Suppression of the soybean phytoglobin *GmPgb1* enhances tolerance to iron deficiency

by

Bethany Asmundson

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

Copyright © 2020 by Bethany Asmundson

ACKNOWLEDGEMENTS

My thesis would not have been possible without the support from many people. First and foremost, I would like to thank my advisor, Dr. Claudio Stasolla. Thank you for your guidance and encouragement, for always having an open door, and for trusting me throughout this experience. You truly made the past two years worthwhile, I am so lucky to have completed my research under your supervision. To my committee member Dr. Robert Hill, thank you for motivating me and for sharing your wisdom and experience. Thank you to my other committee member Dr. Sylvie Renault for sharing your knowledge and helpful suggestions.

A special thanks to Dr. Mohammed Mira for knowing every procedure and for teaching me them, for always being so patient with me, and for never letting me forget that I am capable. You were my “lifeline”! To Mr. Doug Durnin, thank you for your everyday help whether it be assisting with an experiment, editing a paper, or chatting over a cup of coffee. Your generosity never went unnoticed! And finally, to my lab mates, Sam Day and James De Castro, thank you for becoming great friends! This experience would have been so different without you two. Good luck with your future endeavours!

I am so grateful to have been a part of such an amazing lab and to have met so many incredible people. You all helped make this experience unforgettable! I will miss you all!

Thank you to my parents and my boyfriend James Carriere for celebrating all the big and small victories with me and for picking me up when research and writing seemed impossible! The past two years would not have been possible without you! I love you all!!

And to one last very special person, my Grandpa. Thank you for sharing your love for agriculture with me. I wish we could celebrate this accomplishment together. I miss you!

Lastly, I would like to thank Manitoba Pulse and Soybean Growers for funding this research.

TABLE OF CONTENTS

ACKNOWLEDGMENTS ii
LIST OF TABLES vi
LIST OF FIGURES vi
ABBREVIATIONS vii
ABSTRACT ix
FOREWORD x
1 LITERATURE REVIEW 1
1.1 Introduction 1
1.2 Mineral nutrition and iron requirement 4
1.2.1 Iron acquisition 5
1.2.2 Translocation of iron into chloroplasts 7
1.3 Iron deficiency in soybean 8
1.3.1 Environmental factors influencing iron availability 10
1.3.2 Changes in plant structure and physiology during iron deficiency 11
1.3.2.1 Root morphology 11
1.3.2.2 Chlorophyll and chloroplasts 12
1.3.2.3 Reactive oxygen species accumulation during iron deficiency 13
1.4 Ascorbic acid-glutathione redox system 14
1.5 Nitric oxide and iron deficiency 15
1.6 Phytoglobins 17
1.6.1 Phytoglobin and stress response 21
1.6.2 Phytoglobin and the antioxidant machinery 22
2 CHAPTER ONE: Suppression of the soybean phytoglobin *GmPgb1* enhances tolerance to iron deficiency chlorosis 24
2.1 Abstract 24
2.2 Introduction 25
2.3 Materials and methods 28
2.3.1 Plant material and growth conditions 28
2.3.2 Iron deficiency treatments 29
2.3.3 Screening varieties and chlorophyll measurements 29

2.3.4 Pharmacological treatments 30
2.3.5 Antioxidant enzyme activity and quantification of ascorbic acid and glutathione 30
2.3.6 Measurements of ascorbic acid and glutathione 32
2.3.7 Gene expression studies 32
2.3.8 Measurement of hydrogen peroxide 34
2.3.9 Localization of reactive oxygen species 34
2.3.10 Fe(II) and Fe(II) assays 35
2.3.11 Statistical analysis 35
2.4 Results 36
2.4.1 Response of soybean plants to iron deficiency and *GmPgb1* expression 36
2.4.2 Tolerance to IDC is associated with variation in the antioxidant system and elevated levels of ascorbic acid 38
2.4.3 Expression of *GmPgb1* affects soybean response to iron deficiency 46
2.4.4 Suppression of *GmPgb1* elevates the levels of ASC and reduces ROS 49
2.4.5 The Fe(II)/Fe(III) ratio is influenced by *GmPgb1* through ASC and NO 54
2.5 Discussion 56
3 GENERAL CONCLUSION 61
3.1 Future Direction 62
4 SUPPLEMENTAL MATERIAL 63
5 LITERATURE CITED 67

LIST OF TABLES

Supplementary Table 4.1 66

LIST OF FIGURES

Figure 1.1 9
Figure 1.2 19
Figure 2.1 37
Figure 2.2 39
Figure 2.3 42
Figure 2.4 44
Figure 2.5 45
Figure 2.6 48
Figure 2.7 51
Figure 2.8 52
Figure 2.9 53
Figure 2.10 55
Figure 4.1 63
Figure 4.2 64
Figure 4.3 64
Figure 4.4 65

ABBREVIATIONS

ABA, Abscisic acid

ASC, Ascorbic acid

ASC-GSH cycle, Ascorbic acid-glutathione redox cycle

APX, Ascorbate peroxidase

CAT, Catalase

cGMP, Cyclic GMP

cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

DAB, Diaminobenzidine

DHA, Dehydroascorbate

DHAR, Dehydroascorbate reductase

Fe(II), Ferrous iron

Fe(III), Ferric iron

FRO2 and FRO7, Ferric-chelate reductase oxidase

FRO3, Ferric Reductase Defective 3

FPN1, Ferroportin 1

GmPgb1, Glycine max Phytoglobin 1

GR, Glutathione reductase

GSH, Glutathione

GSSG, Oxidize glutathione

IDC, Iron deficiency chlorosis

IRT1, Iron-regulated transport protein

MDHA, Monohydroascorbate

MDHAR, Monohydroascrobate reductase

NBT, Nitroblue tetrazolium

NOD, Nitric oxide dioxygenase

NOS, Nitric oxide synthase

NR, Nitrate reductase

OPT3, Oligopeptide transporter 3

PCD, Programmed cell death

Pgb, Phytoglobin

PIC1, Permease chloroplast 1

RAM, Root apical meristem

ROS, Reactive oxygen species

sGC, Soluble guanylyl cyclase

SOD, Superoxide dismutase

SNP, Sodium nitroprusside

WT, Wild type

YS1, Yellow stripe 1

ABSTRACT

Asmundson, Bethany. M.Sc., The University of Manitoba, November 2020.

Suppression of the soybean phytoglobin *GmPgb1* enhances tolerance to iron deficiency

Supervisor: Dr. Claudio Stasolla

The effect of iron deficiency in soybean (*Glycine max* (L.) merr) seedlings over-expressing or down-regulating *Glycine max Phytoglobin 1* (*GmPgb1*) was examined using an established hydroponic system which mimicked iron deficiency in soil. Iron deficient soybean plants down-regulating *GmPgb1* displayed greater chlorophyll retention and decreased accumulation of reactive oxygen species relative to the *GmPbg1* over-expressing and wild type (WT) plants. Suppression of *GmPgb1* under iron deficiency conditions was also accompanied by elevated levels of ascorbic acid (ASC), and increased activities of catalase, superoxide dismutase and ASC recycling enzymes. When compared to WT and *GmPgb1* over-expressing lines, iron deficient *GmPgb1* suppressing plants also exhibited a higher Fe(II)/Fe(III) ratio. Pharmacological treatments altering the level of nitric oxide (NO) and ASC were further utilized to dissect the effects of *GmPgb1* during iron deficiency. Applications of the NO donor, sodium nitroprusside (SNP), enhanced tolerance to iron deficiency by elevating the levels of ASC, the activities of several antioxidant enzymes, and the Fe(II)/Fe(III) ratio. A similar effect on this ratio was also observed when ASC was administered exogenously. Collectively this study reveals that suppression of *GmPgb1* in soybean attenuates the effects of iron deficiency through changes in NO and antioxidant responses linked to the higher availability of Fe(II).

FOREWORD

This thesis follows the paper style outlined by the Department of Plant Science and the Faculty of Graduate Studies at the University of Manitoba. The manuscript follows the style recommended by the Journal of Plant Physiology. This thesis is presented as a single manuscript, containing an abstract, introduction, materials and methods, results, and a discussion. Supplementary tables and figures are places immediately following the body of the manuscript. A literature review precedes the manuscript and conclusion follows the manuscript.

LITERATURE REVIEW

1.1 Introduction

Soybean (*Glycine max* (L.) merr) is a very important commercial crop belonging to the legume family (Aksoy et al., 2017). Soybean is an excellent source of food, protein and oil and, therefore, more research is essential to increase its yield under various conditions, and ameliorate its response to various forms of stress (Pagano and Miransari, 2016). Soybean yields are heavily affected by nutrient deficiencies worldwide, especially iron, as they are the second most iron rich crop (Aksoy et al., 2017). Iron is an essential micronutrient, as it is a component of the electron transport chain in the chloroplasts and mitochondria, and is vital for the development of chloroplasts and synthesis of chlorophyll (Connorton et al., 2017). Although abundant in the Earth's crust, iron is not present in the readily available form, Fe (II), resulting in iron limitation which severely affects plant growth and productivity (Connorton et al., 2017). Iron deficiency is characteristic of calcareous soils, well-known for their high pH, high levels of calcium carbonates and poor drainage (Franzen and Richardson, 2000; Goos et al., 2019). Plants exposed to limited iron exhibit iron deficiency chlorosis (IDC), characterized by interveinal yellowing, stunted growth and reduced final yield (Barker, 2018). To mitigate adverse effects and symptoms, plants have developed strategies to enhance iron acquisition from the soil. This includes Strategy 1 accomplished by non-graminaceous species, and Strategy 2 common to grasses. Non-graminaceous plants, like soybean, acquire iron in two-step mechanism. Firstly the rhizosphere is acidified by the activities of H^+-ATPases, a process facilitating the reduction of Fe(III) to the more soluble form Fe(II). This reduction is mediated by the membrane-bound ferric-chelate reductase oxidase protein (FRO2). The subsequent uptake of Fe(II) is governed by the iron-regulated transport protein (IRT1) (Santi and Schmidt, 2009). Grasses utilize a

chelation-uptake mechanism, which is accomplished through the release of phytosiderophores into the rhizosphere (Kobayashi and Nishizawa, 2012). However, iron acquisition from the soil is not the only limiting step in iron usage; plants may contain sufficient endogenous iron and yet display IDC due to the presence of insoluble Fe(III). As in the soil, Fe(III) must be reduced to Fe(II) to facilitate deliverability to iron-requiring cells and organelles like chloroplasts (Guerinot and Yi, 1994). Mechanisms by which iron is reduced and transported into the chloroplast remain obscure; however, it is believed that the activities of Ferric-chelate reductase oxidase (FRO7) and Permease in Chloroplast 1 (PIC1) are essential (Solti et al., 2012). Iron reduction mechanisms have also been noted to be influenced by nitric oxide (NO), a bioactive free radicle that is implicated in various physiological processes related to abiotic and biotic stress conditions. Nitric oxide reacts with insoluble Fe(III), producing active Fe(II) and NO^+, this was demonstrated by Graziano et al (2002) when exogenous application of NO prevented interveinal chlorosis of corn (*Zea mays*) leaves exposed to iron stress.

Apart from the reducing mechanisms utilized by plants, iron movement and deliverability are also dependent upon the integrity of cellular membranes, which are jeopardized under stress conditions due to the production and accumulation of reactive oxygen species (ROS) (Gill and Tuteja, 2010). Nitric oxide can mitigate levels of ROS directly (Palavan-Unsal and Arisan, 2009) or indirectly, by inducing signal cascades which activate the antioxidant system (Siddiqui et al., 2011). The antioxidant system comprises enzymatic and non-enzymatic components (Ramírez et al., 2013), and relies mainly on the ascorbic acid-glutathione (ASC-GSH) redox cycle. Besides alleviating the effects of ROS, the activation of the antioxidant machinery can also influence plant responses to IDC as ASC has been shown to reduce Fe(III) to Fe(II) (Teucher et al., 2004).

A common factor linking abiotic stress, NO and the antioxidant system are phytoglobins (Pgbs), heme-containing proteins that participate in NO-scavenging, consequently regulating NO-mediated responses (reviewed by Hill, 2012). Their expression level influences plant response to abiotic and biotic stress. Specifically, by limiting accumulation of NO, the over-expression of *Pgb* has been noted to exercise a protective role during conditions of hypoxia, drought, and excess moisture (Dordas, 2009; Mira et al., 2017; 2016; Youssef et al., 2016). In contrast, an elevation in NO as a result of suppressed *Pgb* levels enhances plant response to biotic stress (Owusu et al., 2019).

The objective of this thesis was to investigate the role of Pgb during IDC in soybean by exposing commercial varieties and plants over-expressing or down-regulating the *Glycine max Pgb1* (*GmPgb1*) to iron-sufficient and iron-deficient conditions using an optimized hydroponic system. The hypothesis is that an increase in NO, as a result of reduced expression of *GmPgb1*, confers tolerance to IDC by changes in the ascorbate-glutathione redox system leading to elevated levels of Fe(II).

1.2 Mineral nutrition and iron requirement

Macro and micronutrients are vital for a plant to achieve optimal growth and development, and to complete its life cycle. From an agricultural standpoint, nutrients greatly impact plant productivity and ultimately final yield. To date, 14 elements have been deemed as essential for plant nutrition, these include 6 macronutrients: N, P, K, S, Ca and Mg, and 8 micronutrients: Fe, Zn, Cu, Mn, Cl, Mo, Co and Ni, which are taken up by the roots as ionic forms (Maathuis and Diatloff, 2013). Although micronutrients are present at a much lower concentration in the plant and in the soil, they are of equal importance to that of macronutrients (Halvin et al., 2014). In the case that one or more of these elements is lacking, plants will fail to meet optimal yields (Alloway, 2008). Therefore, understanding how micronutrients are taken up and utilized is essential for optimizing crop production and managing micronutrient deficient soils.

Iron (Fe), is a micronutrient that is essential for plant growth and development, and helps regulate many cellular processes (Zhang et al., 2019). Iron serves as a structural component of porphyrin molecules, which are involved in the redox-oxidation processes of photosynthesis and respiration. Additionally, Fe is required for chlorophyll synthesis and implicated in chloroplast development (Connorton et al., 2017).

Despite the fact that iron is abundant in cultivated soils, its solubility, and thus its availability for plants, is typically very low in calcareous soils characterized by high pH (Kim and Guerinot, 2007). When exposed to low soil iron levels, or insoluble iron, plants develop IDC, a limiting factor in crop production. Symptoms of IDC include interveinal chlorosis,

decreased photosynthesis, stunted growth, and ultimately reduced yield (Barker, 2018). As for any form of abiotic stress condition, plants have developed various mechanisms to alleviate the effect of iron deficiency and increase the amount of iron forms that can be utilized for key biochemical and physiological processes (Zhang et al., 2019). These mechanisms will be described in the next section of this thesis.

1.2.1 Iron acquisition

Plants acquire iron from the rhizosphere as both Fe(III) and Fe(II). However, availability and solubility depends on soil pH. The uptake of iron is impaired in soils with a neutral or basic pH, where iron is immobilized and not available for plant uptake. To cope with the limitations, plants have evolved various structural, physiological and molecular adaptations (Guerinot and Yi, 1994; Krohling et al., 2016). Over time, to facilitate the intake of iron, plants have developed two strategies. Strategy 1 is common in all dicots and non-graminaceous monocots, whereas strategy 2 is typical of grasses (Guerinot and Yi, 1994).

Non-graminaceous plants, such as soybean and *Arabidopsis*, are known to rely on a reductive-uptake mechanism converting Fe(III) to the more mobile Fe(II). Reduction of iron is slow in neutral pH soil and therefore, the rhizosphere is acidified via H^+-ATPase activities extruding protons in the apoplast lowering the pH of the soil, a condition favouring the reduction of Fe(III) (Guerinot and Yi, 1994). Mechanisms of iron reduction rely upon the action of several genes, including *ferric-chelate reductase oxidase* (*FRO2*) and *iron-regulated transport* (*IRT1*) (Kobayashi and Nishizawa, 2012). *FRO2*, is responsible for the reduction step converting Fe(III) to Fe(II), while *IRT1* translocates Fe(II) across the plasma membrane initiating its symplastic

movement (Mukherjee et al., 2006). Perception of iron deficiency upregulates the expression of *FRO2* in the epidermal cells of the roots, and *IRT1* on plasma membranes of the epidermal cells. Increased expression of both genes, contributes to enhanced tolerance to iron deficiency (Kim and Guerinot, 2007). This increase was not observed in susceptible genotypes exhibiting chlorotic symptoms of iron deficiency.

The second strategy of iron acquisition is typical of grasses and involves a chelation-uptake mechanism, which is characterized by the release of phytosiderophores (Zhang et al., 2019). Phytosiderophores are members of the mugineic acid family, and are biosynthesised in response to iron-deficient conditions. The type and amount of phytosiderophores released in the rhizosphere is genotype and species specific. Plants tolerant to iron deficiency are generally able to release a greater amount of phytosiderophores than susceptible plants (Kim and Guerinot, 2007). Once secreted into the rhizosphere, phytosiderophores form strong chelates with Fe(III) to increase solubility. The Fe(III)-phytosiderophore complexes are then imported into the root epidermis cells by specific transporters, the most characterized of which is Yellow Stripe 1 (YS1) (Kobayashi and Nishizawa, 2012).

Once iron is imported in the epidermal cells it needs to be translocated to above ground plant tissues and organs via long distance transport. Through symplastic movement, iron reaches the pericycle from where it is loaded into the xylem and transported upward through the transpiration stream (Morrissey and Guerinot, 2009). Efficient movement of iron in the xylem is facilitated by the formation of iron-citrate complexes (Green and Rogers, 2004), and the participation of several transporters such as Ferric Reductase Defective 3 (FRD3), Ferroportin1

(FPN1), and Oligopeptide transporter 3 (OPT3) (Kim and Guerinot, 2007). The *Arabidopsis FRD3* gene encodes plasma membrane efflux transporter that mediate the translocation of iron-citrate complexes in the xylem. Mutation of this gene causes phenotypic defects at different stages of development that can be rescued by exogenous supplementations of iron and citrate (Roschzttardtz et al., 2011). *Ferroportin1* is also involved in vascular loading, and iron deficiency symptoms are observed if this gene is mutated (Morrissey and Guerinot, 2009). Distribution of iron within plant tissues/organs is mainly mediated by the phloem, and several plasma membrane transporters including OPT3 are required for the loading of iron into companion cells (Mendoza-Cózatl et al., 2014).

1.2.2. Translocation of iron into chloroplasts

Roughly 80-90% of iron within the plant is located in chloroplasts (Solti et al., 2012). Unlike root iron acquisition, the mechanisms by which iron is translocated into chloroplasts are not well defined. The presence of two distinct membranes in the chloroplasts might require specific iron intake mechanisms that differ from those of eukaryotic cells (Solti et al., 2014). It is suggested that factors participating in iron translocation across the outer and inner chloroplast membranes include the activity of H^+-ATPase and Fe(III) reductase, and the involvement of Fe(II) transporters, similar to that of the root (López-Millán et al., 2016; Morrissey and Guerinot, 2009) . Despite the limited information available, it has been suggested that translocation of iron through the inner membrane of chloroplasts requires Ferric-Chelate Reductase Oxidase 7 (FRO7) and Permease in Chloroplast 1 (PIC1). In sugar beet (*Beta vulgaris*) FRO7 is localized on the inner envelope of the chloroplast, and is responsible for the reduction Fe(III) to Fe(II) (Solti et al., 2012).

The reduction step utilizes NADPH likely produced during the Hill reaction; an observation linking iron acquisition to photosynthesis (Solti et al., 2014, 2012). PIC1 is a chloroplast inner envelope iron transporter that facilitates the movement of both, Fe (III) and Fe(II) across the inner envelope (Jain et al., 2014; Solti et al., 2014, 2012).

1.3 Iron deficiency in soybean

Iron is an essential micronutrient for many plant processes. However, although abundant, iron cannot be readily used by plants, making iron deficiency one of the most widespread nutritional deficiencies. It has been estimated that roughly 30% of the world's agricultural land is affected by iron deficiency. Producers sowing into iron-deficient soils will often observe IDC (Aksoy et al., 2017), which contributes to reduced yield typically observed in calcareous soils (Miller et al., 1984).

Soybean is known to be exceptionally rich in iron, and thus highly susceptible to iron deficit. Soybean plants grown under iron-deficient conditions exhibit chlorotic leaves shortly after emergence and can remain yellow for an extended period of time prior to maturity (Aksoy et al., 2017; Franzen and Richardson, 2000). Soybean grown in iron-deficient soils are prone to significant yield loss. MacMillan (2018) documented that in growing seasons in plants which experience severe IDC, soybean yields were reduced by 20 bu/ac with each 1-unit increase in visual IDC rating (rating assigned from 1 to 5, with 1 being the most tolerant and 5 being the most susceptible) at the fifth trifoliate through to the beginning of blooming (Fig. 1.1). It has been noted that yield losses are limited if recovery from iron deficiency occurs before the fifth-

sixth trifoliate stage; however, if chlorosis persists longer, yield is severely penalized (Franzen and Goos, 2016)

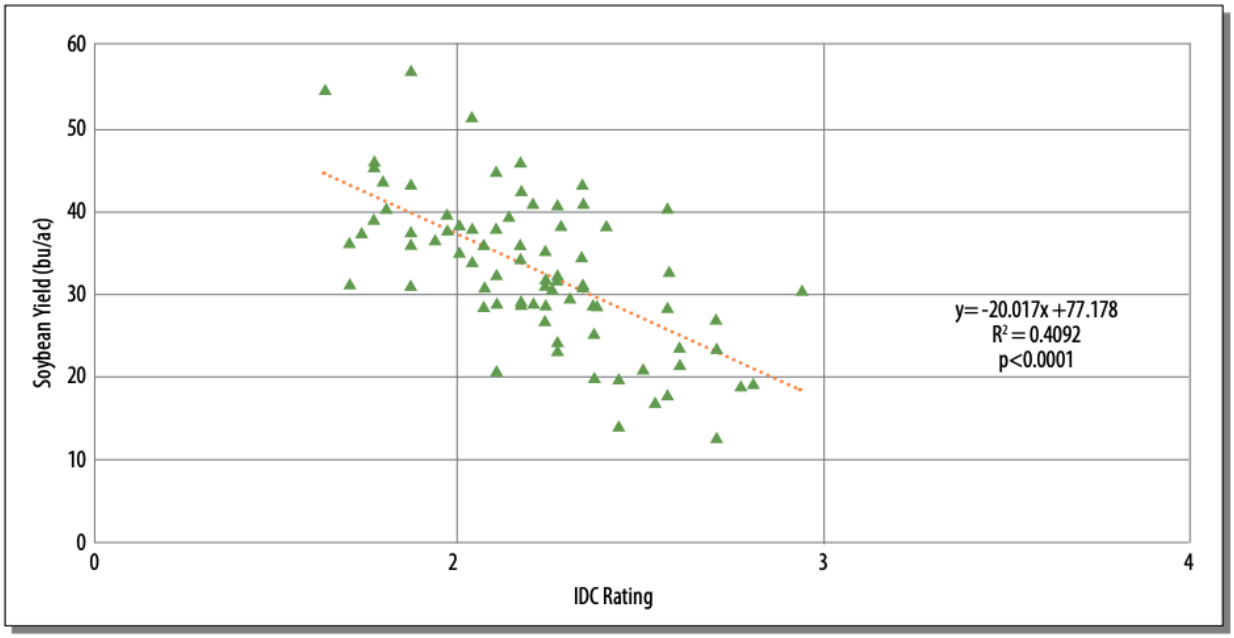


Figure 1.1. Soybean yield decrease with increasing iron deficiency chlorosis. Data was collected in 2017 in Manitoba. Figure is from MacMillan (2018) and permisson has been obtained from the copyright holder to incoporate it in this thesis.

Economic loss to iron deficit can be relevant. It has been estimated that annual yield loss in soybean surpasses $120 million in the western Corn Belt and the Great Plain Region of United States (Santos et al., 2019). Despite the efforts to develop iron-tolerant varieties through the implementation of molecular techniques and breeding programs, IDC remains one of the most predominant factor limiting soybean production (Hansen et al., 2004)

1.3.1. Environmental factors influencing iron availability

Soil characteristics and environment are determining factors for iron availability. Crops grown in calcareous alkaline soils, characterized by coarse texture and low organic matter are more prone to exhibit symptoms of iron deficiency (Boodi et al., 2016). The pH and redox state of the soil often determine the forms of iron present. For example at neutral pH iron tends to precipitate and become less available, while acidic environments favor its solubility and mobilization (Colombo et al., 2014). Calcareous soils, conducive to IDC, are characterized by a high level of calcium carbonates and a high pH (Franzen and Richardson, 2000). It has been well documented that bicarbonates have the ability to induce iron deficiency stress and their concentration is affected by soil carbonates, exchangeable bases (Mg, Ca, P and Na), soil moisture and CO_2 concentration. A clear positive correlation between soil carbonate content and chlorosis was established by Hansen et al. (2004). Introduction of bicarbonates into the soil occurs mainly through limestone and dolomite degradation, as well as irrigation water (Poschenrieder et al., 2018). In grapevine it has been established that the bicarbonate induction of IDC occurs through alterations in expression of genes encoding ferric reductase, iron transporters and H^+-ATPase, thereby preventing the movement of iron into the plant (Covarrubias and Rombolà, 2013).

Drainage can also affect iron availability. Plants grown in soils that are poorly drained and waterlogged typically exhibit iron-deficient symptoms. This is due to the inability of oxygen to diffuse in the soil, and more importantly to the retention of CO_2 which dissolves in the soil contributing to the formation of bicarbonate ions. The resulting elevation in pH inhibits the

activity of iron-reducing proteins thus limiting iron availability (Goos et al., 2019; Halvin et al., 2014).

1.3.2 Changes in plant structure and physiology during iron deficiency

As described above, iron is fundamental for many key processes including photosynthesis, respiration and nitrogen metabolism. It is therefore not surprising that major structural and physiological processes are altered by the level of available iron. The following section deals with major changes observed at the root level and in photosynthetic tissues.

1.3.2.1 Root morphology

Conditions of stress related to nutrient availability have a significant impact on the root system, the main organ perceiving the initial stress. Plants respond to adverse environmental conditions by inducing a morphogenic response. Restriction in nutrient availability has a significant effect on the root system architecture, including alteration in root number, length, orientation, diameter and root hairs (Li et al., 2016). Changes in morphology are highly dependent on species and/or genotype, as well as on the severity of nutrient deficiency. In corn (*Zea mays*), it was observed that severe iron deficiency can cause stunting of the entire root system, whereas in sunflower (*Helianthus annuus*) there was no sign of reduced cell division but rather a change in the direction of cell division, resulting in an increase in diameter at the expense of root length (Römheld and Marschner, 1981). In general, in response to moderate IDC plants increase their absorptive surfaces by triggering the formation of root hairs, and by promoting the elongation of lateral roots through changes in auxin synthesis and response. Both phenotypical adjustments are essential for the root to exploit more iron patches in the soil (Li et al., 2016).

1.3.2.2 Chlorophyll and chloroplasts

Within the cell, iron is complexed with chelators, molecules that tightly bind metal ions and mediate their distribution in sink tissues including chloroplasts. Besides being an essential component in the photosynthetic electron transport chain, iron is required as a cofactor for many enzymes including those involved in chlorophyll biosynthesis (Connorton et al., 2017; López-Millán et al., 2016). Among the most prominent iron compounds found in the chloroplast are the Fe-S centers of Photosystem II, heme groups and Rieske Fe-S proteins of Cytochrome b_6-f, and several iron-containing constituents including Ferredoxin of Photosystem I (Norman and Abadia, 1986). Since several enzymatic processes utilize soluble Fe(II), it is not surprising that impaired reduction activity can aggravate conditions of iron deficiency even in the presence of elevated Fe (III) levels (Graziano et al., 2002). Insufficient levels of Fe(II) have been linked to low amounts of photosynthetic pigments including chlorophyll a, chlorophyll b, carotene and xanthophyll. This decrease in photosynthetic pigments was also accompanied by a reduction in photosynthetic electron transport, an observation confirming the requirement of iron for the production and function of electron carriers (Graziano et al., 2002).

Structural changes in chloroplast membranes also occur under conditions of iron deficit. Norman (1980) documented a reduction in granal and stomatal lamellae of chloroplasts of various monocots and dicots grown with insufficient iron. These changes were associated with a 50% reduction in overall chloroplast proteins, including structural components of the lipid bilayer (Shetty and Miller, 1966)

One of the most prominent symptoms of IDC is a reduction in the content of chlorophyll, the synthesis of which requires Fe(II) (Norman and Abadia, 1986). Ferrous iron is needed for the function of both non-heme enzymes and iron porphyrin enzymes of chlorophyll, and under iron limiting conditions the quantity of these proteins is significantly reduced. Many studies highlight the importance of iron for the synthesis of δ-aminolevulinic acid, which is a key precursor of porphyrin (Marsh et al., 1963; Norman and Abadia, 1986)

1.3.2.3 Reactive oxygen species accumulation during iron deficiency

Adverse growth conditions, including those observed during iron deficit, often result in an imbalance of the cellular redox status contributing to the overproduction of reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical (OH^-). As iron is fundamental for several components of the electron transport chain in the chloroplast and mitochondria, under iron deficiency normal electron transfer is disrupted, a condition contributing to the overproduction of ROS (Ramírez et al., 2013). This is detrimental to many cellular components as ROS can cause damage to proteins, lipids, carbohydrates, and DNA, and ultimately lead to cell death and crop yield loss (Gill and Tuteja, 2010). Besides elevating the level of ROS, IDC also influences the cellular antioxidant machinery which is utilized to scavenge ROS, thus limiting their negative effects (Sun et al., 2007).

Activated under conditions of abiotic and biotic stress, the antioxidant machinery comprises various enzymes including catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), as well as antioxidants such as ascorbic acid (ASC) and glutathione

(GSH). The combined action of these components mitigates oxidative stress (Ramírez et al., 2013). Superoxide dismutase catalyses the dismutation of O_2^- to H_2O_2, while CAT and APX catalyzes the conversion of H_2O_2 to H_2O. The latter enzyme requires ASC as a substrate.

Iron is a cofactor of various antioxidant enzymes, and therefore iron-deficient plants are most vulnerable to oxidative damage. On the other hand, those that are more iron-efficient have the ability to activate the antioxidant system to mediate ROS production (Santos et al., 2019). The ascorbic acid-glutathione redox system plays a key role during IDC, and one of its components, ASC, not only scavenges ROS, but also increases iron availability by reducing Fe(III) to Fe(II) (Teucher et al., 2004).

1.4 Ascorbic acid-glutathione redox system

The overproduction of ROS stimulates the synthesis of ASC and GSH, and the activity of antioxidant enzymes (Ramírez et al., 2013). Iron-efficient plants have the ability to utilize this mechanism to detoxify ROS and restore redox homeostasis. The ASC-GSH cycle works to reduce H_2O_2 to H_2O by oxidizing ASC and GSH. Firstly, APX utilizes ASC as an electron donor to reduce H_2O_2 to H_2O in a reaction generating monodehydroascorbate (MDHA), which is then converted to dehydroascorbate (DHA). Reformation of ASC from the oxidized forms is mediated by the NADPH-dependant reducing enzyme, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) utilizing GSH. Formation of GSH can also occur from oxidized GSH (GSSG) through the action of glutathione reductase (GR) using NADPH (Foyer and Shigeoka, 2011; Ramírez et al., 2013). One of the main functions of this cycle is to replenish ASC and GSH, effective antioxidants keeping ROS below toxic levels. As

stated above, besides its antioxidative properties, ASC can reduce iron. This was initially discovered in humans where alterations in iron homeostasis led to serious clinical issues that can contribute to iron deficiency anaemia and haemochromatosis (Atanassova and Tzatchev, 2008). The high occurrence of these disorders led to extensive research regarding intestinal iron absorption, which maintains homeostasis of the element. Previous knowledge regarding the role of ASC in enhanced absorption of dietary iron in humans, contributed to the discoveries that ASC plays a significant role in reducing Fe(III) to Fe(II) in the intestinal lumen allowing for increased solubility and absorption of iron. Similar mechanisms might operate in plants as iron-deficient *Arabidopsis* seedlings supplemented with exogenous ASC or GSH displayed high levels of chlorophyll (Ramírez et al., 2013). This study, however, does not provide any link related to the ability of ASC and GSH to reduce iron. While not studied in the context of IDC, the ability of ASC to alter iron availability through reduction of Fe(III) to Fe(II) has been documented during pea seed development where, unlike other organs, iron reduction is not catalyzed by membrane-bound ferric reductases (Grillet et al., 2013).

1.5 Nitric oxide and iron deficiency

Nitric oxide (NO) is a bioactive free radical implicated in numerous physiological functions where it acts as a signalling molecule regulating developmental and stress responses (Graziano et al., 2002; Palavan-Unsal and Arisan, 2009). Due to its gaseous nature, NO can diffuse freely through lipid membranes and most of our knowledge on this molecule derives from studies on animal systems. While the mechanisms of NO synthesis in plants are not fully understood (Wilson et al., 2008), NO synthesis in animals is mediated by nitric oxide synthase (NOS), which catalyses the oxidation of arginine to NO and citrulline. Plants however, utilize a

different enzyme, Nitrate Reductase (NR) which can generate NO from nitrite (NO_2^-) using NADH as an electron donor (Palavan-Unsal and Arisan, 2009). Production of NO by NR is enhanced under stress conditions, as observed in experiments conducted under anaerobic conditions and pathogen infection (Palavan-Unsal and Arisan, 2009; Wilson et al., 2008).

Perception of NO in animals is mediated by soluble guanylyl cyclase (sGC). Binding of NO to sGC triggers an increase in cyclic GMP (cGMP), which activates numerous downstream signalling processes, including the modulation of intracellular Ca^{2+}-channels to elevate cytosolic calcium levels important for many signal cascades (Arasimowicz and Floryszak-Wieczorek, 2007). A drought related study argues for a similar mechanism in plants. It was observed that cGMP is positioned downstream of ABA-induced changes in NO and upstream of the increase in cytosolic Ca^{2+} concentration in the signaling pathway responsible for stomatal closure (Dubovskaya et al., 2011).

Among the many functions played by NO during abiotic and biotic stress conditions, including IDC, are those related to its ability to influence ROS homeostasis and reduce iron. A significant amount of work involving the pharmacological application of the NO donor sodium nitroprusside (SNP), and the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) has been used to discern the role of NO (Wilson et al., 2008). Research suggests that NO can mitigate the level of ROS by 1) reacting with ROS directly (Palavan-Unsal and Arisan, 2009) or 2) inducing signals which activate ROS scavenging enzymes (Siddiqui et al., 2011). Due to the existence of an unpaired electron, NO can react directly with some ROS, thereby protecting the plant from oxidative damage. On the other hand,

iron-deficient corn (*Zea mays*) plants supplemented with SNP exhibited a significant reduction in the accumulation of H_2O_2 and O_2^-. This was attributed to the ability of NO to increase the activity of SOD, CAT and APX, thereby attenuating oxidative damage (Siddiqui et al., 2011).

Due to its very reactive nature, NO also has a high affinity for transition metals including iron, which allows for the formation of complexes known as iron-nitrosyl complexes. For instance, Fe(III) can form a complex with NO, which undergoes a charge transfer reaction to form $Fe(II)NO^+$. This property is beneficial to iron-deficient plants, where endogenous iron may be present as Fe(III) which is not readily usable by chloroplasts unless reduced. This was demonstrated in corn where applications of exogenous NO completely prevented leaf interveinal chlorosis by rendering iron more available (Graziano et al., 2002). Based on these observations it is apparent that factors influencing NO levels and homeostasis can have an impact on how plants respond to iron deficiency.

1.6 Phytoglobins

Phytoglobins (Pgbs), or plant hemoglobins, are heme-containing proteins present in plants (Hill et al., 2016). They are commonly recognized for their ability to reversibly bind oxygen, and most importantly scavenge NO, thus interfering with many NO-related processes (reviewed by Hill, 2012). Most organisms contain several different Pgbs that range in size, structure and function (Dordas, 2009). Overall, Pgbs can be subdivided into symbiotic, non-symbiotic and truncated (Gupta et al., 2011).

Symbiotic Pgbs, also known as leghemoglobins, were first isolated from soybean root nodules (Kubo, 1939). These proteins are found in the root nodules of nitrogen-fixing plants which partake in a symbiotic relationship with *Rhizobium* bacteria (Gupta et al., 2011). Leghemoglobins are responsible for maintaining low levels of oxygen in the root nodules to ensure optimal functionality of the nitrogen-fixing enzyme, nitrogenase; an enzyme highly sensitive to oxygen. Leghemoglobins buffer nodule oxygen concentrations preventing nitrogenase breakdown, while supporting mitochondrial oxidation to promote fixation within the nodules (Appleby, 1992).

In contrast to the symbiotic Pbgs, non-symbiotic Pgbs are thought to exist throughout the entire plant kingdom (Dordas, 2009), where they participate in many developmental and stress responses, including seed development and germination, flowering, root development and differentiation, as well as abiotic and biotic stress (Hill, 2012). Based on phylogenic analyses and expression patterns, non-symbiotic Pgbs have been divided into two main classes, Class 1 and Class 2.

Class 1 Pgbs have a weak hexacoordinate structure and are known to have a very high affinity for O_2, which makes them incapable of transporting O_2 or being sensors and storage molecules due to the fact that they remain oxygenated under low oxygen levels (Dordas, 2009). Rather, the main function of class 1 Pbgs is to scavenge NO, thus regulating many NO-mediated responses (Hill, 2012). Expression of class 1 Pbgs is induced in response to exposure to various stresses including osmotic stress, nutrient deficiency, hypoxia, cold stress, exposure to darkness and fungal infection, and to plant hormones such as jasmonic acid, cytokinin, abscisic acid and

ethylene (Dordas, 2009). Pgbs scavenge NO through the oxygenation of NO to form nitrate (NO_3^-) and methemoglobin, in a cycle outlined in Figure 1.2.

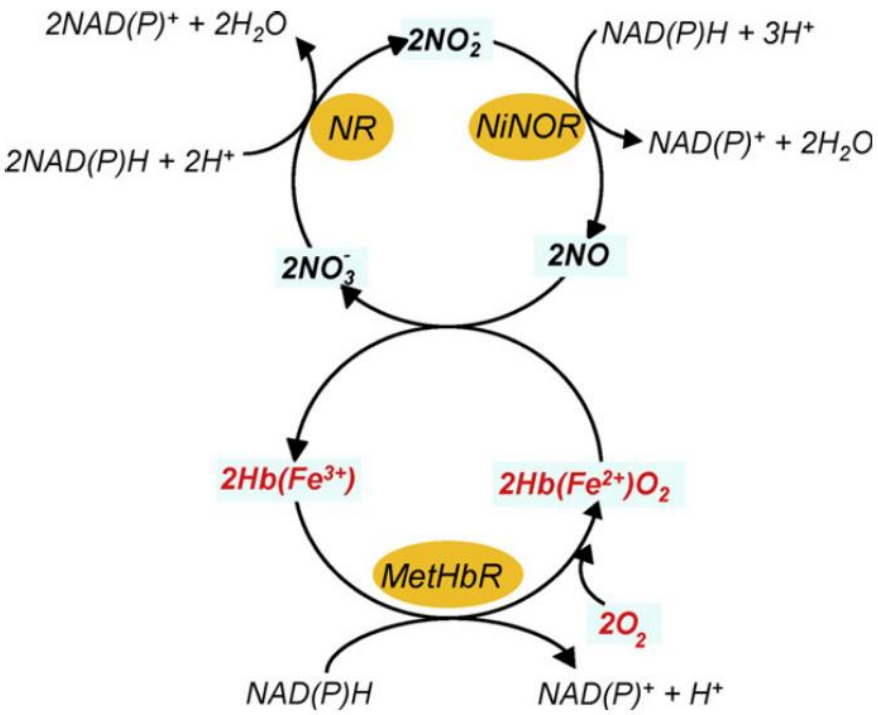


Figure 1.2. NO scavenging mechanism of class 1 Pgb under stress conditions. Conversion of NO to nitrate via the oxygenation of Fe^{2+} hemoglobin. (Gupta et al., 2011)

How Pgbs influence plant behavior under stress is best exemplified during hypoxic conditions. In corn plants exposed to flooding, Pgb expression increases rapidly and high levels of Pgbs confer tolerance to hypoxia by reducing the levels of NO (Youssef et al., 2016). The Pgb protective effect is operative under other types of stress, including drought, as described by (Mira et al., 2017). Class-1 Pgbs also modulate plant pathogen interaction, as suppression of Pbgs reduced disease severity symptoms in corn plants infected with *Clavibacter nebraskensis*. The increased levels of NO promoted enhanced tolerance through ROS-mediated programmed cell death (Owusu et al., 2019).

Unlike class 1 Pgbs, the less studied class 2 Pgbs have a fully hexacoordinate structure and a much lower affinity for O_2 (Hill, 2012), although they have demonstrated NO-scavenging properties. Class 2 Pgbs are less widely inducible by stress, yet have been found to be expressed under cold stress and following cytokinin treatment (Dordas, 2009). Their expression is related to the maintenance of the cellular energy status, as observed during *Arabidopsis* seed development, where their seed-specific over-expression enhances oil synthesis through an elevation in ATP levels (Vigeolas et al., 2011).

The third type of Pgbs are known as truncated Pgbs, due to their variation in structure. These proteins have a "2-on-2" helical fold rather than the typical "3-on-3" alpha helical "sandwich fold" (Dordas, 2009). Truncated Pgbs are the most recently discovered type of Pgbs and are believed to be expressed throughout the plant. Truncated Pgbs were first recognized for their ability to bind and transport O_2 (Watts et al., 2001). However, more recent research in the green alga *(Chlamydomonas reinhardtii)* suggests that truncated Pgbs play a role in cell signaling that affect the nitrogen assimilation pathway by simultaneously modulating NO levels and NR activity (Sanz-Luque et al., 2015b; 2015a). Expression of truncated Pgb is upregulated in response to NO, similarly to Class-1 Pbgs; however, detoxification of NO occurs through NO dioxygenase (NOD) activity, producing NO_3^- from NO and O_2. Activity of NOD is maintained through a mechanism that removes electrons from NR, thereby altering NR activity and ultimately NO production (Sanz-Luque et al., 2015b).

1.6.1 Phytoglobin and stress response

It is widely accepted that class 1 Pgb gene expression is regulated rapidly following exposure to various forms of biotic and abiotic stress, and this influences plant response and behavior (Hill, 2012). Much of the early stress-related work focuses on the effect of Pgbs on plant metabolism and growth under hypoxia. The protective role of Pgbs through the reduction of NO and the maintenance of a high energy status has been well documented under oxygen deficit (reviewed by Hill, 2012). The effects of altered Pgb expression are manifested in many organs, including shoot and root. Youssef et al. (2016) observed increased tolerance of corn plants to waterlogging when *Pgb* was upregulated. Over-expression of Pgb alleviated stress and contributed to the retention of photosynthetic rate, possibly by attenuating the RO-induced damage to photosynthetic tissue and increasing the antioxidant machinery, as evidenced by the elevated activities of SOD and APX (Youssef et al., 2016). Recent studies also showed that the expression of *Pgb* is essential for plant growth under water-deficient, as Pgbs participate in acclimation of the root cells to stress. In corn, the over-expression of *Pgb* contributes to the retention of meristematic function of the root apical meristem (RAM) by suppressing programmed cell death (PCD) triggered by lack of oxygen. The protective role of Pgbs during drought is linked to their ability to reduce PCD in the root tip, likely by modifying NO levels, which influence ethylene synthesis and response, and ultimately ROS accumulation (Mira et al., 2017).

The role of Pgbs in plant pathogen interaction has also been documented, although the mechanisms are not fully understood perhaps as a result of often contradictory observations (Owusu et al., 2019). While Pgb was induced following inoculation with *Verticillium dahlia* (Qu et al., 2006), its suppression was associated with enhanced pathogen-resistance in *Psedomonas*

syringae and *Botrytis cinerea* (Mur et al., 2012). More recently, a similar trend was observed in corn plants infected with *Clavibacter nebraskensis*, where suppression of *Pgb* reduced lesion size (Owusu et al., 2019). Interaction between plants and pathogens is mediated by many signaling molecules like NO, and plant growth regulators such as ethylene, salicylic acid and jasmonic acid. Nitric oxide is essential in plant defense against pathogens, as it influences the hypersensitive response, a rapid and localized PCD at the infection site (Mur et al., 2012; Owusu et al., 2019). Following inoculation with *Clavibacter nebraskensis,* corn cells down-regulating *Pgb* exhibited elevated NO levels responsible for a rise in the transcript levels of ethylene biosynthesis and response genes. The accumulation of ethylene activated NADPH oxidase, a major source of ROS, which contributed to the induction of PCD, a process inhibiting the spread of the pathogen (Owusu et al., 2019).

1.6.2 Phytoglobin and the antioxidant machinery

As elaborated above, elevated levels of ROS are major consequences of many forms of abiotic and biotic stress, and their levels can be lowered by cellular antioxidant mechanisms, the most important of which is the ascorbic acid-glutathione cycle (Yordanova et al., 2004; Youssef et al., 2016). Several studies suggest an intimate link between the NO-scavenging properties of Pgbs and ascorbic acid, as well as the ability of Pgbs to influence the activity of many antioxidant enzymes, thus controlling cellular ROS homeostasis (Igamberdiev et al., 2006; Youssef et al., 2016). Methemoglobin is a product of the reaction of oxyhemoglobin with NO, and in order to sustain NO-scavenging the regeneration of oxyhemoglobin is essential. In barley *(Hordeum vulgares),* it has been suggested that MDHAR may play a role in regeneration of oxyhemoglobin from methemoglobin (Igamberdiev et al., 2006).

Altered expression of *Pgb* can modulate the activity of several antioxidant enzymes. Igamberdiev et al. (2006) observed that the up-regulation of *Pgb1* in hypoxic alfalfa roots enhanced the antioxidant status by increasing ASC levels and the activity of ROS-scavenging enzymes, APX, MHDAR and CAT. A similar trend was observed in waterlogged corn where up-regulation of *Pgb* stimulated the activities of APX and SOD responsible for catalyzing the disproportion of O_2^- to H_2O_2. Likewise, this study concluded that up-regulation of *Pgb* was essential for relieving ROS accumulation and associated damage, consequently increasing tolerance (Youssef et al., 2016). Taken together these studies suggest that experimental manipulations of Pgb expression can be used as a tool to alter the cellular antioxidant machinery and ultimately the level of ROS.

Based on the above information, the focus of this thesis is to investigate the role of Pgb during IDC in soybean with the hypothesis that the increase in NO as a result of suppression of *GmPgb1* enhances plant tolerance through changes in antioxidant responses, ROS accumulation, and iron availability.

2 CHAPTER ONE: Suppression of the soybean phytoglobin *GmPgb1* enhances tolerance to iron deficiency

2.1 Abstract

The effect of iron deficiency in soybean (*Glycine max* (L.) merr) seedlings over-expressing or down-regulating *Glycine max Phytoglobin 1* (*GmPgb1*) was examined using an established hydroponic system which mimicked iron deficiency in soil. Iron deficient soybean plants down-regulating *GmPgb1* displayed greater chlorophyll retention and decreased accumulation of reactive oxygen species relative to the *GmPbg1* over-expressing and wild type (WT) plants. Suppression of *GmPgb1* under iron deficiency conditions was also accompanied by elevated levels of ascorbic acid (ASC), and increased activities of catalase, superoxide dismutase and ASC recycling enzymes. When compared to WT and *GmPgb1* over-expressing lines, iron deficient *GmPgb1* suppressing plants also exhibited a higher Fe(II)/Fe(III) ratio. Pharmacological treatments altering the level of nitric oxide (NO) and ASC were further utilized to dissect the effects of *GmPgb1* during iron deficiency. Applications of the NO donor, sodium nitroprusside (SNP), enhanced tolerance to iron deficiency by elevating the levels of ASC, the activities of several antioxidant enzymes, and the Fe(II)/Fe(III) ratio. A similar effect on this ratio was also observed when ASC was administered exogenously. Collectively this study reveals that suppression of *GmPgb1* attenuates the effects of iron deficiency through changes in NO and antioxidant responses linked to the a higher availability of Fe(II).

2.2 Introduction

Soybean plants are susceptible to iron deficiency chlorosis (IDC) especially when grown on calcareous soils limiting the availability and acquisition of iron. Yield loss due to IDC can be significant (Froehlich and Fehr, 1981). Common methods for selecting IDC tolerant varieties are based on observations of foliar-chlorosis, although this practice can be often inaccurate due to the genotype by environment interactions (Charlson et al., 2005). An environment-independent approach includes the simulation of iron deficiency in controlled growth conditions such as hydroponic systems, where the effects of iron depletion can be dissociated from other confounding factors (Chaney et al., 1992). Plants use several physiological mechanisms to cope with iron deficit and have evolved two separate strategies favoring iron acquisition from the soil: acidification of the rhizosphere, carried out by non-grass species (Santi and Schmidt, 2009) or secretion of phytosiderophores, accomplished by grasses (Kobayashi and Nishizawa, 2012) . Furthermore, cellular iron can be present as Fe(III) or Fe(II) and in order to be translocated effectively within the plant tissue, Fe(III) must be reduced to Fe(II) (Guerinot and Yi, 1994). This reduction step is mainly controlled by membrane-bound ferric-chelate reductases (González-Vallejo et al., 2000; Solti et al., 2012), although nitric oxide (NO) (Stamler et al., 1992) and ascorbic-acid (ASC) (Grillet et al., 2013) may also serve as reducing agents.

Nitric oxide is an important signal molecule regulating a diverse range of physiological processes interfering with growth, development and adaptive responses to multiple forms of stress (Beligni and Lamattina, 2001). Exposure to stress, including iron deficiency, is associated with cellular redox imbalance as a result of toxic levels of ROS production. Nitric oxide is a free radical and can consequently exerts a protective effect against oxidative stress provoked by an

increase in ROS concentration (Lamattina et al., 2003). Nitric oxide has the ability to restore cellular redox homeostasis by either reacting with ROS directly, or by activating antioxidant enzymes (Sun et al., 2007). Furthermore it has been suggested that NO can prevent leaf chlorosis and improve internal iron availability in iron deficient plants (Graziano et al., 2002).

Another contributor to IDC tolerance is the antioxidant system, comprised of enzymatic and non-enzymatic components. Stress conditions are often associated with an elevation in antioxidants, especially ascorbic acid (ASC) and glutathione (GSH), and activities of redox enzymes required to limit the undesirable accumulation of ROS (Noctor and Foyer, 1998). Similar to the effects of NO, studies indicate the ability of ASC and GSH to attenuate chlorosis in plant subjected to iron deficiency (Graziano and Lamattina, 2005; M'sehli et al., 2014), with one study documenting that ASC increases seed iron availability through the reduction of Fe(III) to Fe(II) (Grillet et al., 2013).

Cellular components linking abiotic stress, NO and the antioxidant system are Phytoglobins (Pgbs), heme-containing proteins characterized mainly for their ability to scavenge NO, thus modulating NO-mediated responses (reviewed by Hill, 2012). Phytoglobins are divided into three categories: symbiotic, non-symbiotic (further divided into subclasses, 1 and 2) and truncated (Gupta et al., 2011). Under conditions of stress, *Pgbs* are transcriptionally induced and able to modulate plant response through adjustments in NO homeostasis (reviewed by Hill, 2012). It has been well documented that cellular NO levels decrease as a result of *Pgb* over-expression, while increase in those environments where *Pgbs* are suppressed (Dordas et al., 2003a; Mira et al., 2016).

To integrate and assess the role of Pgbs with NO and antioxidant responses during iron deficit, IDC was induced in soybean plants over-expressing or down-regulating the *Glycine max Pgb1* gene, *GmPgb1*. Plants were grown in a hydroponic system enabling precise manipulations in iron levels (Chaney et al., 1992), and targeted alterations of NO and antioxidants (Graziano et al., 2002; Ramírez et al., 2013). This experimental system was used to test the hypothesis that an increase in NO levels, as a result of *GmPgb1* suppression, enhances tolerance to iron deficit through changes in the antioxidant system and ROS accumulation.

2.3 Materials and Methods

2.3.1 Plant material and growth conditions

Fifteen commercially varieties of soybean commonly grown in Western Canada were provided by the Manitoba Pulse and Soybean Growers and Dennis Lange (Manitoba Agriculture). Soybean lines over-expressing or down-regulating the phytoglobin gene, *GmPgb1*, were both generated previously in the Williams 82 background, and available for this research. The over-expressing lines were generated through sense transformation, whereas the down-regulating lines were generated through RNA interference. The entire *GmPgb1* open reading frame (486 bp) was used for the over-expressing line and a short unique fragment (165 bp) was utilized for generating the down-regulating line. The two fragments were amplified from soybean cDNA using gene specific primers using Phusion™ High-Fidelity DNA Polymerase for amplification. The amplicons were inserted into pENTRT1A Dual Selection Vector (Thermo Fisher Scientific) as per the manufacturer's protocol, and the sequences were then confirmed through DNA sequencing. The fusion constructs were cloned into the final destination vector (PB2GW7 for the over-expressing line or PB7GWIWG2(II) for the down-regulating line) using the Gateway® LR Clonase® II Enzyme mix (Thermo Fisher Scientific), and then transformed into *A. tumefaciens* cells. The transformation was performed at the Iowa State University Transformation Facility as per Luth et al. (2014).

All commercial varieties were compared to control varieties, near-isogenic lines, Clark and Iso-Clark. Clark is tolerant to iron stress and does not exhibit interveinal chlorosis, whereas Iso-Clark is an iron-inefficient line and displays severe IDC symptomology (Severin et al., 2010).

Seeds were germinated on filter papers soaked with sterile distilled water. At the unifoliate stage, uniform seedlings were transferred into a hydroponic system described by Chaney et al. (1992), in which the iron concentration was manipulated to mimic either iron deficient or iron sufficient conditions. Plants were grown in a growth chamber under a 16 hour photoperiod at 22°C light/18°C dark and a light intensity of 400 μmol s^{-1} m^{-2}. Depending on requirements for the given experiment plants were in hydroponic for 7 (first trifoliate; V1 stage of development) to 21 days (third trifoliate; V3 stage of development).

2.3.2 Iron deficiency treatments

The hydroponic system described by Chaney et al. (1992) was representative of a calcareous soil type, containing bicarbonates which further influence iron deficiency in soybean. The system contained all required macro- and micronutrients (Chaney et al., 1992). Iron was the uniquely manipulated variable, in which two treatments were established: iron sufficient (control) conditions was represented by 60 μM Fe and deficient condition by 15 μM Fe. These two concentrations were selected empirically and small scale preliminary trials were conducted to verify the efficiency of noted iron concentrations. Hydroponic solutions were topped up as needed throughout the course of the experiment. Plants were randomly placed into the hydroponic system.

2.3.3 Screening and chlorophyll measurements

All commercial varieties were screened for their ability to tolerate low iron levels. Plants were collected at V1, V2 and V3 developmental stages and IDC was measured/assessed through chlorophyll quantification (Lichtenthaler, 1987). Tissue (80-110 mg fresh weight) from the

newest trifoliate developed in the treatment was sampled from each plant for chlorophyll quantification which was measured using a spectrophotometer after extraction in 80% acetone, as described by Sudhakar et al. (2016). Total chlorophyll content was quantified by measuring the absorbance at 645 and 663 nm and concentration was calculated using the following formulas:

$$\text{Chlorophyll a } (\mu g/ml) = 12.7\ (A_{663}) - 2.69\ (A_{645})$$

$$\text{Chlorophyll b } (\mu g/ml) = 22.9\ (A_{645}) - 4.68\ (A_{663})$$

$$\text{Total chlorophyll } (\mu g/ml) = 20.2\ (A_{645}) + 8.02\ (A_{663})$$

2.3.4 Pharmacological treatments

Ascorbic acid (ASC, 0.5 mM and 1.0 mM), glutathione (GSH, 0.5 mM and 1.0 mM), the nitric oxide (NO) donor sodium nitroprusside (SNP,100 μM), and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 200 μM) were prepared in water and used for foliar applications. Each solution (10ml) was sprayed uniformly on the leaves every other day during the treatment. Water was used as a control. Leaves were collected 24h after final application for analyses. The concentrations of the chemicals were selected based on previous studies (Graziano et al., 2002; Ramirez et al., 2013).

2.3.5. Antioxidant enzyme activity and quantification of ascorbic acid and glutathione

Activity of APX, DHAR, MDHAR, and GR were analyzed exactly as described by Zhang and Kirkham (1996). Leaf tissue (100-150 mg fresh weight) was collected at day 1 and immediately homogenized on ice with 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA and 1% (w/v) polyvinylpyrrolidone. The homogenate was then centrifuged at 18000 xg at

4°C for 15 minutes. The supernatant was collected for the analysis of enzyme activities (Zhang and Kirkham, 1996). Analysis was completed using a Bio-Tek Synergy H4 Microplate Reader.

APX activity was estimated by measuring the H_2O_2-dependent oxidation of ascorbic acid following the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}). The reaction mixture contained 0.5 mM ASC, 0.1 mM H_2O_2, 0.1 mM EDTA, and 50 mM sodium-phosphate buffer (pH 7.0).

DHAR activity was measured by following the GSH-dependent production of ASC at 265 nm (extinction coefficient 14 mM^{-1} cm^{-1}). The reaction mixture contained 2.5 mM GSH, 0.1 mM EDTA, 0.2 mM dehydroascorbate (DHA), and 50 mM sodium-phosphate buffer (pH 7.0).

MDHAR activity was measured by observing the change in absorbance at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 0.1 mM NADH, 2.5 mM ASC, 50 mM sodium-phosphate buffer (pH 7.6), and 4 units of ASC oxidase.

GR activity was determined by observing the NADPH-dependent oxidation of GSSG following the decrease in absorbance at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and 0.1 M sodium-phosphate buffer (pH 7.8).

For CAT and SOD enzyme assays (performed by a collaborator at the University of Calgary), leaf tissue (100-150 mg fresh weight) was homogenized on ice-cold 50 mM sodium-

phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 1% (w/v) polyvinylpyrrolidone. Homogenate was centrifuged at 15000 xg at 4°C for 20 minutes. Supernatant was then collected for enzyme activity analysis which was performed as described by Zhang and Kirkham (1996).

Protein contents of the crude enzyme extract were determined according to Bradford (1976).

2.3.6 Measurements of ascorbic acid and glutathione

For the analysis of ascorbic acid (ASC), dehydroascorbate (DHA), reduced (GSH) and oxidized (GSSG) glutathione, about 100-150 mg (fresh weight) of leaf tissue was first powdered in liquid nitrogen, and then homogenized with 2 mL of ice-cold 5% meta-phosphoric acid. Homogenate was centrifuged at 21000 xg at 4°C for 15 minutes. The supernatant was collected for spectrophotometrical analyses (Zhang and Kirkham, 1996).

2.3.7 Gene expression studies

For gene expression studies tissue from day 0, 1, 3 and 7 was collected and frozen in liquid nitrogen and stored at -80°C. TRI Reagent RNA Isolation Reagent (Invitrogen) was used to extract the RNA, and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis.

About 50 – 100 mg of fresh tissue was ground in liquid nitrogen and then was homogenized in 1 mL of TRI Reagent RNA Isolation Reagent (Invitrogen) with ceramic beads using a TissueLyser (6500 rpm for 45 seconds). Samples were then incubated at room temperature for 15 minutes and centrifuged at 12000 xg at 4°C for 15 minutes. The supernatant

was transferred to a clean 2 mL microcentrifuge tube, then 500 μL chloroform and 500 μL phenol (pH 4.3) were added. The solution was vortexed and then incubated at room temperature for 15 minutes. Tubes were centrifuged at 12000 xg at 4°C for 15 minutes. The aqueous phase was carefully transferred to a clean 1.5 mL microcentrifuge tube. 500 μL of 2-propanol and 3 μL NaOAc was added, the samples were then inverted and left to incubate for 10 minutes at room temperature. Samples were centrifuged again at 12000xg at 4°C for 15 minutes. The supernatant was then removed and discarded, leaving the RNA as a pellet. The RNA pellets were washed with 1 mL of 70% ethanol/DEPC water, vortexed and then centrifuged at 7500xg at 4°C for 5 minutes. The ethanol was removed and the pellet was air dried for ~5 minutes. Finally, the pellet was dissolved in 22 μL DEPC water.

DNase digestion was preformed using the DNase I recombinant RNase-free kit (Thermo Fisher Scientific). Samples were placed on ice, 3 μL of buffer and 1.5 μL of DNase I were added to each sample and then vortexed. The samples were incubated at 37°C for 30 minutes and then at 65°C for 5 minutes. Prior to the 65°C incubation period, 2 μL of 0.2 mM EDTA was added to each sample. Following incubation, 2 μL of NaOAc and 40 μL of 95% ethanol were added to each sample and vortexed; samples were then incubated at -20°C for a minimum of 1 hour and centrifuged at 21000 xg at 4°C for 45 minutes. The supernatant was removed and the pellets were washed with 1 mL 70% ethanol. Pellets were dissolved in 11.5 μL DEPC water.

The concentration and quality of RNA were determined using a Nanodrop spectrophotometer. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA. From the kit, 2 μL buffer, 0.8 μL dNTPs, 2 μL primer, 1 μL

enzyme and 4.2 μL DEPC water were added to each sample. Tubes were spun down and then incubated at 25°C for 15 minutes, 37°C for 2 hours, and then at 85°C for 5 minutes.

Quantitative real time PCR (qRT-PCR) was used to measure the relative gene expression of *GmPgb1*, which was then analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using helicase as the reference gene. (See list of primers in Supplementary Table 4.1).

2.3.8 Measurement of hydrogen peroxide

The concentration of hydrogen peroxide (H_2O_2) was determined by grinding tissue in 3 mL 10% acetone. The homogenate was centrifuged at 10000 xg at 4°C for 10 minutes. 1 mL of the supernatant was mixed with 0.1 mL 5% $Ti(SO_4)_2$ and 0.2 mL 35% ammonia. Following the formation of the precipitate, the reaction mixture was centrifuged at 10000 xg at 4°C for 10 minutes. The pellet was dissolved in 2 M H_2SO_4 and then the absorbance was measured at 415 nm. Levels of H_2O_2 were calculated according to the standard curve of H_2O_2.

2.3.9 Localization of reactive oxygen species

Visualization of ROS in leaf tissue was performed using nitroblue tetrazolium (NBT) to stain superoxide and diaminobenzidine (DAB) to stain H_2O_2, as described by Campbell et al. (2015). The first trifoliate leaves were removed from plants and placed into a 50 mL foil-wrapped falcon tube containing either 0.5mg/mL NBT solution in 10mM potassium phosphate buffer (pH 7.6) or 1 mg/mL DAB in 50 mM Tris-acetate buffer (pH 5). All tubes were incubated in the dark at 25°C (3 hours for NBT or 24 hours for DAB). Following the incubation period, the

leaves were boiled in 95% ethanol to remove the chlorophyll, and then rehydrated in a 10% glycerol solution for 24 hours. Leaves were then gently spread out and photographed.

2.3.10 Fe(II) and Fe (III) assays

Measurements were carried out on leaf tissue using the Iron Assay Kit (Sigma-Aldrich, MAK025), following the manufacturer's instructions. Briefly, about 30 – 40 mg of tissue was collected from the first trifoliate leaves and homogenized with the Iron Assay buffer. The samples were then centrifuged at 16000 xg at 4°C for 10 minutes. 20 μL of sample was transferred to the flat-bottom plate sample wells. To measure Fe(II), 85 μL of Iron Assay Buffer were added to each well. To measure total iron, 80 μL Iron Assay Buffer and 5 μL Iron Reducer were added to each well. Individual sample wells were mixed using a horizontal shaker and incubated in the dark at 25°C for 30 minutes. 100 μL of the Iron Probe was added and mixed to each well before incubation in the dark at 25°C for 60 minutes. Absorbance at 593 nm was measured using a SpectraMAX 340 Microplate Reader. The concentrations of iron were determined using a standard curve (as per manufacturer's instructions). The amount of Fe(III) was calculated by subtracting Fe(II) from the total iron.

2.3.11 Statistical analysis

Analysis of variance of the data was conducted by one way ANOVA using the SPSS program (IMB Corp. SPSS Statistics for Windows, Version 27.0). Treatment means were compared by Duncan test ($\alpha = 0.05$) to compare the significant differences. All experiments were conducted in three biological replicates, each consisting of trifoliate leaf tissue from at least three plants.

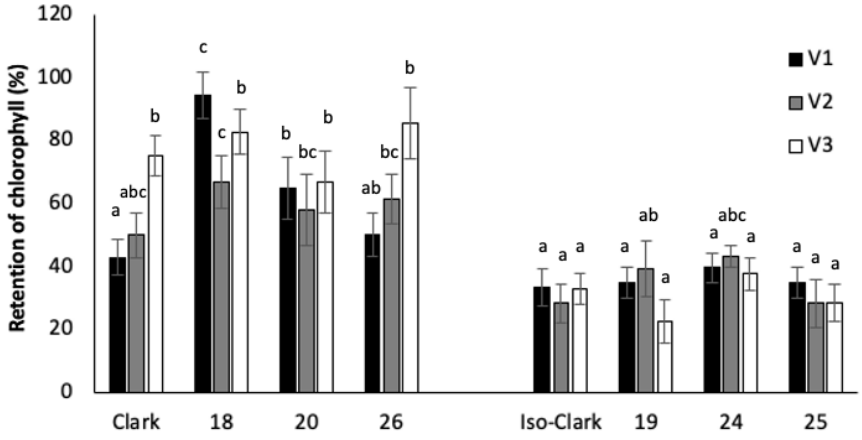
2.4 Results

2.4.1 Response of soybean plants to iron deficiency and *GmPgb1* expression

Chlorosis as a result of chlorophyll degradation, one of the first symptoms exhibited by plants growing under iron deficiency, was examined in the 15 commercial varieties and the two checks (Clark and Iso-Clark) at three different stages of development: V1-V3, corresponding to the formation of first, second and third trifoliate leaf set. Relative to iron sufficient conditions (60 µM Fe), chlorophyll content in the 15 varieties and two checks was reduced to different degrees under iron deficiency (15 µM Fe) (Supplemental Fig. 4.1). Based on these results, three varieties exhibiting the greatest retention of chlorophyll (18, 20 and 26), three varieties exhibiting the greatest reduction in chlorophyll (19, 24 and 25), as well as the two checks (Clark and Iso-Clark) were selected (Fig. 2.1A). These 8 varieties were utilized for all further experiments at the V1 stage of development (corresponding to 7 days of treatment), where the symptoms were first manifested.

To determine the potential relationships between chlorophyll retention and expression of *GmPgb1*, the transcript levels of this gene were measured over the course of 7 days in all selected commercial varieties exposed to iron deficit. As shown in Fig. 2.1B, the *GmPgb1* gene is down-regulated in tolerant varieties (18, 20, 26 and Clark) at 24 hours of treatment relative to the susceptible varieties (19, 24 25, and Iso-Clark). This difference was more pronounced at day 7 (Fig. 2.1B).

A



B

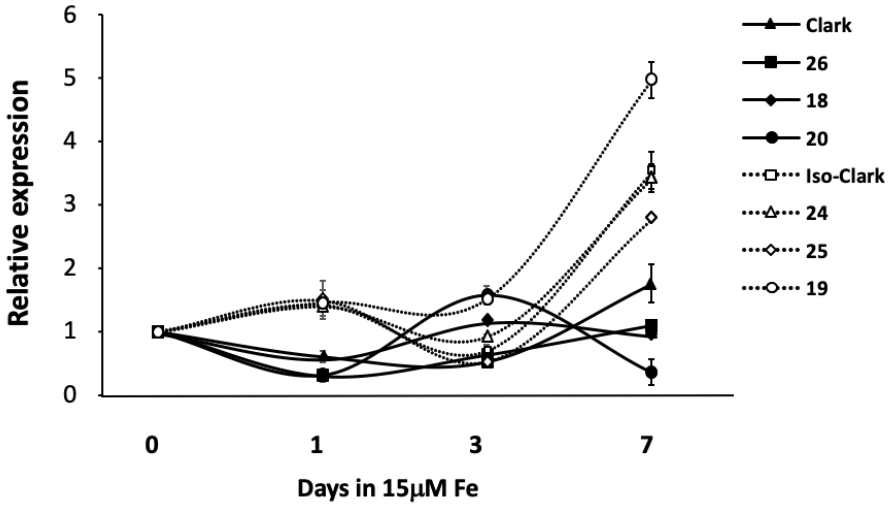


Figure 2.1. (A) Percentage retention of total chlorophyll measured in leaves after 7 days of treatment (Fe 60µM or Fe 15µM) in selected soybean varieties tolerant (Clark, 18, 20, and 26) or susceptible (Iso-Clark, 19, 24, and 25) to IDC. Percent retention indicates chlorophyll retained in deficient (Fe 15µM) plants in comparison to control (Fe 60µM) plants. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$). **(B)** Relative transcript levels of *GmPgb1* measured during 7 days of treatment (Fe 15µM) in the leaves of the same varieties. Values (normalized to the respective values of day 0 set at 1) are means $\pm$ SE of three biological replicates each consisting of at least three plants.

2.4.2 Tolerance to IDC is associated with variations in the antioxidant system and elevated levels of ascorbic acid

To assess if the differences in *GmPgb1* expression observed at day 1 (Fig. 2.1B) between the tolerant and susceptible varieties correlate to changes in the antioxidant system at the same day of treatment, the activities of the ascorbate-glutathione redox enzymes, as well as catalase (CAT) and superoxide dismutase (SOD), all implicated in IDC responses (Santos et al., 2019) were measured (Fig. 2.2).

In the susceptible varieties (19, 24 25, and Iso-Clark) the activity of ascorbate peroxidase (APX), which scavenges H_2O_2 by oxidizing ASC to dehydroascorbic acid (DHA), increased under conditions of iron deficit (Fig. 2.2). This was in contrast to the tolerant varieties, which also exhibited an increase in activities of the ascorbic acid oxidizing enzymes, monodehydroascrobate reductase (MDHAR), and dehydroascrobate reductase (DHAR), as well as glutathione reductase (GR) generating GSH from GSSG, when grown under iron deficit. The activities of SOD and to some degree CAT, increased in the tolerant varieties in comparison to the susceptible varieties when exposed to low iron levels (Fig. 2.2)

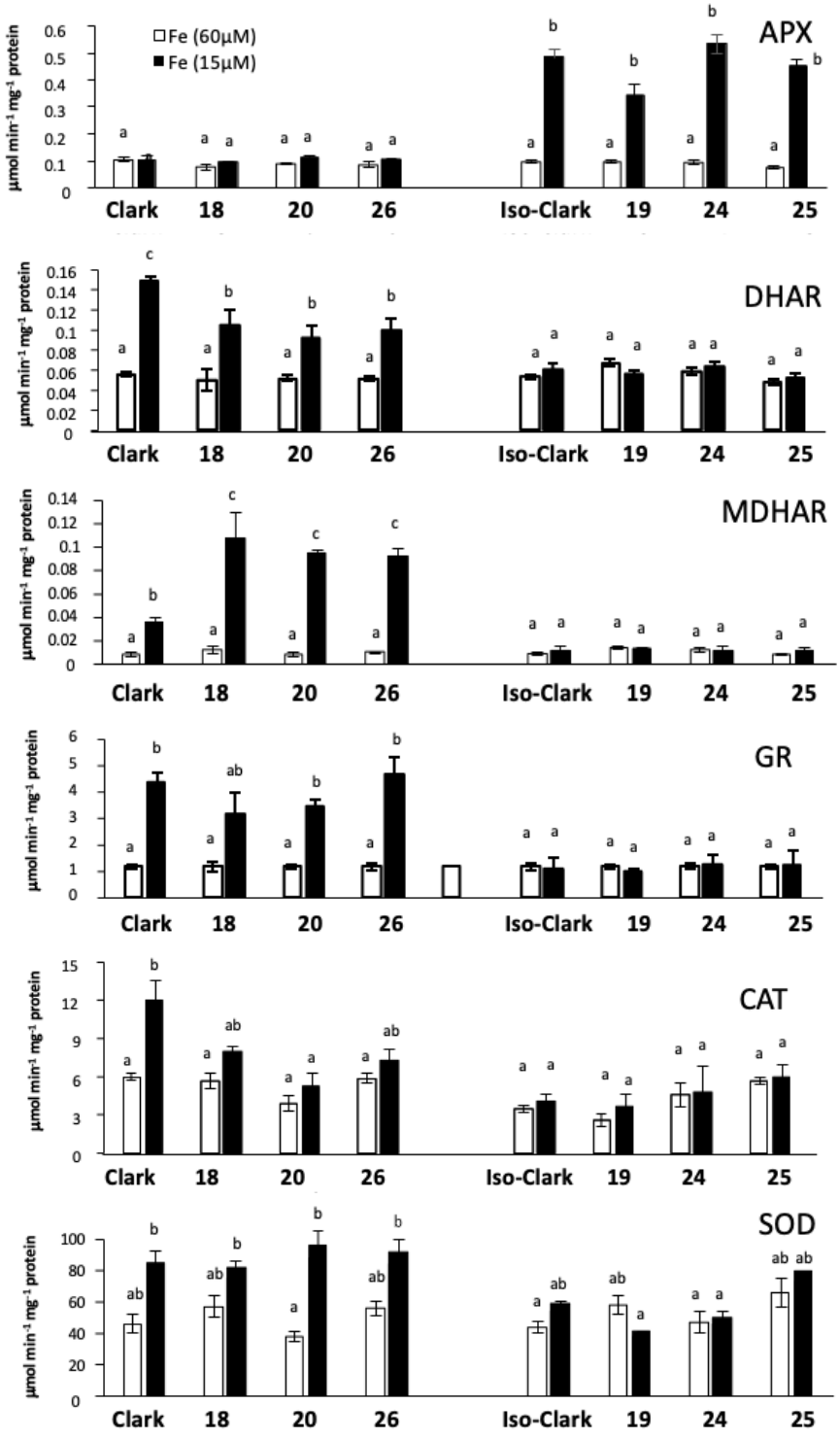


Figure 2.2. Enzymatic activity of the major antioxidant enzymes (ascorbate peroxidase, APX; dehydroascorbate reductase, DHAR; monohydroascrobate reductase, MDHAR; glutathione

reductase, GR; catalase, CAT; and superoxide dismutase, SOD) measured in leaves of commercial varieties following one day of exposure to iron-deficit. Letters indicate statistically significant differences ($P \leq 0.05$). Values are means $\pm$ SE of three biological replicates each consisting of at least three plants.

To determine whether the alterations in enzyme activities observed in the trifoliate leaves at day 1 were followed by changes in antioxidants and ROS, the cellular levels of ascorbate, glutathione, and hydrogen peroxide were measured during the subsequent days (3 and 7) of treatment (Fig. 2.3). Relative to iron sufficient conditions, exposure to iron deficit in the tolerant varieties elevated the content of ASC at day 7 and decreased that of DHA at both day 3 and 7. This trend was not observed in susceptible varieties, which showed no significant differences in ASC and DHA levels (with the exception of Iso-Clark and variety 25 at day 3 for DHA) at day 3 and 7.

No differences were observed at day 3 and 7 for GSH levels in tolerant varieties. Overall, the susceptible varieties displayed lower levels of GSH at day 3 compared to day 7. Regardless of the iron levels, tolerant varieties had less GSSG at day 3 relative to the susceptible varieties. Iron deficit reduced GSSG content in the tolerant varieties at day 7 (Fig. 2.3).

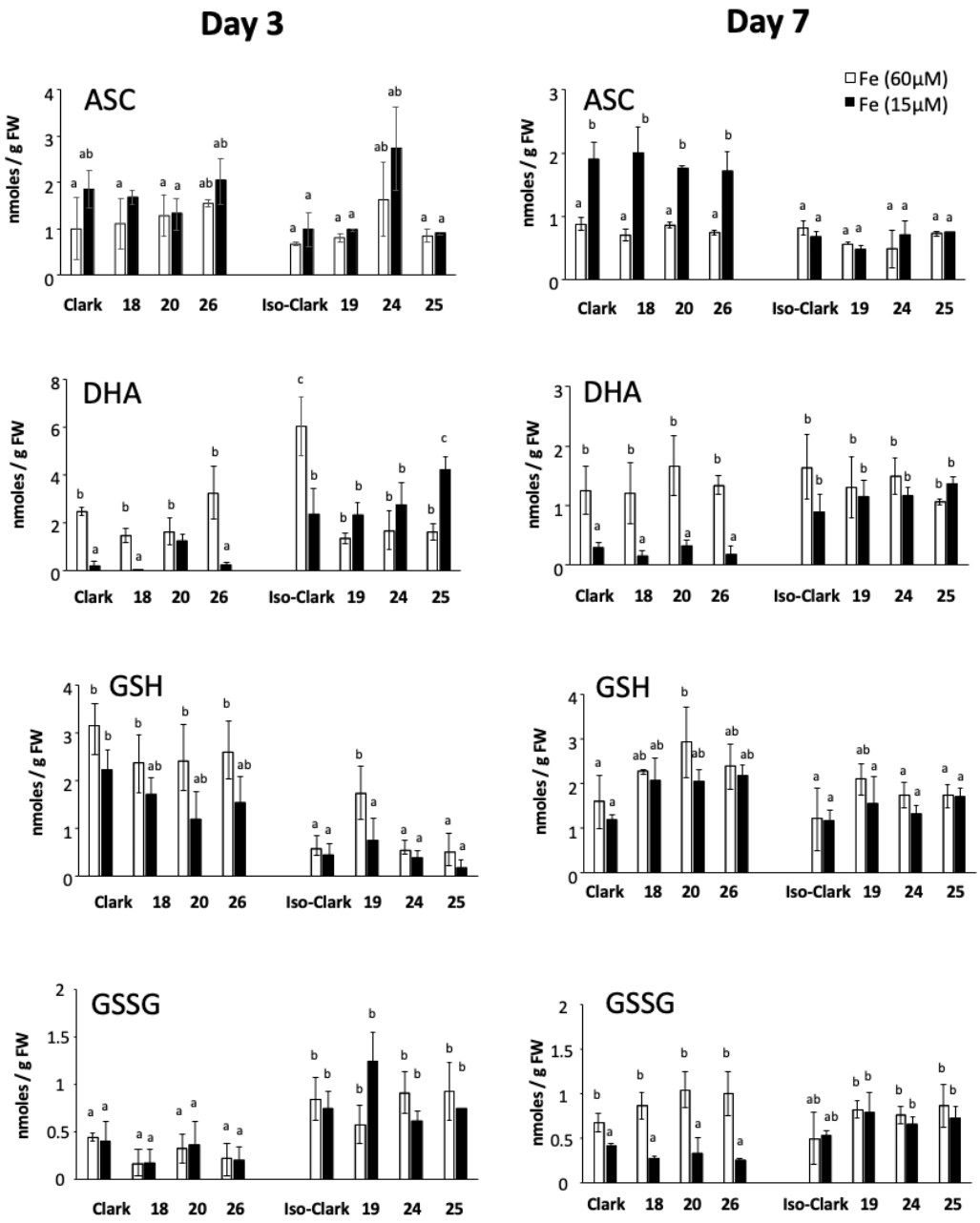


Figure 2.3. Levels of ascorbic acid (ASC), dehydroascorbic acid (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) measured in leaves of soybean plants exposed for 3 and 7 days to different iron levels (Fe 60µM or Fe 15µM). Letters indicate statistically significant differences ($P \leq 0.05$). Values are means $\pm$ SE of three biological replicates each consisting of at least three plants.

Imposition of iron deficiency augmented the level of H_2O_2 in the susceptible varieties at day 3 and 7 (Fig. 2.4A). No significant differences were observed in tolerant varieties. An increase in H_2O_2 and especially superoxide staining intensity was observed in leaves of susceptible varieties exposed to iron deficit (Fig. 2.4B, C). This effect was mitigated in the tolerant varieties.

The elevation in ASC levels observed in iron deficient tolerant lines at day 7, but not in their susceptible counterparts (Fig. 2.3) prompted to assess if the susceptibility to IDC could be improved by exogenous applications of ASC. Foliar applications of 0.5 mM of ASC in the susceptible lines mitigated the loss in chlorophyll content (Fig. 2.5A) and partially rescued the chlorotic phenotype (Fig. 2.5B). Applications of ASC under iron sufficient conditions had an added beneficial effect on chlorophyll content (Supplemental Fig. 4.2).

Although iron deficiency did not cause major changes in GSH levels in the varieties (Fig. 2.3), previous studies documented the ability of applications of GSH to enhance IDC tolerance (Ramírez et al., 2013). Exogenous GSH (0.5 mM or 1.0 mM, as per Ramirez et al., 2013) did not show any beneficial effects, but rather resulted in abnormal curling of the leaves in all varieties (Supplemental Fig. 4.3).

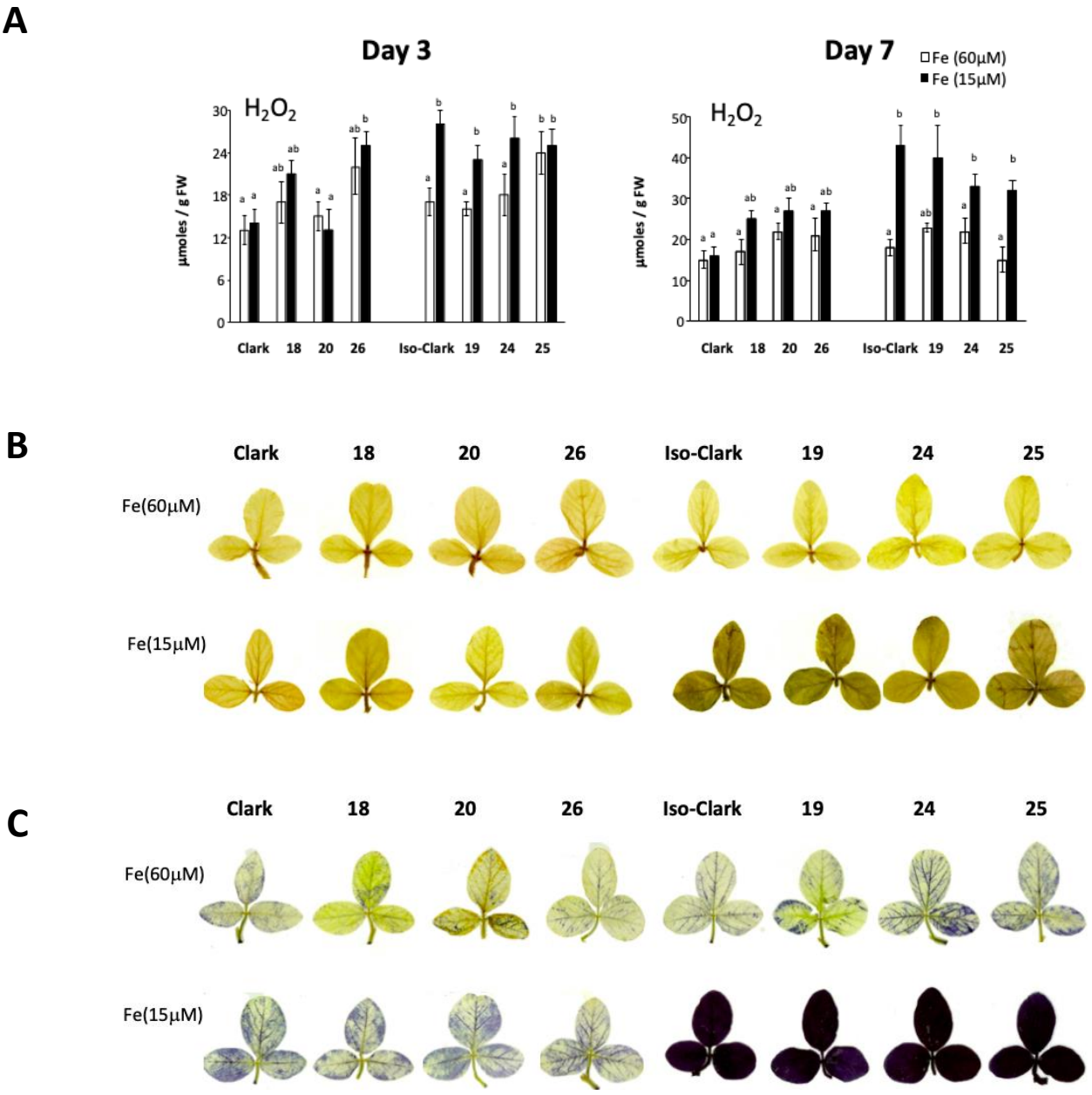


Figure 2.4. Levels of reactive oxygen species (ROS) in leaves of soybean plants exposed to different iron concentrations (Fe 60µM or Fe 15µM). Plants included varieties tolerant (Clark, 18, 20, and 26) or susceptible (Iso-Clark, 19, 24, and 25) to IDC. **(A)** Hydrogen peroxide level measured at day 3 and 7. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$). Leaves were also stained for **(B)** hydrogen peroxide and **(C)** superoxide after 7 days of treatment.

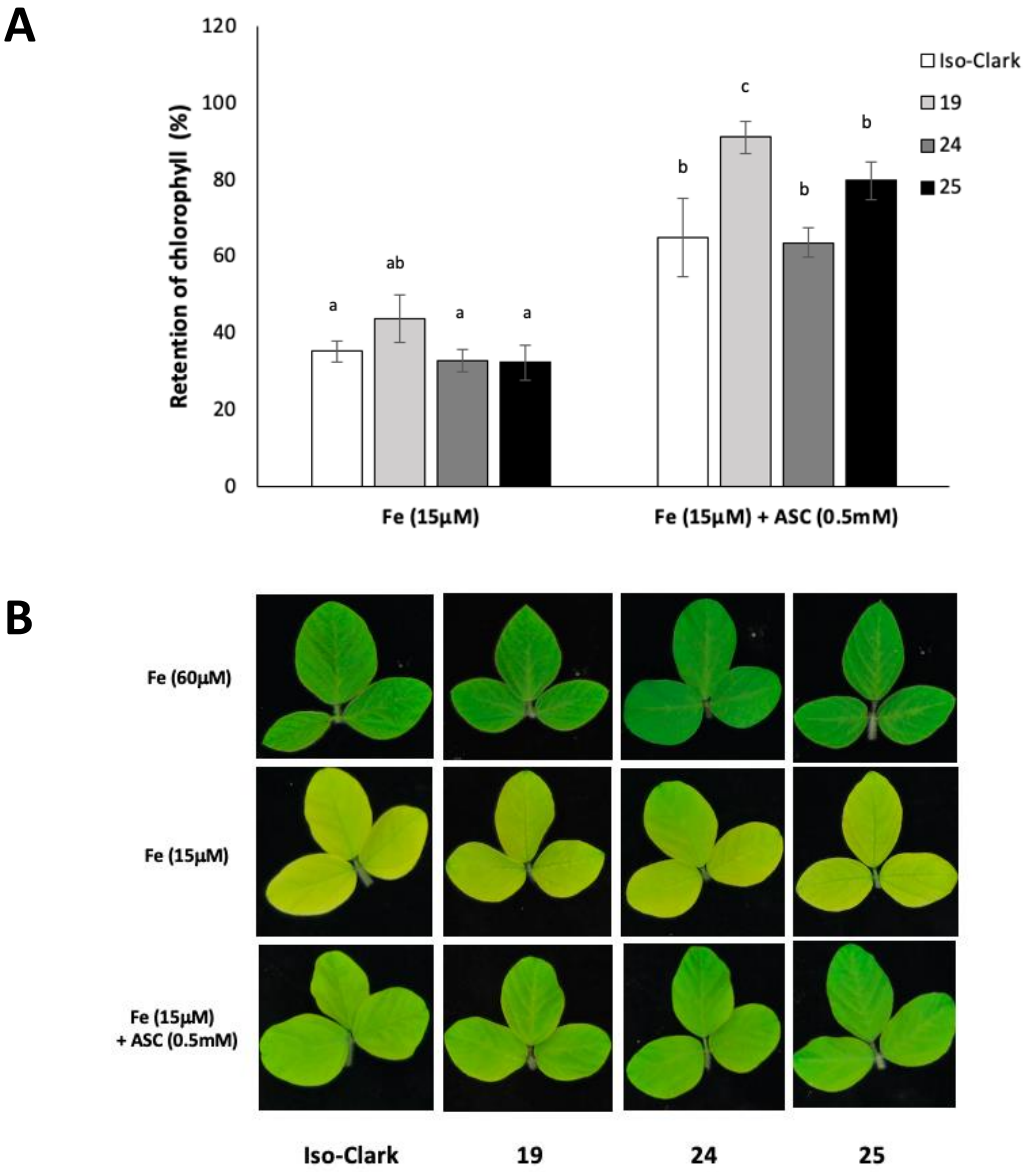


Figure 2.5. (A) Effects of foliar applications of ascorbic acid (ASC) on chlorophyll retention measured in leaves after 7 days of treatment (Fe 15µM) in soybean varieties susceptible to IDC. Percent retention indicates chlorophyll retained in deficient (Fe 15µM) plants in comparison to control (Fe 60µM) plants. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$). **(B)** Effects of foliar applications of ascorbic acid (ASC) on leaf chlorosis in susceptible varieties.

2.4.3 Expression of *GmPgb1* affects soybean response to iron deficiency

Transgenic lines with altered levels of *GmPgb1* were also exposed to iron deficiency. Seven lines were used for this experiment: a wild type (WT) line, three down-regulating lines RNAi lines: GmPgb1(RNAi)-1, -18, and -23, and three over-expressing lines: GmPgb1(S)-2, -9, and -17. Note that line GmPgb1(RNAi)-1 had an expression level of *GmPgb1* comparable to that of the WT (Supplemental Fig. 4.4), thus serving as an additional control. Under iron deficient conditions, plants down-regulating *GmPgb1* [GmPgb1(RNAi)-18, and -23] exhibited the greatest retention of chlorophyll, with retention levels exceeding 80% of control (iron sufficient) values. In contrast, in all lines up-regulating *GmPgb1*, chlorophyll retention was significantly diminished when plants were exposed to iron deficit (Fig. 2.6A). The line GmPgb1(RNAi)-1 exhibited a retention of chlorophyll similar to that measured in the WT line. Based on these results, the *GmPgb1* down-regulating line GmPgb1(RNAi)-23 and the *GmPgb1* over-expressing line GmPgb1(S)-17, exhibiting the best and the worst behavior under iron deficit, were utilized for further experiments.

The major function of Pgbs is to scavenge NO and manipulations in their expression levels have been used routinely to modulate cellular NO in a pattern consistent with their function; that is, environments enriched with Pgbs are depleted in NO while those limited in Pgbs accumulate NO (reviewed by Hill 2012). With this premise, it was assessed if targeted alterations of NO in the transgenic lines could influence the response to IDC. This was done by increasing NO (with SNP) in the *GmPgb1* over-expressing line GmPgb1(S)-17, and reducing NO (with cPTIO) in the *GmPgb1* suppressing line GmPgb1(RNAi)-23. An increase in NO (SNP) in the *GmPgb1* over-expressing line increased chlorophyll retention during iron

deficiency, whereas the application of the NO scavenger (by cPTIO) to the *GmPgb1* suppressing line reduced chlorophyll retention (Fig. 2.6B). The NO effect was also observed in WT plants.

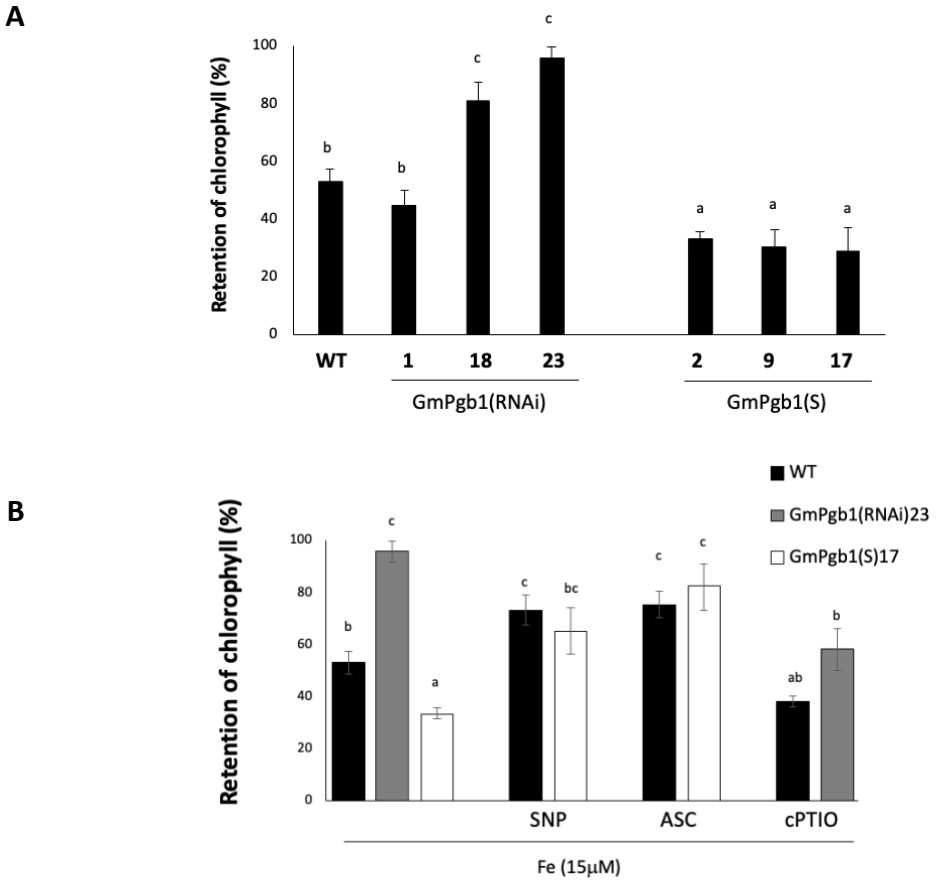


Figure 2.6. (A) Percentage retention of total chlorophyll measured in leaves after 7 days of treatment (Fe 60µM or Fe 15µM) in soybean plants suppressing [GmPgb1(RNAi)-1,-18, and -23] or over-expressing [GmPgb1(S)-2, -9, and -17] *GmPgb1*. Values are means ± SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$). **(B)** Effects of applications of ascorbic acid (ASC), nitric oxide donor SNP, or the nitric oxide scavenger cPTIO on chlorophyll retention measured in leaves of the WT, *GmPgb1* over-expressing [GmPgb1(S)-17] and *GmPgb1* down-regulating [GmPgb1(RNAi)-23] lines after 7 days of treatment (Fe 15µM). Values are means ± SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$).

2.4.4 Suppression of *GmPgb1* alters the antioxidant system and reduces ROS

By using similar time points adopted for the soybean varieties, the activities of the several antioxidant enzymes were measured in the transgenic lines after 1 day of treatment, while the levels of antioxidants and ROS were measured after 7 days. Exposure to iron deficit augmented the activity of APX in the GmPgb1(S)-17 line relative to the WT and GmPgb1(RNAi)-23 lines (Fig. 2.7A). This was in contrast to the activities of DHAR, SOD and CAT which increased in the iron deficient GmPgb1(RNAi)-23 line.

Conditions of iron deficiency caused an elevation in ASC and a reduction in DHA in the *GmPgb1* down-regulating line; this was in contrast to the *GmPgb1* over-expressing line displaying an increase in DHA (Fig. 2.7B). Applications of the NO donor SNP to the *GmPgb1* over-expressing line augmented ASC and reduced DHA, while the NO scavenger cPTIO diminished ASC and elevated DHA in the *GmPgb1* down-regulating line. A similar NO effect, albeit less significant, was also observed in the WT line (Fig. 2.7B).

The increased level of ASC observed in the iron deficient GmPgb1(RNAi)23 line (Fig. 2.7B) exhibiting the highest retention of chlorophyll content (Fig. 2.6A) prompted to determine if the susceptibility to IDC in the *GmPgb1* up-regulating line GmPgb1(S)17 could be reversed by exogenous ASC. Foliar applications of ASC were sufficient to increase the chlorophyll retention in this line; an observation also noted for the WT line (Fig. 2.6B).

Relative to the WT and GmPgb1(S)17 lines, imposition of iron deficiency decreased the levels of H_2O_2 in the *GmPgb1* down-regulating line [GmPgb1(RNAi)23] (Fig. 2.8A), a trend consistent with the staining pattern of superoxide and H_2O_2 (Fig. 2.8B, C).

The staining intensity of both ROS under iron deficit conditions was dependent upon the levels of NO and ASC. Staining was reduced in environments enriched in NO (*GmPgb1* down-regulating line or SNP treatments) or ASC (*GmPgb1* down-regulating line (Fig. 2.7B) or ASC treatments), whereas it was increased in those in which NO was reduced (*GmPgb1* over-expressing line or cPTIO treatments) (Fig. 2.9). In WT leaves the increased ROS staining by cPTIO was partially rescued by applications of ASC (compared cPTIO and ASC+cPTIO).

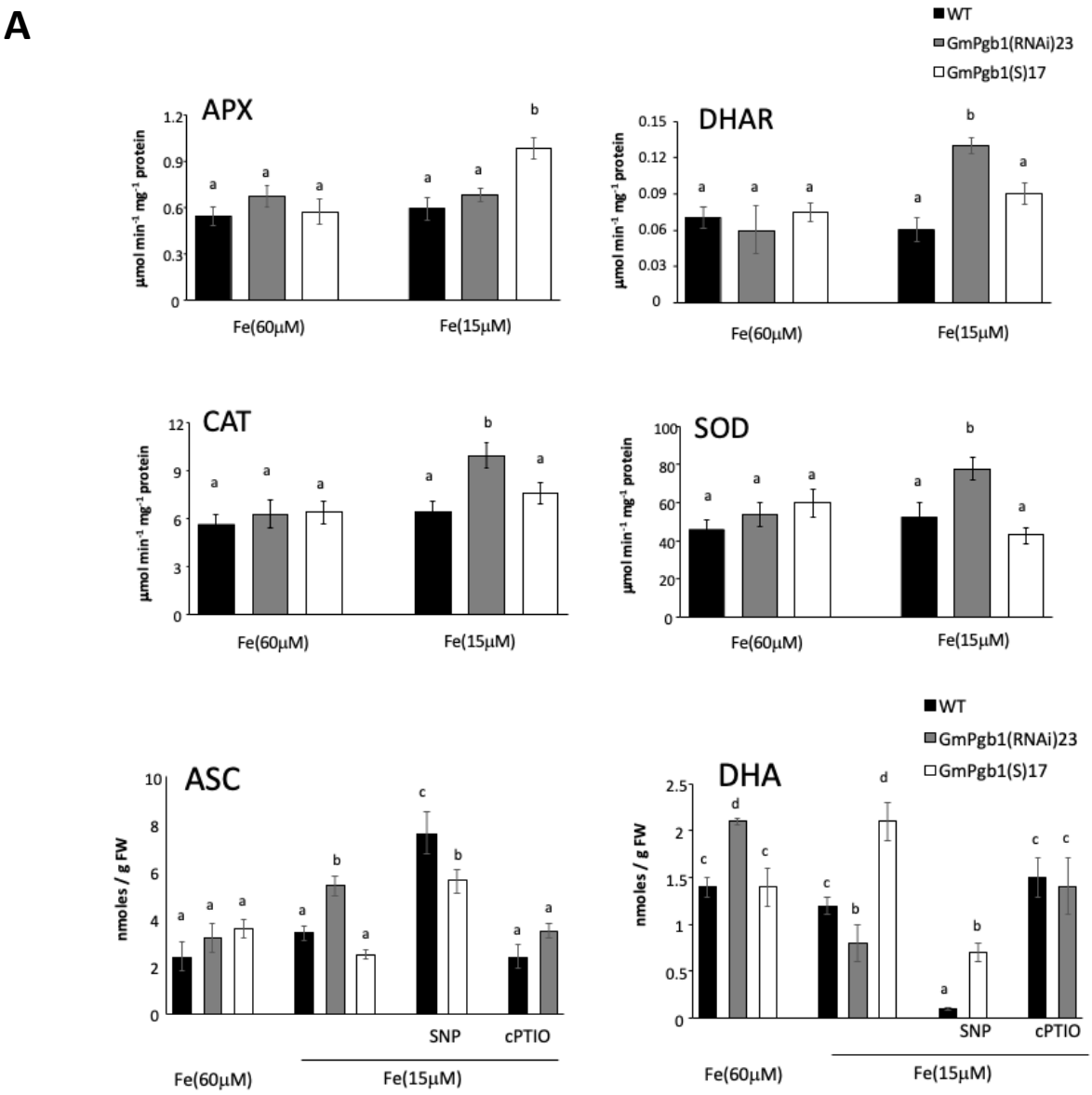


Figure 2.7. Enzymatic activity of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), catalase (CAT) and superoxide dismutase (SOD) **(A)** and levels of ascorbic acid (ASC), and dehydroascorbic acid (DHA) **(B)** measured in leaves of the WT, *GmPgb1* over-expressing [GmPgb1(S)17] and *GmPgb1* down-regulating [GmPgb1(RNAi)23] lines after 7 days of treatment (Fe 60µM or Fe 15µM). Values are means ± SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$).

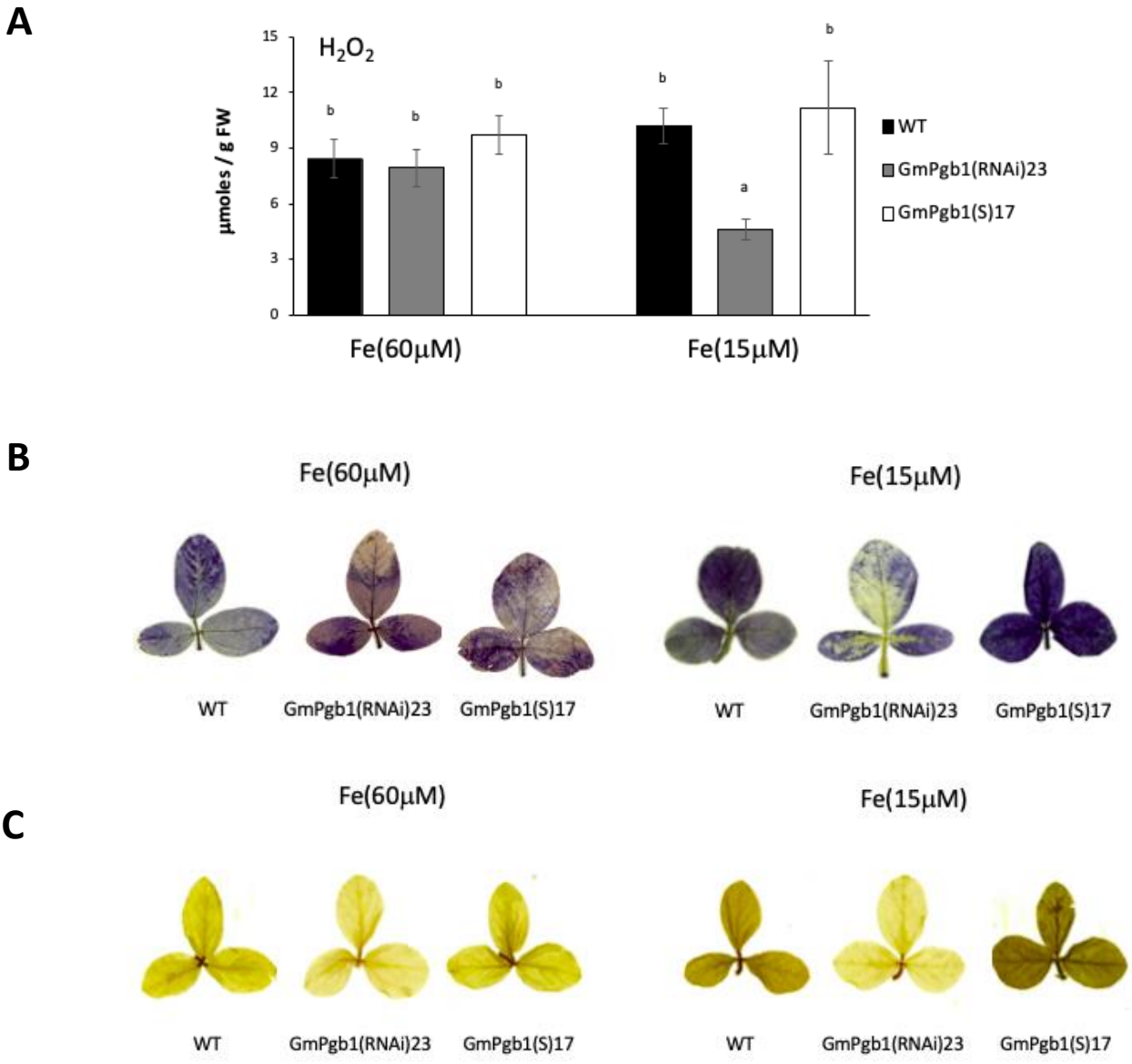


Figure 2.8. Levels of reactive oxygen species (ROS) in leaves of soybean plants exposed for 7 days to different iron concentrations (Fe 60µM or Fe 15µM). Measurements were taken from leaves of the WT, *GmPgb1* over-expressing [GmPgb1(S)17] and *GmPgb1* down-regulating [GmPgb1(RNAi)23] lines. **(A)** Hydrogen peroxide levels. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$). Leaves were also stained for superoxide **(B)** and hydrogen peroxide **(C)**.

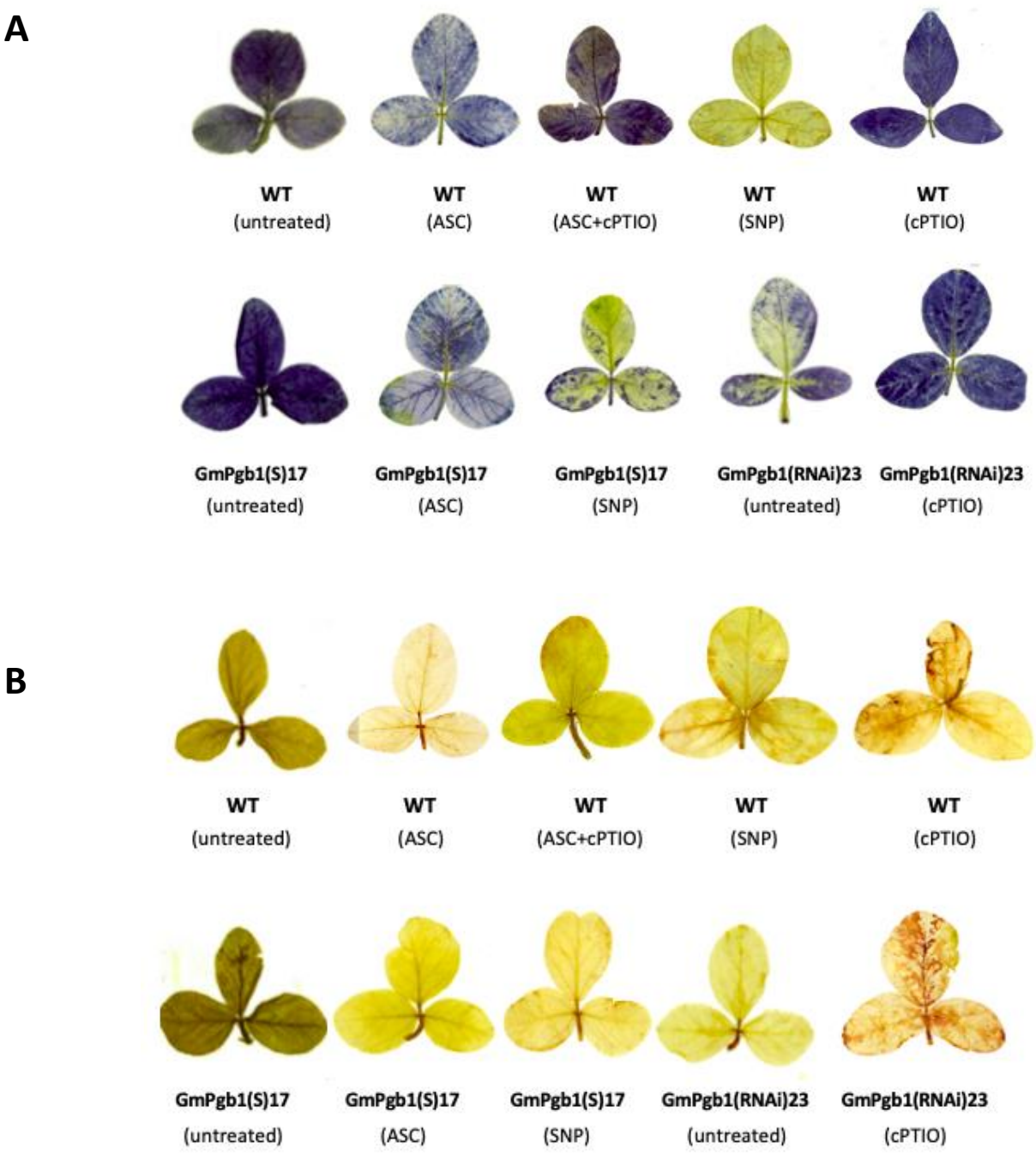


Figure 2.9. Staining for superoxide **(A)** and hydrogen peroxide **(B)** in leaves of transgenic lines suppressing [GmPgb1(RNAi)23] or over-expressing [GmPgb1(S)17] *GmPgb1*. Plants were grown for 7 days in low iron levels (Fe 15µM). Treatments with ascorbic acid (ASC), the nitric oxide donor (SNP) and nitric oxide scavenger (cPTIO) are also shown.

2.4.5 The Fe(II) / Fe(III) ratio is influenced by *GmPgb1* through ASC and NO

Reduction of Fe(III) to the more soluble and mobile Fe(II) is a strategy that alleviates the effects of IDC by facilitating iron transport across membranes. In iron deficient WT and GmPgb1(S)17 lines the Fe(II)/Fe(III) ratio favored the oxidized [Fe(III)] form, an effect not observed in the GmPgb1(RNAi)-23 line exhibiting a high Fe(II)/Fe(III) ratio (Fig. 2.10). The Fe(II)/Fe(III) ratio increased following applications of SNP or ASC in the *GmPgb1* up-regulating line, while it decreased when cPTIO was administered to the *GmPgb1* down-regulating line.

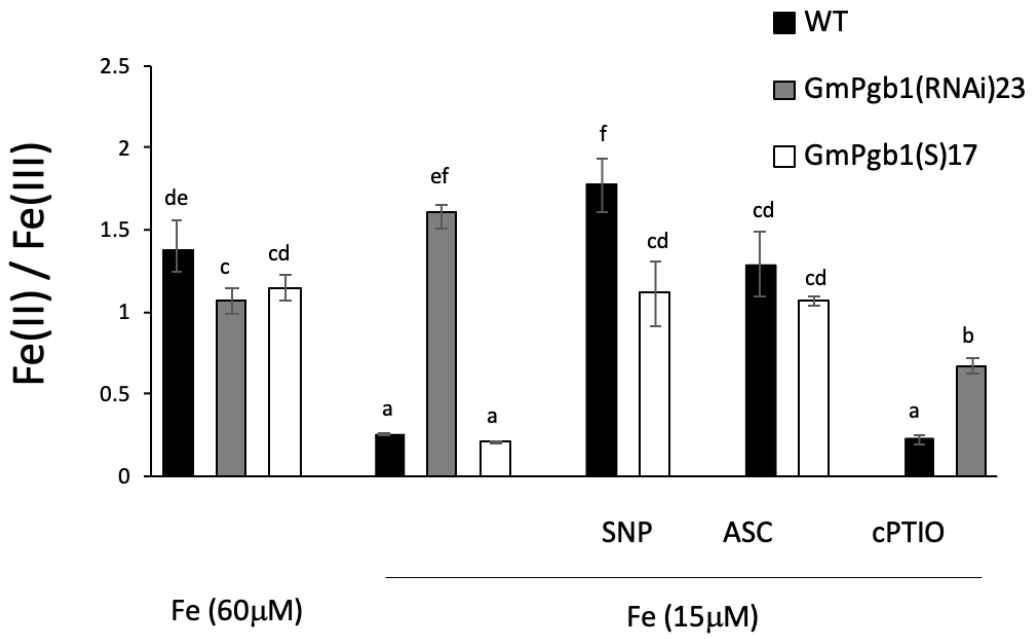


Figure 2.10. Ratio of Fe(II)/Fe(III) measured in leaves of the WT, *GmPgb1* over-expressing [GmPgb1(S)17] and *GmPgb1* down-regulating [GmPgb1(RNAi)23] lines after 7 days of treatment (Fe 60µM or Fe 15µM). Leaves were also treated with ascorbic acid (ASC), the nitric oxide donor SNP, or the nitric oxide scavenger cPTIO. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicate statistically significant differences ($P \leq 0.05$).

2.5 Discussion

This thesis presents strong evidence supporting the role of Phytoglobins (Pgbs) in altering plant response to IDC. The observation that soybean varieties tolerant to IDC are characterized by low levels of *GmPgb1* at day 1 and 7 (Fig. 2.1) is further supported by the enhanced tolerance acquired when the same gene is experimentally downregulated (Fig. 2.6A). These effects are linked to the level of NO, as shown by the ability of SNP to increase and cPTIO decrease tolerance to iron deficit (Fig. 2.6B), in a model consistent with the function of Pgbs as NO scavengers (reviewed by Hill, 2012).

In response to stressful environments, plant tissues express *Pgbs,* mitigating the levels of NO, also produced in response to stress (Dordas et al., 2003a). NO is reduced by oxygenated Pgb to form nitrate and methemoglobin (Igamberdiev and Hill, 2004). Due to this property, it has been well demonstrated that plants over-expressing *Pgbs* have reduced levels of NO while plants down-regulating the same gene accumulate NO (Dordas et al., 2003b; Igamberdiev and Hill, 2004). Previous studies have shown the ability of NO to mitigate the effects of IDC. Graziano et al. (2002) observed that applications of exogenous NO, through SNP, were able to revert the chlorotic phenotype of iron-deficient corn plants and restore chloroplast development and structure impaired by iron deficit. This was in contrast to cPTIO applications causing interveinal chlorosis in the younger leaves. Comparable research was also conducted in peanut (Kong et al., 2014; Song et al., 2018). These studies, however, do not provide an explanation for the observed effects, with no indications on how variations in NO levels modulate plant response to IDC.

By further analysing the function of NO, results from this thesis demonstrate that an elevation in NO either through genetic manipulations (suppression of *GmPgb1*) or pharmacological treatments (SNP) is sufficient to enhance resilience to iron deficit through changes in the antioxidant system suppressing the accumulation of ROS and increasing the availability of Fe(II).

In plants, ROS are important signal transduction molecules regulating various developmental responses (Miller et al., 2010). However, over-production of ROS in response to stress, can be extremely toxic and can lead to the oxidative damage of proteins, DNA and lipids (Choudhury et al., 2017). Reactive oxygen species tend to accumulate especially in environments depleted of iron as this microelement is an essential component of the electron transport chain. It is well established that iron limitation disrupts electron flows resulting in the over production of ROS and ultimately to the degradation of the photosynthetic machinery contributing to the chlorotic symptoms (Ramírez et al., 2013). Capable of modulating multiple plant responses, NO has the capacity to regulate the level and toxicity of ROS directly or indirectly. Sun et al. (2007) suggested that NO alleviates iron deficiency induced oxidative stress by reacting directly with ROS or by inducing changes in ROS-scavenging enzymes. In soybean, tolerance to IDC is associated with an induction of CAT and SOD and a concomitant reduction in H_2O_2 and ROS staining. This pattern was observed in tolerant lines, characterized by a lower expression of *GmPgb1* at day 1 and 7, and the transgenic line GmPgb1(RNAi)23 suppressing *GmPgb1*. The dependence of IDC tolerance from NO level was further demonstrated by experimental manipulations of NO, with elevated levels augmenting chlorophyll retention and decreasing ROS staining in leaves of iron-deficient plants. The ability

of the NO donor SNP to reduce ROS was also reported in peanut (*Arachis hypogaea*) and corn (*Zea mays*) where the authors also observed an elevation in activities for CAT and SOD (Song et al., 2018; Sun et al., 2007). In the same studies, however, NO also elevated the activity of another ROS-scavenging enzyme, APX; an observation not consistent with this thesis. Our study saw that in soybean, APX activity increased in iron deficient susceptible plants or under conditions in which NO was reduced (by cPTIO or up-regulation of *GmPgb1*). Although the reasons for this discrepancy are difficult to explain, it is suggested that the low activity of APX (using ASC as a substrate) in tolerant varieties and lines suppressing *GmPgb1* contributes to a rise in cellular ASC which might play a key role in alleviating the detrimental effects of iron deficit. In the same varieties and line the higher activity of DHAR (converting DHA to ASC), can also contribute to elevate the levels of ASC.

Research in *Medicago ciliaris* also shows that plants tolerant to iron deficit exhibit significantly larger pools of ASC compared to susceptible plants (M'sehli et al., 2014). The requirement of high levels of ASC for tolerance to IDC was also confirmed by exogenous applications of ASC, or SNP (which increases ASC, Fig. 2.7A); an observation also documented in Ramirez et al. (2013). Of interest, the authors also showed that applications of GSH phenocopied the effects of ASC, something not apparent in our studies, where similar concentrations of GSH inhibited growth by causing abnormal leaf curling. The inability of exogenous GSH to enhance IDC tolerance was also reflected by unclear differences in glutathione metabolism (Fig. 2.3), thus suggesting that this antioxidant might not be key in soybean response to iron deficit.

Ascorbic acid may play a key role in regulating iron uptake. This phenomenon was originally researched in humans who suffer from iron deficiency anemia, a common and widespread nutrient deficiency affecting roughly two million people worldwide (Macknight et al., 2017). Evidence strongly suggests that dietary ASC has the ability to enhance nonheme iron absorption in the gut as well as regulate cellular iron uptake and metabolism (Lane and Richardson, 2014). Specifically, in the intestinal lumen ASC plays an important role in reducing Fe(III) to Fe(II) thereby increasing iron solubility, availability and absorption (Atanassova and Tzatchev, 2008). In plants there are fewer studies; however, one in particular provides insights on the role of ASC in iron reduction in pea seeds. Rather than utilizing a membrane bound protein to reduce iron, pea embryos rely on apoplastic ASC to reduce Fe(III) to Fe(II) thereby increasing iron availability (Grillet et al., 2013). The reducing ability of ASC might contribute to IDC tolerance. Conditions favoring higher levels of ASC (indirectly through elevation in NO by suppression of *GmPgb1* and treatments with SNP, or directly through exogenous ASC applications) increased the cellular Fe(II)/Fe(III) ratio, while a reduction in the same ratio was measured when ASC was lowered as a result of *GmpPgb1* over-expression or cPTIO applications. This mechanism, which was suggested (Stamler et al., 1992) but never demonstrated *in planta* before, might represent a strategy to enhance iron utilization during conditions of iron deficiency, as Fe(II) is more mobile relative to Fe (III).

Together, results from this thesis demonstrate a clear connection between *GmPgb1* expression and tolerance to iron deficiency. A model is provided in which suppression of *GmPgb1* (elevating NO levels) induces changes in the antioxidant system scavenging ROS through the activation of CAT and SOD while elevating the level of ASC (by suppression of

APX) needed to mobilize iron by reduction of Fe(III) to Fe(II). Furthermore, work conducted on the commercial varieties suggests that measurements of *GmPgb1* correlate to the behaviour of the plants to iron deficiency, thus providing a tool for screening varieties at the early stages of development.

3 GENERAL CONCLUSION

Plants exposed to stressful conditions, such as iron deficit, respond by altering *Pgb* expression, which in turn regulates the level of NO present in the plant. Among the many properties of NO, it has the ability to reverse symptoms associated with iron deficiency. This thesis demonstrates the means by which NO enhances tolerance to IDC in soybean. Through pharmacological application of NO donor, SNP, and by experimentally down-regulating *GmPgb1* in soybean, it was confirmed that NO has the ability to revert the chlorotic phenotype of iron deficient plants through increasing iron availability. This can be explained through the induction of the antioxidant system, which is influenced by the increase in NO. The enzymatic and non-enzymatic components of the antioxidant system not only mitigate oxidative stress but also IDC symptoms. In this study, it was observed that enzyme activity of CAT and SOD increase in response to iron deficit and work to minimize ROS accumulation and damage through scavenging ROS. The level of ASC, a vital antioxidant, also increased which aids in the detoxication of H_2O_2 and facilitates the reduction of Fe(III). Our research observed that in iron-efficient plants, tolerance to IDC is associated with high levels of ASC. Though the application of exogenous NO or ASC and by down-regulating *GmPgb1*, IDC symptoms were attenuated. Further iron-assay results confirm that pharmacologically treated plants and those down-regulating *GmPgb1* contain greater amounts of Fe(II) in comparison to the non-treated and iron-inefficient plants, and therefore can confirm that ASC has the ability to facilitate the reduction of insoluble, unavailable Fe(III) to the plant-usable form, Fe(II).

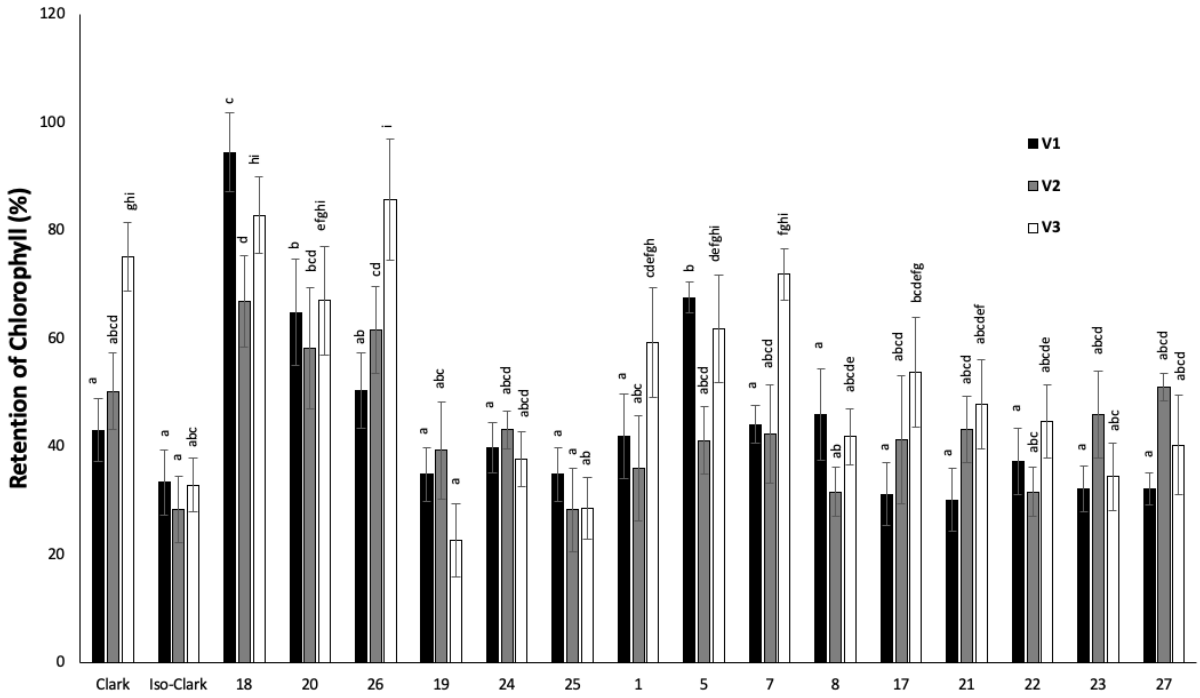
Overall, we can conclude that based on these results and previous literature there is a clear association between *GmPgb1* expression and tolerance to IDC in soybean. Through the

suppression of *GmPgb1*, there is increased levels of NO present in the plant which promote the activation of the antioxidant system, which enables the removal of ROS and elevates levels of ASC, responsible for reducing unusable Fe(III) to Fe(II).

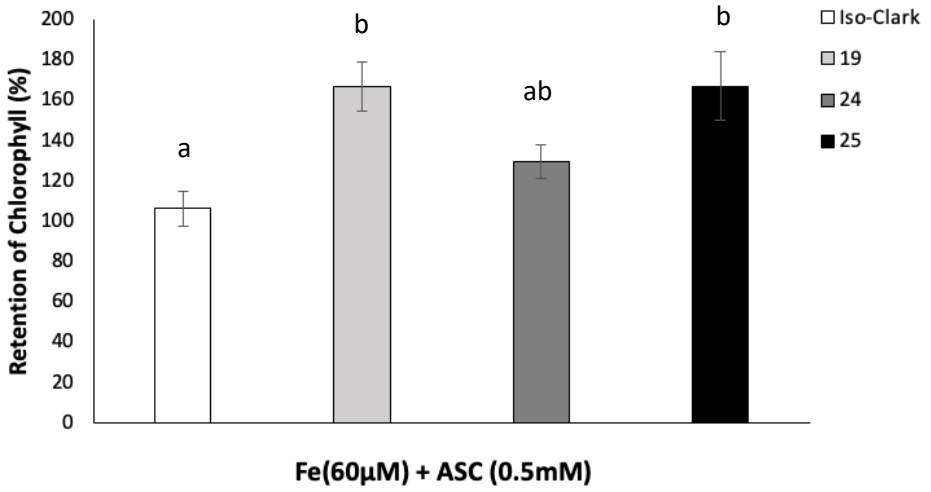
3.1 Future Direction

Development of IDC-tolerant varieties in soybean is slow in comparison to other crop species and many soybean studies depend solely on physiological observations (Aksoy et al., 2017). In addition to the phenotypic based selection, future breeding decisions could employ more molecular based studies utilizing *GmPgb1* as a molecular marker for the selection and identification of genotypes able to cope with iron deficit. Development of non-transgenic lines suppressing *GmPgb1* might contribute to alleviate the effects of IDC. It is important, however, that these selected lines be tested for other agronomical traits, such as tolerance to other types of biotic and abiotic stress, as suppression of *GmPgb1* and a concomitant elevation in NO might interfere with other processes.

4 SUPPLEMENTAL MATERIAL



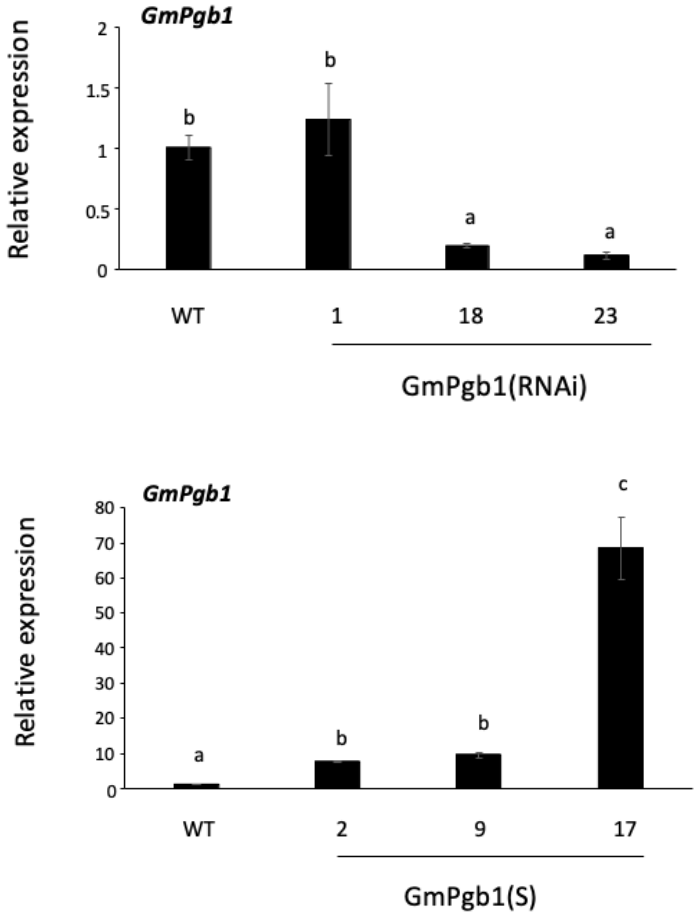
Supplementary Figure 4.1. Retention of chlorophyll in all commercial soybean varieties at the V1-V3 stage of development. Plants were exposed to iron deficient (15 μM) or sufficient (60 μM, control) conditions. Percent retention indicates chlorophyll retained in deficient (Fe 15μM) plants in comparison to control (Fe 60μM) plants. Values are mean ± SE of 3 biological replicates each consisting of at least three plants. Letters indicates statistically significant differences at individual developmental stages. ($P \leq 0.05$).



Supplementary Figure 4.2. Effects of foliar applications of ascorbic acid (ASC) on chlorophyll retention measured in leaves after 7 days of treatment (Fe 60µM) in soybean varieties susceptible to IDC. Values are means ± SE of 3 biological replicates each consisting of at least three plants. Letters indicates statistically significant differences ($P \leq 0.05$).



Supplementary Figure 4.3. Leaf curling exhibited by soybean varieties susceptible to IDC when treated with glutathione (GSH).



Supplementary Figure 4.4. Level of *GmPgb1* in leaves of transgenic lines suppressing [GmPgb1(RNAi)] or over-expressing [GmPgb1(S)] *GmPgb1*. Values are normalized to the WT value set at 1. Values are means $\pm$ SE of three biological replicates each consisting of at least three plants. Letters indicates statistically significant differences ($P \leq 0.05$).

Supplementary Table 4.1. Primers used in gene expression study.

Gene	**Name**	**Sequence**
PGB1-RT-PCR	Gm11g12980-RT-F	AGTTCAGCTGCGGAAGGCCG
	Gm11g12980-RT-R	TGCTCGTTTGCTACGCCGGT
Helicase Reference	Gm04g07180.4-RT-F	TAACCCTAGCCCCTTCGCCT
	Gm04g07180.4-RT-R	GCCTTGTCGTCTTCCTCCTCG

5 LITERATURE CITED

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

Alloway BJ (2008) Micronutrients and Crop Production: An Introduction. In: Alloway, BJ. Micronutrient Deficiencies in Global Crop Production. Springer. p. 1–39.

Appleby CA (1992) The origin and functions of haemoglobin in plants. Science Progress **76**: 365–398.

Arasimowicz M, Floryszak-Wieczorek J (2007) Nitric oxide as a bioactive signalling molecule in plant stress responses. Plant Science **172**: 876–887.

Atanassova BD, Tzatchev KN (2008) Ascorbic acid-important for iron metabolism. Folia Medica **50**: 11–16.

Barker B (2018) Micronutrients in Pulse and Soybean Production. In: Pulse Advisor Newsletter. Saskatchewan Pulse Growers. p. 1-2.

Beligni MV, Lamattina L (2001) Nitric oxide in plants: The history is just beginning. Plant, Cell and Environment **24**: 267–278.

Boodi IH, Pattanashetti SK, Biradar BD, Naidu GK, Chimmad VP, Kanatti A, Kumar V, Debnath MK (2016) Morpho-physiological parameters associated with iron deficiency chlorosis resistance and their effect on yield and its related traits in groundnut. Journal of Crop Science and Biotechnology **19**: 177-187.

Bradford, MM (1976) A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry **72**: 248–254.

Campbell MT, Proctor CA, Dou Y, Schmitz AJ, Phansak P, Kruger GR, Zhang C, Walia H (2015) Genetic and molecular characterization of submergence response identifies *Subtol6* as a major submergence tolerance locus in maize. PLoS One **10**: 1–25.

Chaney RL, Coulumbe BA, Bell PF, Angle JS (1992) Detailed method to screen dicot cultivars for resistance to fe-chlorosis using FeDTPA and bicarbonate in nutrient solutions. Journal of Plant Nutrition **15**: 2063–2083.

Charlson DV, Bailey TB, Cianzio SR, Shoemaker RC (2005) Molecular marker Satt481 is associated with iron-deficiency chlorosis resistance in a soybean breeding population. Crop Science **45**: 2394–2399.

Choudhury FK, Rivero RM, Blumwald E, Mittler R (2017) Reactive oxygen species, abiotic stress and stress combination. The Plant Journal **90**: 856–867.

Colombo, C, Palumbo, G, He JZ, Pinton, R, Cesco, S (2014) Review on iron availability in soil: Interaction of Fe minerals, plants, and microbes. Journal of Soils and Sediments **14**: 538–548.

Connorton JM, Balk J, Rodríguez-Celma J (2017) Iron homeostasis in plants- a brief overview. Metallomics **9**: 813–823.

Covarrubias JI, Rombolà AD (2013) Physiological and biochemical responses of the iron chlorosis tolerant grapevine rootstock 140 Ruggeri to iron deficiency and bicarbonate. Plant and Soil **370**: 305–315.

Dordas C (2009) Nonsymbiotic hemoglobins and stress tolerance in plants. Plant Science **176**: 433–440.

Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J, Hill RD (2003) Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. The Plant Journal **35**: 763–770.

Dordas C, Rivoal J, Hill, RD (2003) Plant haemoglobins, nitric oxide and hypoxic stress. Annals of Botany **91**: 173–178.

Dubovskaya LV, Bakakina YS, Kolesneva EV, Sodel DL, Mcainsh MR, Hetherington AM, Volotovski ID (2011) cGMP-dependent ABA-induced stomatal closure in the ABA-insensitive Arabidopsis mutant *abi1-1*. New Phytologist **191**: 57–69.

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiology **155:** 93–100.

Franzen D, Goos RJ (2016) How Much Does IDC Reduce Soybean Yield? NDSU Extension Service.

Franzen DW, Richardson JL (2000) Soil factors affecting iron chlorosis of soybean in the Red River Valley of North Dakota and Minnesota. Journal of Plant Nutrition **23**: 67–78.

Froehlich DM, Fehr WR (1981) Agronomic Performance of Soybeans with Differing Levels of Iron Deficiency Chlorosis on Calcareous Soil. Crop Science **21**: 438–441.

Gill SS, Tuteja N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48**: 909–930.

González-Vallejo EB, Morales F, Cistué L, Abadía A , Abadía J. (2000) Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. Plant Physiology **122**: 337–344.

Goos RJ, Kandel H, Teboh J, Endres G (2019) Soybean Soil Fertility. NDSU Extension Service.

Graziano M, Lamattina L (2005) Nitric oxide and iron in plants: An emerging and converging story. TRENDS in Plance Science **10**: 4–8.

Graziano M, Beligni MV, Lamattina L (2002) Nitric oxide improves internal iron availability in plants. Plant Physiology **130**: 1852–1859.

Green LS, Rogers EE (2004) FRD3 controls iron localization in Arabidopsis. Plant Physiology **136**: 2523–2531.

Grillet L, Ouerdane L, Flis P, Hoang MTT, Isaure MP, Lobinsk R, Curie C, Mari S (2013) Ascorbate efflux as a new strategy for iron reduction and transport in plants. Journal of Biological Chemistry **289**: 2515–2525.

Guerinot ML, Yi Y (1994) Iron: Nutritious, noxious, and not readily available. Plant Physiology **104**: 815–820.

Gupta KJ, Hebelstrup KH, Mur LAJ, Igamberdiev AU (2011) Plant hemoglobins: Important players at the crossroads between oxygen and nitric oxide. FEBS Letters **585:** 3843–3849.

Halvin JL, Tisdale SL, Nelson WL, Beaton JD (2014) Micronutrients. In: Soil Fertility and Fertilizers. Pearson. p. 261–305.

Hansen NC, Jolley VD, Naeve SL, Goos RJ (2004) Iron deficiency of soybean in the North Central U.S. and associated soil properties. Soil Science and Plant Nutrition **50**: 983–987.

Hill RD, Hargrove M, Arredondo-Peter R (2016) Phytoglobin: A novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on Oxygen-Binding and Sensing Proteins. F1000 Research **5**: 1–8.

Hill RD (2012) Non-symbiotic haemoglobins-what's happening beyond nitric oxide scavenging? AoB PLANTS **12**: 1–13.

Igamberdiev AU, Hill RD (2004) Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. Journal of Experimental Botany **55**: 2473–2482.

Igamberdiev AU, Stoimenova M, Seregélyes C, Hill RD (2006) Class-1 hemoglobin and antioxidant metabolism in alfalfa roots. Planta **223**: 1041–1046.

Jain A, Wilson GT, Connolly EL (2014) The diverse roles of FRO family metalloreductases in iron and copper homeostasis. Frontiers in Plant Science **5**: 1–6.

Kim SA , Guerinot ML (2007) Mining iron: Iron uptake and transport in plants. FEBS Letters **581**: 2273–2280.

Kobayashi T, Nishizawa NK (2012) Iron Uptake, Translocation, and Regulation in Higher Plants. Annual Reviews of Plant Biology **63**: 131–152.

Kong J, Dong Y, Xu L, Liu S, Bai X (2014) Effects of foliar application of salicylic acid and nitric oxide in alleviating iron deficiency induced chlorosis of *Arachis hypogaea* L. Botanical Studies **55**: 1–12.

Krohling CA, Eutrópio FJ, Bertolazi AA, Dobbss LB, Campostrini E, Dias T, Ramos AC. (2016) Ecophysiology of iron homeostasis in plants. Soil Science and Plant Nutrition **62**: 39–47.

Kubo H (1939) Über das Hämoprotein aus den Wurzelknöllchen von Leguminosen. Acta Phytochimica **11**: 195–200.

Lamattina L, García-Mata C, Graziano M, Pagnussat G (2003) Nitric Oxide: The Versatility of an Extensive Signal Molecule. Annual Review of Plant Biology **54**: 109–136.

Lane DJR, Richardson DR (2014) The active role of vitamin C in mammalian iron metabolism: Much more than just enhanced iron absorption. Free Radical Biology Medicine **75**: 69–83.

Li G, Kronzucker HJ, Shi W (2016) The response of the root apex in plant adaptation to iron heterogeneity in soil. Frontiers in Plant Science **7**: 1-7.

Lichtenthaler HK (1987) Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. Methods Enzymology **148**: 350–382.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods **25**: 402–408.

López-Millán AF, Duy D, Philippar K (2016) Chloroplast iron transport proteins – Function and impact on plant physiology. Frontiers in Plant Science **7**: 1-12

Luth D, Warnberg K, Wang K (2014) Soybean (*Glycine max* (L.) Merr.). In: Wang K. Agrobacterium Protocols: Third Edition. New York, NY, Springer. p. 275–284.

M'sehli W, Houmani H, Donnini S, Zocchi G, Abdelly C, Gharsalli M (2014) Iron Deficiency Tolerance at Leaf Level in *Medicago ciliaris* Plants. American Journal of Plant Sciences **5**: 2541–2553.

Maathuis FJM, Diatloff E (2013) Roles and Functions of Plant Mineral Nutrients. In: Maathuis FJM. Plant Mineral Nutrients. York, UK, Humana Press. p. 1–22.

Macknight RC, Laing WA, Bulley SM, Broad RC, Johnson AA, Hellens RP (2017) Increasing ascorbate levels in crops to enhance human nutrition and plant abiotic stress tolerance. Current Opinion in Biotechnology **44**: 153–160.

Macmillan KP (2018) Yield Impact of Yellow Soybeans and Management Strategies. Manitoba Pulse and Soybean Growers.

Marsh HV, Evans HJ, Matrone G (1963) Investigations of the Role of Iron in Chlorophyll Metabolism - Effect of Iron Deficiency on Chlorophyll and Heme Content and on the Activities of Certain Enzymes in Leaves. Plant Physiology **38**: 632–638.

Mendoza-Cózatl DG, Xie Q, Akmakjian GZ, Jobe TO, Patel A, Stacey MG, Song L, Demoin DW, Jurisson SS, Stacey G, Schroeder JI (2014) OPT3 is a component of the iron-signaling network between leaves and roots and misregulation of OPT3 leads to an over-accumulation of cadmium in seeds. Molecular Plant **7**: 1455–1469.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell and Environment **33**: 453–467.

Miller GW, Pushnik JC, Welkie GW (1984) Iron chlorosis, a world wide problem, the relation of chlorophyll biosynthesis to iron. Journal of Plant Nutrition **7**: 1–22.

Mira MM, Hill RD, Stasolla C (2016) Phytoglobins improve hypoxic root growth by alleviating apical meristem cell death. Plant Physiology **172**: 2044–2056.

Mira MM, Huang S, Kapoor K, Hammond C, Hill RD, Stasolla C (2017) Expression of Arabidopsis class 1 phytoglobin (*AtPgb1*) delays death and degradation of the root apical meristem during severe PEG-induced water deficit. Journal of Experimental Botany **68**: 5653–5668.

Morrissey J, Guerinot ML (2009) Iron uptake and transport in plants: The good, the bad, and the ionome. Chemical Reviews **109**: 4553–4567.

Mukherjee I, Campbell NH, Ash JS, Connolly EL (2006) Expression profiling of the Arabidopsis ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. Planta **223**: 1178–1190.

Mur LAJ, Sivakumaran A, Mandon J, Cristescu SM, Harren FJM, Hebelstrup KH (2012) Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanism against pathogens. Journal of Experimental Botany **63**: 4375–4387.

Noctor G, Foyer CH (1998) Ascorbate and Glutathione: Keeping Active Oxygen under Control. Annual Review of Plant Biology **49**: 249–279.

Norman T (1980) Limiting Factors in Photosynthesis. Plant Physiology **65**: 114–120.

Norman T, Abadia J (1986) Function of iron in chloroplasts. Journal of Plant Nutrition **9**: 1532-4087.

Owusu V, Mira M, Soliman A, Adam LR, Daayf F, Hill RD, Stasolla C (2019) Suppression of the maize phytoglobin *ZmPgb1.1* promotes plant tolerance against *Clavibacter nebraskensis*. Planta **250**: 1803–1818.

Pagano, M.C., Miransari, M., 2016. The importance of soybean production worldwide. In: Miransari M. Abiotic and Biotic Stresses in Soybean Production. Elsevier. p. 1–26.

Palavan-Unsal N, Arisan D (2009) Nitric oxide signalling in plants. Botanical Review **75**: 203–229.

Poschenrieder C, Fernández JA, Rubio L, Pérez L, Terés J, Barceló J (2018) Transport and use of bicarbonate in plants: Current knowledge and challenges ahead. International Journal of Molecular Science **19**: 1352.

Qu ZL, Zhong NQ, Wang HY, Chen AP, Jian GL, Xia GX (2006) Ectopic expression of the cotton non-symbiotic hemoglobin gene *GhHbd1* triggers defense responses and increases disease tolerance in Arabidopsis. Plant and Cell Physiology **47**: 1058–1068.

Ramírez L, Bartoli CG, Lamattina L (2013) Glutathione and ascorbic acid protect Arabidopsis plants against detrimental effects of iron deficiency. Journal of Experimental Botany **64**: 3169–3178.

Römheld V, Marschner H (1981) Iron deficiency stress induced morphological and physiological changes in root tips of sunflower. Physiologia Plantarum **53**: 354–360.

Roschzttardtz H, Séguéla-Arnaud M, Briat JF, Vert G, Curie C (2011) The FRD3 citrate effluxer promotes iron nutrition between symplastically disconnected tissues throughout *Arabidopsis* development. The Plant Cell **23**: 2725–2737.

Santi S, Schmidt W (2009) Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. New Phytologist **183**: 1072–1084.

Santos CS, Ozgur R, Uzilday B, Turkan I, Roriz M, Rangel AOSS, Carvalho SMP, Vasconcelos MW (2019) Understanding the role of the antioxidant system and the tetrapyrrole cycle in iron deficiency chlorosis. Plants **8**: 1-19.

Sanz-Luque E, Chamizo-Ampudia A, Llamas A, Galvan A, Fernandez E (2015) Understanding nitrate assimilation and its regulation in microalgae. Frontiers in Plant Science **6**: 1-17.

Sanz-Luque E, Ocaña-Calahorro F, De Montaigu A, Chamizo-Ampudia A, Llamas A, Galván A, Fernández E (2015) THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity. The Plant Journal **81**: 467–479.

Severin AJ, Peiffer GA, Xu WW, Hyten DL, Bucciarelli B, O'Rourke JA, Bolon YT, Grant D, Farmer AD, May GD, Vance CP, Shoemaker RC, Stupar RM (2010) An integrative approach to genomic introgression mapping. Plant Physiology **154**: 3–12.

Shetty AS, Miller GW (1966) Influence of Iron Chlorosis on Pigment and Protein Metabolism in Leaves of *Nicotiana tabacum* L.. Plant Physiology **41**: 415–421.

Siddiqui MH, Al-Whaibi MH, Basalah MO (2011) Role of nitric oxide in tolerance of plants to abiotic stress. Protoplasma **248**: 447–455.

Solti Á, Kovács K, Basa B, Vértes A, Sárvári,É, Fodor F (2012) Uptake and incorporation of iron in sugar beet chloroplasts. Plant Physiolohy and Biochemistry **52**: 91–97.

Solti Á, Müller B, Czech V, Sárvári É, Fodor F (2014) Functional characterization of the chloroplast ferric chelate oxidoreductase enzyme. New Phytologist **202**: 920–928.

Song Y, Dong Y, Tian X, Wang W, He Z (2018) Mechanisms of Exogenous Nitric Oxide and 24-Epibrassinolide Alleviating Chlorosis of Peanut Plants Under Iron Deficiency. Pedosphere **28**: 926–942.

Stamler JS, Singel DJ, Loscalzo J (1992) Biochemistry of nitric oxide and its redox-activated forms. Science **258**: 1898–1902.

Sudhakar P, Latha P, Reddy PV (2016) Plant Pigments. In: Phenotyping Crop Plants for Physiological and Biochemical Traits. Elsevier Science & Technology. p. 121–128.

Sun B, Jing Y, Chen K, Song L, Chen F, Zhang L (2007) Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). Journal of Plant Physiology **164**: 536–543.

Teucher B, Olivares M, Cori H (2004) Enhancers of iron absorption: Ascorbic acid and other organic acids. International Journal of Vitamin and Nutrition Research **74**: 403–419.

Vigeolas H, Hühn DH, Geigenberger P (2011) Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic arabidopsis plants. Plant Physiology **155**: 1435–1444.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ, Dennis ES (2001) A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proceedings of the National Academy of Sciences of the United States of America **98**: 10119–10124.

Wilson ID, Neill SJ, Hancock JT (2008) Nitric oxide synthesis and signalling in plants. Plant, Cell and Environment **31**: 622–631.

Yordanova RY, Christov KN, Popova LP (2004) Antioxidative enzymes in barley plants subjected to soil flooding. Environmental and Experimental Botany **51**: 93–101.

Youssef MS, Mira MM, Renault S, Hill RD, Stasolla C (2016) Phytoglobin expression influences soil flooding response of corn plants. Annals of Botany **118**: 919–931.

Zhang J, Kirkham BM (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytologist **132**: 361–373.

Zhang X, Zhang D, Sun W, Wang T (2019) The adaptive mechanism of plants to iron deficiency via iron uptake, transport, and homeostasis. International Journal of Moleculaur Sciences **20**: 1-4.