Estimation of phytosiderophores in wheat-*Aegilops* addition/substitution lines under iron deficiency

By
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CERTIFICATE – I

This is to certify that this thesis entitled, **"Estimation of phytosiderophores in wheat-*Aegilops* addition/substitution lines under iron deficiency**" submitted for the degree of **Master of Science,** in the subject of **Molecular Biology, Biotechnology & Bioinformatics** to the CCS Haryana Agricultural University, is a bonafide research work carried out by **Miss Bindu Bisla** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Per cent
µl	Microlitre
$Ca(NO_3)_2$	Calcium nitrate
CS	Chinese Spring
$CuSO_4$	Copper sulphate
DMAS	Deoxymugineic acid synthase
e.g.	For example
et al.	*et alia* (and others)
$Fe(OH)_3$	Ferric oxide
Fig.	Figure
g	Gram
H_3BO_3	Boric acid
HCl	Hydrochloric acid
i.e.	That is
Ids3	Iron-deficiency-clone 3
KNO_3	Potassium nitrate
M	Molar
mg/kg	Milligram per kilogram
$MgSO_4$	Magnesium sulphate
min.	Minute
ml	Millilitre
$MnCl_2$	Manganese chloride
Na_2MoO_4	Sodium molybdate
NAAT	Nicotinamine amino transferase
NaFeEDTA	Ferric SodiumEthylenediaminetetraacetate
NAS	Nicotinamine synthase
$NH_4H_2PO_4$	Ammonium dihydrogen phosphate
pH	Negative logarithm of hydrogen ion concentration
qRT-PCR	Quantitative Real Time Polymerase Chain
RNA	Ribonucleic acid
RNase	Ribonuclease
sec	Seconds
T_m	Melting Temperature
$ZnSO_4$	Zinc sulphate

CHAPTER – I

INTRODUCTION

Wheat (*Triticum aestivum* L., 2n = 6x = 42) is a grass that belongs to family Gramineae. It is a major cereal crop grown all over the world, second only to maize in terms of production. It is India's second most significant food crop after rice, and it is prized for its nutritional qualities. It is native to Turkey's south-eastern region and is grown primarily in India's northern and northwestern regions (Heun *et al.*, 1997). According to the Second Advance Estimates for 2020-21 published by the Department of Agriculture, Cooperation and Farmer Welfare, Govt. of India, it is probable that the country will reach a total production of food grain of 303.34 million tons and which is higher by 5.84 million tons to the previous production of food grain of 297.50 million tons in 2019-20. Wheat production of 2020-21 is estimated at 109.24 million tons 8.81 million tons higher than the record wheat production of 100.42 million tons achieved in 2019-20. Wheat is the world's most traded crop, according to several surveys and researches, and its production plays a vital part in the administration of the Indian economy. India is currently in surplus and is able to export grain to the international market and earn foreign exchange. Wheat, in addition to being a food, is a good source of carbohydrates, macro and micro minerals, protein, vitamin B and E, and dietary fibers (Divite *et al*., 2019). *Triticum aestivum*, *Triticum durum*, and *Triticum dicoccum* are the three main wheat species cultivated in the region, *T. aestivum*, also known as "common" or "bread" wheat, and is most widely grown wheat species worldwide. *Triticum sphaerococcum*, or Indian wheat, is no longer grown and has been substituted by *Triticum aestivum*, or bread wheat, and *Triticum turgidum*, or durum wheat. Bread and durum wheat account for 90% of the world's wheat supply (Shewry and Hey, 2015). Bread wheat is used to make bread, chapattis, pies, cakes, pastries, and other baked goods, while durum wheat is used to make pasta.

Micronutrients are necessary for plant development and play a key role in crop and human nutrition. In most Indian soils, total macronutrient content is adequate, but micronutrient concentrations in the soil solution are essentially poor to fulfill the demand of growing crops (Singh, 1998). Crops grown in the majority of Indian soils are deficient in multiple micronutrients. Deficiencies vary in nature and severity depending on soil type, soil composition, crop genotype, and agro-ecological conditions. Intensive cultivation of high-yield rice and wheat species resulted in zinc (Zn), iron (Fe) and manganese (Mn) deficiencies being a serious threat to the maintenance of high food grain output. Primary wheat growing areas of the country i.e., Madhya Pradesh, Punjab, Haryana and Uttar Pradesh have the highest Fe and Zn deficit (Divte *et al*., 2019). Fe is a significant micronutrient that limits plant growth and grain production. It is essential for life-sustaining processes such as respiration

and photosynthesis, where it participates in electron transport via reversible redox reactions, cycling between Fe^{2+} and Fe^{3+}. Fe insufficiency symptoms include interveinal chlorosis in leaves and reduced crop yield due to insufficient Fe uptake. Plants require Fe concentrations of 109–104 M for optimal growth, although Fe uptake is difficult due to its poor solubility in soil solution (Abbaspour*et al*., 2014).

Although Fe is the fourth most prevalent element in the earth's crust, it is not easily accessible by plants (Kim *et al*., 2007). Fe is inadequate in more than 50 percent of the world's cultivated soil. Fe deficiency is common on high pH, calcareous, or sandy soils, and is caused mostly by their inaccessible forms rather than its amount in the soil (Marschner, 1995). In Indian soils, accessible Fe ranges from trace amount to 234 mg/kg. Fertilizers cannot be used to compensate for Fe deficiency in upland crops because the ferrous (Fe^{2+}) form is oxidised to ferric (Fe^{3+}) form, which is not accessible to crops (Malewar and Ismail, 1995). The recurrent conversion of Fe^{2+} to Fe^{3+} reduces Fe^{2+} availability to plants, particularly during rice-wheat crop cycles (Katyal and Deb, 1982). It is also a critical micronutrient for human nutrition. Micronutrient deficiency is listed as the fifth significant worldwide concern to human health by the World Health Organization (WHO) in the Copenhagen Consensus of 2020. Over three billion people, mostly women and children, suffer from micronutrient malnutrition, notably Fe deficiency (Wang *et al*., 2012). Fe deficiency leads to poor general health, anemia, and higher rates of morbidity and death. It is a particular issue in developing nations since cereals are the mainstay of the diet (Peleg *et al*.,2008).

However, plants have evolved different ways to deal with Fe deficiency by making the Fe^{3+} form of Fe easier to absorb. In the context of low Fe supplies, plants activate Fe acquisition mechanisms (Ogo *et al*., 2011). Dicots can release proton, activating Fe(III) chelate reductase, and then transporting Fe^{2+} ions across the root plasma membrane to make the insolubilized form of Fe accessible (Stratergy I) (Kobayashi *et al*., 2010). Grasses release non proteinaceous amino acids termed as phytosiderophores (PS), that are the natural chelating agents produced particularly under Fe deficiency (Takagi, 1976) and PS coordinate Fe with their amino and carboxyl group. Various monocot species generate and release PS of various classes, including mugineic acid (MA), 2'-deoxymugineic acid (DMA), 3-hydroxymugineic acid (HMA), and 3-epihydroxy 2'-deoxymugineic acid (epi-HMA). The MA, epi-HMA, and HMA are mostly produced by barley, whereas the DMA is primarily produced by wheat, and avenic acid (AVA) is produced by oats. Mugineic acid dissolves inorganic Fe^{3+} efficiently, forming Fe^{3+}-mugineic acid complex which is then absorbed by specialized transporters (Masuda *et al*., 2017). The quantity of PSs secreted by the plant is significantly controlled by the proportion of PSs produced in the root and accessible for release within the rhizosphere (Singh and Singh, 2011). The root system architecture (RSA) regulates PS release, whereas Fe scarcity promotes the activities of nicotianamine synthase

(*NAS*) and nicotianamine amino transferase (*NAAT*), which regulate PS synthesis (Oburger *et al*., 2014). Low Fe accessibility causes root epidermal cells to undergo morphological alterations similar to those seen in phosphorus deficit. Under Fe-restricted growth circumstances, root hair production and elongation increase (Lopez-Bucio *et al*., 2003). In *Arabidopsis*, a moderate Fe deficit increased the length of primary roots by 15% to 17%, whereas a mild Fe deficiency promoted root elongation and a severe Fe deficit resulted in stunting. Bernards and coworkers (2002) discovered that the nodal root system in maize produces more PSs than that of the primary root system, while Higuchi *et al*. (1999) discovered that overexpression of the *NAS* gene in barley promotes PS-mediated Fe deficiency resistance. PS production has been linked to Fe deficit tolerance in cereal species. In addition to Fe, PSs have been found to chelate zinc (Zn), copper (Cu), manganese (Mn), and cadmium (Cd). PS, a natural plant product, may therefore be another potential phytoextraction method for mobilising metals from polluted soils, as it is biodegradable and has an advantage over synthetic chelators (Divte *et al*., 2019). Thus, the current work focuses on PSs biosynthesis in order to get a better knowledge of its involvement in controlling Fe deficiency tolerance in wheat.

In view of the above, the current study entitled **"Estimation of phytosiderophores in wheat-*Aegilops* addition/substitution lines under Fe deficiency"** was planned with the following objectives:

1. **To quantify the phytosiderophores (DMA and NA) under Fe deficiency in wheat-*Aegilops* addition/substitution lines**
2. **Relative expression analysis of the putative gene(s) responsible for Fe uptake in wheat-*Aegilops* addition/substitution lines**

CHAPTER – II

REVIEW OF LITERATURE

2.1 Wheat: An important cereal crop

Wheat (*Triticum aestivum*) is one of the "big three" cereal crops. Wheat, maize, and rice provide more than 85 percent of all grain output globally and more than half of total food calories. Wheat has been grown in India since 5000 years ago (Shrewry, 2001), and it is a staple meal for millions of people, mostly in India's northern and northwestern areas. Wheat is the world's most traded crop (worth $50 billion each year), and it is crucial to the Indian economy's management. In 2019, global wheat output was around 765.5 million tons from a cultivated area of 215.9 million hectares, with India producing 103.5 million tons (FAOS, 2020). In terms of wheat production, India is second only to China. India is currently self-sufficient in wheat production and will be able to earn forging exchange from wheat cultivation in the near future.

Wheat is the most significant crop in temperate areas, where it is utilized for human consumption and animal feed. Even though there are several wheat species recognized across the globe, the commercially cultivated species in India are *T. aestivum* (Bread wheat), *T. durum* (Macaroni wheat), and *T. dicoccum* (Emmer wheat). To a certain extent, the success of wheat cultivation depends on its high adaptability and potential for yield and mainly because of the presence of gluten (protein fraction), which provides the viscoelastic properties to process dough into various products including bread, pasta, noodles, and other nutritional products (Shewry, 2009). Wheat grains also include all important elements, such as all essential amino acids (with the exception of lysine, tryptophan, and methionine), lipids (1.5-2%), minerals (1.5-2%), vitamins (vitamin E and B complex), 2.2 percent crude fibres, and beneficial phytochemicals (Godfrey *et al*., 2010).

In wheat, Fe and Zn content is low, and genetic diversity is limited across cultivated bread and durum wheat varieties (Cakmak *et al*., 2004; White and Broadley, 2005). However, when comparing wild progenitor and non-progenitor wheat species to contemporary wheat cultivars, a broad range of variance for grain micro and macronutrients has been noted (Neelam *et al*., 2012). The related wild progenitor and non-progenitor Triticum and *Aegilops* species have two to three times greater grain Fe and Zn content (Rawat*et al*., 2009).

As micronutrients are lost during processing, knowing where Fe is located is crucial in determining grain nutritional quality. The majority of research on micronutrient variability in wheat germplasm used entire grains, which can be misleading because the ratios of bran and endosperm in hexaploid wheat and its wild relatives can vary significantly. As a result,

selecting hexaploid wheat cultivars, related wild species, and wheat *Ae. Kotschyi* addition/substitution lines is critical for useful variability of high Fe content in endosperm and bran section, which could be useful in identifying potential donors for biofortification of endosperm/bran fraction of hexaploid wheat.

Wheat germplasm from wild progenitor and non-progenitor lines has been widely used for wheat improvement (Tiwari *et al*., 2010), and useful variability for grain micronutrients has recently been transferred to elite wheat cultivars via the development of various wheat-*Aegilops* addition, substitution, and translocation lines for different chromosomes with higher Fe content (Schnedier *et al*., 2008). A number of established wheat-*Aegilops* translocation, addition, and substitution lines have grain Fe levels that are 50 to 150 percent greater than recipient wheat cultivars, with grain yield and harvest index that are comparable to wheat cultivars (Tiwari *et al*., 2010; Neelam *et al*., 2012). Wheat varieties with increased absorption efficiency and greater Fe translocation into grains may be useful in improving crop productivity and overcoming Fe deficiency in the developing countries.

2.2 Mineral nutrients in cereal crops

In developing countries, agriculture plays an important role in maintaining the country's economy since they require long-term food grain production to maintain their rapidly growing populations. However, grain production is directly influenced by the physical and chemical qualities of the soil, as well as the environment in which it grows. Apart from that, soil nutrients are important in influencing crop grain production and regulating their vegetative growth and development. Plants require 17 key nutritional elements to complete their life cycle, viz. C, H, O, N, P, K, Ca, Mg, S (macronutrients) and Fe, Cu, Mn, Zn, B, Mo, Cl, Ni (micronutrients) (Arnon and Stout, 1939). Despite the fact that micronutrients are required in much smaller quantities by plants, they are just as important for the plant as macronutrients.

Micronutrients have a substantial impact on the plant, animal, and human growth, metabolism, and reproductive vigour (Lyons *et al*., 2004). Micronutrient deficits are prevalent in soils across the world, including India, limiting agricultural output. It also makes a significant contribution to malnutrition in humans. Micronutrient deficiency is estimated to affect more than three billion individuals globally (Singh, 2001). It is therefore crucial that efforts of research focus on dealing with micronutrient deficiencies, as micronutrient deficiencies may seriously restrict agricultural production if they are not addressed promptly. To feed a growing population, we require more food grains from a confined soil and natural resources, which will necessitate a genuine scientific and managerial approach (Fageria, 2002). More than half of the soils in the world were found to be deficient in Fe and Zn (Sillanpaa, 1982; White and Zasoski, 1999; Salwa *et al*., 2010). Despite the fact that these nutrients are physically present in the soil, they are not accessible for plant absorption. The

rhizospheric circulation and absorption of these nutrients are influenced by a variety of soil parameters including electrical conductivity (EC), pH and soil texture (Divte *et al*., 2019).

2.3 Micronutrients for nutritional food security

Crop productivity and growth, as well as human development, are affected by micronutrient scarcity. It also affects the nutritional content of food grain and seed production (Sanchez and Swaminathan, 2005). There are several ways to enhance the nutritional uptake in humans like a post-harvest nutritional fortification, mineral enrichment and dietary diversification. However, in developed countries, the present degree of Fe and Zn post-harvest fortification is difficult to enforce (Cakmak *et al*., 2001; Irshad *et al*., 2004). To combat micronutrient starvation, biofortification through conventional breeding, molecular breeding, genome editing and transgenic techniques are used (Divte *et al*., 2019). To improve the nutritional content of food crops we can adopt biofortification process, in which conventional practices like plant breeding are used for the enhancement of vitamins and minerals etc. (Bouis *et al*., 2011).

Fe deficiency is a significant cause of infant mortality worldwide, and also threaten the wellbeing of adults (Hotz and Brown, 2004). It is a well-known public health issue in the countries where majority of people rely on a cereal-based diet to fulfill the basic calorie needs. Cereal crops have lower Fe concentrations by nature, and growing them on Fe-deficient soil decreases the grain Fe concentration even further. The major portion of India's soil is insufficient in Fe, and the country ranks first among countries with the most Fe-deficient soils in the world. The low level of soil Fe leads to a low level of crop Fe and hence does not make a major contribution to biofortification. Fe malnutrition is a big concern in rural India, where rice-wheat accounts for 75 percent of daily calorie intake. There is a critical need for the improvement of grain Fe content nationally as well as globally (Welch and Graham, 2004). Biofortification is a promising and sustainable agriculture-based strategy for addressing Fe deficiency, especially in high risk populations with constricted resources (Petry *et al*.,2016; Vasconcelos *et al*., 2017).

2.4 Fe in human nutrition

Fe is an abundant element on earth and a biologically important component for all living organisms (Aisen *et al*., 2001; Lieu *et al*., 2001). Fe levels in adult women and man are around 35 and 45 mg/kg of body weight, respectively. Around 60-70 percent, of total body Fe, is found in haemoglobin. A further 10% of necessary Fe is present in the form of myoglobins, cytochromes, and Fe-containing enzymes. In healthy people, the rest 20-30% of spare Fe is retained in hepatocytes and reticuloendothelial macrophages as ferritins and hemosiderins (Lieu *et al*., 2001). Fe metabolism disorders are among the most prevalent human illnesses, encompassing a wide range of conditions with varying clinical manifestations, ranging from anemia to Fe excess and perhaps neurodegenerative diseases

(Lieu *et al*., 2001). Fe plays a vital role in the formation of haemoglobin and oxygen delivery in human body (Abbaspour *et al*., 2014). Low Fe consumption and bioavailability are now the leading causes of anemia in developing countries (Dary *et al*., 2006), as well as infectious and inflammatory diseases (particularly malaria), and other nutrient deficiencies like vitamin A and B12, riboflavin, folic acid (Grantham *et al*., 2001). Fe deficiency anemia (IDA) can cause fatigue, exhaustion, and irritability in adults, but the effects of long-term IDA in children are much greater, including decreased brain function and exposure to multiple diseases (Kennedy *et al*., 2003). Fe deficiency in females results in the development of autism spectrum disease in infants (Schmidt *et al*., 2014). Fe content in bodily tissue, on the other hand, must be closely controlled since too much Fe causes tissue damage due to the production of free radicals. The ability to identify the underlying causes of each disease and provide correct diagnosis and therapy requires a molecular understanding of Fe control in the body (Leiu *et al*.,2001).

2.5 Role of Fe in plants

The importance of mineral nutrition in plant development cannot be overstated. However, information on the acquisition, assimilation and storage of most important metal ions is limited at the molecular level. Fe is significant among these elements because of its physicochemical properties: it participates in most of the fundamental redox processes necessary in both the synthesis and consumption of oxygen, and it is coordinated at metalloprotein active sites. Fe also has a role in nitrogen fixation, DNA synthesis (ribonucleotide reductase), and hormone production (Briat and Lobreaux, 1997). Fe is a part of heme, the Fe-sulfur complex, nitrogen fixation, growth and development of plants, and participates in several metabolic processes like DNA synthesis, photosynthesis, and respiration (Rout and Sahoo, 2015). It is reported in many important enzymes, including ribonucleotide reductase, lipoxygenase, as well as nitrate reductase, and electron transport chain cytochromes. It is also involved in biosynthesis of chlorophyll precursor, aminolevulinic acid (ALA), which plays important role in maintenance of chlorophyll structure and proper functioning (Rout and Sahoo, 2015). Plants closely regulate uptake, utilisation, and storage in response to the environmental abundance to accumulate adequate Fe while minimising toxicity (Marschner, 1995). Fe scarcity also has an impact on the photosynthetic system structure and function, specifically Photosystem-I (Moseley *et al*., 2002).

2.6 Fe deficiency in plants

Fe deficiency in cultivable soils has been recorded in India. There is not one cause of Fe limitation in plants. Instead, it's the consequence of a number of intricate interactions between the plant and its surroundings. The relatively poor solubility of Fe(III) oxides is probably the most significant factor causing Fe scarcity in plants (Lindsay, 1995). The major

symptom of Fe deficiency in plants is interveinal chlorosis in which the numbers of photosynthetic pigments are decreased and the leaves turn yellow-brown at the margins between the green veins (Spiller and Terry, 1980; Morales *et al*., 1998). Fe deficiency chlorosis often termed "lime induced chlorosis," is a major issue in wheat plants, particularly in calcareous soil, leading to a considerable loss in output (Lindsay, 1995). Severe deficiency results in complete yellowing of the leaves, which eventually turn white, resulting in crop development and output being hampered (Benke and Stich, 2011). Fe deficiency effects are primarily seen on young leaves and shoots since Fe is stationary in plants (Marschner,1995).

2.7 Fe toxicity in plants

Fe toxicity in crops is generally not widespread except in damp soil, where it is the second-most serious yield-constraining factor for rice (Nikolic & Pavlovic, 2018). Drought can produce oxidative damage in photosynthetic tissue due to Fe-catalyzed reactive oxygen species (ROS) production in the chloroplasts, therefore Fe toxicity can occur in dryland environment as well (Price & Hendry, 1991; Broadley *et al*., 2012). The most common cause of Fe toxicity is decrease in soil pH that results in an excess of available Fe. The passage of atmospheric oxygen into the soil is restricted under submerged conditions, which encourages the growth of anaerobic microorganisms and lowers the pH of the soil solution owing to increased CO_2 buildup. Excess Fe can precipitate as Fe(III) hydroxides or phosphates under aerobic conditions, producing a large Fe pool in the root apoplast that provides 95% of the total Fe content in plant roots and can be reused when Fe is limited (Cesco *et al*., 2002). Plants increase the activities of anti-oxidants like superoxide dismutase (SOD), catalases (CAT) and peroxidase to avoid the effect of oxidative damage caused by ROS on the cells, in particular on the lipid contents of the cell membrane. The removal of ROS is also supported by antioxidant molecules like carotenoids, glutathione, ubiquinol, tocopherol, ascorbate, and lipote (Nikolic & Pavlovic, 2018). Fe toxicity can also occur if the soil is deficient in Zn and is in a "reduced" state as a result of flooding or damp condition. The obvious signs of Fe poisoning are probably another nutritional deficit (Tanaka *et al*., 1976). Dark green leaves, the steep development of roots and the dark brown to purple leaves (e.g. rice bronze) are all indicators of Fe toxicity (Tanaka *et al*., 1976). Fe-tolerant genotypes along with appropriate techniques of soil, water and fertilizer management are necessary for overcoming the loss in yield from Fe toxicity.

2.8 Fe acquisition strategies in plants

Fe deficiency, a worldwide agricultural concern in calcareous soil with insufficient Fe availability, is also a serious human nutritional shortfall. Plants induce Fe acquisition mechanisms in low Fe accessibility (Ogo *et al*., 2011). Under aerobic circumstances, Fe is very minimally soluble, especially in high pH and calcareous soils (Marschner, 1995). Fe as a free ion causes two primary issues for plants: insolubility and toxicity (Haydon *et al*., 2012).

Romheld and Marschner (1986) investigated Fe acquisition methods in different plants and discovered that they followed two distinct strategies: strategy I, found in nongraminaceous plants, and strategy II, in graminaceous plants (Figure 2.1).

Takagi (1976) was the former to identify phytosiderophores as compounds released by roots to solubilize and chelate inaccessible Fe form (Fe^{3+}) in Fe deficient graminaceous plants. These PSs aid transporter proteins like Yellow Stripe 1 (*YS1*) in the absorption of Fe^{3+}-PS complexes. Dicotyledonous and nongraminaceous monocotyledonous species (strategy- I) can make unsolubilized Fe accessible by liberating proton, activating Fe^{3+}chelate reductase, and thentransferring Fe^{2+} ions through the root plasma membrane using Fe-regulated transporter, *IRT1* (Kobayashi *et al.*, 2010). Grasses (Strategy II) use efflux transporters, transporter of mugineic acid (*TOM1)* to release mugineic acids or PSs into the rhizosphere. MA effectively solubilizes Fe^{3+}, forming Fe^{3+}-MA complex which is then taken up by specific transporters i.e., YELLOW STRIPE 1 (*YS1*) and YELLOW STRIPE 1-like (*YSL*) (Kobayashi *et al*., 2010). PSs are released in response to root properties (Bernards *et al*.,2002).

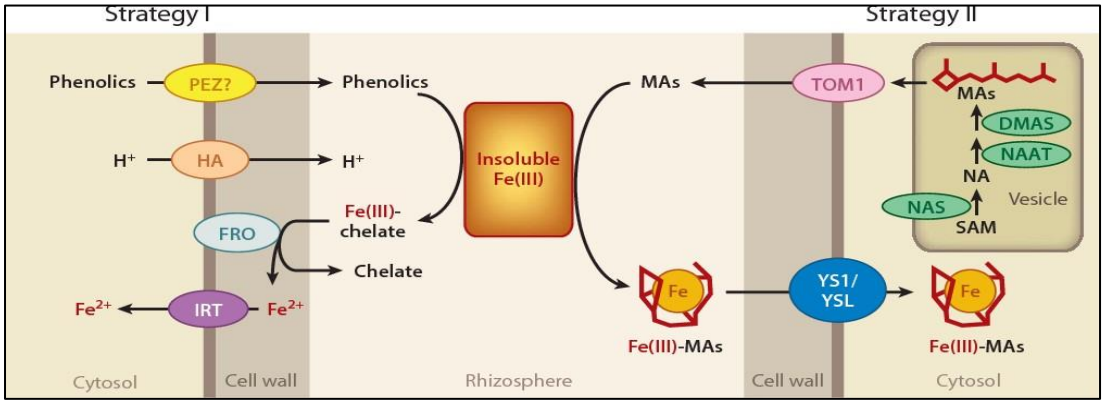

Fig. 2.1: Fe acquisition strategy in higher plants: Strategy I in nongraminaceous plants and Strategy II in graminaceousplants

2.8.1 Strategy I

Strategy I is observed in almost all plants other than grasses. Several dicots like *Lycopersicon esculentum* (tomato), *Arabidopsis thaliana*, and *Pisum sativum* (pea) are used as model plants for studying strategy I. Fe intake involves proton excretion through plasma membrane P-type ATPase to acidify the surrounding rhizospheric solution and thus enhance Fe^{3+} solubility by reduction of Fe^{3+} to the more soluble Fe^{2+} form by a Fe^{3+} chelate reductase. During Fe scarcity the activity of all the components of strategy-I are enhanced. Other adaptation metrics consist of changes in root hair and shape, transfer cell development (Santi and Schmidt, 2008), and alleviation in phloem citrate concentrations (Schmidt, 1999). The main reason of acidity in plant is increased number of H^+-ATPase family protein (Palmgren, 2001). The ferric chelate reductase oxidase gene (*FRO2*) (Robinson *et al*., 1999, Waters *et al*., 2002) and Fe-regulated transporter gene (*IRT1*) were the first gene to be cloned in *Arabidopsis* (Eide *et al*., 1996). Fe^{3+}chelate reductase are intrinsic membrane proteins that fall

in the protein family that transport electrons from cytosolic NADPH to FAD and then to electron acceptors on the plasmalemma's outer side through heme groups. In response to Fe deprivation, the FRO genes are increased in roots (Robinson *et al*., 1999; Waters *et al*., 2002). A handful of the many known H^+-ATPase (HA) genes are increased in the presence of Fe scarcity and play a major part in determining strategy I responses, as demonstrated by cucumber *CsHA1* (Santi *et al*., 2005) and *Arabidopsis AHA2* (Santi and Schmidt, 2009) (Fig. 2.1). In non-graminaceous plants genes that regulate the production and release of phenolic chemicals are still to be decoded.

2.8.2 Strategy II

Bernards *et al*., (2002) investigated the release of PSs from the primary, and entire root systems of maize and discovered a substantial link among Fe deficiency tolerance and PSs release. The monocot species-specific Strategy II response is regulated by the effectiveness of PS production and release. There are many forms of PSs in the mugineic acid family, all of which are synthesised from S-adenosyl-L-methionine (SAM). The three enzymes nicotianamine synthase (*NAS*), nicotianamine aminotransferase (*NAAT*), and deoxymugineic acid synthase (*DMAS*) carry out three successive enzymatic processes that culminate in DMA, the precursor to all other MAs. (Bashir *et al*., 2006; Higuchi *et al*., 1999; Takahashi *et al*., 1999). Under Fe shortage, expression of several genes such as *NAS*, *NAAT*, and *DMAS* substantially promote MAs biosynthesis. The methionine cycle, also known as the Yang cycle, is a collection of recycling processes that provides methionine for the manufacture of several PSs (Ma *et al*., 1995). MA secretion has a diurnal rhythm, with a significant surge early in the morning (Takagi *et al*., 1984).

Negishi *et al*., 2002 found that root cells which were deficient in Fe contain vesicles that swell in the morning but shrink by sunset. As previously stated, MAs secreted by the roots solubilize and chelate Fe^{3+} in the rhizosphere, forming Fe^{3+}-MA complexes which are absorbed by the roots with the help of metal-PS complex transporters, *YS1* and *YSL* (Curie *et al*., 2001; Murata *et al*., 2006; Inoue *et al*., 2009; Lee *et al*.,2009) (Fig. 2.1).

2.9 Genes regulating phytosiderophores biosynthesis under Fe deficiency

Plants that use the Strategy II Fe absorption mechanism produce Fe chelators termed PSs from their roots to solubilize sparingly soluble Fe in the rhizosphere. Under Fe deficiency stress, the amount of PS released rises. MAs have a biosynthetic route that has been discovered. SAM synthetase converts Met to SAM, and nicotianamine synthase (*NAS*) combines three molecules of SAM to make one molecule of Nicotianamine (NA). Nicotianamine amino transferase (*NAAT*) converts NA to [399-keto acid] and by subsequent reductase activity deoxymugineic acid (DMA) is formed (Figure 2.2). Han *et al*., (2014) found that *NAS* is critical for balancing heavy metal concentrations in plants. Under Fe deficiency, *NAS* catalyses the synthesis of nicotianamine (NA) from S adenosyl methionine

(SAM), a PS intermediary molecule (Higuchi *et al*., 1999). Plants with low levels of NA acquire less Fe, Cu, Zn, and Mn, and have altered heavy metal distribution (Haydon *et al*.,2012) (Fig. 2.0).

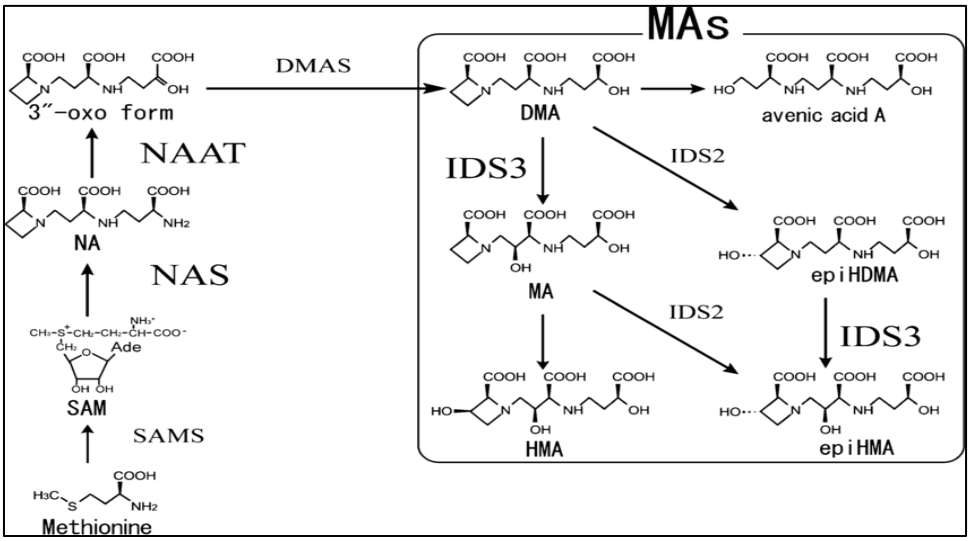

Fig. 2.2: Biosynthetic pathway of Mugineic acid (MA) and its derivatives

2.10 COEXISTENCE OF STRATEGIES-I & II

Rice is an unusual graminaceous plant as it uses both reduction and chelation strategy for Fe uptake. This is not odd considering that rice and its wild progenitors thrive in highly reductive wetlands. *OsYSL15*, the main transporter, which is responsible for rhizosphere Fe-PS absorption, is activated by Fe deprivation, that can arise when rice is grown in upland conditions. It is found in the root epidermis and developing seeds where it is involved in Fe homeostasis. As a result, it appears that Fe loading into seeds is more essential than Fe-PS absorption by roots (Morrissey & Guerinot, 2009). Unlike maize and barley, rice generates significantly less PS and is less tolerant of Fe deficit on calcareous soils (Nikolic & Pavlovic, 2018). In response to Fe deficit, rice roots have overexpressed the transporters *OsIRT1* and *OSIRT2*, apparently to adapt to an environment where oxygen concentration is reduced due to water-logging and Fe is primarily in Fe(II) form. Despite the discovery of two ferric reductase oxidase (*FRO*) genes (*OsFRO1* and *OsFRO2*) in the rice genome (Hindt & Guerinot, 2012), rice roots have limited ability for Fe(III) reduction. As a result, the reduction approach alone is insufficient under Fe(II) restricted upland conditions (Morrissey & Guerinot, 2009). Rice plants, on the other hand, are more vulnerable to Fe toxicity when grown in soils rich in Fe^{2+} (Hindt & Guerinot, 2012). Moreover, a recently discovered *IRT1* ortholog in barley has a strong affinity for Fe^{2+}, as well as other divalent metals including Mn^{2+}, Zn^{2+}, and Cd^{2+} (Kobayashi & Nishizawa, 2012). Even though the location of *HvIRT1* is yet to be identified, it seems to be enhanced under Fe-restricted situation; nonetheless, it is too early to conclude that barley carries Fe^{2+} into the root epidermis just as non graminacious species (Morrissey & Guerinot,2009).

CHAPTER – III

MATERIALS AND METHODS

The present study entitled "**Estimation of different phytosiderophores in wheat-*Aegilops* addition/substitution lines under iron deficiency"** was conducted during the year of 2020–2021 in the Department of Molecular Biology, Biotechnology and Bioinformatics, CCSHAU Hisar. The experiment was planned to quantify PSs under Fe deficiency and to find out the relative expression analysis of putative genes responsible for Fe uptake in wheat-*Aegilops* addition/substitution lines (UP HAU-3 and Chinese Spring).

3.1 Plant Material and Growth conditions

Wheat- *Aegilops* addition/substitution lines (UP HAU-3) and Chinese Spring were taken and germinated for 5 days in petriplate at room temperature and then they were transferred to plastic containers having water. After 10 days of germination the seedlings were cultured hydroponically in plastic containers having half strength Hoagland nutrient solution (composition is shown in Table 3.1). The pH of the solution was kept at 5.7 with the help of 1N NaOH solution.

Table 3.1: Chemical composition of Hoagland nutrient solution

Sr. No.	Nutrients	Concentration (mM)
1.	KNO_3	3 mM
2.	$Ca(NO_3)_2$	2 mM
3.	$NH_4H_2PO_4$	0.5 mM
4.	$MgSO_4$	1 mM
5.	$ZnSO_4$	0.0008 mM
6.	$CuSO_4$	0.0003 mM
7.	$MnCl_2$	9.1 mM
8.	H_3BO_3	0.046 mM
9.	Na_2MoO_4	0.0008 mM
10.	NaFeEDTA	0.005 mM
11.	Ferrozine	0.005 mM

3.1.1 Collection of root exudates

10 days old plants were subjected to nutrient solutions with and without Fe for the collection of root exudates. To induce Fe deficiency, ferrozine was use instead of NaFeEDTA. The nutrient solution was changed after three days to keep the pH at 5.7 throughout the experiment. Root exudates were collected after 5 days of treatment. The plants were removed from the nutrient solution 2 hours after the onset of the light period and the roots were rinsed several times with distilled water before root exudates were collected. The root exudates were collected after immersing the plant roots in 500 mL distilled water for 4

hours. The root exudates were for collected for five times after every 2 days. Root exudates were collected and filtered through filter paper (WhatmanNo.1 filter paper), concentrated to 50 ml in a lyophilizer at -80°C, and stored at -20°C until analysis.

3.1.2 Determination of phytosiderophores content in root exudates

The Fe-mobilization test was used to evaluate the PS content in root exudates (Takagi, 1976). 2 ml of freshly precipitated $Fe(OH)_3$ and 0.5 ml of 0.5 M Na acetate buffer (pH 5.6) were added to 8 ml of the collection solution, agitated for 2 h, and filtered using Whatman No.1 filter paper into 0.2 ml of 6N HCl. By adding 0.5 mL of 8% hydroxylamine hydrochloride and heating to 60 °C for 20 minutes, ferric ions were reduced. After adding 0.2 ml of 0.25 percent ferrozine and 1 ml of 2 M Na-acetate buffer (pH 4.7), the amount of ferrous ion was measured spectrophotometrically by measuring absorbance at 562 nm. Amount of PS was calculated using the following formula and expressed as nmol of Fe equivalent g-:

$$PS= \frac{A_{562}X \text{ final volume of collected root exudates}}{2.15 \text{ X sample volume used in the assay}}$$

3.2 Expression analysis of *NAS*, *NAAT*, *DMAS* and *Ids3* genes under Fe sufficient and deficient conditions

Transcript expression level of genes *NAS*, *NAAT*, *DMAS* and *Ids3* were studied in roots of Fe sufficient and deficient plants after 14 days of treatment.

3.2.1 RNA isolation-TRIzol method

Root sample was crushed to a fine powder in liquid nitrogen in a pre-chilled mortar and pestle. To convert the powered sample into a liquid, 1 ml of TRIzol reagent was added. The solution was transferred to a 2 mL vial and spun for 5 seconds. To allow full dissociation of the nucleoprotein complex, it was incubated horizontally on ice for 10 minutes. With vigorous shaking for 30 seconds, 200 µl of chloroform was added. The sample was centrifuged at 13,000 rpm for 10 minutes at 4 °C after being incubated on ice for 5 minutes. The mixture was divided into three phases: a lower red phenol-chloroform phase, an interphase, and an upper aqueous phase that was colourless. The supernatant was transferred to a new 1.5 ml vial with an equal amount of isopropyl alcohol and maintained on ice for 10 minutes. It was centrifuged for 10 minutes at 4 °C at 13,000 rpm. The supernatant was discarded, taking care not to lose the pellet, and 800 µl of 70 percent ethanol were added. The pellet was treated with ethanol and then centrifuged for 10 minutes at 4°C at 13,000 rpm. The supernatant was decanted while being careful not to lose the pellet and it was allowed to dry for 10 to 15 minutes at room temperature. Later, the pellet was dissolved in 10-30 µl DEPC treated water. DNase treatment was given at 37°C for 30 minutes to remove the genomic DNA from RNA samples.

3.2.2 cDNA synthesis:

cDNA synthesis was carried out using Thermo Scientific Verso cDNA synthesis Kit. cDNA synthesis was carried out at 42 °C for 30 minutes and inactivation was done at 92°C for 2 minutes. All the reagents are given in Table 3.2.

Table 3.2 Reaction mix for cDNA synthesis

Sr. No.	Components	Volume (µl)
1.	5X cDNA synthesis buffer	4 µl
2.	dNTP Mix	2 µl
3.	RNA Primer	1 µl
4.	RT Enhancer	1 µl
5.	Verso Enzyme Mix	1 µl
6.	RNA Template (500ng)	1-5 µl
7.	Nuclease free water	To 20µl
	Total volume	20 µl

3.2.3 Primer Designing

The cDNA sequence of *NAS, NAAT, DMAS* and *Ids3* were retrieved from Ensembl Plants (Appendix). Forward and reverse primers of the target genes were synthesized using Primer Quest software with following parameters: Tm range was set from 55-60°C and GC content was adjusted to 40-60% and product size ranges from 150-200 bp. Further hairpin and primer-dimer formation was checked using oligoanalyzer. (Table 4.3).

Table 3.3 Primers used in RT-PCR

Sr. No.	Gene	Forward Primer (5'-3')	Reverse Primer (5'-3;)	T_m (°C)
1.	*NAS*	GCTACGTATGATGTCGTCTTCC	TGCACGTCGTTGGTCTTT'	56°C
2.	*NAAT*	CTCAAGCGATCGAGGTCATAAT	TCGCGGTTGTGTTCTTGT	54°C
3.	*DMAS*	AGCTAGTGTCTTCTGGGAATTG	ACTGCTAGGAAGGAGTGAAATG	56°C
4.	*Ids3*	TTAGACCTCAAGTTCACCTGTATC	GATCTTACCCGGACACACATT	54°C

3.2.4 Quantitative real-time PCR:

Quantitative RT-PCR analysis was done using Applied Biosystem SYBR Green Master Mix (Thermo Fisher Scientific) on an Applied Biosystems (Quant Studio 6 Flex) instrument. Wheat-actin gene was used as an internal control. The relative expression levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method. The reaction mixture contained the following components (Table 3.4). The following standard cycle programme was used to conduct the reactions on an Applied Biosystems machine (Table 3.5).

Table 3.4 Reaction mix for RT-PCR

Sr. No.	Component	Volume (µl)
1.	PowerUp SYBR Green Master Mix (2x)	10 µl
2.	cDNA	2 µl
3.	Gene specific forward primer	0.5 µl
4.	Gene specific reverse primer	0.5 µl
5.	Nuclease free H_2O	7 µl
	Total Volume	20 µl

Table 3.5: Protocol used for RT-PCR

Sr. No.	Step	T_m (°C)	Duration	Cycles
1	UDG activation	50°C	2 minutes	Hold
2	Dual-Lock™ DNA polymerase	95°C	2 minutes	Hold
3	Denature	95°C	15 seconds	40 cycles
4	Anneal	54-60°C	15 seconds	
5	Extend	72°C	1 minute	

3.2.5 Expression analysis of *NAS, NAAT, DMAS* and *Ids3* genes

The C_t (Cycle Threshold) comparative method was used to quantify genes, which is expressed as n-fold up or down regulation of transcription in contrast to a calibrator, which represents the lowest signal observed for that specific gene. For comparative C_t quantification, the results were represented relative to the calibrator. At each time point, the expression of selected genes was calibrated against that of the reference gene, actin, and the relative expression ratio was calculated as fold of expression,

$$\text{Fold of Expression} = 2^{-\Delta\Delta Ct}$$

Where,

$\Delta\Delta C_t$ =Average ΔC_t of target - Average ΔC_t of calibrator

ΔC_t =Average C_t of target - Average C_t of endogenous control

3.3 Analysis of Fe content in grains

The grains were washed for 1 minute in N/10 hydrochloric acid to remove all dust particles from the grain surface, and then dried in a hot air oven at 80°C with continuous weight measurements until the next day of drying. After removing the embryos with a scalpel, wheat grains were submerged in distilled water at room temperature for 12 hours (Antoine *et al*., 2004). The grains were dried in an oven at 60°C until they were uniform in weight. In a microwave digestion system, 0.5 g of dried seeds was treated with 2.0 ml hydrogen peroxide (30% w/w) and 5.0 ml nitric acid (65% w/w). The digested samples were put in a 50 ml falcon tube and diluted to a final concentration of 25 ml with deionized water. The blanks were diluted in the same manner as the test samples. Atomic Absorption Spectrophotometer (AAS) was used to examine the samples. On the basis of dry weight, Fe concentration was determined in mg/kg (ppm).

CHAPTER – IV

RESULTS

4.1 Phytosiderophores content in root exudates

Phytosiderophores (PS) content was measured in root exudates of UP HAU-3 and CS is shown in Table 4.1. Under Fe sufficient conditions, there was no significant difference in PS content between UP HAU-3 and CS. Under Fe deficient conditions PS release was higher in both the lines. However, the PS content in UP HAU-3 was higher as compared to CS. Under Fe deficiency, 29.71% and 9.62% increase was observed in UP HAU-3 and CS respectively.

Table 4.1: Percent increase of phytosiderophores content in root exudates of UPHAU-3 and CS

Sr. No.	Genotype	Fe^{+} (nmol of Fe equivalent/g)	Fe^{-} (nmol of Fe equivalent/g)	% increase
1.	UP HAU-3	1.38	1.79	29.71%
2.	Chinese Spring	1.35	1.48	9.62%

4.2 Relative expression profiling for Fe transporter and storage genes in wheat

The relative gene expression of four Fe transporter and storage associated genes, *NAS, NAAT DMAS* and *Ids3* were analyzed in roots, of two wheat genotypes (UP HAU-3 & CS). In this study, the actin gene was used as an endogenous control.

4.2.1 *NAS* Gene Expression

Nicotianamine plays an important function in the uptake of Fe from the soil in graminaceous plants such as wheat, barley, rice, and maize. It is a precursor of the mugineic acid family PSs. Fe deficiency in the root activates *NAS* genes involved in PS synthesis. The transporter of type *YS1* from maize, which is upregulated by Fe shortage in roots, imports PS-Fe^{3+} complexes into the rhizodermis. The primer's specificity was validated by the lack of multiple T_m peaks in melt curve analysis and no amplification in NTC (non template control) shown by the real-time PCR amplification plot as shown in Fig. 4.1. In UP HAU-3 high level of *NAS* gene expression was recorded as compared to CS (Fig. 4.2). Under Fe deficient condition the Relative Quantification value (RQ) showed fold increase of 3.797 and 2.242 for UP HAU-3 and CS respectively.

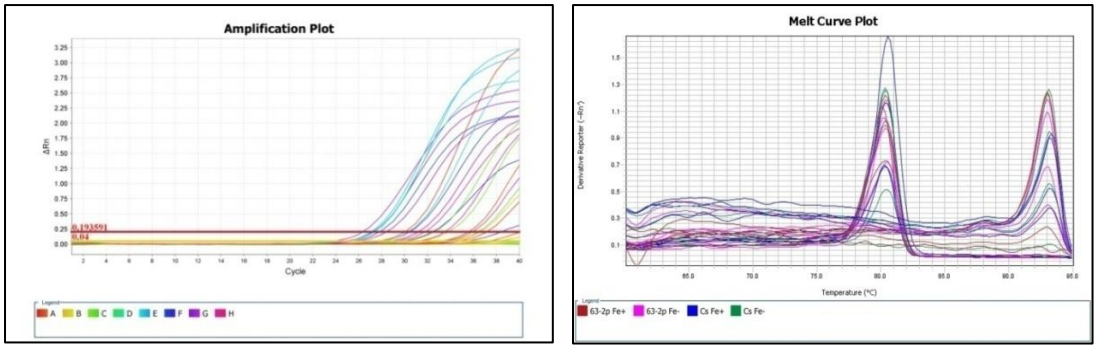

Figure 4.1: Amplification plot and melt curve of *NAS* gene

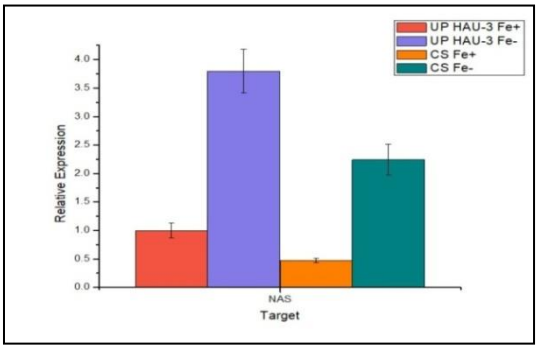

Figure 4.2: Relative expression of *NAS* gene

4.2.2 *NAAT* Gene expression

The *NAAT* gene involved in the production of PSs that are reseponsible for absorption of micronutrients in cereals (Strategy II). The absence of multiple T_m peaks in melt curve analysis, as well as no amplification in NTC as demonstrated by the amplification plot obtained by the real-time PCR, suggested that the primer was specific (Fig. 4.3). High level of NAAT was found in UP HAU-3 as compared to CS (Fig. 4.4). Under Fe deficient condition the RQ value for *NAAT* showed fold increase of 1.711 and 1.511 for UP HAU-3 and CS respectively.

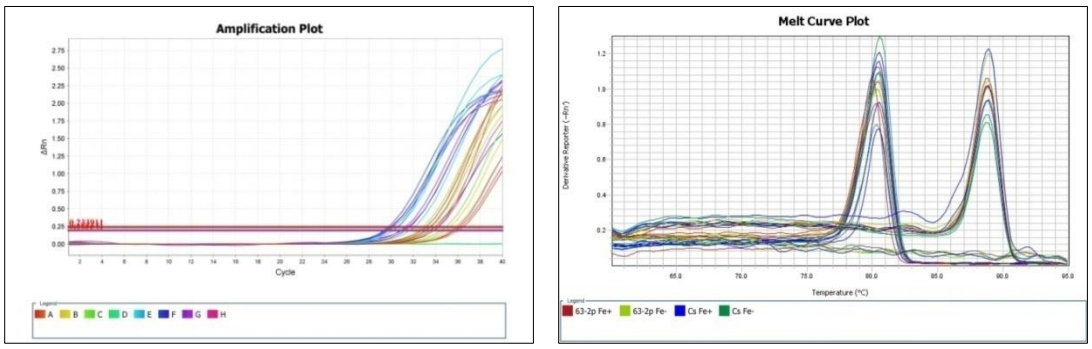

Figure 4.3: Amplification plot and melt curve of *NAAT* gene

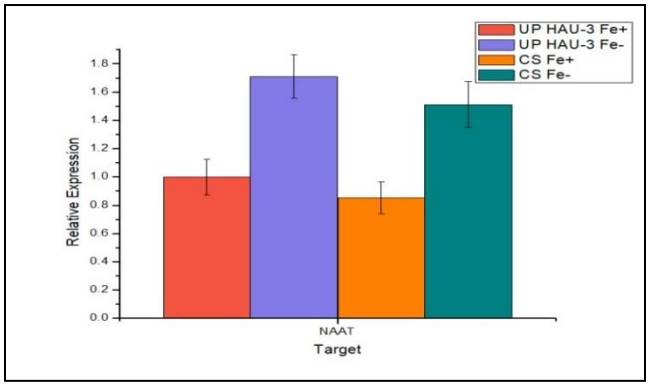

Figure 4.4: Relative expression of *NAAT* gene

4.2.3 *DMAS* Gene expression

DMA is synthesised from nicotianamine (NA) via a 3"-oxo intermediate utilising the enzymes *NAAT* and *DMAS*. Absence of multiple T_m peaks in melt curve analysis and no amplification in NTC indicated by the amplification plot generated by the real-time PCR suggested the specificity of the primer. Relative normalized expression of *DMAS* using actin as endogenous control is shown in Fig.4.5. There was a gradual increase in the expression of *DMAS* gene in both the genotypes under Fe deficiency. However, UP HAU-3 has high rate of gene expression as compared to CS (Fig.4.6). Under Fe deficient condition the RQ value for *DMAS* showed fold increase of 2.115 and 1.292 for UP HAU-3 and CS respectively.

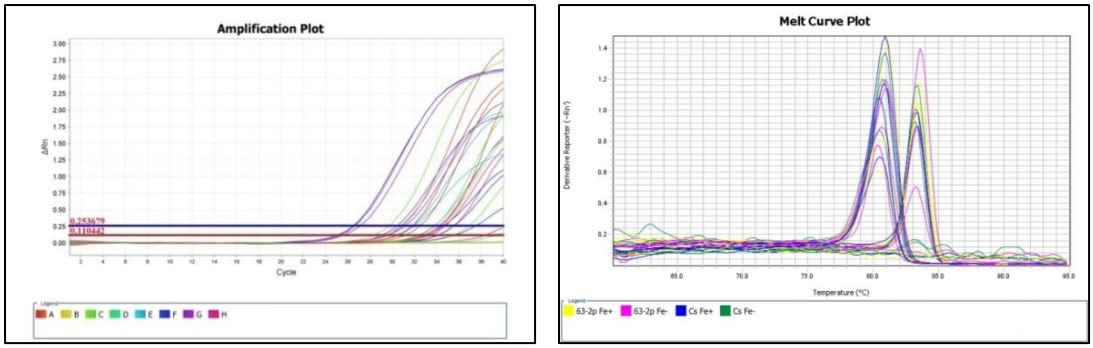

Figure 4.5: Amplification plot and melt curve of *DMAS* gene

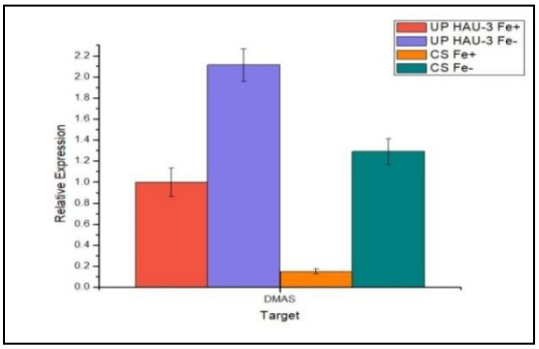

Figure 4.6: Relative expression of *DMAS* gene

4.2.4 *Ids3* Gene expression

The iron-deficiency-specific clone 2 (*Ids2*) and iron-deficiency-specific clone 3 (*Ids3*) genes encode hydroxylated PSs, which are secreted by Graminaceous plants. The *Ids3* gene produces a dioxygenase enzyme that hydroxylates the C-2′ position of DMA and 3-epihydroxy-2′-deoxymugineic acid (epi-HDMA) and convert DMA to MA or epi-HDMA to epi-HMA. The real-time PCR amplification plot and melt curve amplified a specific product (Fig. 4.7). The expression of Ids3 was up regulated in root of UP HAU-3 as compared to CS (Fig. 4.8). Under Fe deficient condition the RQ value for *Ids3* showed fold increase of 16.336 and 1.429 for UP HAU-3 and CS respectively.

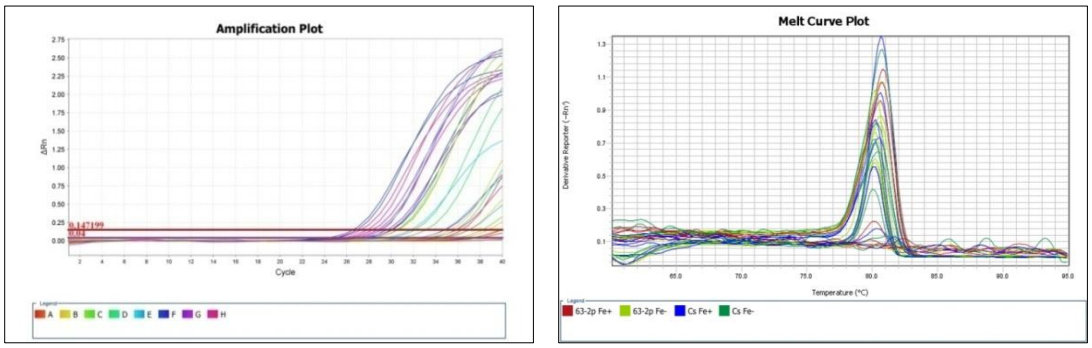

Figure 4.7: Amplification plot and melt curve of *Ids3* gene

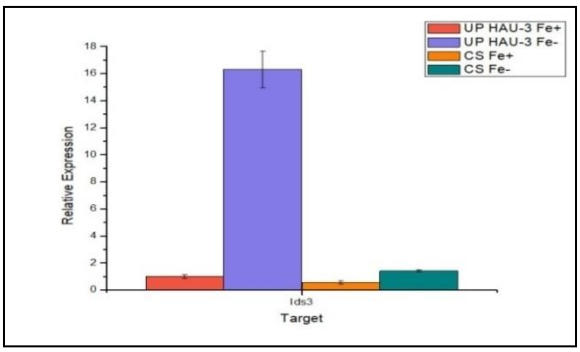

Figure 4.8: Relative expression of *Ids3* gene

4.3 Fe content in whole grain of UP HAU-3 and CS

The analyzed wheat- *Aegilops* addition/substitution lines (UP HAU-3 and CS) differed significantly with respect to the concentration of Fe in their grain (Table 4.2). The grain of CS contained significantly less Fe than the grain of UP HAU-3. The Fe concentration in UP HAU-3 and CS varied from 47.55 ± 0.45 to 40.31 ± 0.33 mg/kg in whole grain respectively. As a result of higher gene expression in UP HAU-3 the grain Fe content of UP HAU-3 was higher as compared to CS.

Table 4.2 Grain Fe content of UP HAU-3 and CS

Sr.No.	Genotype	Whole grainFe(mg/kg)
1.	UP HAU-3	47.55 ± 0.45
2.	Chinese Spring	40.31 ± 0.33

CHAPTER –V

DISCUSSION

To cope with changes in mineral nutrient supply, plants rely on a vast range of adaptive responses. These adaptive responses are mediated by a complex sensing and signalling network involving hormones; however, changes in mineral nutrients alter hormone biosynthesis, reducing the hormones ability to sense and signal the induction of metabolic and physiological responses (Rubio *et al*., 2009). The availability of one or more nutrients can change the root system architecture (RSA) and impact plant performance under conditions of low nutrient availability (Shahzad and Amtmann, 2017). Micronutrients have been shown to influence lateral root growth (Gruber *et al*., 2013). These variations in RSA in response to nutrient availability assist the plant in surviving under unfavourable growth and development conditions, and are intended to ensure plant survival (Dorlodot *et al*., 2007). The most frequent abiotic stress that impacts root growth across plant species is Fe deficiency (Yan *et al*., 2011). However, the majority of the data on this topic pertains to strategy-II plants (monocots). Under Fe shortage, the involvement of secondary root and root hair growth is also described (Bernards *et al*., 2002).

The extent of a plant's influence on the rhizospheric activities that determine the nutrient availability dynamics in the root zone is likely to vary if the RSA is modified. Plants can change the morphology of their roots, increase the affinity of nutrient transporters within their cell membranes, and release organic compounds (phenols, enzymes, carboxylates, and carbohydrates), proton and non-protein amino acids (PSs) to improve nutrient availability in the rhizosphere and thus the abundance and diversity of the microbial community in the rhizosphere, allowing them to thrive (Rengel and Marschner, 2005).

Fe scarcity in cultivable soils is widespread across the world, particularly in high pH calcareous soil, sandy soil, and high phosphorus treated soil (Marschner, 1995), and it is a major constraint to grain output. Lately, the mineral levels of wheat varieties, landraces, and wild species have been investigated, and genotypes with high mineral contents have been used to transfer gene(s) responsible for high mineral contents in selected hexaploid wheat species, genotypes, and accessions (Kumar *et al*., 2015). Grain iron concentration was relatively high in addition lines of group 2 chromosomes of various Aegilops species followed by group 7 chromosomes, although group 7 chromosomes exhibited greater zinc concentration. According to Tiwari *et al*. (2010), wheat grain Fe and Zn content increased when group 2 and 7 of *Ae. kotschyi* were substituted. Neelam *et al*. (2012) observed that adding *Ae. peregrina*group 4 and 7 to wheat increased grain iron and zinc density.

Monocots have adapted to low Fe accessibility by secreting PS, which have been found to mobilise not only Fe but also Zn, Mn, and Cu. The release of the low molecular weight exudates in the nutrient-deficient soils might be crucial for Fe absorption. Exudates from the roots may also help in the mobilisation and absorption of Fe from the apoplasmic pool, where it can be exchangeably bonded or precipitated as apoplasmic Fe (Zhang *et al*., 1991; Suzuki *et al*., 2006). According to studies on grain and other grass crops variations in tolerance of Fe deficiencies are strongly linked with PSs that can make inorganic Fe easier to mobilise (Chaignon *et al*., 2002; Meda *et al*., 2007; Neelam *et al*., 2010). Cereal species that release a higher amount of PS are thought to be more Fe efficient than those that release a lower amount. Another important but understudied variables that are known to impact the response of plants to Fe deficit include tissue Fe requirement for chlorophyll synthesis, root/shoot ratio, tissue Fe, and Fe use efficiency for biomass production, which can change between species and genotypes within a species (Crowley *et al*., 2002; Krouma *et al*., 2003 and Mahmoudi *et al*., 2007).

PS release, on the other hand, is influenced by PS biosynthesis efficiency, and not all PS synthesised in the root is released in the rhizosphere to aid Fe mobilisation. SAM is a precursor in the biosynthesis of polyamines, PSs, and ethylene and it is synthesized from Met by SAM synthase (Mori and Nishizawa, 1987). It is a metabolically important molecule because it acts as donor of methyl group in numerous biological processes. In order to retain Met physiological level, continual recovery of the various metabolites on the Met salvage path is important. To meet the increasing PS biosynthesis demands, the Met cycle recycles Met actively (Ma *et al*., 1995). Met cycle enzymes are transcriptionally upregulated under Fe deficiency.

In the current investigation, UP HAU-3 accumulated more PS under Fe deficit condition than CS.The biosynthesis and release of PSs are important in the strategy II of Fe absorption and translocation from the rhizosphere to the grains. Several researchers have found increased PS release from the roots of graminaceous species subjected to micronutrient deficit (Khobra & Singh, 2019; Divte *et al*., 2019). For both strategy-I and strategy-II plants, a relationship between physiological responses to Fe deficit has been shown (Brown and Jolly, 1988; Walter *et al*., 1994). Several studies have found that wheat genotypes that are sensitive to micronutrient deficit have a higher root to shoot ratio, indicating that food is portioned more towards the root than the shoot under Fe deficiency stress. Differential expression of genes involved in PS production can be linked to variations in PS accumulation. In this study, qRT-PCR expression analysis revealed that genes involved in PS biosynthesis, such as *NAS*, *NAAT*, *DMAS*, and *Ids3*, were upregulated in both genotypes, UP HAU-3 and CS, and with upregulation being greater in UP HAU-3. Increased up-regulation of most Met salvage pathway genes ensures a constant supply of various metabolites for PS synthesis in

the presence of Fe deficiency. These findings suggest that activation of the Met salvage pathway is one of the mechanisms that lead to increased PS buildup and effective Fe absorption. However, in UP HAU-3, up-regulation of these genes was higher than in CS, which might explain why PS biosynthesis and release were enhanced, resulting in effective Fe absorption and transportation.

Other research has discovered that when Fe deficiency is prevalent, PS biosynthesis genes are up-regulated in a variety of crops, including rice, wheat, barley, and oats (Ahmadzadeh and Khoshgoftarmanesh, 2019; Gupta *et al*., 2020). Several studies have found that Fe deficiency enhances the expression of genes involved in NA and DMA production in rice, barley, and maize (Bashir *et al*., 2006; Zheng *et al*., 2009; Zhou *et al*., 2013). Mathpal *et al*. (2018) showed that that the regulation of expression of *HvIds3* like gene in wheat is Fe dependent, which might be associated with iron utilization by plant. Similarly, during the vegetative development stage, increased expression of *TaNAS* genes, *TaNAAT* genes, and *TaDMAS1* has been observed in wheat roots in response to Fe deficiency (Bonneau *et al*., 2016; Beasley *et al*., 2017).NA is intermediary in the synthesis of DMA and chelates Fe for long-distance and intracellular transport (Takahashi *et al*., 2003; Schuler *et al*., 2012). Further studies have revealed that, DMA, in addition to its well-known involvement in Fe absorption from the rhizosphere, chelates Fe in the xylem and phloem for long-distance transport (Kakei *et al*., 2009; Nishiyama *et al*., 2012). According to Tsednee *et al*. (2012), DMA and MA are mainly involved in the translocation of Fe to other parts of plants rather than in Fe absorption from soil. However, due to high degree of hydroxylation or particular chemical structures, AVA and HAVA contribute to Fe acquisition when there is a Fe deficiency on the root surface by forming a stable Fe (III) complex in the rhizosphere.

Different divalent cations, such as nickel, have been found to compete with Fe for PS binding accessibility, indicating a regulatory crosstalk sequence. Nickel deficiency substantially increased wheat shoot Fe concentrations while decreasing triticale shoot Fe concentrations (Ahmadzadeh and Khoshgoftarmanesh, 2019). As a result, studying the binding kinetics of different divalent cations, such as Fe and Zn, with the available PS is essential for better understanding their uptake and transportation mechanisms.

CHAPTER –VI

SUMMARY AND CONCLUSION

Survival of plants and their performance under abiotic stress are predominantly focused on their capacity to seek and use soil nutrients efficiently. The latter is aided by a preferential allocation of resources to the roots and a favorable alteration in root system architecture (RSA), both of which are produced by a hormone imbalance. Understanding how plants respond to Fe deficiency is crucial for plant health and the human diet, which is heavily reliant on Fe derived from plants. To compensate for the nutritional shortage, plants undergo a variety of physiological and morphological changes. These responses include (i) extracellular medium acidification, (ii) ferric ion reduction, and (iii) enhanced ferrous ion transit into root cells. Since Fe is an important mineral element for plant growth and development, plants have developed systems to ensure that Fe homeostasis is kept precisely regulated at all levels. The roots improve the nutrient availability in the rhizosphere by removing proton and phenolic compounds from their roots into the rhizosphere which is intended to improve the solubility of ferric ions (Strategy I in dicots) or the solubilization of Fe^{3+} and other metal micronutrients by PS of the mugineic acid (MA) family secreted from roots (Strategy II, in monocots). These processes of root-mediated nutrient uptake take on a major importance under situations of low availability of nutrients in cultivable soil. Under conditions of low nutrient availability in cultivable soils, these root-mediated nutrient-uptake processes become more important. It's important to note that India's soils are severely deficient in nutrients, with deficiencies in micronutrients like Zn, Fe, Cu, Mn, B, Mo, and S found in more than 48, 12, 4, 5, 33, 13, and 41% of cultivable soils, respectively. In cereal species, biosynthesis and release of PSs by roots have been linked to Fe deficiency tolerance. While nicotianamine synthase (*NAS*) and nicotianamine amino transferase (*NAAT*) have been implicated in PS production, relative activation of the PS transporter gene, *TOM1*, determines PS release by the roots into the rhizosphere. In view of the foregoing facts and knowledge gaps, the current work was designed to better understand the control of PS production in the presence of Fe shortage. This study was done on two wheat genotypes viz. UP HAU-3 and CS under hydroponics with the following two objectives:

1. To quantify the phytosiderophores (DMA and NA) under Fe deficiency in wheat-*Aegilops* addition/substitution lines and,

2. Relative expression analysis of the putative gene(s) responsible for Fe uptake in wheat-*Aegilops* addition/substitution lines.

While the first experiment helped to explore the Fe deficiency response of the UP HAU-3 and CS wheat cultivars in terms of PS level of the root exudates; another experiment was conducted to address the objective 2. which involved the relative expression analysis of PS biosynthesis regulatory genes *NAS*, *NAAT*, *DMAS* and *Ids3*. The results of the above experiments are summarized as follows:

- Fe use efficiency was considerably greater in the Fe^- treatment than in the Fe^+ treatment.
- An insignificant level of PS release was measured under the Fe sufficient treatment.
- PS production and release were triggered by Fe deficit in UP HAU-3 and CS wheat cultivars, with UP HAU-3 producing and releasing much more PS than CS.
- Significantly higher expression of *NAS*, *NAAT*, *DMAS* and *Ids3* was measured in UP HAU-3 than CS under Fe deficiency.
- The relative expression of *Ids3* gene which is responsible for the production of hydroxylated form of PS (Mugineic acid family) and grain Fe content were higher in UP HAU-3 as compared to CS.

CONCLUSION

From the above results, it can be concluded that UP HAU-3 is more efficient in Fe uptake under Fe deficient conditions as the expression of genes involved in Fe uptake was higher in UP HAU-3 as compared to CS. A reduced Fe absorption by CS compared to UP HAU-3 might be linked to decreased PS synthesis and release, as a result of a lower induction of root tips, a location for PS release, under Fe deficiency.

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APPENDIX

cDNA Sequence of *NAS* gene retrieved from Ensembl Plants

>TraesCS6A02G386200.1 cdna:protein_coding

ATGGACGCCCAGAACAAGGAGGTGGACGCCCTGGTCCAGAAGATCACCGGCCTCCACGCC
GCCATCGCCAAGCTGCCCTCGCTCAGCCCGTGCCCCGCCGTCGACGCGCTCTTCACCGAC
CTCGTCACCGCGTGCGTCCCCCCGAGCCCCGTGGACGTGACCGAGCTCGGCCCGGAGGCG
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CACTACTCCGACATGCTCGCCGCCTTCGACAACCCGCTCGACCACCTCGGCGTCTTCCCT
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GCCGTGTGTCACCCAGACGACGACGTGGTGAACTCCGTCATCATCGCGCAAAAGACCAAC
GACGTGCATGAGTATGGACTTGGCAACGGGCGTGGTGGACGGTACGCGCGGGGCACGGTG
CCGGTGGTCAGCCCGCCCTGCAGGTTCGGCGAGATGGTGGCGGACGTGACCCAGAAGAGA
GAGGAGTTTGCCAACGCGGAACTGGCCTTCTGA

cDNA Sequence of *NAAT* gene retrieved from Ensembl Plants

>TraesCS1A02G291200.1 cdna:protein_coding

ACAGCAACGGCTATATTAGGACAGTCAATCACCGCTATTCGATCCACCGCGGCACCAGTT
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CAAGATCAGCGCGAGCGTGCGGGAGAGCGGGCCGCGGCCCGTGCTGCCGCTGGCCCACGG
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CGCGCTGCGCACCGGCCAGTTCAACTGCTACCCCGCCGGCGTCGGCCTGCCCGCCGCACG
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GTTCAACAAGCTGGAGGTTCGACATTTCGACCTTATCCCGGAGAAGGGGTGGGAGATCGA
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TTAGTTCTCTATGAATAGAAGTCAACATCTCCTTGAATATGTTATGGTTGCCATGGCCTG
GACGAAACAATAGTGAATGTTAGTGAAGTTACATTGGTGTCGAAGATCTTTAAAGTTTGT
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cDNA Sequence of *DMAS* gene retrieved from Ensembl Plants

>TraesCS4A02G074800.1 cdna:protein_coding

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CGGCCGCGGGCATGCCGCGCATCGGCATGGGCACGGCGGTGCAGGGGCCCAAGCCGGACC
CCATCCGCCGCGCCGTCCTCCGCGCCATCGAGGTAGGGTACCGCCACTTCGACACGGCCG
CGCACTACGAGACCGAGGCCCCCATCGGCGAGGCCGCCGCCGAGGCCGTGCGCTCCGGCG
CCGTCGCCTCCCGGGACGACCTCTTCATCACCTCCAAGCTCTGGTGCAGCGACGCGCACC
GCGACAGGGTCGTCCCCGCCCTCAGGCAGACGCTCCGGAATCTCCAGATGGAGTACGTGG
ACCTGTACCTCGTCCACTGGCCGGTGTCCATGAAGCCCGGGCGGTTCAAGGCCCCCTTCA
CGGCGGAGGACTTCGTGCCGTTCGACATGCGGGCCGTGTGGGAGGCCATGGAGGAGTGCC
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CGCCGCTGGGGGCCAAGGGCACGCACTGGGGCAGCGACGCCGTGATGGACGCGGGCGTCC
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TGGACGTGGACGGCTGGGAGCTCACCGAGGAGGAGCACCGCAGGATCGCGGAGATCCCGC
AGCGCAAGATCAACCTCGGCAAGCGCTACGTGTCCGAGCACGGGCCCTACAAGTCCCTCG
AGGAGCTCTGGGACGGTGAGATATGAGTGATCACTGGAGAGGCTGGCTGGCCGGTGCGAC
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CTTTGAATAAAATCATGTTCTGCATTGTGTTTCAGTGAATAAATGGAGATGTTTACTACC
GGATTTAAGGAGAGTTACTAAGAGATTGGGATCATCTCATCATGTTATTATGCGTACTTT
CCTGGCAATGTAAGTTGTTACAATGGCAGAACAAAAAGAAGGTGTATAAATAAAGAGAGG
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cDNA Sequence of *Ids3* gene retrieved from Ensembl Plants

>TraesCS7A02G026100.1 cdna:protein_coding

ATGGCCAAACTGCTCTCCTCCGCCTCGTCTCACGAGCTGCTCGCCCTGCCACCGGCCGTC
TCCCTCCCCGTTGTCGACCTCTCCCTCAGTCATGACGAGGTCCGCCGCGCCATCCTCGAC
GCCGGCAAGGAGCTGGGCTTCTTCCTGGTGGTCAACCACGGCGTTCCTGAGCATGCGATG
CGGGACATGGAGGCAGTGTGCGAGGAGTTCTTCCGCCTCCCGGTGCTGGACGTGGCACCC
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TGGATTAACGTCGTAACCAACGGGGTGCTGAAGAGCATAGAGCACCGGGTGATGACTAAT
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CGTGAGTTCTTGCGCGTCCACAATGTTGTAAAGCTGGGCTCATCGCTCAATCTTACAACC
AATCTCAGCAACTCTCAACAAACACAAGACAAAAATACATGA

ABSTRACT

Title of Thesis	:	Estimation of phytosiderophores in wheat-*Aegilops* addition/substitution lines under iron deficiency
Full Name of the Degree Holder	:	BINDU BISLA
Admission Number	:	2019BS29M
Title of the Degree	:	Master of Science
Name of Discipline	:	Molecular Biology and Biotechnology
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Wheat is a major cereal crop all over the world in terms of cultivated area and food source. Most elite wheat cultivars, on the other hand, have low grain iron (Fe) content. Over three billion people, mostly women and children, suffer from micronutrient deficiency, mainly Fe deficiency. However, plants have developed systems to ensure that Fe homeostasis is kept precisely regulated at all levels. The roots improve the nutrient availability in the rhizosphere by removing proton and phenolic compounds from their roots into the rhizosphere which is intended to improve the solubility of ferric ions (Strategy I in dicots) or the solubilization of Fe^{3+} and other metal micronutrients by phytosiderophores (PS) of the mugineic acid (MA) family secreted from roots (Strategy II, in monocots). In the present investigation, two wheat-*Aegilops* addition/substitution lines (UP HAU-3 and CS) were grown hydroponically under Fe sufficient and deficient condition. Phytosiderophore release in the root exudates was induced under Fe deficiency and was higher for UP HAU-3 as compared to CS. Significantly higher expression of *NAS, NAAT, DMAS* and *Ids3* was measured in UP HAU-3 than CS under Fe deficiency. We can use biotechnological tools and techniques to introduce the gene responsible for phytosiderophores secretion into field crops. It will enhance the crop uptake in nutrient deficient conditions and indirectly crop yield.

MAJOR ADVISOR **DEGREE HOLDER**

HEAD OF THE DEPARTMENT

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2. Webinar: Preventing Future Zoonotic Pandemics, ICAR-NRCE, Hisar
3. International Web Conference: Innovative and Current Advances in Agriculture & Allied Sciences

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I, **BINDU BISLA** , Admission No. **2019BS29M** undertakes that I give copyright to the CCS Haryana Agricultural University, Hisar of my thesis entitled, **"Estimation of phytosiderophores in wheat-*Aegilops* addition/ substitution lines under iron deficiency".** I also undertake that, patent, if any, arising out of the research work conducted during the programme shall be filed by me only with due permission of the competent authority of CCS Haryana Agricultural University, Hisar.

Signature of student