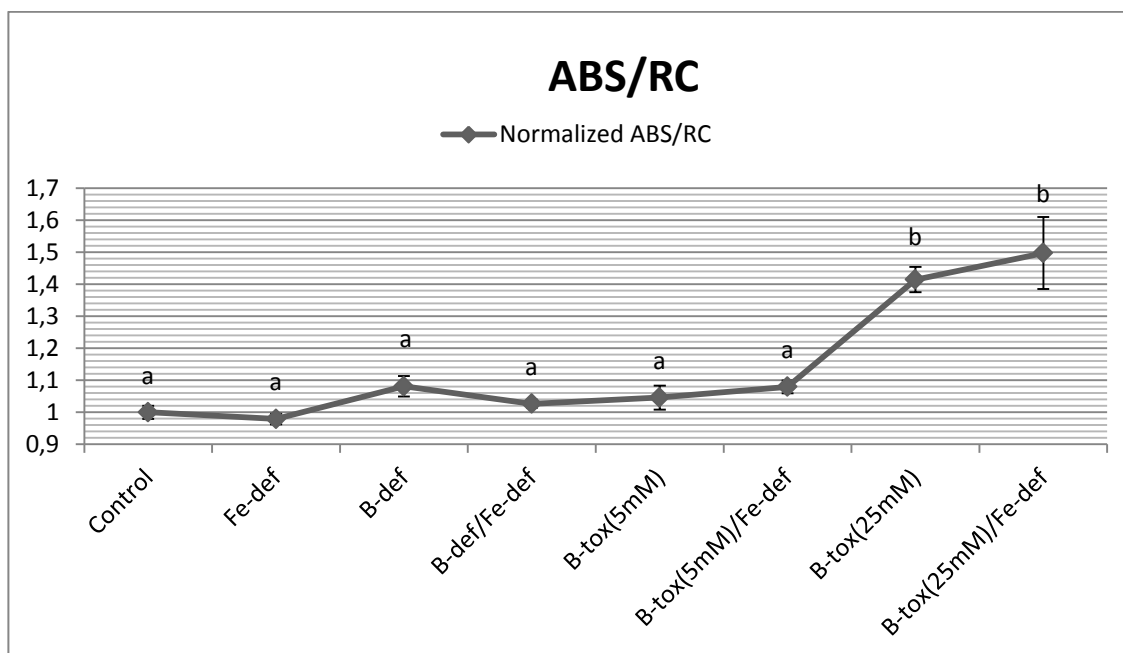


Figure 3.17 The normalized ABS/RC values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's test* in conjunction with *ANOVA*

Effective antenna size of an active RC ($ABS/RC = [TR_0/RC]/[TR_0/ABS]$) can be expressed as the total number of photons absorbed by the Chl molecules divided by the total number of active RCs [215]. Therefore, it gives the average absorption per RC. The high value of ABS/RC means that total number of RCs is greater than the number of active RCs. However, the photons are absorbed by Chl molecules associated with both active and inactive RCs. Significantly high ABS/RC values of conditions with 25 mM B concentration indicate a decrease in the antenna size and could result from PSII inactivation and excitation energy transfer from inactive PSII to active PSII units (Figure 3.17).

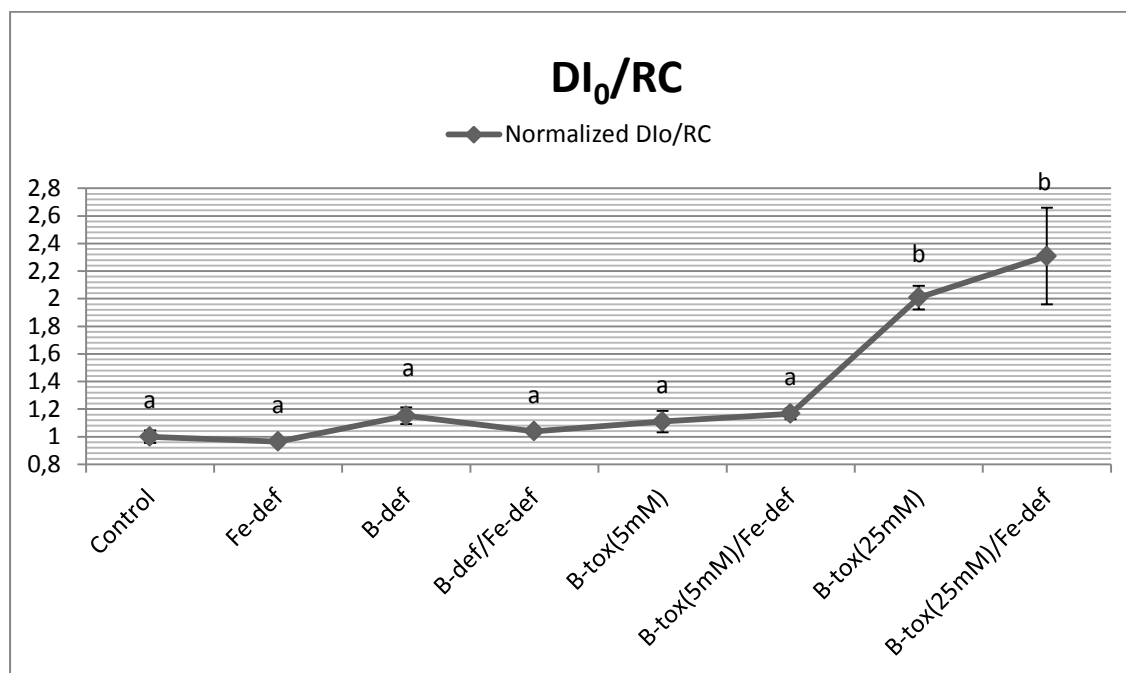


Figure 3.18 The normalized DI_0/RC values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

Absorbed energy may be lost by heat and fluorescence emission and energy transfer to systems other than electron transport, which refers to dissipation. The equation, $DI_0/RC = (ABS/RC) - (TR_0/RC)$, represents the dissipation per RC, the ratio of the total dissipation of untrapped excitation energy from all RCs to the number of active RCs [225]. In the present study, a significant increase of DI_0/RC meaning an activation of dissipation process was observed under high toxic (25 mM) B concentrations (Figure 3.18). It reflects the loss of connectivity where the trapped energy does not go to RC and is dissipated. When the excitation energy in the antenna of the RCs is in excess of required for trapping, excess energy is probably dissipated as heat. The reductions in F_v/F_m and RE_0/ET_0 were correlated with an increase in DI_0/RC suggesting that B treatments induced the dissipation of damaging excess energy.

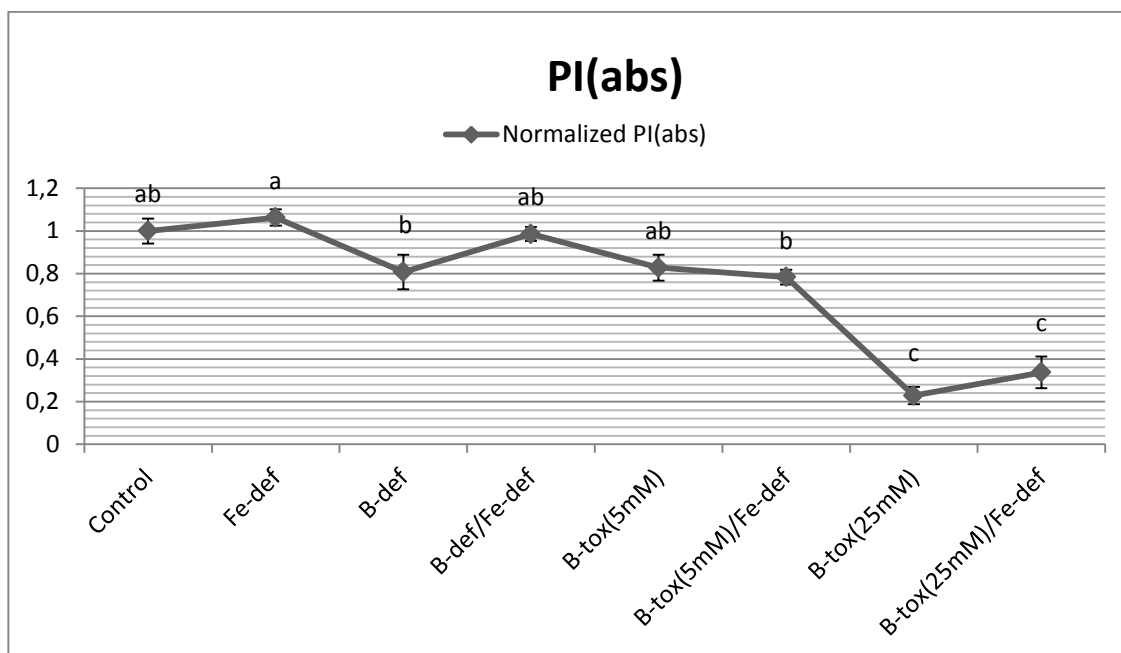


Figure 3.19 The normalized PI(abs) values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

The performance index (PI) can be a suitable parameter for evaluating a large number of genotypes for stress tolerance [235]. In the present study, the effect of given stress conditions on photosynthetic activity was quantified by the values for PI(abs), the overall expression for PSII activity and PI(total), the overall expression of both PSII and PSI. The PI expresses the three functional steps (energy absorption, energy trapping, and energy conversion into the electron transport). The PI(total), measuring the performance from PSII to the PSI end electron acceptors, is the combination of all of the aforementioned parameters [235]. Thus, the overall change in OJIP parameters can be deducted in the expression of PI(total). The effect of stress treatments on PI(abs) and PI(total) values was found to be insignificant except for 25 mM B containing conditions (Figure 3.19 and Figure 3.20). In addition, such a similarity between PI(abs) and PI(total) values indicates that the main damage is on PSII rather than PSI activity.

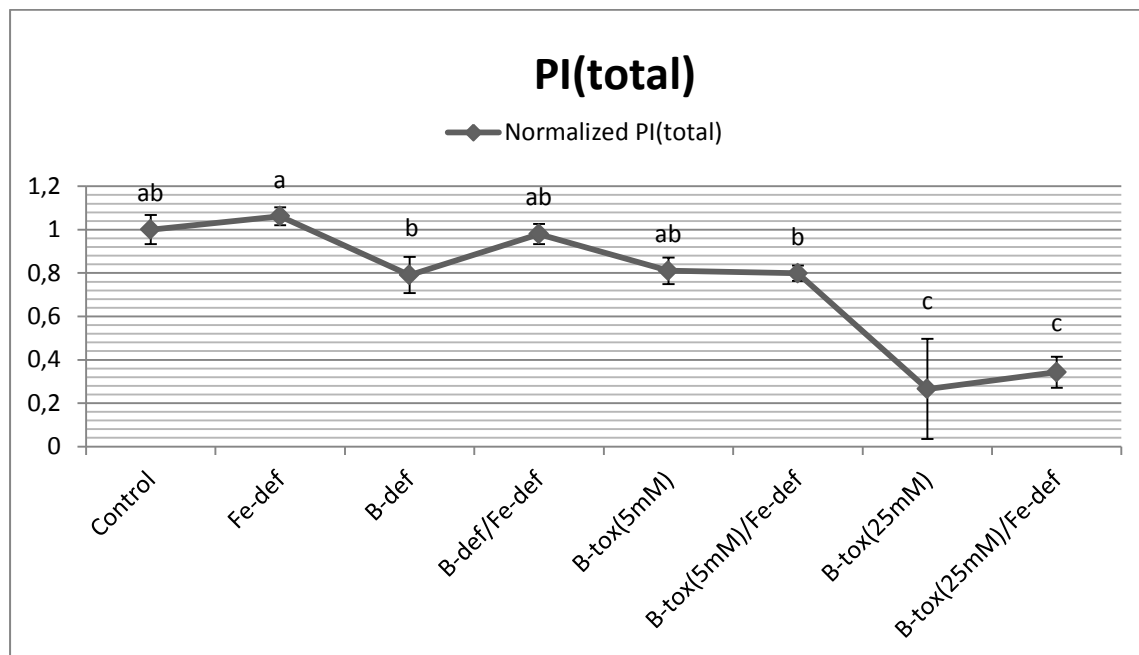


Figure 3.20 The normalized PI(total) values shoot tissues of barley cultivar Tarm-92 exposed to given stress conditions after 5 day. Each value represents the mean $\pm$ SEM (treatment/control) of eight independent replicates. Values that do not share a letter are significantly ($P < 0.05$) different from each other according to the *Tukey's* test in conjunction with ANOVA

The overall evaluation of photosynthetic activity by JIP test revealed that photosynthetic performance of Tarm-92 cultivar was affected significantly only by the 25 mM B treatment conditions. As a B tolerant cultivar, it can sustain its photosynthetic activity even in 5 mM B treatments. In addition, although slight decreases in PSI activity were observed under Fe deficiency, they are not significant to decrease its overall photosynthetic activity.

3.4 Gene Expression Analysis

Previous studies have reported that 5 d of B deficiency results in decrease between 15 to 35 fold in expression of NAS, NAAT, and IDS3 genes at mRNA level [207] (Table 1.1), and Fe deficiency results in increase of IDS2 and IDS3 proteins in B tolerant barley [176]. Expression analyses of NAS and IDS3 genes were performed in order to predict phytosiderophore production rate under given stress conditions. For this

purpose, *HvNAS* and *HvIDS3* gene expression profiles were monitored under Fe-deficiency, B-deficiency, B-toxicity (5mM), Fe-deficiency/ B deficiency, and Fe-deficiency/B-toxicity at 0th, 1st, and 5th days of stress application. Analysis of data was done according to relative quantification method as explained in section 2.2.7.

3.4.1 RNA Integrity

Integrity of RNA is the main factor affecting the results of Q-PCR. The integrity of RNA was checked by separation of rRNA species using agarose gel electrophoresis (Figure 3.21). Discrete bands of rRNA species, with a little smearing between bands in electrophoresis demonstrated integrity of RNA samples.

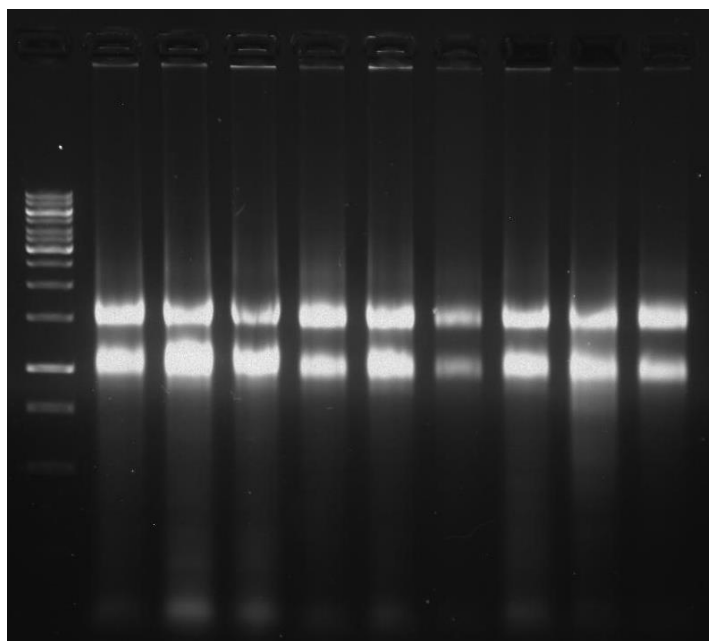


Figure 3.21 Electrophoretic separation of representative total RNA samples isolated from root tissues used for Q-PCR in agarose gel

3.4.2 Conventional PCR

Before beginning Q-PCR, conventional PCR was performed with the primer pairs of *IDS3*, *NAS*, and *GAPDH* and pool cDNA. Three different annealing temperatures (50°C - 53°C - 55°C) were tried in order to optimize the annealing temperature for Q-

PCR, and 55°C was selected as optimal (Figure 3.22). Because primer dimers were seen in the gel, primer concentration for all three genes was decreased in half when Q-PCRs were performed. Other optimization attempts like changing concentration of reaction mixture components other than the primers were not tried, since the Q-PCR mixture has one fixed reaction mixture including all reagents except primers. Thus, considering the fact that amplification of desired products is achieved, it was decided to continue Q-PCR with only decreased primer concentrations.

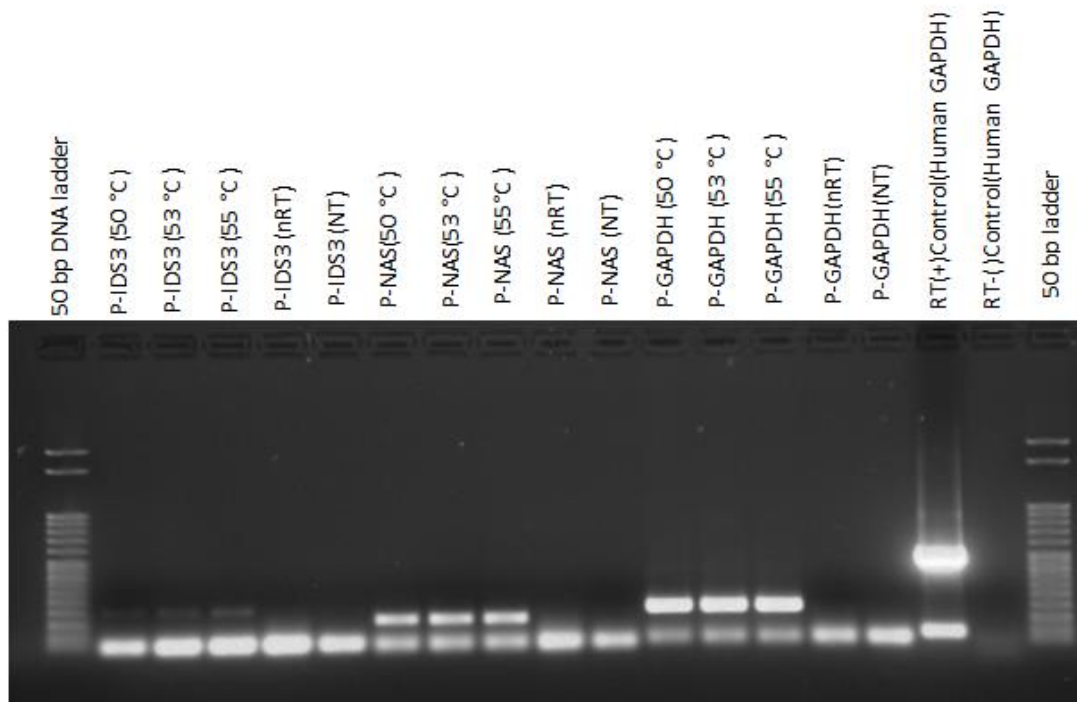


Figure 3.22 Conventional PCR results done by primers designed for *HVIDS3* (195 bp), *HvNAS* (151 bp), and *HvGAPDH* (198 bp). No template (NT) and no reverse transcription (nRT) controls were included. As a control for efficiency of RT reaction, Human GAPDH RNA and primers (496 bp) provided by the manufacturer were also used.

3.4.3 Q-PCR

After conventional PCR optimization, a brief optimization labor with Q-PCR kit was took place. Three different primer concentrations (0.075, 0.15, and 0.3 μ M) were

tried at 55°C annealing for 40 cycles. Because SYBR green detection is based on the ability of SYBR green to bind double stranded DNA including primer dimers, contaminating DNA, and unspecific PCR products, melting curve was constructed to confirm only desired amplicon is detected following every Q-PCR. Agarose gel electrophoresis results and the melt curves are given in Figure 3.23. According to these results; it was decided to use 0.15 μ M primers.

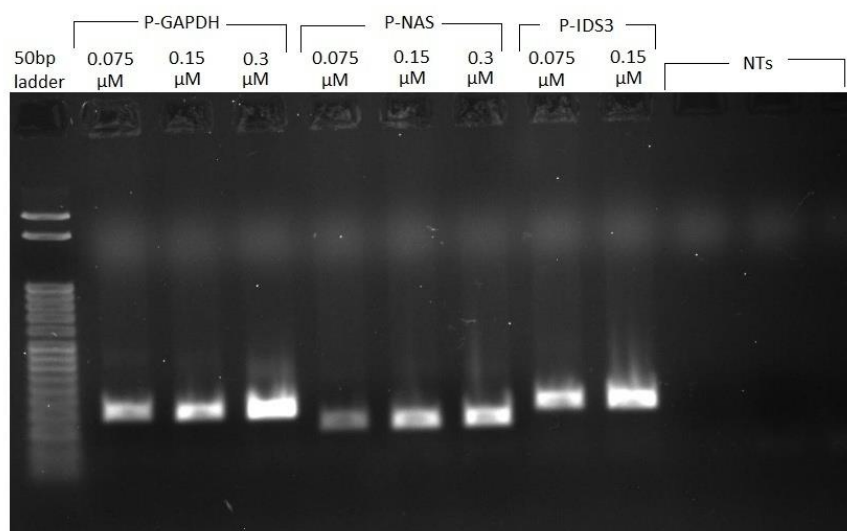


Figure 3.23 Agarose gel of PCR products shows that only the desired amplicons were obtained

3.4.3.1 Standard Curve Construction

For standard curve construction of target genes (NAS and IDS3) and control gene (GAPDH), all of the isolated RNA samples were pooled into a single tube. A dilution series from the cDNA pool were amplified in Q-PCR and the Ct values for each dilution and each gene were obtained (Table 3.1) Standard curves were constructed for all three genes by using Ct values obtained (Figure 3.25).

Melt curve analysis of GAPDH and NAS (Figure 3.26a,c) showed only narrow peaks indicating amplification of only the desired amplicons. However, IDS3 melt curve analysis (Figure 3.26b) showed wider peaks. As indicated earlier in conventional PCR results (Figure 3.22), the peaks were probably indication of primer dimers. The peaks

become higher when the concentration of the cDNA added into reaction decreased (Figure 3.26). Therefore, primers bind each other if there is not enough cDNA to bind, confirming the formation of primer dimers.

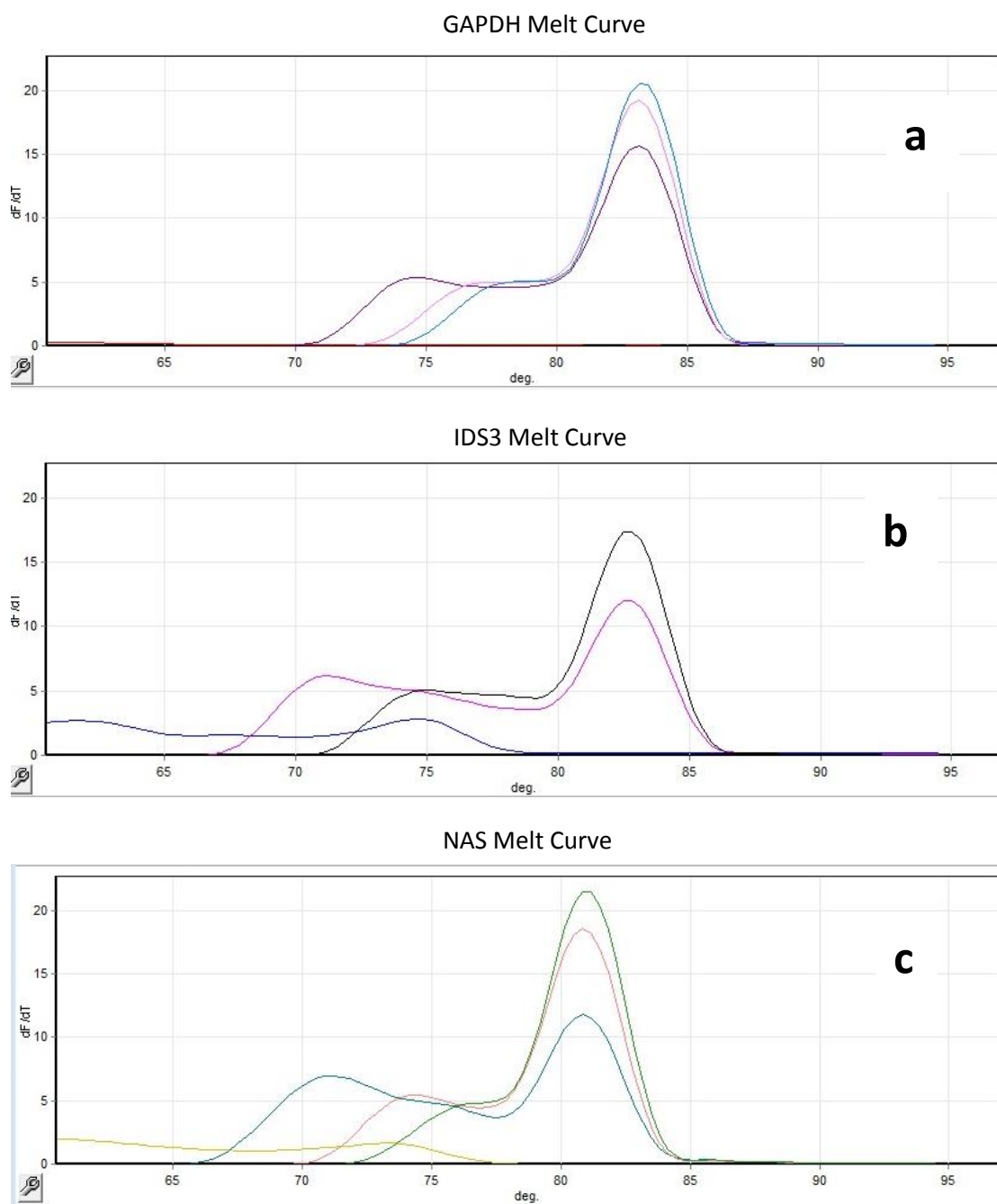


Figure 3.24 Optimization of primer concentrations in Q-PCR. Melt curve of GAPDH (a), IDS3 (b), NAS (c); the curves show 0.3, 0.15, 0.075 μM primer concentrations respectively from up to down

Table 3.1 The Ct values of standards

Log ng total RNA	Ct GAPDH	Ct NAS	Ct IDS3	Δ Ct NAS	Δ Ct IDS3
4	12.65	16.06	20.14	3.41	7.49
4	12.66	16.35	19.83	3.69	7.17
4	12.51	16.34	20.2	3.83	7.69
3	15.76	20.94	22.24	5.18	6.48
3	15.89	20.88	22.51	4.99	6.62
3	15.83	20.63	22.16	4.8	6.33
2	18.79	22.68	24.55	3.89	5.76
2	18.96	23.02	24.93	4.06	5.97
2	19.05	23.75	25.31	4.7	6.26
1	21.84	25.42	28.31	3.58	6.47
1	22.31	25.76	28.29	3.45	5.98
1	22.08	26.4	28	4.32	5.92
0	25.07	28.24	29.82	3.17	4.75
0	25.22	29.99	28.69	4.77	3.47
0	25.43	29.21	30	3.78	4.57

Table 3.2 Correlation Statistics of Standard Curves

	GAPDH	IDS3	NAS
R	0.99960	0.98973	0.98903
R ²	0.99919	0.97957	0.97819
M	-3.152	-2.479	-3.084
B	25.242	29.955	29.213
E	1.08	1.53	1.11

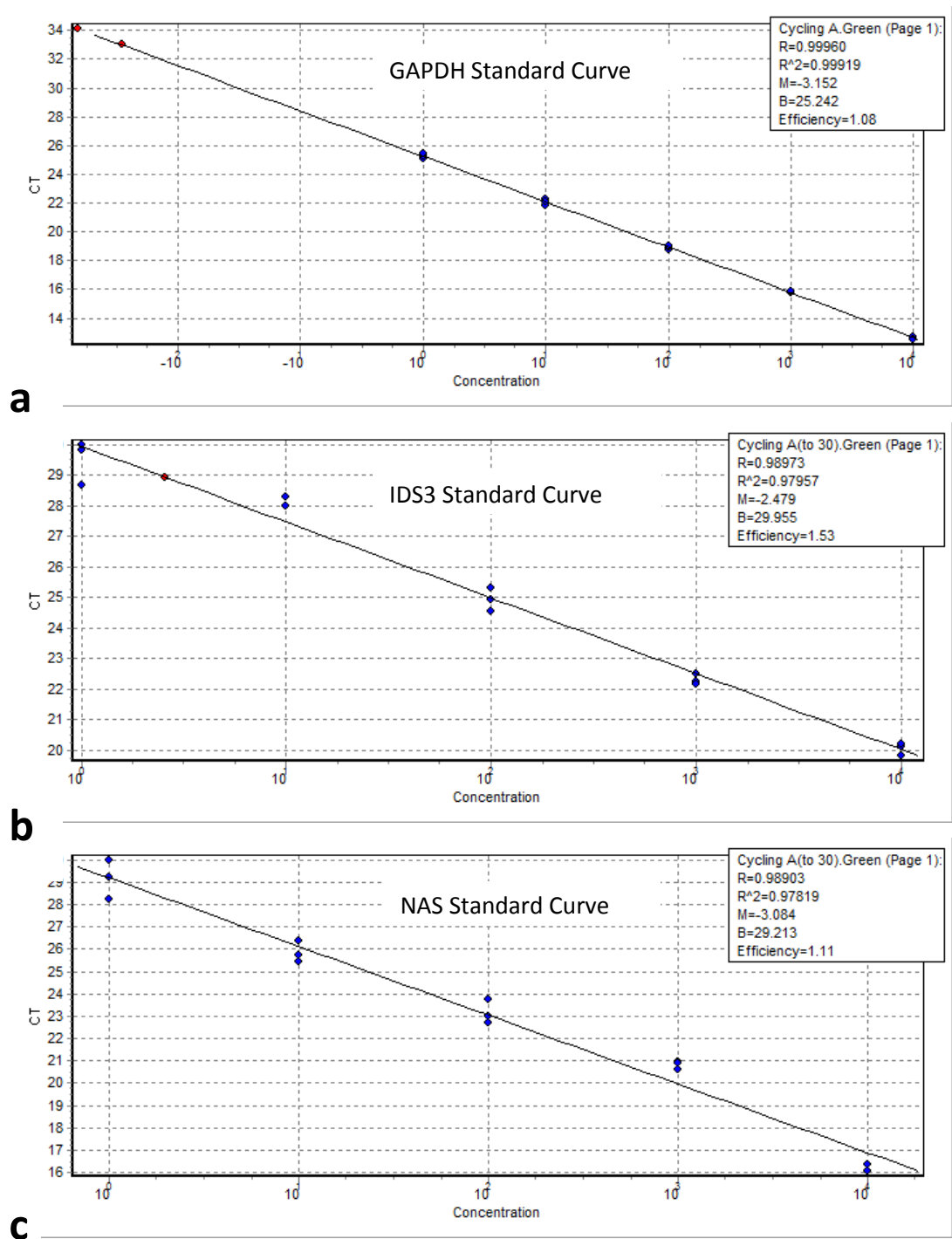


Figure 3.25 Standard curve of GAPDH(a), IDS3 (b), and NAS(c)

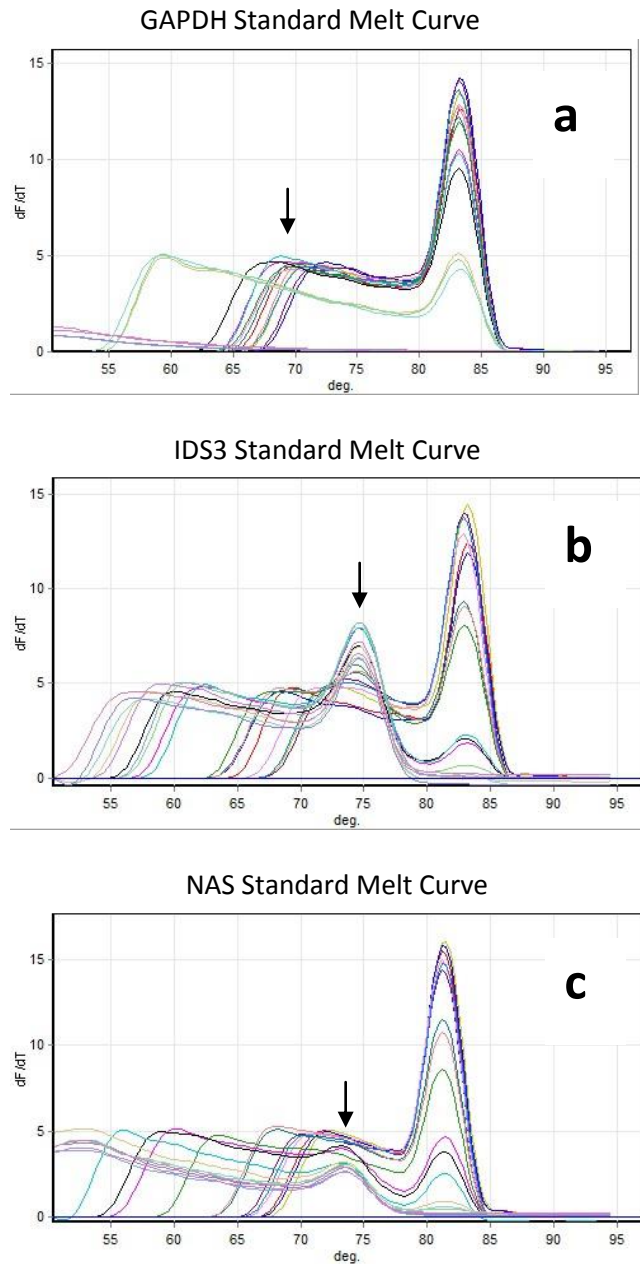


Figure 3.26 Melt curve of standards; GAPDH (a), IDS3 (b), and NAS(c); arrows show primer dimers

3.4.3.2 Calculation and Comparison of Amplification Efficiencies

Amplification efficiencies were calculated in order to decide which relative quantification method will be used. For this purpose, first the amplification efficiencies (E) for each gene were calculated as indicated in the section 2.2.7. Then, efficiency

comparison was done by plotting the ΔC_t values against log template amount. Slope of the line was used to determine the difference in PCR efficiency (Figure 3.27). Amplification efficiencies were accepted as comparable, if the slope is <0.1 . According to this method, the amplification efficiencies of GAPDH and NAS were comparable, while they were different for GAPDH and IDS3.

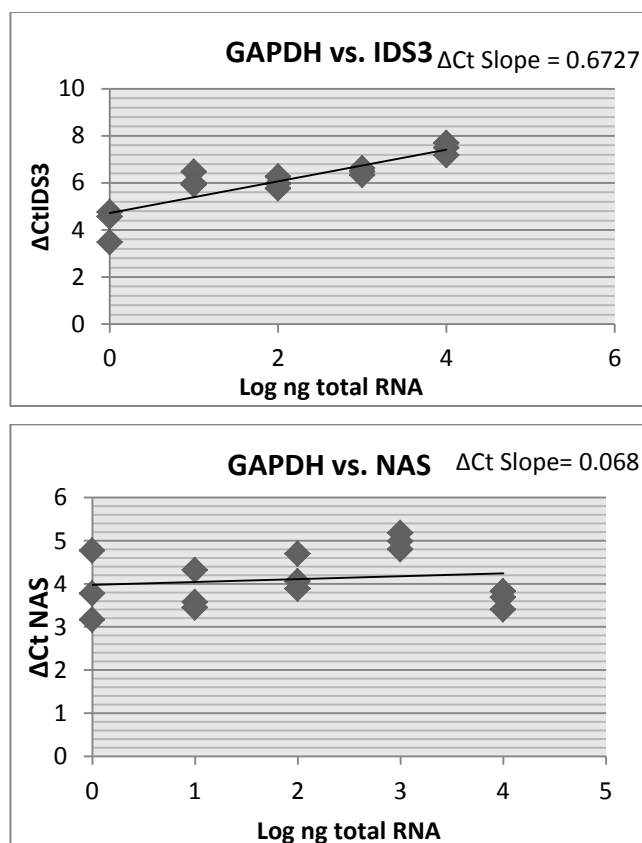


Figure 3.27 Efficiency comparison GAPDH vs. IDS3 and GAPDH vs. NAS

3.4.3.3 IDS3 and NAS Expression Level Changes

Different amplification efficiencies of the target gene and the endogenous reference gene are usually seen because efficiency of primer annealing, GC-content of the sequences to be amplified, and PCR product size of two genes are usually quite

different. This is the case for IDS3 and GAPDH amplification efficiencies. Therefore, standard curves were prepared for the IDS3 and GAPDH separately.

After calculation of amount of IDS3 and GAPDH in the samples by using their Ct values and the corresponding standard curves (Appendix D), the average amount of IDS3 was divided by the average amount of GAPDH to calculate the normalized amount of IDS3 in samples. Then, the relative expression level of IDS3 was calculated by dividing the normalized amounts of IDS3 by the value of the assigned calibrators which are control in Day-0, Day-1, and Day-5. Calculated fold changes according to corresponding calibrator samples were given in Appendix D.

For NAS, amplification efficiencies were comparable with a slope of 0.068 (Figure 3.27). $\Delta\Delta\text{Ct}$ method, an analysis for comparable amplification efficiencies, uses an approach in which ΔCt value of each sample was calculated by subtracting GAPDH Ct value from NAS Ct value. Three calibrator samples (control of Day-0, Day-1, and Day-5) were defined and the $\Delta\Delta\text{Ct}$ value was calculated by subtracting the calibrator ΔCt from the sample ΔCt . Then, the normalized level of NAS expression was calculated by using the formula $2^{-\Delta\Delta\text{Ct}}$.

The expectation was down-regulation of IDS3 and NAS under B-deficiency up-regulation under Fe-deficiency when the previously mentioned studies were considered [176], [207]. Set-2 and Set-3 samples gave the similar results almost in all conditions and time scales. However, the results of Set-1 have not internal consistency and are also quite incompatible with other sets (Appendix D). The different and incompatible results of Set-1 may be contamination of samples during RNA isolation or even in growing stage. RNA isolation of samples was performed for every set at different times, so the difference may be explained by that. In addition, a contamination in growing solution might be the answer, because sets were grown at different times with independently prepared growing solutions. Any contamination that was seen in Set-1 growing solution would not affect the other two sets. Aside from the incompatible results of Set-1, the results of other two seem compatible. Therefore, further comments will be on the average results of Set-2 and Set-3 only.

3.4.3.3.1 Fold changes of IDS3 and NAS in Day-1

Fe-deficiency: As expected, up-regulation of IDS3 and NAS is observable after 1d of Fe deficiency. When the calibrator sample was assigned as control in Day-0, fold changes of IDS3 in Day-1 are 16.21 and 4.87 for Set-2 and Set 3 respectively with an average value of 10.54 (Table 3.3 and Appendix D). Results resemble each other even more when control sample of Day-1 was assigned as calibrator. In that case, the fold changes are 16.87 and 18.02, 17.45 in average. The same goes for NAS expression levels. The fold changes are 9.03-6.08 (av.7.56) according to Day-0 and 9.78-10.93 (av. 10.35) according to Day-1 calibrator.

B-deficiency: The expected results for samples under B deficiency were not compatible with the obtained ones. Up-regulation of IDS3 and NAS with similar results is clear for both calibrators. However, it should be noted that mRNA was obtained after 5 d of stress treatment in the previous study [207]

Fe-deficiency/B-deficiency: The analysis of barley seedlings under Fe-deficiency accompanied with B-deficiency was done in order to observe combined effects of two stress conditions on phyto siderophore producing genes. The results obtained from Fe-deficiency and B-deficiency alone supported to expect even a higher number in fold change. The fold changes for IDS3 were 24.78 and 15.23 (av. 20.01) for calibrator Day-0, and 25.79-56.30 (av.41.04) for Set-2 and Set 3 respectively. For NAS, the numbers are 9.29-17.94 (av.13.61) according to Day-0 and 10.06-32.22 (av. 21.14) according to Day-1 calibrator.

B-toxicity: There is no data in literature on expression levels of phyto siderophore producing genes under B-toxicity to best of our knowledge. The results show up-regulation of IDS3 and NAS with average values of 2.42-4.77 for IDS3 and 5.32-7.48 for NAS according to calibrator Day-0 and Day-1 respectively.

Fe-deficiency/ B-toxicity: Up-regulations of IDS3 and NAS were also observed when both Fe-deficiency and B-toxicity applied to seedlings. Similar results to B-toxicity were seen with average values of fold changes 1.95 - 4.48 for IDS3 and 4.03 – 5.98 for NAS according to calibrator Day-0 and Day-1 respectively.

The results of fold changes of IDS3 and NAS in Day-1 showed that both IDS3 and NAS were up-regulated in all stress conditions given. It is also notable that the values for both genes are quite compatible (Table 3.3 and Figure 3.28). However, application of B-toxicity accompanied with Fe-deficiency was lowered the numbers compared to Fe-deficiency alone. In addition, the results of Fe-deficiency/B-toxicity grown samples were slightly lower than the B-toxicity alone. Fe- deficiency is seen if the soil is too alkaline ($\text{pH} > 6.5$). The similar case is also applied for B-toxicity ($\text{pH} > 7.5$). A common mechanism for barley to cope with B-toxicity and Fe-deficiency may evolve, since the two stress condition may occur simultaneously in nature. In addition, it was reported that Fe- deficiency results in increased uptake of Zn [176]. It is also known that B-toxicity symptoms were partially alleviated in tomato plants grown with applied Zn [210]. In the absence of applied Zn, plants were collected significantly higher amounts of B under B-toxicity. Furthermore, Zinc fertilization of B-toxic soil significantly increased grain yield of a great variety of wheat cultivars up to 40 % [209]. However, there was no significant change in Zn content of stress induced plants in our study (see section 3.2.2). Maybe, this mechanism is useful only for long term stresses or is used as a minor action to cope with stress.

Table 3.3 Average fold changes (AFC) of NAS and IDS3 according to calibrators Day-0, Day-1, and Day-5

Samples(Day)	AFC Day-0		AFC Day-1		AFC Day-5	
	IDS3	NAS	IDS3	NAS	IDS3	NAS
Control (0)	1,00	1,00	-	-	-	-
Control (1)	0,62	0,74	1,00	1,00	-	-
Fe- def (1)	10,54	7,56	17,45	10,35	-	-
B-def (1)	4,37	4,71	10,51	6,33	-	-
Fe- def / B-def (1)	20,01	13,61	41,04	21,14	-	-
B- tox (1)	2,42	5,32	4,77	7,48	-	-
Fe-def/B-tox (1)	1,95	4,03	4,48	5,98	-	-
Control (5)	95,01	16,19	-	-	1,00	1,00
Fe- def (5)	177,08	22,33	-	-	4,44	1,81
B-def (5)	69,67	17,98	-	-	3,63	1,17
Fe- def / B-def (5)	6,86	1,20	-	-	2,39	0,09
B- tox (5)	2,56	3,37	-	-	1,26	0,23
Fe-def/b-tox (5)	3,22	5,84	-	-	0,03	0,83

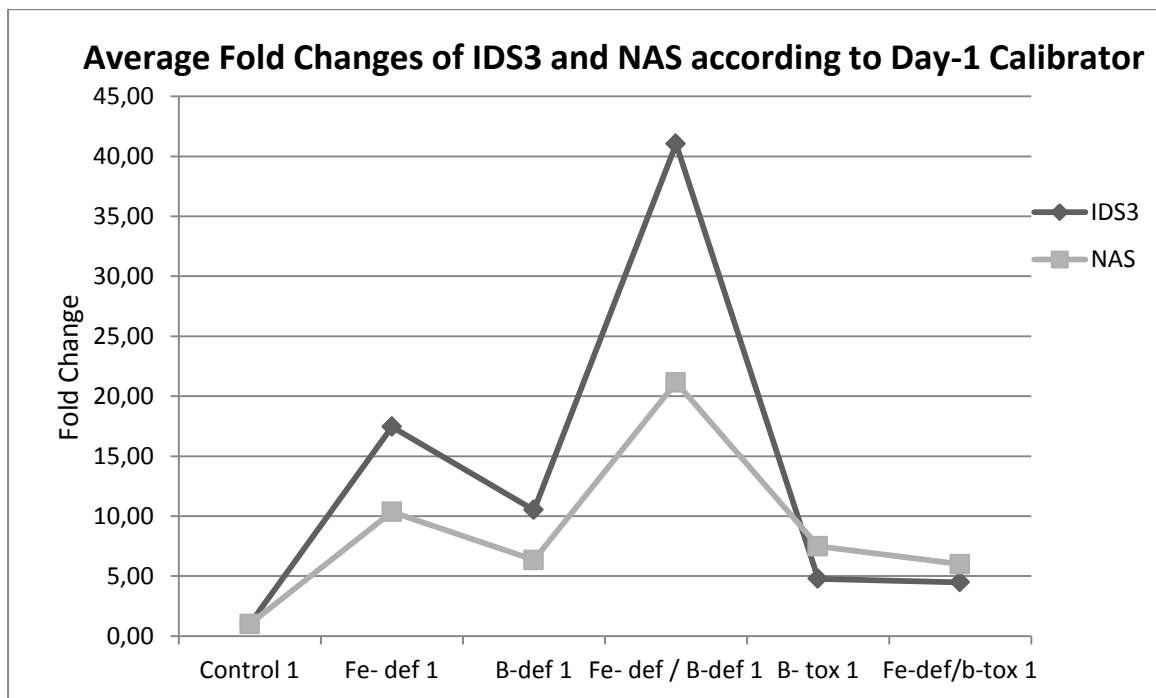
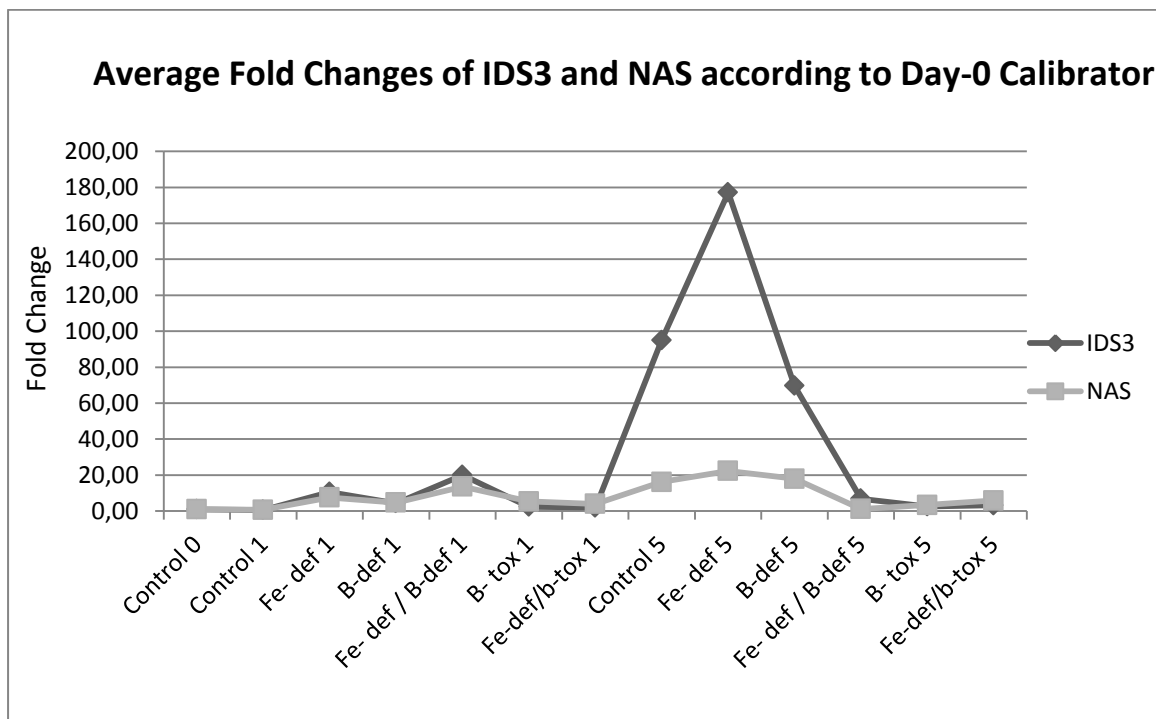


Figure 3.28 Average fold changes (AFC) of NAS & IDS3 according to calibrators Day-0 & Day-1

3.4.3.3.2 Fold changes of IDS3 and NAS in Day-5

Unlike the results of Day-1, results of Set-2 and Set-3 samples were quite different from each other when calibrator sample was defined as control of Day-0. However, when control of Day-5 was designated as calibrator, the results resemble each other (Figure 3.29 and Table 3.3). Since the ratio between 5th day's control sample and stress induced sample is used in calculation of fold change according to calibrator Day-5, it was not observed in that calculation.

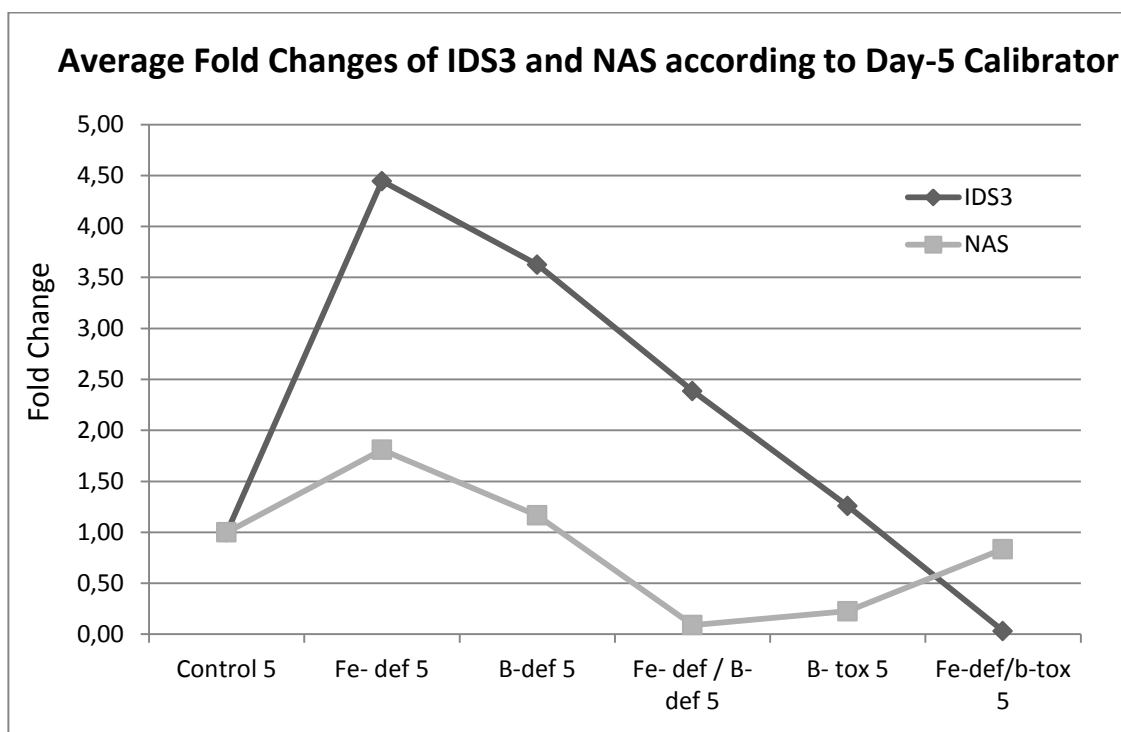


Figure 3.29 Average fold changes (AFC) of NAS & IDS3 according to calibrator Day-5

Fe-deficiency: As in Day-1, up-regulation of IDS3 and NAS is still observable in Day-5. However, as mentioned earlier, the IDS3 values for Set-2 and Set-3, which are 12.23-341.93 respectively, are quite different from each other according to Day-0 calibrator. Similar situation is seen for NAS of which values are 6.87-37.79 respectively

for Day-0 calibrator. The average results according to calibrator control of Day-5 are 4.44-1.81 for IDS3 and NAS respectively.

B-deficiency: Again, up-regulation of IDS3 and NAS seen in Day-1 continues for both calibrators.

Fe-deficiency/B-deficiency: Unlike Day-1, the values for both genes are smaller than the values of samples which are treated with only one stress. So much that, NAS seems down-regulated in both Set-2 and Set-3 when the calibrator sample is assigned as control of Day-5. In addition, IDS3 expression decreases dramatically in Set-3. Use of average values for this situation may be quite confusing. Average value of 4.74 and 0.03 is 2.39 and still suggests up-regulation of gene; however, the value of 0.03 means that the expression decreased about 30 fold. Therefore, average values may not reflect the actual situation because of the calculation method.

B-toxicity: Similar situation with Fe-deficiency/B-deficiency is also observed for B-toxicity. Down-regulation of NAS for both Set-2 and Set3, and IDS3 for only Set-3 were observed. However, the IDS3 value of Set-2's sample still suggests it is up-regulated. Again, the average values are useless when the mathematical method behind it is considered.

Fe-deficiency/ B-toxicity: The results show inconsistency like B-toxicity and Fe-deficiency/B-deficiency. However, the values of IDS3 according to calibrator Day-5 significantly decreased as an indication of down-regulation about 30 fold.

The evaluation of the gene expression analyses of NAS and IDS3 showed the increase is higher in Day 1 and following a similar pattern for both of the genes. However, this is not applicable in Day 5. The first response of seedlings after detection of stress conditions may be increase in expression of corresponding genes to collect the remaining Fe molecules, so more regular increase in day 1 expression can be explained. However, the mission of the phytosiderophores may be ceased after 5 day, and especially first enzyme in the biosynthesis of phytosiderophores, NAS did not continue to increase as in day 1.

CHAPTER 4

CONCLUSION

B level in toxic concentrations is a big obstacle for agricultural production in arid and semi-arid regions in the world including Turkey, California, Chile, and South Australia. The co-existence of B-toxicity and Fe- deficiency because of the soil pH and water content requires co-evaluation of those conditions. Recent studies revealed that there might be a relationship between B content of barley and phytosiderophores responsible for Fe uptake in grasses both in mRNA and protein levels, confirming the co-evaluation of those stress conditions are necessary. The relation between B and Fe might lead us not only understanding the B tolerance mechanisms of grasses, but also potential use of B fertilizers in abnormal Fe conditions in soil. In this study, we investigated the potential link between iron, boron, and phytosiderophore production in barley.

For this purpose the B-tolerant barley cultivar Tarm-92 seedlings were treated with deficiency or toxicity of boron and iron as well as combinations of these and expression levels of two of the phytosiderophore hydroxyl mugineic acid (HMA) producing genes namely NAS and IDS3 were examined. In addition to gene expression analysis, physiological parameters, total B, Fe and Zn content and photosynthetic performance of seedlings were considered.

Changes in physiological parameters were detected in all of the given stress conditions. Especially, the conditions with 25 mM boric acid treatment disrupted the growth of seedling in every aspect, where Fe-deficiency did not significantly affect the growth when applied with other stress conditions. In addition, as a B-tolerant barley cultivar, Tarm-92 showed fairly a good growth performance under 5 mM B treatment.

Further, B deficiency did not result in significant decrease in any of the growth parameters, except root fresh weight.

Element analyses revealed that toxic levels of B resulted in significant increase in B-uptake for both 1 day and 5 day after stress induction. However, other stress conditions did not affect the B status of plants. In addition, Fe-deficiency did not result in significant increase of B accumulation after 120 h of stress application according to our data, conflicting with previous studies. It was also not affected B-content when applied with B-toxicity. The evaluations of Fe and Zn content also conflict with the previous studies. Our results did not show any significant difference for both Zn and Fe content in any of the induced stress conditions.

The photosynthetic activity evaluation by JIP test revealed that photosynthetic performance of Tarm-92 cultivar was affected significantly only by the 25 mM B treatment conditions. As a B tolerant cultivar, it can sustain its photosynthetic activity even in 5 mM B treatments. In addition, although slight decreases in PSI activity were observed under Fe deficiency, they are not significant to decrease its overall photosynthetic activity.

The gene expression analyses of IDS3 and NAS in Day-1 showed that both IDS3 and NAS were up-regulated in all stress conditions given. It is also notable that the values for both genes are quite compatible. However, application of B-toxicity accompanied with Fe-deficiency was lowered the numbers compared to Fe-deficiency alone. In addition, the results of Fe-deficiency/B-toxicity grown samples were slightly lower than the B-toxicity alone. Moreover, increase of NAS and IDS3 expression were higher in Day 1 than the Day 5 and followed a similar pattern for both of the genes. Although changes in expression levels of Day 5 were present, they were not regular.

The overall evaluation of physiological parameters, element analyses, photosynthetic activity, and gene expression confirmed that Tarm-92 is a B-tolerant cultivar. Although deadly concentration of B (25 mM) caused significant reduce in growth and photosynthetic activity of the cultivar, its responses against high B concentration (5 mM) for especially physiological parameters and photosynthetic

activity were quite remarkable. In addition, other stress conditions, Fe deficiency and B deficiency did not affect the cultivar at all, showing strength of its response mechanisms against those stresses.

Future studies may include testing other B-tolerant barley cultivars and/or species against Fe deficiency. In addition, investigations of B-sensitive cultivars and/or species for similar parameters are also necessary to understand the differences. For gene expression analyses, a narrower time scale, not in days but in hours (e.g. 1h, 6h, and 12h) can be used to determine changes at earlier stages of stress conditions. Further, the analyses of related miRNAs may give important information about the process. In the case of phytosiderophore production determination, direct detection of phytosiderophores will be more useful to understand the response and its correlation with corresponding gene expression. Changing the stress conditions and concentrations of minerals is also an option. For instance, application of one day of Fe deficiency followed by one day of Fe toxicity to 9d old seedlings may help to detect element content changes easily. Finally, including Zn in the system as Zn deficiency and/or Zn toxicity is also important to understand the effects of Zn on the relation with B and phytosiderophore production.

Although further investigations to understand the relation between Fe, B and phytosiderophore production are needed, the results of this thesis confirmed: (i) Tarm-92 is B-tolerant both in terms of physiological parameters and photosynthetic performance; (ii) Tarm-92 is also tolerant to B and Fe deficiency; (iii) up-regulation of PS genes under Fe deficiency occurs; and (iv) tolerance to Fe deficiency may be an indication of common response mechanism against B toxicity and Fe deficiency.

REFERENCES

- [1] D. Zohary and M. Hopf, *The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley*. Oxford, England: Clarendon Press, 1993.
- [2] S. Abbo, S. Lev-Yadun, and A. Gopher, "Agricultural origins: Centers and noncenters; a Near Eastern reappraisal," *Crit. Rev. Plant Sci.*, vol. 29, pp. 317–328, 2010.
- [3] GRAMENE, "Cereal Statistics," *Gramene Species*, 2005. [Online]. Available: <http://archive.gramene.org/species/cereals.html>. [Accessed: 12-Dec-2014].
- [4] B. . Baik and S. Ulrich, "Barley for food: Characteristics, improvement, and renewed interest.," *J. Cereal Sci*, vol. 48, pp. 233–242, 2008.
- [5] A. MacGregor, "Barley," in *Encyclopaedia of Food Science, Food Technology, and Nutrition*, R. Macrae, R. Robinson, and M. Sadler, Eds. New York: Academic Press, 1993, p. 308.
- [6] FAOSTAT, "Barley Statistics," *Food and Agriculture Organization of The United Nations (FAO) Statistical Databases*, 2015. [Online]. Available: <http://faostat.fao.org/>. [Accessed: 12-Dec-2014].
- [7] M. Koornneef, C. Alonso-Blanco, and A. Peeters, "Genetic approaches in plant physiology," *New Phytol.*, vol. 137, pp. 1–8, 1997.
- [8] K. F. Mayer, R. Waugh, P. Langridge, T. J. Close, R. P. Wise, A. Graner, T. Matsumoto, and K. Sato, "A physical, genetic and functional sequence assembly of the barley genome," *Nature*, 2012.
- [9] NCBI, "Mapview," *barley genome*, 2012. [Online]. Available: http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?chr=barley.inf. [Accessed: 12-Dec-2014].
- [10] O. Maloy and I. Debra, "Powdery Mildew," *Diseases of Washington Crops*, 1993. [Online]. Available: [http://pnw-ag.wsu.edu/smallgrains/Powdery Mildew.html](http://pnw-ag.wsu.edu/smallgrains/Powdery_Mildew.html). [Accessed: 12-Dec-2014].

- [11] J. . Davis, "Notes on parasitic fungi in Wisconsin," in *VIII. Transactions of the Wisconsin Academy of Science*, 1922, pp. 413–431.
- [12] T. Murray and D. Parry, *Diseases of Small Grain Cereal Crops*. Manson Publishing, 2008, p. 132.
- [13] G. Bai and G. Shaner, "Management and resistance in wheat and barley to Fusarium head blight," *Annu. Rev. Phytopathol.*, vol. 42, pp. 135–161, 2004.
- [14] D. Jones and B. Clifford, *Cereal diseases, their pathology and control*. John Wiley & Sons Ltd, 1983.
- [15] W. Huth, D. E. Lesemann, and H. . Paul, "Barley yellow mosaic virus purification, electron microscopy, serology, and other properties of 2 types of the virus," *Phytopath. Z*, vol. 111, p. 37, 1984.
- [16] J.-P. Gao, D.-Y. Chao, and H.-X. Lin, "Understanding Abiotic Stress Tolerance Mechanisms: Recent Studies on Stress Response in Rice," *J. Integr. Plant Biol.*, vol. 49, no. 6, pp. 742–750, 2007.
- [17] R. Dolferus, X. Ji, and R. Richards, "Abiotic stress and control of grain number in cereals," *Plant Sci.*, vol. 181, pp. 331–341, 2011.
- [18] R. Clark, "Plant response to mineral element toxicity and deficiency," in *Breeding Plants for Less Favourable Environments*, M. Christiansen and C. Lewis, Eds. New York: John Wiley & Sons, 1982, pp. 71–142.
- [19] J. P. Lynch and S. B. St.Clair, "Mineral stress: the missing link in understanding how global climate change will affect plants in real world soils," *F. Crop. Res.*, vol. 90, pp. 101–115, 2004.
- [20] J. Marten and D. Westermann, "Fertilizer applications for correcting micronutrient deficiencies," in *Micronutrients in Agriculture*, 2nd ed., J. Mordtvedt, F. Cox, L. Shuman, and R. Welch, Eds. 1991, pp. 549–592.
- [21] K. Ambak and T. Tadano, "Effect of micronutrient application on the growth and occurrence of sterility in barley and rice in a Malaysian deep peat soil," *Soil Sci. Plant Nutr*, vol. 37, pp. 715–724, 1991.
- [22] S. Jamjod and B. Rerkasem, "Genotypic variation in response of barley to boron deficiency," *Plant Soil*, vol. 215, pp. 65–72, 1999.

- [23] K. Mengel and E. Kirkby, *Principles of Plant Nutrition*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 2001, pp. 621 – 638.
- [24] B. Torun, M. Kalayci, L. Ozturk, A. Torun, M. Aydin, and I. Cakmak, “Differences in shoot boron concentrations, leaf symptoms, and yield of Turkish barley cultivars grown on boron-toxic soil in field,” *J. Plant Nutr.*, vol. 26, pp. 1735 – 1747, 2003.
- [25] H. Marschner, “Role of root growth, arbuscular mycorrhiza and root exudates for the efficiency in nutrient acquisition,” *F. Crop. Res.*, vol. 56, pp. 203–207, 1998.
- [26] C. Curie, Z. Panaviene, C. Loulergue, S. L. Dellaporta, J. F. Briat, and E. L. Walker, “Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake,” *Nature*, vol. 409, pp. 250–253, 2001.
- [27] S. Fraire-Velázquez, R. Rodríguez-Guerra, and L. Sánchez-Calderón, “Abiotic and Biotic Stress Response Crosstalk in Plants-Physiological, Biochemical and Genetic Perspectives,” in *InTech Open Access Company*, A. Shanker, Ed. 2011, pp. 1–26.
- [28] C. Laloi, K. Appel, and A. Danon, “A. Reactive oxygen signalling: The latest news,” *Curr. Opin. Plant Biol.*, vol. 7, pp. 323–328, 2004.
- [29] S. H. Spoel and X. Dong, “Making sense of hormone crosstalk during plant immune response,” *Cell Host Microbe*, vol. 3, pp. 348–351, 2008.
- [30] M. Fujita, Y. Fijita, Y. Noutoshi, F. Takahashi, Y. Narusaka, K. Yamaguchi-Shinozaki, and K. Shinozaki, “Crosstalk between abiotic and biotic stress responses: A current view from the points of convergence in the stress signaling networks,” *Curr. Opin. Plant Biol.*, vol. 9, pp. 436–442, 2006.
- [31] P. Wojtaszek, “Oxidative burst: An early plant response to pathogen infection,” *Biochem. J.*, vol. 322, pp. 681–692, 1997.
- [32] C. Foyer and G. Noctor, “Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses,” *Plant Cell*, vol. 17, pp. 1866–1875, 2005.
- [33] D. Zhang, C. J. Martyniuk, and V. L. Trudeau, “SANTA domain: A novel conserved protein module in Eukaryote with potential involvement in chromatin regulation,” *Bioinformatics*, vol. 22, pp. 2459–2462, 2006.

- [34] B. Wurzinger, A. Mair, B. Pfister, and M. Teige, “Cross-talk of calcium-dependent protein kinase and MAP kinase signaling,” *Plant Signal. Behav.*, vol. 6, pp. 8–12, 2011.
- [35] S. . Choudhury, P. . Panda, L. . Sahoo, and S. K. Panda, “Reactive oxygen species signaling in plants under abiotic stress,” *Plant Signal. Behav.*, vol. 8, p. e23681, 2013.
- [36] X.-J. . Xia, Y.-J. . Wang, Y.-H. . Zhou, Y. . Tao, W.-H. . Mao, K. . Shi, T. . Asami, Z. . Chen, and J.-Q. Yu, “Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber,” *Plant Physiol.*, vol. 158, pp. 1034–1045, 2012.
- [37] G. . Miller, N. . Suzuki, S. . Ciftci-Yilmaz, and R. Mittler, “Reactive oxygen species homeostasis and signalling during drought and salinity stresses,” *Plant Cell Environ.*, vol. 33, pp. 453–467, 2010.
- [38] S. H. . Spoel and G. J. Loake, “Redox-based protein modifications: The missing link in plant immune signalling,” *Curr. Opin. Plant Biol.*, vol. 14, pp. 358–364, 2011.
- [39] S. H. . Spoel, Y. . Tad, and G. J. Loake, “Post-translational protein modification as a tool for transcription reprogramming,” *New Phytol.*, vol. 186, pp. 333–339, 2010.
- [40] K. Apel and H. Hirt, “Reactive oxygen species: metabolism, oxidative stress, and signal transduction,” *Annu. Rev. Plant Biol.*, vol. 55, pp. 373–399, 2004.
- [41] M. A. Hossain and M. Fujita, “Regulatory role of components of ascorbate-glutathione (AsA-GSH) pathway in plant tolerance to oxidative stress,” in *Oxidative Stress in Plants: Causes, Consequences and Tolerance*, N. A. Anjum, S. Umar, and A. Ahmed, Eds. India: IK International Publishing House Pvt. Ltd., 2011, pp. 81–147.
- [42] D. M. Pellet, D. L. Grunes, and L. V. Kochian, “Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.),” *Planta.*, vol. 196, pp. 788–795, 1995.
- [43] E. Delhaize, P. R. Ryan, and P. J. Randall, “Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices,” *Plant Physiol.*, vol. 103, pp. 695–702, 1993.

- [44] J. W. Huang, D. M. Pellet, L. A. Papernik, and L. V. Kochian, "Aluminum interactions with voltage-dependent calcium transport in plasma membrane vesicles isolated from roots of aluminum-sensitive and -resistant wheat cultivars.," *Plant Physiol.*, vol. 110, pp. 561–569, 1996.
- [45] S. C. Miyasaka, J. G. Buta, R. K. Howell, and C. D. Foy, "Mechanism of aluminum tolerance in snapbeans. Root exudation of citric acid.," *Plant Physiol.*, vol. 96, pp. 737–743, 1991.
- [46] S. J. Zheng, J. F. Ma, and H. Matsumoto, "High aluminum resistance in buckwheat. I. Al-induced specific secretion of oxalic acid from root tips.," *Plant Physiol.*, vol. 117, pp. 745–751, 1998.
- [47] J. E. Hayes and R. J. Reid, "Boron tolerance in barley is mediated by efflux of boron from the roots.," *Plant Physiol.*, vol. 136, pp. 3376–3382, 2004.
- [48] K. Miwa, J. Takano, H. Omori, M. Seki, K. Shinozaki, and T. Fujiwara, "Plants tolerant of high boron levels.," *Science (80-.)*, vol. 318, p. 1417, 2007.
- [49] C. M. Luna, C. A. Gonzalez, and V. S. Trippi, "Oxidative damage caused by an excess of copper in oat leaves.," *Plant Cell Physiol.*, vol. 35, pp. 11–15, 1994.
- [50] A. Brune, W. Urbach, and K. . Dietz, "Compartmentation and transport of zinc in barley primary leaves as basic mechanisms involved in zinc tolerance.," *Plant, Cell Environ.*, vol. 17, pp. 153–162, 1994.
- [51] R. Munns and M. Tester, "Mechanisms of salinity tolerance.," *Annu. Rev. Plant Biol.*, vol. 59, pp. 651–681, 2008.
- [52] M. D. Vazquez, C. Poschenrieder, I. Corrales, and J. Barcelo, "Change in apoplastic aluminum during the initial growth response to aluminum by roots of a tolerant maize variety.," *Plant Physiol.*, vol. 119, pp. 435–444, 1999.
- [53] R. F. M. van Steveninck, M. E. van Steveninck, D. R. Fernando, W. J. Horst, and H. Marschner, "Deposition of zinc phytate in globular bodies in roots of *Deschampsia caespitosa*; a detoxification mechanism?," *J. Plant Physiol.*, vol. 131, pp. 247–257, 1987.
- [54] R. Vogeli-Lange and G. J. Wagner, "Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Implication of a transport function for cadmium-binding peptides.," *Plant Physiol.*, vol. 92, pp. 1086–1093, 1990.

- [55] A. A. Torun, A. Yazici, H. Erdem, and I. Cakmak, "Genotypic Variation in Tolerance to Boron Toxicity in 70 Durum Wheat Genotypes," *Turk J Agric*, vol. 30, pp. 49–58, 2006.
- [56] N. J. Robinson and P. J. Jackson, "'Metallothionein-like' metal complexes in angiosperms; their structure and function.," *Physiol. Plantarum.*, vol. 67, pp. 499–506, 1986.
- [57] W. E. Rauser, "Partial purification and characterization of copper-binding protein from roots of *Agrostis gigantea* Roth.," *J. Plant Physiol.*, vol. 115, pp. 143–152, 1984.
- [58] A. Murphy and L. Taiz, "Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis* ecotypes. Correlation with copper tolerance.," *Plant Physiol.*, vol. 109, pp. 945–954, 1995.
- [59] A. Tukendorf, S. Lyszczyk, and T. Baszynski, "Copper binding proteins in spinach tolerant to excess copper.," *J. Plant Physiol.*, vol. 115, pp. 351–360, 1984.
- [60] H. Schat and M. M. A. Kalif, "Are phytochelatins involved in differential metal tolerance or do they merely reflect metal-imposed strain?," *Plant Physiol.*, vol. 99, pp. 1475–1480, 1992.
- [61] U. Galli, H. Schuepp, and C. Brunold, "Thiols in cadmium- and copper-treated maize (*Zea mays* L.).," *Planta*, vol. 198, pp. 139–143, 1996.
- [62] T. Maitani, H. Kubota, K. Sato, and T. Yamada, "The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*.," *Plant Physiol.*, vol. 110, pp. 1145–1150, 1996.
- [63] I. Kishinami and J. M. Widholm, "Characterization of Cu and Zn resistant *Nicotiana glauca* suspension cultures.," *Plant Cell Physiol.*, vol. 28, pp. 203–210, 1987.
- [64] H. Marschner, *Mineral Nutrition of Higher Plants*, 2nd ed. London: Academic Press, 1995.
- [65] V. Shorrocks, "The occurrence and correction of boron deficiency," *Plant Soil*, vol. 193, pp. 121–148, 1997.
- [66] P. Power and W. Woods, "The chemistry of boron and its speciation in plants," *Plant Soil*, vol. 193, pp. 1–13, 1997.

- [67] D. Lemarchand, J. Gaillardet, E. Lewin, and C. Allegre, "The influence of rivers on marine boron isotopes and implications for reconstructing past ocean pH," *Nature*, vol. 408, pp. 951–954, 2000.
- [68] K. Warington, "The effect of boric acid and borax on the broad bean and certain other plants," *Ann. Bot.*, vol. 37, pp. 629–672, 1923.
- [69] D. J. Pilbeam and E. A. Kirkby, "The physiological role of boron in plants," *J. Plant Nutr.*, vol. 6, no. 7, pp. 563–582, 1983.
- [70] A. Parr and B. Loughman, "Boron and membrane function in plants," in *Metals and Micronutrients: Uptake and Utilization by Plants*, D. Robb and W. Pierpoint, Eds. London: Academic Press, 1983, pp. 87–107.
- [71] T. Matoh, K. Ishigaki, K. Ohno, and J. Azuma, "Isolation and characterization of a boronpolysaccharide complex from radish roots," *Plant Cell Physiol.*, vol. 34, pp. 639–342, 1993.
- [72] M. Kobayashi, T. Matoh, and J. Azuma, "Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls," *Plant Physiol.*, vol. 110, pp. 1017–1020, 1996.
- [73] M. O'Neill, T. Ishii, P. Albersheim, and A. Darvill, "Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide," *Annu. Rev. Plant Biol.*, vol. 55, pp. 109–139, 2004.
- [74] I. Cakmak and V. Römheld, "Boron deficiency-induced impairments of cellular functions in plants," *Plant Soil*, vol. 193, pp. 71–83, 1997.
- [75] A. Pollard, A. Parr, and B. Loughman, "Boron in relation to membrane-function in higher-plants," *J. Exp. Bot.*, vol. 28, no. 105, pp. 831–841, 1977.
- [76] I. Cakmak, H. Kurz, and H. Marschner, "Short-term effects of boron, germanium and high light intensity on membrane permeability in boron deficient leaves of sunflower," *Physiol. Plant.*, vol. 95, pp. 11–18, 1995.
- [77] R. Reid, J. Hayes, A. Post, J. Stangoulis, and R. Graham, "A critical analysis of the causes of boron toxicity in plants," *Plant Cell Env.*, vol. 25, pp. 1405–1414, 2004.
- [78] J. Landers, R. Oda, and M. Schuchard, "Separation of boron-complexed diol compounds using high performance capillary electrophoresis," *Anal. Chem.*, vol. 64, pp. 2846–2851, 1992.

- [79] C. Hunt, "Boron-binding-biomolecules: a key to understanding the beneficial physiologic effects of dietary boron from prokaryotes to humans," in *Boron in Plant and Animal Nutrition*, H. Goldbach, B. Rerkasem, M. Wimmer, P. Brown, M. Thellier, and R. Bell, Eds. New York: Kluwer Academic Publishers, 2002, pp. 21–36.
- [80] X. Chen, S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. Bassler, and F. Hughson, "Structural identification of a bacterial quorum-sensing signal containing boron," *Nature*, vol. 415, pp. 545–549, 2002.
- [81] B. Dell and L. Huang, "Physiological response of plants to low boron," *Plant Soil*, vol. 193, pp. 103–120, 1997.
- [82] H. Gauch and W. Dugger, "The physiological action of boron in higher plants: review and interpretation," *Univ. Maryl. AES Techn. Bull*, vol. 80, pp. 1–43, 1954.
- [83] E. Hanson, M. Chaplin, and P. Breen, "Movement of foliar applied boron out of leaves and accumulation in flower buds and flower parts of 'Italian' prune," *HortScience*, vol. 20, pp. 747–748, 1985.
- [84] H. Rawson, "The developmental stage during which boron limitation causes sterility in wheat genotypes and the recovery of fertility," *Aust. J. Plant Physiol*, vol. 23, pp. 709–717, 1996.
- [85] B. Rerkasem and J. Loneragan, "Boron deficiency in two wheat genotypes in a warm, subtropical region," *Agron. J.*, vol. 86, pp. 887–890, 1994.
- [86] S. Agarwala, P. Sharma, C. Chatterjee, and C. Sharma, "Development and enzymatic changes during pollen development in boron deficient maize plants," *J. Plant Nutr.*, vol. 3, pp. 329–336, 1981.
- [87] P. Brown, N. Bellaloui, M. Wimmer, E. Bassil, J. Ruiz, and H. Hu, "Boron in plant biology," *Plant Biol*, vol. 4, pp. 205–223, 2002.
- [88] J. Raven, "Short- and long-distance transport of boric acid in plants," *New Phytol.*, vol. 84, pp. 231–249, 1980.
- [89] C. Dordas and P. Brown, "Permeability of boric acid across lipid bilayers and factors affecting it," *J. Membr. Biol*, vol. 124, pp. 1349–1361, 2000.
- [90] J. Stangoulis, R. Reid, P. Brown, and R. Graham, "Kinetic analysis of boron transport in *Chara*," *Planta*, vol. 213, pp. 142–146, 2001.

- [91] F. Dannel, H. Pfeffer, and V. Romheld, "Update on boron in higher plant-uptake, primary translocation and compartmentation," *Plant Biol.*, vol. 4, pp. 193–204, 2002.
- [92] C. Dordas, M. Chrispeels, and P. Brown, "Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots," *Plant Physiol.*, vol. 124, pp. 1349–1361, 2000.
- [93] J. Takano, M. Wada, U. Ludewig, G. Schaaf, N. von Wiren, and T. Fujiwara, "The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation.," *Plant Cell*, vol. 18, pp. 1498–1509, 2006.
- [94] H. Hanaoka and Fujiwara, "Channel-mediated boron transport in rice," *Plant Cell Physiol.*, vol. 48, p. 844, 2007.
- [95] K. L. Fitzpatrick and R. J. Reid, "The involvement of aquaglycoporins in transport of boron in barley roots," *Plant Cell Env.*, vol. 32, no. 10, pp. 1357–1365, 2009.
- [96] F. Dannel, H. Pfeffer, and V. Romheld, "Characterization of root boron pool, boron uptake and boron translocation in sunflower using the stable isotopes ^{10}B and ^{11}B ," *Aust. J. Plant Physiol.*, vol. 27, pp. 397–405, 2000.
- [97] Y. Nakagawa, H. Hanaoka, M. Kobayashi, K. Miyoshi, K. Miwa, Fujiwara, and T, "Cell-type specificity of the expression of Os BOR1, a rice efflux boron transporter gene, is regulated in response to boron availability for efficient boron uptake and xylem loading," *Plant Cell*, vol. 19, pp. 2624–2635, 2007.
- [98] S. Goldberg, "Reactions of boron with soils.," *Plant Soil*, vol. 193, pp. 35–48, 1997.
- [99] R. Reid, "Update on boron toxicity and tolerance in plants," in *Advances in Plant and Animal Boron Nutrition*, F. Xu, H. Goldbach, P. Brown, I R. Bel, T. Fujiwara, C. Hunt, S. Goldberg, and L. Shi, Eds. Dordrecht, The Netherlands: Springer, 2007, pp. 83–90.
- [100] M. Tanaka and T. Fujiwara, "Physiological roles and transport mechanisms of boron: perspectives from plants," *Eur. J. Physiol*, vol. 456, pp. 671–677, 2008.
- [101] M. Avci and T. Akar, "Severity and Spatial Distribution of Boron Toxicity in Barley Cultivated Areas of Central Anatolia and Transitional Zones," *Turk J Agric*, vol. 29, pp. 377–382, 2005.

- [102] B. Cartwright, B. Zarcinas, and A. Mayfield, "Toxic concentrations of boron in red-brown earth at Gladstone, South Australia," *Aust. J. Soil Res*, vol. 22, pp. 261–272, 1984.
- [103] R. Nable, G. Banuelos, and J. Paull, "Boron toxicity," *Plant Soil*, vol. 193, pp. 181–198, 1997.
- [104] H. Hu, P. Brown, and J. Labavitch, "Species variability in boron requirement is correlated with cell wall pectin," *J. Exp. Bot.*, vol. 47, pp. 227–232, 1996.
- [105] D. Blevins and K. Lukaszewski, "Boron in plant structure and function," *Annu. Rev. Plant Physiol. Mol. Biol.*, vol. 49, pp. 481–500, 1998.
- [106] E. Karabal, M. Yucel, and H. . Oktem, "Antioxidants responses of tolerant and sensitive barley cultivars to boron toxicity," *Plant Sci.*, vol. 164, pp. 925–933, 2003.
- [107] R. J. Reid and K. L. Fitzpatrick, "Influence of leaf tolerance mechanisms and rain on boron toxicity in barley and wheat," *Plant Physiol.*, vol. 151, pp. 413–420, 2009.
- [108] W. Loomis and R. Durst, "Chemistry and biology of boron," *BioFactors*, vol. 3, pp. 229–239, 1992.
- [109] S. Stavrianakou, G. Liakopoulos, and G. Karabourniotis, "Boron deficiency effects on growth, photosynthesis and relative concentrations of phenolics of *Dittrichia viscosa* (Asteraceae)," *Environ. Exp. Bot.*, vol. 56, no. 3, pp. 293–300, Jul. 2006.
- [110] S. Han, N. Tang, H.-X. Jiang, L.-T. Yang, Y. Li, and L.-S. Chen, "CO₂ assimilation, photosystem II photochemistry, carbohydrate metabolism and antioxidant system of citrus leaves in response to boron stress," *Plant Sci.*, vol. 176, no. 1, pp. 143–153, Jan. 2009.
- [111] M. T. Öz, Ö. Turan, C. Kayihan, F. Eyidoğan, Y. Ekmekçi, M. Yücel, and H. a. Öktem, "Evaluation of photosynthetic performance of wheat cultivars exposed to boron toxicity by the JIP fluorescence test," *Photosynthetica*, vol. 52, no. 4, pp. 555–563, Sep. 2014.
- [112] M. Redondo-Nieto, R. Rivilla, A. El-Hamdaoui, I. Bonilla, and L. Bolanos, "Boron deficiency affects early infection events in the pea- *Rhizobium* symbiotic interaction," *Aust. J. Plant Physiol.*, vol. 28, pp. 819–823, 2001.

- [113] J. Takano, M. Wada, U. Ludewig, G. Schaaf, N. von Wiren, and T. Fujiwara, "The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation," *Plant Cell*, vol. 18, pp. 1498–1509, 2006.
- [114] J. Camacho-Cristobal, M. Herrera-Rodriguez, V. Beato, J. Rexach, M. Navarro-Gochicoa, and J. Maldonado, "The expression of several cell wall-related genes in Arabidopsis roots is down-regulated under boron deficiency.," *Env. Exp. Bot.*, vol. 63, pp. 351–358, 2008.
- [115] M. Kobayashi, T. Mutoh, and T. Matoh, "Boron nutrition of cultured tobacco BY-2 cells. IV. Genes induced under low boron supply.," *J. Exp. Bot.*, vol. 55, pp. 1441–1443, 2004.
- [116] H. Goldbach and M. Wimmer, "Boron in plants and animals: Is there a role beyond cell-wall structure?" *J. Plant Nutr. Soil Sci.*, vol. 170, pp. 39–48, 2007.
- [117] A. Gonzalez-Fontes, J. Rexach, M. Navarro-Gochicoa, M. Herrera-Rodriguez, V. Beato, and J. Maldonado, "Is boron involved solely in structural roles in vascular plants?" *Plant Signal. Behav.*, vol. 3, pp. 24–26, 2008.
- [118] Q. Yu, R. Wingender, M. Schulz, F. Baluska, and H. Goldbach, "Short-term boron deprivation induces increased levels of cytoskeletal proteins in Arabidopsis roots.," *Plant Biol.*, vol. 3, pp. 335–340, 2001.
- [119] Q. Yu, F. Baluska, F. Jasper, D. Menzel, and H. Goldbach, "Shortterm boron deprivation enhances levels of cytoskeletal proteins in maize, but not zucchini, root apices.," *Physiol. Plant.*, vol. 117, pp. 270–278, 2003.
- [120] Q. Yu, A. Hlavacka, T. Matoh, D. Volkmann, D. Menzel, and H. Goldbach, "Short-term boron deprivation inhibits endocytosis of cell wall pectins in meristematic cells of maize and wheat root apices.," *Plant Physiol.*, vol. 130, pp. 415–421, 2002.
- [121] I. Kasajima and T. Fujiwara, "Identification of novel Arabidopsis thaliana genes which are induced by high levels of boron.," *Plant Biotech.*, vol. 24, pp. 355–360, 2007.
- [122] M. T. Oz, R. Yilmaz, F. Eyidogan, L. De Graaff, M. Yucel, and H. A. Oktem, "Microarray Analysis of Late Response to Boron Toxicity in Barley (*Hordeum vulgare* L.) Leaves," *Turk J Agric*, vol. 33, pp. 191–202, 2009.

- [123] N. Terry and J. J. Abadia, "Function of Iron in Chloroplasts," *Plant Nutr*, vol. 9, p. 609, 1986.
- [124] J. Briat, I. Fobis-Loisy, N. Grignon, S. Lobreaux, N. Pascal, G. Savino, S. Thoiron, N. von Wiren, and O. Van Wuytswinkel, "Cellular and molecular aspects of iron metabolism in plants.," *Biol Cell*, vol. 84, pp. 69–81, 1995.
- [125] J. Balk and S. Lobreaux, "Biosynthesis of iron-sulfur proteins in plants," *Trends Plant Sci*, vol. 10, p. 324, 2005.
- [126] S. . Mori, N. . Nishizawa, M. . Hayashi, M. . Chino, E. . Yoshimura, and J. Ishihara, "Why are young rice plants highly susceptible to iron deficiency?," in *Iron Nutrition and Interactions in Plants*, Y. Chen and Y. Hadar, Eds. The Netherlands: Kluwer Academic Publishers, 1991, p. 175.
- [127] R. Rose, D. McCurdy, and M. Sheahan, "Plant Mitochondria.," *Encycl. Life Sci.*, 2007.
- [128] G. Schaaf, A. Honsbein, A. Meda, S. Kirchner, D. Wipf, and N. von Wiren, "AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in Arabidopsis thaliana roots," *J. Biol. Chem*, vol. 281, no. 35, pp. 25532–25540, 2006.
- [129] M. Takahashi, Y. Terada, I. Nakai, H. Nakanishi, E. Yoshimura, S. Mori, and N. Nishizawa, "Role of nicotianamine in the intracellular delivery of metals and plant reproductive development," *Plant Cell*, vol. 15, no. 6, pp. 1263–1280, 2003.
- [130] B. Waters, H. Chu, R. Didonato, L. Roberts, R. Easley, and E. Walker, "Mutations in Arabidopsis Yellow Stripe-Like1 and Yellow Stripe-Like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds," *Plant Physiol*, vol. 141, no. 4, pp. 1446–1458, 2006.
- [131] M. Stacey, H. Osawa, A. Patel, W. Gassmann, and G. Stacey, "Expression analyses of Arabidopsis oligopeptide transporters during seed germination, vegetative growth and reproduction," *Planta*, vol. 223, no. 2, pp. 291–305, 2006.
- [132] M. L. Guerinot and Y. Yi, "Iron: nutritious, noxious, and not readily available.," *Plant Physiol*, vol. 104, pp. 815–820, 1994.
- [133] S. Santi, S. Cesco, Z. Varanini, and R. Pinton, "Two plasma membrane H⁺-ATPase genes are differentially expressed in iron-deficient cucumber plants.," *Plant Physiol. Biochem.*, vol. 43, pp. 287–292, 2005.

- [134] R. A. Olsen, R. B. Clark, and J. H. Bennett, "The enhancement of soil fertility by plant roots.," *Am. Sci.*, vol. 69, pp. 378–384, 1981.
- [135] E. P. Colangelo and M. L. Guerinot, "The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response.," *Plant Cell*, vol. 16, no. 3400–3412, 2004.
- [136] S. Santi and W. Schmidt, "Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots," *New Phytol*, vol. 183, no. 4, pp. 1072–1084, 2009.
- [137] Y. Yi and M. L. Guerinot, "Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency.," *Plant J.*, vol. 10, pp. 835–844, 1996.
- [138] E. L. Connolly, N. Campbell, N. Grotz, C. L. Prichard, and M. L. Guerinot, "Overexpression of the FRO2 iron reductase confers tolerance to growth on low iron and uncovers posttranscriptional control.," *Plant Physiol*, vol. 133, pp. 1102–1110, 2003.
- [139] B. M. Waters, D. G. Blevins, and D. J. Eide, "Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition.," *Plant Physiol.*, vol. 129, pp. 85–94, 2002.
- [140] L. Li, X. Cheng, and H. . Ling, "Isolation and characterization of Fe(III)-chelate reductase gene LeFRO1 in tomato.," *Plant Mol. Biol.*, vol. 54, pp. 125–136, 2004.
- [141] R. Henriques, J. Jasik, M. Klein, E. Martinoia, U. Feller, J. Schell, M. S. Pais, and C. Koncz, "Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects.," *Plant Mol. Biol.*, vol. 50, pp. 587–597, 2002.
- [142] J. Lasswell, L. E. Rogg, D. C. Nelson, C. Rongey, and B. Bartel, "Cloning and characterization of IAR1, a gene required for auxin conjugate sensitivity in Arabidopsis.," *Plant Cell*, vol. 12, pp. 2395–2408, 2000.
- [143] G. Vert, J.-F. Briat, and C. Curie, "Arabidopsis IRT2 gene encodes a root-periphery transporter.," *Plant J.*, vol. 26, pp. 181–189, 2001.
- [144] U. Eckhardt, A. M. Marques, and T. . Buckhout, "Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants.," *Plant Mol. Biol.*, vol. 45, pp. 437–448, 2001.

- [145] E. L. Connolly, J. P. Fett, and M. . Guerinot, “Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation.,” *Plant Cell*, vol. 14, pp. 1337–1357, 2002.
- [146] Y. Ishimaru, M. Suzuki, T. Tsukamoto, K. Suzuki, M. Nakazono, T. Kobayashi, Y. Wada, S. Watanabe, S. Matsushashi, M. Takahashi, H. Nakanishi, S. Mori, and N. K. Nishizawa, “Rice plants take up iron as an Fe³⁺-phytosiderophore and as Fe²⁺.,” *Plant J.*, vol. 45, pp. 335–346, 2006.
- [147] G. Winklemann, “Fungal siderophores membrane reactive ionophores.,” *J. Plant. Nutr.*, vol. 5, pp. 645–651, 1982.
- [148] H. Marschner, *Mineral nutrition of higher plants*. New York: Academic Press, 1986.
- [149] S. Takagi, K. Namoto, and T. Takemoto, “Physiological aspects of mugineic acid, a possible phytosiderophore of graminaceous plants.,” *J. Plant Nutr*, vol. 7, pp. 469–477, 1984.
- [150] S. Mori, “Iron acquisition by plants,” *Curr. Opin. Plant Biol.*, vol. 2, pp. 250–253, 1999.
- [151] K. Bashir, H. Inoue, S. Nagasaka, M. Takahashi, H. Nakanishi, S. Mori, and N. K. Nishizawa, “Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants.,” *J. Biol. Chem.*, vol. 281, pp. 32395–32402, 2006.
- [152] K. Kanazawa, K. Higuchi, N. Nishizawa, S. Fushiya, M. Chino, and S. Mori, “Nicotianamine aminotransferase activities are correlated to the phytosiderophore secretion under Fedeficient conditions in Gramineae. .,” *J. Exp. Biol*, vol. 46, pp. 1903–1906, 1994.
- [153] H. Nakanishi, H. Yamaguchi, T. Sasakuma, N. K. Nishizawa, and S. Mori, “Two dioxygenase genes, Ids3 and Ids2, from *Hordeum vulgare* are involved in the biosynthesis of mugineic acid family phytosiderophores.,” *Plant Mol. Biol.*, vol. 44, pp. 199–207, 2000.
- [154] M. Suzuki, M. Takahashi, T. Tsukamoto, S. Watanabe, S. Matsushashi, J. Yazaki, N. Kishimoto, S. Kikuchi, H. Nakanishi, S. Mori, and N. K. Nishizawa, “Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley.,” *Plant J.*, vol. 48, no. 1, pp. 85–97, Oct. 2006.

- [155] N. K. Fageria, V. C. Baligar, and R. . Wright, "Iron nutrition of plants: An overview on the chemistry and physiology of its deficiency and toxicity.," *Pesq.Agropec. Bras*, vol. 25, no. 4, pp. 553–570, 1990.
- [156] W. M. Latimer, *The oxidation status of the elements and their potentials in aqueous solutions.*, 2nd ed. New York: Prentice Hall Inc, 1952.
- [157] Y. Chen and P. Barak, "Iron nutrition of plants in calcareous soils.," *Adv.Agron.*, vol. 35, pp. 217–240, 1982.
- [158] R. Dudal, "Inventory of the major soils of the world with special reference to mineral stress hazards.," in *Plant adaptation to mineral stress in problem soils.*, M. J. Wright, Ed. Beltsville: Cornell Univ Press, 1977, pp. 3–13.
- [159] C. D. Foy, R. L. Chaney, and M. C. White, "The physiology of metal toxicity in plants.," *Ann. Rev. Plant Physiol.*, vol. 29, pp. 511–566, 1978.
- [160] W. . Anderson, "Diagnosis and correction of iron deficiency in field crops- An overview.," *J. Plant Nutr*, vol. 5, pp. 785–795, 1982.
- [161] T. Tadano, "Naturally occurring iron-chelating compounds in oat and rice root washing.," *Soil Sci.Plant Nutr.*, vol. 22, pp. 423–433, 1976.
- [162] M. P. Barbosa Filho, N. K. Fageria, and L. . Stone, "Manejo d'agua e calagem em relaao a produtividade e toxicidade de ferro arroz.," in *An overview on the chemistry and physiology of its deficiency and toxicity.*, N. K. Fageria, V. C. Baligar, and R. J. Wright, Eds. Pesq.Agropec. Bras., 1983.
- [163] D. . Proudhon, J. . Wei, J. F. . Briat, and E. C. Theil, "1996 Ferritin gene organization: Differences between plants and animals suggest possible kingdom-specific selective constraints," *J. Mol. Evol.*, vol. 42, pp. 325–336, 1996.
- [164] K. . Platt-Aloia, W. W. . Thomson, and N. Terry, "Changes in plastid ultrastructure during iron nutrition-mediated chloroplast development," *Protoplasma*, vol. 114, pp. 85–92, 1983.
- [165] T. L. . Winder and N. J. Nishio, "Early iron deficiency stress response in leaves of sugar beat," *Plant Physiol.*, vol. 108, pp. 1487–1494, 1995.
- [166] M. Hippler, J. Klein, A. Fink, T. Allinger, and P. Hoerth, "Towards functional proteomics of membrane protein complexes: analysis of thylakoid membranes from *Chlamydomonas reinhardtii*," *Plant J*, vol. 28, pp. 595–606, 2001.

- [167] B. Naumann, E. Stauber, A. Busch, F. Sommer, and M. Hippler, “N-terminal processing of Lhca3 is a key step in remodeling of the photosystem I-light-harvesting complex under iron deficiency in *Chlamydomonas reinhardtii*,” *J Biol Chem*, vol. 280, pp. 20431–20441, 2005.
- [168] C. K. . Mathews and K. . van Holde, *Biochemistry*, 2nd ed. The Benjamin Cummings Publishing, 1996, pp. 588–617.
- [169] N. Robinson, C. Proctor, E. Connolly, and M. Guerinot, “A ferricchelate reductase for iron uptake from soils,” *Nature*, vol. 397, pp. 694–697, 1999.
- [170] D. Eide, M. Broderius, J. Fett, and M. Guerinot, “A novel iron-regulated metal transporter from plants identified by functional expression in yeast,” *Proc Natl Acad Sci USA*, vol. 93, pp. 5624–5628, 1996.
- [171] G. Vert, N. Grotz, F. Dédaldéchamp, F. Gaymard, M. Guerinot, J. Briat, and C. Curie, “IRT1, an Arabidopsis transporter essential for iron uptake from the soil and plant growth,” *Plant Cell*, vol. 14, pp. 1223–1233, 2002.
- [172] A. Herbig, A. Giritch, C. Horstmann, R. Becker, H. Balzer, H. Bäumlein, and U. Stephan, “Iron and copper nutrition-dependent changes in protein expression in a tomato wild type and the nicotianamine- free mutant chloronerva,” *Plant Physiol*, vol. 111, pp. 533–540, 1996.
- [173] G. Rabotti, P. De Nisi, and G. Zocchi, “Metabolic implications in the biochemical responses to iron deficiency in cucumber (*Cucumis sativus* L.) roots,” *Plant Physiol*, vol. 107, pp. 1195–1199, 1995.
- [174] O. Thimm, B. Essigmann, S. Kloska, T. Altmann, and T. Buckhout, “Response of Arabidopsis to iron deficiency stress as revealed by microarray analysis,” *Plant Physiol*, vol. 127, p. 10301043, 2001.
- [175] T. Negishi, H. Nakanishi, J. Yazaki, N. Kishimoto, F. Fujii, K. Shimbo, K. Yamamoto, K. Sakata, T. Sasaki, and S. Kikuchi, “cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore secretion in Fe-deficient barley roots,” *Plant J*, vol. 30, pp. 83–94, 2002.
- [176] J. Patterson, K. Ford, A. Cassin, S. Natera, and A. Bacic, “Increased abundance of proteins involved in phytosiderophore production in boron-tolerant barley,” *Plant Physiol.*, vol. 144, no. 3, pp. 1612–31, Jul. 2007.

- [177] T. Kobayashi, T. Yoshihara, R. N. Itai, H. Nakanishi, M. Takahashi, S. Mori, and N. K. Nishizawa, "Promoter analysis of iron-deficiency-inducible barley IDS3 gene in Arabidopsis and tobacco plants.," *Plant Physiol. Biochem.*, vol. 45, no. 5, pp. 262–9, May 2007.
- [178] J. Petit, J. Briat, and S. Lobréaux, "Structure and differential expression of the four members of the Arabidopsis thaliana ferritin gene family.," *Biochem J*, vol. 359, pp. 575–582, 2001.
- [179] S. Lobréaux, T. Hardy, and J. Briat, "Absciscic acid is involved in the iron-induced synthesis of maize ferritin.," *EMBO J*, vol. 12, pp. 651–657, 1993.
- [180] J. Briat and S. Lobréaux, "Iron transport and storage in plants.," *Trends Plant Sci*, vol. 2, pp. 187–193, 1997.
- [181] N. Kawagashira, Y. Ohtomo, K. Murakami, K. Matsubara, J. Kawai, R. Carninci, P. Hayashizaki, S. Kikuchi, and K. Higo, "Multiple zinc finger motifs with comparison of plant and insects.," in *12th International Conference on Genome Informatics*, 2001, pp. 368–369.
- [182] U. Kramer and S. Clemens, "Function and homeostasis of zinc, copper, and nickel in plants.," *Top. Curr Genet*, vol. 14, pp. 215–271, 2005.
- [183] G. Richter, S. Lamppa, "Structural properties of the chloroplast stromal processing peptidase required for its function in transit peptide removal.," *J Biol Chem*, vol. 278, pp. 39497–39502, 2003.
- [184] P. Luciano, K. Tokatlidis, I. Chambre, J. Germanique, and V. Geli, "The mitochondrial processing peptidase behaves as a zincmetallopeptidase.," *J Mol Biol*, vol. 280, pp. 193–199, 1998.
- [185] A. Stahl, P. Moberg, J. Ytterberg, O. Panfilov, V. Brockenhuus, H. Lowenhielm, F. Nilsson, and E. Glaser, "Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants.," *J Biol Chem*, vol. 277, pp. 41931–41939, 2002.
- [186] S. Bailey, E. Thompson, P. Nixon, P. Horton, C. Mullineaux, C. Robinson, and N. Mann, "A critical role for the Var2 FtsH homologue of Arabidopsis thaliana in the photosystem II repair cycle in vivo.," *J Biol Chem*, vol. 277, pp. 2006–2011, 2002.

- [187] A. Serero, C. Giglione, and T. Meinel, "Structure and catalytic mechanism of the cytosolic D-ribulose-5-phosphate 3-epimerase from rice.," *J Mol Biol*, vol. 314, pp. 695–708, 2001.
- [188] J. Sullivan, K. Shirasu, and X. Deng, "The diverse roles of ubiquitin and the 26S proteasome in the life of plants.," *Nat Rev Genet*, vol. 4, pp. 948–958, 2003.
- [189] S. Snaith and G. Levvy, "Alpha-mannosidase as a zinc-dependent enzyme.," *Nature*, vol. 218, pp. 91–92, 1968.
- [190] J. Maidment, D. Moore, G. Murphy, G. Murphy, and I. Clark, "Matrix metalloproteinase homologues from *Arabidopsis thaliana*. Expression and activity.," *J Biol Chem*, vol. 274, pp. 34706–34710, 1999.
- [191] J. P. Singh, D. J. Dahiya, and R. P. Narwal, "Boron uptake and toxicity in wheat in relation to zinc supply.," *Fert. Res.*, vol. 24, no. 105–110, 1990.
- [192] R. D. Graham, "Breeding for Nutritional Characteristics in Cereals.," *Adv. Plant Nutr.*, vol. 1, pp. 57–102, 1984.
- [193] J. G. White and R. H. Zasoski, "Mapping Soil Micronutrients.," *F. Crop. Res.*, vol. 60, pp. 11–26, 1999.
- [194] C. Cohen, T. Fox, D. Garvin, and L. Kochian, "The role of iron-deficiency stress responses in stimulating heavymetal transport in plants.," *Plant Physiol.*, vol. 116, pp. 1063–1072, 1998.
- [195] Y. Korshunova, D. Eide, W. Clark, M. Guerinot, and H. Pakrasi, "The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range.," *Plant Mol. Biol.*, vol. 40, pp. 37–44, 1999.
- [196] L. Roberts, A. Pierson, Z. Panaviene, and E. Walker, "Yellow stripe 1. Expanded roles for the maize iron-phytosiderophore transporter.," *Plant Physiol.*, vol. 135, pp. 112–120, 2004.
- [197] G. Schaaf, U. Ludewig, B. Erenoglu, S. Mori, T. Kitahara, and N. von Wiren, "ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianaminechelated metals.," *J. Biol. Chem*, vol. 279, pp. 9091–9096, 2004.
- [198] S. Alam, S. Kamei, and S. Kawai, "Effect of iron deficiency on the chemical composition of the xylem sap of barley.," *Soil Sci. Plant Nutr*, vol. 47, pp. 643–649, 2001.

- [199] T. Kobayashi, T. Yoshihara, and T. Jiang, “Combined deficiency of iron and other divalent cations mitigates the symptoms of iron deficiency in tobacco plants.,” *Physiol. Plant*, vol. 119, pp. 400–408, 2003.
- [200] A. Mozafar, “Distribution of nutrient elements along the maize leaf: Alteration by iron deficiency.,” *J. Plant Nutr*, vol. 20, pp. 999–1005, 1997.
- [201] M. Kanai, M. Hirai, M. Yoshiba, T. Tadano, and K. Higuchi, “Iron deficiency causes zinc excess in *Zea mays*,” *Soil Sci. Plant Nutr.*, vol. 55, no. 2, pp. 271–276, Apr. 2009.
- [202] S. Thomine, F. Lelièvre, E. Debarbieux, J. Schroeder, and H. Barbier-Brygoo, “AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency.,” *Plant J.*, vol. 34, pp. 685–695, 2003.
- [203] P. Pédas, C. Ytting, A. Fuglsang, T. Jahn, J. Schjoerring, and S. Husted, “Manganese efficiency in barley: identification and characterization of the metal ion transporter HvIRT1.,” *Plant Physiol*, vol. 148, pp. 455–466, 2008.
- [204] Y. Murata, J. Ma, N. Yamaji, D. Ueno, K. Nomoto, and T. Iwashita, “A specific transporter for iron(III)-phytosiderophore in barley roots.,” *Plant J*, vol. 46, pp. 563–572, 2006.
- [205] N. von Wirén, H. Marschner, and V. Romheld, “Roots of Iron-Efficient Maize also Absorb Phytosiderophore-Chelated Zinc,” *Plant Physiol.*, vol. 111, pp. 1119–1125, 1996.
- [206] K. Lukaszewski and D. Blevins, “Root growth inhibition in boron-deficient or aluminum-stressed squash may be a result of impaired ascorbate metabolism.,” *Plant Physiol.*, vol. 112, pp. 1135–1140, 1996.
- [207] M. T. Oz, “Microarray based expression profiling of barley under boron stress and cloning of 3h boron tolerance gene,” Ph.D. dissertation, Dept. Biotech., METU, Ankara, Turkey, 2012.
- [208] R. D. Graham, R. M. Welch, D. L. Grunes, E. E. Cary, and W. A. Norvell, “Effect of zinc deficiency on the accumulation of boron and other mineral nutrients in barley.,” *Soil Sci. Soc. Am. J*, vol. 51, pp. 652–657, 1987.
- [209] A. Torun, I. Gültekin, M. Kalayci, A. Yilmaz, S. Eker, and I. Cakmak, “Effects of zinc fertilization on grain yield and shoot concentrations of zinc, boron, and phosphorus of 25 wheat cultivars grown on a zinc-deficient and boron-toxic soil” *J. Plant Nutr.*, vol. 24, no. 11, pp. 1817–1829, 2001.

- [210] A. Gunes, M. Alpaslan, Y. Cikili, and H. Ozcan, "Effect of zinc on the alleviation of boron toxicity in tomato," *J. Plant Nutr.*, vol. 22, no. 7, pp. 1061–1068, 1999.
- [211] D. Hoagland and D. Arnon, "The water-culture method for growing plants without soil.," *Cal. Agric. Exp. Sta. Cir.*, vol. 347, pp. 1–32, 1950.
- [212] R. Smart and G. Bingham, "Rapid estimates of relative water content," *Plant Physiol.*, vol. 53, pp. 258–260, 1974.
- [213] P. Chomczynski and N. Sacchi, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.," *Anal. Biochem.*, vol. 162, pp. 156–159, 1987.
- [214] B. J. Strasser and R. . Strasser, "Measuring fast fluorescence transients to address environmental questions: the JIP test.," in *Photosynthesis: from Light to Biosphere*, P. Mathis, Ed. Dordrecht: Kluwer Academic Publishers, 1995, pp. 997–980.
- [215] R. J. Strasser, M. Tsimilli-Michael, and A. Srivastava, "Analysis of the chlorophyll a fluorescence transient.," in *Chlorophyll a Fluorescence: a Signature of Photosynthesis, Advances in Photosynthesis and Respiration Series.*, G. C. Papageorgiou and Govindjee, Eds. Rotterdam: Springer, 2004, pp. 321–362.
- [216] U. . Gupta, "Boron deficiency and toxicity symptoms for several crops as related to tissue boron levels.," *J. Plant Nutr.*, vol. 6, pp. 387–395, 1983.
- [217] T. E. Sotiropoulos, I. N. Therios, and K. N. Dimassi, "Nutritional status, growth, CO₂ assimilation, and leaf anatomical responses in two kiwifruit species under boron toxicity," *J. Plant Nutr.*, vol. 25, pp. 1249–1261, 2002.
- [218] M. Kalayci, A. Alkan, and I. Cakmak, "Studies on differential response of wheat cultivars to boron toxicity," *Euphytica*, vol. 100, pp. 123–129, 1998.
- [219] L. M. Cervilla, M. A. Rosales, and M. . Rubio-Wilhelmi, "Involvement of lignification and membrane permeability in the tomato root response to boron toxicity," *Plant Sci.*, vol. 176, pp. 545–552, 2009.
- [220] D. Ghosh and J. Xu, "Abiotic stress responses in plant roots: a proteomics perspective.," *Front. Plant Sci.*, vol. 5, no. 6, 2014.
- [221] H. D. Barr and P. E. Weatherley, "A re-examination of the relative turgidity technique for estimating water deficit in leaves.," *Aust. J. Biol. Sci.*, vol. 15, pp. 413–428, 1962.

- [222] Y. Chantachume, D. Smith, and G. . Hollamby, "Screening for boron tolerance in wheat (*T. aestivum*) by solution culture in filter paper.," *Plant Soil*, vol. 177, pp. 249–254, 1995.
- [223] K. Oxborough, "Imaging of chlorophyll a fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance," *J. Exp. Bot.*, vol. 55, pp. 1195–1205, 2004.
- [224] F. Bussotti, R. J. Strasser, and M. Schaub, "Photosynthetic behavior of woody species under high ozone exposure probed with the JIP test: a review," *Environ. Pollut.*, vol. 147, pp. 430–437, 2007.
- [225] R. J. Strasser, A. Srivastava, and M. Tsimilli-Michael, "The fluorescence transient as a tool to characterise and screen photosynthetic samples.," in *Probing Photosynthesis: Mechanisms, Regulation and Adaptation.*, M. Yunus, U. Pathre, and P. Mohanty, Eds. London: Taylor and Francis, 2000, pp. 445–483.
- [226] I. E. Papadakis, K. N. Dimassi, and A. M. Bosabalidis, "Effects of B excess on some physiological and anatomical parameters of 'Navelina' orange plants grafted on two rootstocks," *Environ. Exp. Bot.*, vol. 51, pp. 247–257, 2004.
- [227] M. Landi, A. Pardossi, D. Remorini, and L. Guidi, "Antioxidant and photosynthetic response of a purple-leaved and a green-leaved cultivar of sweet basil (*Ocimum basilicum*) to boron excess," *Environ. Exp. Bot.*, vol. 85, pp. 64–75, 2013.
- [228] S. Andaluz, A. Lopez-Millan, J. De Las Rivas, E. Aro, J. Abadia, and A. Abadia, "Proteomic profiles of thylakoid membranes and changes in response to iron deficiency.," *Photosynth Res*, vol. 89, pp. 141–155, 2006.
- [229] J. Moseley, T. Allinger, S. Herzog, P. Hoerth, E. Wehinger, S. Merchant, and M. Hippler, "Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus," *EMBO J*, vol. 21, pp. 6709–6720, 2002.
- [230] G. H. Krause and E. Weis, "Chlorophyll fluorescence and photosynthesis - the basics.," *Annu. Rev. Plant Phys*, vol. 42, pp. 313–349, 1991.
- [231] M. Čajánek, M. Štroch, and I. Lachetová, "Characterization of the photosystem II inactivation of heat-stressed barley leaves as monitored by the various parameters of chlorophyll a fluorescence and delayed fluorescence," *J. Photoch. Photobio*, vol. 47, pp. 39–45, 1998.

- [232] L. Duysens and H. Sweers, "Mechanism of the two photochemical reactions in alga as studied by means of fluorescence," *Jpn. Soc. Plant Physiol.*, pp. 353–372, 1963.
- [233] K. Maxwell and G. . Johnson, "Chlorophyll fluorescence - a practical guide.," *J. Exp. Bot.*, vol. 51, pp. 659–668, 2000.
- [234] L. B. Thach, A. Shapcott, S. Schmidt, and E. Critchley, "The OJIP fast fluorescence rise characterizes Graptophyllum species and their stress responses.," *Photosynth. Res.*, vol. 94, pp. 423–436, 2007.
- [235] A. J. Strauss, G. H. J. Kruger, R. J. Strasser, and P. D. . Van Heerden, "Ranking of dark chilling tolerance in soybean genotypes probed by the chlorophyll a fluorescence transient O-J-I-P.," *Environ. Exp. Bot.*, vol. 56, pp. 147–157, 2006.

APPENDICES

A. COMPOSITION OF HOAGLAND'S SOLUTION

Table A.1 Composition of ½ Hoagland's Solution

Component	Final concentration
MgSO ₄ ·7H ₂ O	0.5 mM
Ca(NO ₃) ₂ ·4H ₂ O	1.15 mM
KH ₂ PO ₄	0.25 mM
KNO ₃	1.25 mM
MnCl ₂ ·4H ₂ O	2.3 µM
ZnSO ₄ ·7H ₂ O	0.2 µM
Na ₂ MoO ₄ ·2H ₂ O	0.1 µM
CuSO ₄ ·5H ₂ O	0.1 µM
NaFe(III)EDTA	50 µM
H ₃ BO ₃	10 µM

Table A.2 Iron and Boron compositions of stress conditions

Conditions	Final NaFe(III)EDTA conc.	Final H ₃ BO ₃ conc.
Control	50 µM	10 µM
Fe-deficient	-	10 µM
B-deficient	50 µM	-
B-toxic(5)	50 µM	5 mM
B-toxic(25)	50 µM	25 mM
Fe-deficient+ B-deficient	-	-
Fe-deficient+ B-toxic(5)	-	5 mM
Fe-deficient+B-toxic(25)	-	25 mM

B. ALIGNMENT RESULTS OF AFFYMETRIX BARLEY1 PROBES AND CORRESPONDING MRNA SEQUENCES

Table B.1 Alignment results of AB011269.1 and Contig10741_at sequences using Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Arrows show the start point of HvNAS primer binding

Score	Expect	Identities	Gaps	Strand
625 bits(338)	0.0	343/345(99%)	1/345(0%)	Plus/Plus
Query 890	TGCATGCCAATGAACGTCCCAACGGGCGTGGTGGACAGTAC-CGGGGCGCGGTACCGGTG	948		
Sbjct 1	TGCATGCCAATGAACGTCCCAACGGGCGTGGTGGACAGTACGCGGGGCGCGGTACCGGTG	60		
Query 949	GTCAGCCCCGCCGTGCAGGTTCCGGTGAGATGGTGGCGGACGTGACCCACAAGAGAGAGGAG	1008		
Sbjct 61	GTCAGCCCCGCCGTGCAGGTTCCGGTGAGATGGTGGCGGACGTGACCCACAAGAGAGAGGAG	120		
Query 1009	TTCACCAACGCGGAAGTGGCCTTCTGATCGTTGCGAGGGAATGAAAATGAAGGTGGACGT	1068		
Sbjct 121	TTCACCAACGCGGAAGTGGCCTTCTGATCGTTGCGAGGGAATGAAAATGAAGGTGGACGT	180		
Query 1069	GTGTGGTCAGCATCCATACGTGGCTGCCTGCTTCATCGCTTGCAATCGTACTACTACCTA	1128		
Sbjct 181	GTGTGGTCAGCATCCATACGTGGCTGCCTGCTTCATCGCTTGCAATCGTACTACTACCTA	240		
Query 1129	CCTATGCAGTTCAAGTCATGTGTTGTCAATGTAAGTGTGATGTTTACACTAGTCTATGAA	1188		
Sbjct 241	CCTATGCAGTTCAAGTCATGTGTTGTCAATGTAAGTGTGATGTTTACACTAGTCTATGAA	300		
Query 1189	AGGCAGGGCAGACGAGGGTAGTGTGCCAAGTAAACAGTGTGTCATT	1233		
Sbjct 301	AGGCAGGGCAGACGAGGGTAGTGTGCCAAGTAAAAAGTGTGTCATT	345		

Table B.2 Alignment results of AF136941.1 and Contig10741_at sequences using Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Arrows show the start point of HvNAS primer binding

Score	Expect	Identities	Gaps	Strand
625 bits(338)	0.0	343/345(99%)	1/345(0%)	Plus/Plus
Query 902	TGCATGCCAATGAACGTCCCAAC - GGCGTGGTGGACAGTACGCCGGGCGCGGTGCCGGTG	960		
Sbjct 1	TGCATGCCAATGAACGTCCCAACGGGCGTGGTGGACAGTACGCCGGGCGCGGTACCGGTG	60		
Query 961	GTCAGCCCGCCGTGCAGGTTCCGGTGAGATGGTGGCGGACGTGACCCACAAGAGAGAGGAG	1020		
Sbjct 61	GTCAGCCCGCCGTGCAGGTTCCGGTGAGATGGTGGCGGACGTGACCCACAAGAGAGAGGAG	120		
Query 1021	TTCACCAACGCGGAAGTGGCCTTCTGATCGTTGCGAGGGAATGAAAATGAAGGTGGACGT	1080		
Sbjct 121	TTCACCAACGCGGAAGTGGCCTTCTGATCGTTGCGAGGGAATGAAAATGAAGGTGGACGT	180		
Query 1081	GTGTGGTCAGCATCCATACG TG GCTGCCTGCTTCATCGCTTGCAATCGTACTACTACCTA	1140		
Sbjct 181	GTGTGGTCAGCATCCATACG TG GCTGCCTGCTTCATCGCTTGCAATCGTACTACTACCTA	240		
Query 1141	CCTATGCAGTTCAAGTCATGTGTTGTCAATGTAAGTGTGATGTTTACACTAGTCTATGAA	1200		
Sbjct 241	CCTATGCAGTTCAAGTCATGTGTTGTCAATGTAAGTGTGATGTTTACACTAGTCTATGAA	300		
Query 1201	AGGCAGGGCAGACGAGGGTAGTGTGCCAAGT AAAAGTGTGTCATT 1245			
Sbjct 301	AGGCAGGGCAGACGAGGGTAGTGTGCCAAGT AAAAGTGTGTCATT 345			

Table B.3 Alignment results of D37796.1 and Contig12916_s_at sequences using Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Arrows show the start point of HVIDS3 primer binding

Score	Expect	Identities	Gaps	Strand
625 bits(338)	0.0	358/368(97%)	0/368(0%)	Plus/Plus
Query 2563	AGGTTGTGACAAATGGGTTGTTAAAGAGCATAGAG	CACCGTGCAATGACAAACTCAGCGC	2622	
Sbjct 160	AGGTTGTGACAAATGGGTTGTTAAGGAGCATAGAGCATCGTGCGATGACAAACTCGGTGC	219		
Query 2623	TTGCACGCACATCGGTGGCTACCTTTATCATGCCTACTCAAGAATGTCTTATTGGTCCCG	2682		
Sbjct 220	TTGCACGCACATCGGTGGCTACCTTTATTATGCCTACTCAAGAATGTCTTATTGGTCCCG	279		
Query 2683	CGAAGGAGTTCCTTAGCAAGAAAAACCCACCGTGCTACCGCACCACCATGTTTCGTGACT	2742		
Sbjct 280	CGAAGGAGTTCCTTAGTGAAGAAAAACCCACCGTGCTACCGCACCACCATGTTTCGTGACT	339		
Query 2743	TCATGCGCATCTACAATGTTGTCAAGCTTG	GGTCATCGCTCAATCTCACAACCAATCTCA	2802	
Sbjct 340	TCATGCGCATCTACAATGTTGTCAAGCTTG	GGTCATCGCTCAATCTCACAACCAATCTCA	399	
Query 2803	AGAACGTCCAGAAGGAGATATAAAGCAAATCTACAAGATTCCTTCAAGATCTGGAATTAT	2862		
Sbjct 400	AGAACGTCCAGAAGGAGATATAAAGCAAATCTACAAGACTCCTTCAAGATCTGGAATTAT	459		
Query 2863	CCTATTCCTCACTTGTATGTTTCTTACCTATGCCATAGATCTCAGTTGTGTCCAATTGTT	2922		
Sbjct 460	CCTATTCCTCACTTGTATGTTTCTTACCTATGCCATAGATCTCAGTTGTGTCCAATTGTT	519		
Query 2923	GCTATGCA	2930		
Sbjct 520	GCTATGCA	527		

C. PHOTOSYNTHETIC PARAMETERS

Table C.1 Summary of the formula and terms of JIP test parameters and their description [215], [225]

Parameter	Formula/Explanation
Extracted and technical fluorescence parameters	
F_0	Initial fluorescence intensity, when all RCs of PSII are open
$F_{300}-F_J-F_I$	Fluorescence intensity at K-step (300 μ s), J-step (2 ms), I-step (30 ms)
F_M	Maximal fluorescence intensity, when all RCs of PSII are closed
$t(F_M)$	Time to reach F_M , in ms
$V_{K-J-I} \cdot V_I$	$(F_{(K-J-I)} - F_0)/(F_M - F_0)$, relative variable fluorescence at the K-J-I step
M_0 or $(dV/dt)_0$	$4 (F_{300} - F_0)/(F_M - F_0)$, approximated initial slope (in ms^{-1}) of the fluorescence transient $V = f(t)$
Area	Total complementary area between fluorescence induction curve and F_M
S_M	$Area/(F_M - F_0)$, normalized total complementary area above the OJIP (reflecting multiple-turnover QA reduction events) or total electron carriers per RC
Quantum efficiencies or flux ratios	
ϕP_0 or TR_0/ABS	$1 - F_0/F_M$ or F_V/F_M , maximum quantum yield of primary photochemistry at $t = 0$
ϕE_0 or ET_0/ABS	$(1 - F_0/F_M) \times \psi_0$, quantum yield for electron transport at $t = 0$
ψ_0 or ET_0/TR_0	$1 - V_J$, probability (at $t = 0$) that a trapped exciton moves an electron into the electron transport chain beyond QA^-
δR_0 or RE_0/ET_0	$(1 - V_J)/(1 - V_I)$, the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end final electron acceptors
ϕR_0 or RE_0/ABS	$\phi P_0 \times \psi_0 \times \delta R_0$, the quantum yield of electron transport from QA^- to the PSI end electron acceptors
Specific fluxes or specific activities	
ABS/RC	$M_0 \times (1/V_J) \times (1/\phi P_0)$, absorption flux per RC at $t = 0$ or a measure for an average antenna size
TR_0/RC	$M_0 \times (1/V_J)$, trapped energy flux per RC at $t = 0$
ET_0/RC	$M_0 \times (1/V_J) \times \psi_0$, electron transport flux per RC at $t = 0$
Phenomenological fluxes or phenomenological activities	
ABS/CS_0	F_0 or other useful expression, absorption flux per CS at $t = 0^*$
TR_0/CS_0	$\phi P_0 \times (ABS/CS_0)$, trapped energy flux per CS at $t = 0$
ET_0/CS_0	$\phi P_0 \times \psi_0 \times (ABS/CS_0)$, electron transport flux per CS at $t = 0$
RC/CS_0	$\phi P_0 \times (V_J/M_0) \times F_0$, amount of active PSII RCs per CS at $t = t_{FM}$
$PI(ABS)$, $PI(total)$	$(RC/ABS) \times [\phi P_0/(1 - \phi P_0)] \times [\psi_0/(1 - \psi_0)] \times [\delta R_0/(1 - \delta R_0)]$, total PI, measuring the performance up to the PSI end electron acceptors

D. Q-PCR SUPPLEMENTARY DATA

Table D.1 Fold changes of IDS3 expression according to Day 0, Day 1, and Day 5 controls

	FC – Day 0 (calibrator)			FC - Day 1 (calibrator)		
Samples(Day)	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Control 0	1.00	1.00	1.00	-	-	-
Control 1	250.31	0.96	0.27	1.00	1.00	1.00
Fe- def 1	94.35	16.21	4.87	0.38	16.87	18.02
B-def 1	14.00	4.26	4.49	0.06	4.43	16.59
Fe-def/B-def 1	4.49	24.78	15.23	0.02	25.79	56.30
B- tox 1	0.40	3.15	1.69	0.00	3.27	6.26
Fe-def/B-tox 1	0.89	2.04	1.85	0.00	2.12	6.84
	FC – Day 0 (calibrator)			FC - Day 5 (calibrator)		
Samples(Day)	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Control 5	19.98	1.73	188.29	1.00	1.00	1.00
Fe- def 5	2525.45	12.23	341.93	126.38	7.07	1.82
B-def 5	39.60	11.36	127.99	1.98	6.57	0.68
Fe-def/B-def 5	94.28	8.20	5.52	4.72	4.74	0.03
B- tox 5	-	4.34	0.77	-	2.51	0.00
Fe-def/B-tox 5	1.66	0.05	6.40	0.08	0.03	0.03

Table D.2 Fold changes of NAS expression according to Day 0, Day 1, and Day 5 controls

	FC – Day 0 (calibrator)			FC - Day 1 (calibrator)		
Samples(Day)	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Control 0	1.00	1.00	1.00	-	-	-
Control 1	30.06	0.92	0.56	1.00	1.00	1.00
Fe- def 1	7.70	9.03	6.08	0.26	9.78	10.93
B-def 1	1.75	5.98	3.45	0.06	6.48	6.19
Fe-def/B-def 1	1.00	9.29	17.94	0.03	10.06	32.22
B- tox 1	1.19	5.84	4.81	0.04	6.32	8.63
Fe-def/B-tox 1	1.34	3.51	4.55	0.04	3.80	8.17
	FC – Day 0 (calibrator)			FC - Day 5 (calibrator)		
Samples(Day)	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Control 5	21.86	2.94	29.45	1.00	1.00	1.00
Fe- def 5	41.93	6.87	37.79	1.92	2.34	1.28
B-def 5	9.35	3.63	32.33	0.43	1.24	1.10
Fe-def/B-def 5	5.45	0.32	2.07	0.25	0.11	0.07
B- tox 5	0.33	0.73	6.00	0.02	0.25	0.20
Fe-def/B-tox 5	1.15	4.16	7.52	0.05	1.41	0.26