गेहूं में लोहे द्वारा सल्फर उदग्रहण और आत्मसात करने का कार्यिकी और आणविक विनियमन

PHYSIOLOGICAL AND MOLECULAR REGULATION OF SULPHUR UPTAKE AND ASSIMILATION BY IRON IN WHEAT

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PHYSIOLOGICAL AND MOLECULAR REGULATION OF SULPHUR UPTAKE AND ASSIMILATION BY IRON IN WHEAT

BY

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This is to certify that the thesis entitled "P**hysiological and molecular regulation of sulphur uptake and assimilation by iron in wheat"** submitted to the Faculty of the Post-Graduate School, ICAR-Indian Agricultural Research Institute, New Delhi, in partial fulfillment of Doctor of Philosophy degree in Plant Physiology, embodies the results of bonafide research work carried out by **Ms. Vasundhara Sharma**, under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by her.

Bhupinder Singh

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Place : New Delhi

Date :

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(Vasundhara Sharma)

DEDICATED TO

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Chapter 1

INTRODUCTION

Wheat is one of the most commonly consumed staple cereals worldwide and is the third most important crop in terms of production after rice and maize. Wheat is the second oldest cultivated plant after barley and mainly three species of wheat are of economical importance in India i.e. *Triticum aestivum* (bread wheat), *Triticum durum* (durum or macaroni wheat) and *Triticum dicoccum* (emmer wheat). It contributes maximum calories and protein to the world diet but contains less bioavailable iron (Fe), zinc (Zn) and other nutrients, when floured. It is, thus, important to improve the grain micro-metal-nutrients which are critical for human health. Physical enrichment of micronutrients in wheat flour is being tried presently to meet the malnutrition challenge, however, the approach is uneconomical and unsustainable going by the large volume of food materials to be fortified. Most practical solution to meet the malnutrition challenge, thus, relies in either a balanced application of nutrients to the soil to ensure optimum availability for plant uptake or in improving the mobilization of the bound and unavailable macro and micro nutrients in the rhizosphere for their facilitated plant uptake. Biofortification efforts are, thus, important to address the challenge of Fe and Zn deficiency anaemia and immunity disorder, major nutritional health problem especially in women and children in area where people are dependent on wheat as major staple cereal. The World Health Organization estimates that around 25 percent of the world's population suffers from anaemia (WHO, 2008), and that Fe-deficiency anaemia has resulted in the loss of more than 46,000 disability adjusted life years (DALYs) in 2010 alone (Murray and Lopez, 2013). Zn and manganese (Mn) deficiency also affects human health as it leads to growth retardation, loss of appetite, poor utilization of vitamins and impaired liver and immune function.

In plants, physiological role of Fe has been shown as cofactor of various enzymes in photosynthesis, respiration and nitrogen fixation as a constituent of cytochromes and non heme Fe-S protein (Balk and Pilon, 2011; Rout and Sahoo, 2015; Brumbarova *et al.,* 2015). Zn and Mn also act as a cofactor for enzymes involved in chlorophyll biosynthesis, photosynthesis, respiration and other metabolic pathways. Fe, Zn and Mn deficiency results in intervienal chlorosis of the new leaves initially which may transcend into necrotic spots arising on the margins or leaf tips at

the later stages. Fe deficiency is a widespread problem particularly in calcareous soils in arid and semiarid regions, which often results in significant reduction in plant growth and yield losses (Mortvedt, 1991; Sánchez-Rodríguez *et al.,* 2014). Plant yield on many soils is limited by poor Fe availability for plant uptake, rather than its content in the soil (Bybordi and Mamedov, 2010). Currently, the hidden hunger for micronutrients represents the most serious "robber" of crop yield and quality. Results of a broad based study conducted in wheat growing regions showed that addition of micronutrient (Fe, Zn, Cu and B) individually or in combination increased grain yield (Malakouti, 2000).

Besides micronutrients, an imperative requirement of sulphur (S) is cropping up in cultivable soils and most of the crops respond to S application. The situation is alarming as more than 41 % of the agri-soils are S deficient. Further, the average S content in the atmosphere has also dropped significantly from 1.38 to 1.00 mg S-SO_4/100 g, between 1995 and 2015 due to reduction in S emission from industries (Siebielec *et al*., 2017). S is an essential nutrient, required for maintaining the gluten protein composition in wheat, which is critical for improving the content of non-replaceable amino acids and baking quality of wheat flour (Jarvan *et al.*, 2008; Podlesna and Cacak-Pietrzak, 2008; Zörb *et al.,* 2010). S is also a constituent of amino acid such as methionine and cysteine and that of various coenzymes and secondary plant products. S deficiency alters plant chlorophyll and protein metabolism and cause stunted growth in plant with reduced tillering. S and Zn nutrition is reported to affect grain yield and grain and dough quality in wheat (Orman and Ok, 2012). S deficiency in wheat appears if N: S ratio, on dry weight basis is greater than 17:1. Without adequate S, crops cannot possibly reach their full potential in terms of grain/yield and quality nor can they make efficient use of N, P, K and other mineral nutrients. S supply with NPK fertilization can be useful in biofortification of spring wheat and also increase the uptake of micronutrients such as Fe, Mn, Zn, and copper (Cu) (Klikocka and Marx, 2018).

Nutrient-nutrient interactions are also a critical determinant of nutrient availability and uptake by plants. These interactions may either be synergistic (presence of a nutrient increase the availability and uptake of other nutrient) or antagonistic (presence of a nutrient decrease the availability and uptake of other nutrient) which ultimately regulate the plant nutrient content and their response to the

prevailing nutrient availability condition. Competition between Fe and other metal micronutrient for plant uptake is known particularly under their limited availability condition. Fe and S interaction has also been studied by different group of researchers owing to their vital role in determining the plant growth and development. Interactively, S is essential for Fe nutrition of plant as S-containing "methionine" is precursor of Fe chelating agent phytosiderophores (PS), which facilitates the uptake of Fe in Strategy II plants (graminaceous species) particularly under alkaline conditions. The increase in rhizospheric S under Fe deficiency condition increase the S-uptake and the production of S-adenosyl methionine, which converts into nicotianmine and PS in the presence of enzymes Nicotianamine synthase (NAS) and Nicotianamine amino transferase (NAAT). The release of PS from roots chelates the bound Fe from rhizosphere and facilitates the Fe uptake as Fe-PS complex (Fig. 1.1A). S deficiency affects the Fe uptake in maize (Astolfi *et al.,* 2003), tomato (Zuchi *et al.,* 2015) and bread and durum wheat (Ciaffi *et al.,* 2013; Sharma *et al.,* 2016). In addition, it is shown that the S supply in form of sulphate can increase synthesis (Kuwajima and Kawai, 1997) and release (Astolfi *et al*., 2006) of PS in Fe-deficient barley roots to improve the capacity of these plants to cope with Fe-deficiency (Römheld, 1987; Römheld and Marschner, 1990). S and Fe also interact in plant as Fe-S clusters which plays an important role in formation of prosthetic groups such as heme and Fe–S clusters, and in assembling them into apoproteins, which are major components of plant metabolism (Briat *et al*., 2010). Fe–S clusters are co-factors of proteins that function in vital processes such as photosynthesis, respiration, S and N metabolism, plant hormone and coenzyme synthesis (Balk and Pilon, 2011).

Interaction of S with Fe may be mediated via nitrogen (N) as N interacts with Fe by increasing the root release of phytosiderophores (PS) and root uptake of Fe (III)-PS in Fe-deficient wheat plants (Aciksoz *et al.,* 2011). Fe deficiency led to a decline in N assimilation in plants (Borlotti *et al.,* 2012). N fertilization helped in enhanced Zn and Fe uptake and increase yield components of wheat and spring barley (Sedlar *et al.,* 2014). Growth and nutrient uptake of maize plant increased by elemental S and N fertilizer application in sandy calcareous soil (Rahman *et al.,* 2011). N also plays regulatory role in sulphur metabolism by participating in the formation of O-acetyl serine, which is a first stable product in S assimilation pathway (Kopriva *et al.,* 2004). OAS (O- acetyl serine) seems to play a major role in linking

the sulphate and the nitrate assimilatory pathways (Koprivova *et al*., 2000) whereas methionine synthesis or degradation forms connection between sulphur and Fe metabolism mainly through the formation of nicotianamine, precursor of iron chelating agent phytosiderophores (Mori and Nishizawa, 1987). Thus, there appears to be a strong interconnection between S, Fe and N metabolism in plants, and improving S efficiency of crops by investigating these interactions is, thus, a major challenge to ensure optimum plant health, grain production and quality.

Role of transporters and activity of key rate limiting assimilatory enzymes are important for governing the uptake and utilization of mineral nutrients in plants. S supply induces the activity of Fe-PS transporter YS1 and increases Fe uptake in plant. S uptake by the plants is in the form of sulphate through specific sulphate transporters (Takahashi *et al.,* 2011). Sulfate is taken up from the soil by two root-specific high-affinity transporters of group 1, SULTR1;1 and SULTR1;2 (Yoshimoto *et al.,* 2002), and is transported to the xylem by two low-affinity transporters of group 2, SULTR2;1 and SULTR2;2 (Takahashi *et al.,* 2000). High affinity sulphate transporters are active under S deficient condition whereas low affinity sulphate transporters are expressed under S sufficient condition. This sulphate is further metabolized in different components and eventually forms cysteine and methionine amino acids which are used in protein synthesis and other plant processes. Any change in the activity of the O-acetyl serine (OAS) and O-acetyl serine thio lyase (OASTL), the key enzymes of S assimilation, is likely to affect the biosynthesis of cysteine and methionine. Further, the S and N interplay may influence the activity of key enzyme of nitrate and carbon assimilation i.e., nitrate reductase and rubisco, respectively. It is apt to indicate the amino acids are basic constituent of enzyme protein and any change in the quantity and quality of the amino acid pool is likely to alter activity of metabolic enzymes.

Despite a significant effort and research focus on S and Fe, although individually, the interactive regulation of S and Fe nutrition and ensuing metabolism are poorly understood. Our previous study on Fe and S interaction in bread and durum wheat indicated that Fe availability is critical for the activity of high affinity sulphate uptake transporter, SULTR1;1 which are induced principally under low S availability condition (Sharma *et al.,* 2018). This helps in increasing S uptake and synthesis of

nicotianamime. Later is known to be involved in sequestration of Fe, Zn and Mn in the plant and regulates the micronutrient partitioning and retranslocation (Fig. 1.1B). Interactive effect of Fe, S and N on plant dry matter accumulation, root exudation and S and Fe partitioning and utilization are poorly understood. The present investigation aims to decipher the effect of altered Fe supply on the S uptake of bread and durum wheat. Further, an effect of altered Fe and S supply was studied on the key enzymes of S, N and C metabolism which includes serine acetyl transferase (SAT), O-acetyl serine lyase (OAS), rubisco and nitrate reductase (NR). Regulated synthesis of cysteine, methionine and other amino acids under variable S supply in presence or absence of Fe was also determined. The studies were culminated with the short term uptake experiment conducted using radiotracers of Fe and S i.e., ^{59}Fe and ^{35}S to deduce conclusive evidence on Fe mediated regulation of S uptake and partitioning in bread and durum wheat.

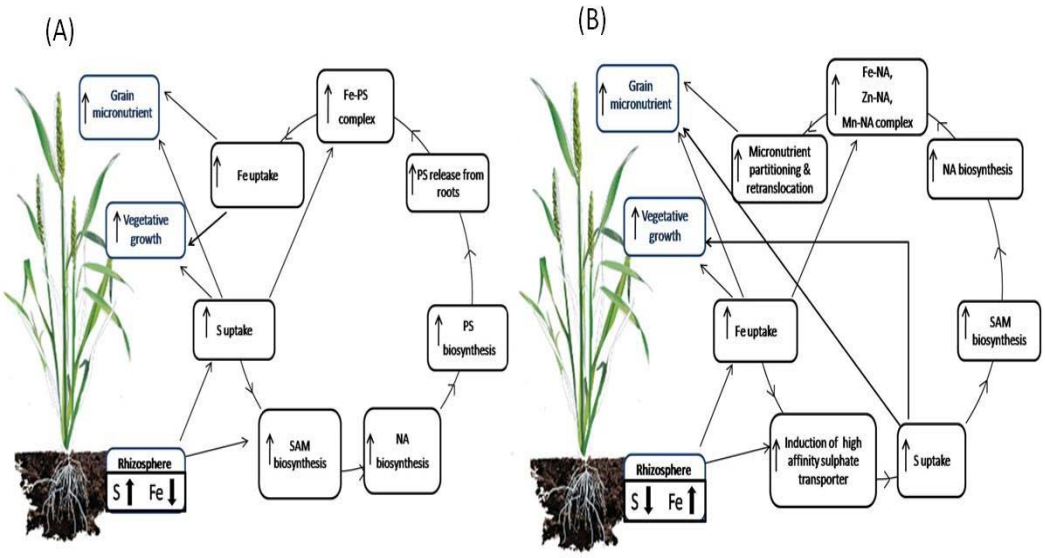

Fig. 1.1: Interaction between sulphur (S) and iron (Fe) in plants in presence of (A) high S and low Fe and (B) low S and high Fe in the rhizosphere (SAM; S-Adenosyl Methionine, NA; Nicotianamine, Zn; Zinc, Mn; Manganese, PS; Phytosiderophores)

In view of the critical analysis of the available literature and gaps in knowledge, the current study is proposed with the following objectives:-

1. To determine the effect of Fe on S uptake, assimilation and partitioning under low and sufficient S availability.
2. To investigate the interactive effect of Fe and S nutrition on plant root architecture, phytosiderophore synthesis and release in relation to $^{35}SO_4^{2-}$ and ^{59}Fe uptake and partitioning.
3. To determine the transcript level expression of sulphate transporters *SULTR1;1* and *SULTR2;1*, Fe-PS transporter *YS1* and nitrogen transporter *NRT2.1* in relation to root and shoot S, N and Fe.

Chapter 2

REVIEW OF LITERATURE

Agriculture is the backbone of the economic system of a country. Most countries have an economy that is dependent on agriculture – either in a small or a big way. From employment generation to contribution to national income, agriculture is important. With the wide economic growth of the country, the economic input of agriculture to India's GDP is gradually declining. The share of agriculture and allied sectors in total Gross Value Added (GVA) at current price has been consistently decreasing and reached to 16.1 percent in 2018-19 and the nominal growth rates of GVA in agriculture and allied sectors have also been falling from 7 per cent in 2017-18 to 4 per cent in 2018-19. On the other hand, the global demand for agricultural crops is rising and it may continue to do so for generations, driven by worldwide population growth of 7.7 billion people and per-capita incomes expected through the mid-century (Tilman *et al*., 2011, https://www.worldometers.info/world-population/). Despite our best effort, however, India is still struggling with food security and according to the FAO more than 190 million of the Indian population remains hungry on a daily basis. India's yield per hectare of wheat at 3 mt is half that of China at 6 mt. With nearly 195 million undernourished people, India shares a quarter of the global hunger burden and is ranked at the 103rd position among 119 countries on the Global Hunger Index (Grebmer *et al*., 2018).

Cereals such as wheat, rice, maize, millets and sorghum have major share in the production and productivity of agricultural crops and also contribute to the human diet and nutrition. Among these, wheat is one of the most staple cereal worldwide and second most important crops in terms of production after rice. Efforts of the scientists and the farmers alike, aided superbly by the advantages of Green revolution technology, have helped us attain self sustainability in terms of grain production to meet the growing demand (Spielman and Pandya-Lorch, 2010) and we realized an average annual production of 99.87 million tonnes (mt) for wheat in the year 2017-2018 (https://economictimes.indiatimes.com/news/economy/agriculture/283-37-million-tonnes-of-food-grains-produced-in-2018-19/articleshow/6963704 1.cms?from=mdr). However, the dream run also caused deficiency and imbalance of both macro and micro nutrients in the soil. Not only the availability of mineral

nutrients in soil but also the nutritional status of the agricultural produce is declining day by day. Both of which are likely to negate grain productivity and lead to health issues such as anaemia, fatigue, slow growth and hormonal imbalance.

2.1. Crop nutrient requirement

For most developing countries, agriculture is the foundation of the economy as they need sustainable food grain production to assist their rapidly growing population. On the other side, grain production relies exclusively on the physical and chemical properties of the soil and soil environment. In addition to these, soil nutrients through right source of nutrients, at right place, right time and right dose, play a key role in determining crop grain productivity and regulating their vegetative growth and development (Johnston and Bruulsema, 2014; Withers *et al.,* 2018). Seventeen essential nutrients are necessary for crops to meet their nutritional requirement (Arnon and Stout, 1939); these are classified into macronutrients and micronutrients based on their requirement in plants. Macronutrients are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S), which are required in larger quantities while micronutrients include iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), boron (B), chlorine (Cl), molybdenum (Mo) and nickel (Ni) which are required in smaller quantities by the plants. N is important constituent of protein, nucleic acid and carbohydrate synthesis and also play role in formation of chlorophyll, alkaloids, hormones, etc. P increases root growth and fruit ripening by translocation of carbohydrates. It is important component of plasma membrane, nucleic acid and energy carrier molecules ATP and NADPH (Epstein and Bloom, 2005). K mediates osmoregulation, stomatal movement, enzyme activation, pH homeostasis and membrane electric potential (Hawkesford *et al.,* 2012; Hawkesford, 2014). S is essential part of cysteine and methionine amino acids, helps to develop vitamins and chlorophyll synthesis. Mg is a key component in chlorophyll biosynthesis and helps in carbohydrate and P metabolism in plants. Ca is an important constituent of middle lamella and acts as a secondary messenger to regulate root and pollen tube growth and also play role in plant defense system (Ortiz-Ramírez *et al.,* 2017; Zhang *et al.,* 2017). It is activator of several enzymes required for respiration, photosynthesis and cell division (Thor, 2019). Micronutrients such as Cu, Fe, Zn, Mn, Mo, B and Ni mainly act as a cofactor for activation of respiratory and photosynthetic

enzymes and facilitate the absorption and utilization of other nutrients (Tripathy *et al.,* 2015; Marschner, 1995).

Plant tissue nutrient requirement varies at different period of the plant growth and is maximum during the pod formation and seed filling stages (Hanway and Weber, 1971; Bender *et al*., 2015) but decreases at late reproductive stages (Harper, 1971). Plants remobilize nutrients from the source to sink according to the demand. At initial growth stages green photosynthesizing leaves act as a source and newly formed plant parts and young leaves act as a sink whereas at later growth stages, the reproductive organs act as a major sink to which the nutrients are remobilized from green leaves and storage organs (Roberts, 1999). Nutrient demand also varies with crop for instance N requirement is more in cereals as compared to legumes. Generally, plant nutrient concentration differs from the concentration of nutrient in the soil solution since the former depends on various factors such as condition of the soil and nutrient supply, nutrient absorption rate by the plants, nutrient assimilation rate, distribution to the nutrient requirement sites and degree of mobilization and remobilization (Loneragan, 1968). A lower plant nutrient uptake despite a sufficient soil nutrient concentration causes a significant reduction in grain yield (Morgan and Connolly, 2013). The yield gaps, however, can be minimized by the use of good nutrient management practices and efficient plant types to achieve optimally higher yield (He *et al.,* 2009; Chen *et al.,* 2010).

2.2. Nutrient availability in soil and crop productivity

In agriculture, sustainable farming is becoming the need of the hour as the conventional farming system depends heavily on chemical fertilizers, which is unsustainable and causes environmental pollution, nutrient deficiencies in soils, negative impact on physical and biological health of soil and are, thus, becoming a major constraint for crop growth, yield and human health (Lyon *et al*., 2004). Widespread nutrient imbalance and non judicious use of NPK fertilizers on cultivable soils is regarded as the major reason for our inability to break grounds in crop productivity. With increasing use of NPK fertilizers, the toxicity of these nutrients in soil has increased to yield a negative impact on the availability of other nutrients. S deficiencies is now becoming a common problem worldwide due to more consumption of S free N and P fertilizers such as urea, triple superphosphate and

ammoniated phosphates instead of single superphosphate and ammonium sulphate, which consist of 12 and 24 percent S respectively, for plant growth and removal of soil available S by increased cropping intensity and higher crop yields (Scherer, 2001). The ICAR system, TSI-FAI-IFA project and other programs have analyzed soil samples from different states and reported that S levels of Indian soils is decreasing with the reduction in industrial pollution (Camberato *et al.,* 2012). Increased agricultural production and different farming practices are causing the loss of S in soil. S loss is also occurring through leaching and runoff in areas with heavy rainfall and flood irrigation system.

Similarly, micronutrient deficiency is also an alarming problem for crop and human health. Globally more than 50% of the soils were found to be low on Fe and Zn (Sillanpaa, 1982; White and Zasoski, 1999; Salwa *et al*., 2010). Fe and Zn deficiency is a most common micronutrient deficiency in human (Bailey *et al.,* 2015) and results in health problems, learning disabilities in children, increased death rates, decreasing human potentials and diminishing national economic development (Welch and Graham, 2004). Various food fortification practices have been adopted for the physical enrichment of the nutrients and vitamins to food but these are uneconomical and have various technological issues such as doses of nutrients, stability of fortificants, nutrient interactions and also alter the taste, appearance and shelf life of the produce, thus, are unacceptable to the consumers (Fletcher *et al*., 2004).

The solution to meet the malnutrition challenge is, either balanced fertilization or mobilizing the unavailable nutrient in the rhizosphere and make them available for plant uptake. Although these nutrients are physically present in the soil, they are not available for uptake by the plants. There is also a necessity of the sustainable agriculture method with eco-friendly practices as a key to achieve food security which can help to minimize the yield gaps, maximize nutrient uptake and utilization efficiency, and efficiently increases the crop productivity (Xu *et al.,* 2015; Rajavat, 2019). Different approaches such as classical breeding, increasing nutrient use efficiency, biofortification through plant genetic engineering and the assessment of nutrient interaction are significant determinants for increasing the available amount of nutrients in the soil or for improving plant uptake.

Genetic improvements and cross breeding methods mainly depend on use of genetic variations, modern selection tools, identification of new genes and genetic transformation methods (Manwaring *et al.,* 2016). The exploitation of plant genetic ability for effective nutrient uptake and use and variability in intra-species characteristics is, therefore, a significant strategy for maintaining grain yield and quality on low nutrient soils (Cakmak *et al.*, 2001; Irshad *et al.,* 2004). Monocots such as bread and durum wheat, triticale and rye, for instance, display an elevated degree of variation in Zn tolerance. Also, in seeds of new wild emmer plant accessions with high levels of Zn (up to 139 mg kg^{-1}) and Fe (up to 88 mg kg^{-1}), an elevated tolerance to drought stress has been recognized (Singh *et al.,* 2005; Peleg *et al.,* 2008). Additionally, increasing nutrient use efficiency (NUE) of crops with appropriate resource management methods will also lead to sustainable farming systems. Inter and intra-specific variation for plant growth and mineral NUE is known to be under genetic and physiological control and is affected by interaction between plants and environmental factors (Baligar *et al*., 2001). Higher plant NUE could considerably reduce the supply cost of fertilizers, decrease the nutrient loss level, and increase crop yields. A high NUE will also boost the biofortification efforts which are critical to address the challenge of Fe and Zn deficiency anaemia, a major nutritional health problem especially in women and children in area where people are dependent on wheat as major staple cereal (Velu *et al.,* 2007). Biofortification which can be described as a method for increasing bioavailability and nutrient concentration in plants through classical plant breeding (White and Broadley, 2005) and recombinant DNA technology (Zimmermann and Hurrell, 2002) is regarded as the most viable and cost-effective strategy to deliver micronutrients to communities with restricted access to various diets and other micronutrient applications, the world over (Bouis, 2018). Any improvement in NUE or grain nutrients through classical or genetic advances would be guided by availability and translocation efficiency of respective nutrients in the rhizosphere and in-plant, both of which are regulated by the prevailing inter-nutrient interactions.

2.3. Inter-nutrient interactions

Nutrient-nutrient interactions are important to facilitate plant nutrient availability in the rhizosphere. A nutrient can interact with one or more than one

nutrient simultaneously and lead to toxicity, deficiency or change in plant growth responses and nutrient composition. Nutrient interactions can occur at various levels such as in rhizosphere, at the root surface, or within the plant system at both the root or shoot level. Nutrient interactions mainly occur between nutrients of similar size, charge, geometry of coordination and electronic configuration (Robson and Pitman, 1983). These nutrient-nutrient interactions are governed by various soil and plant internal factors. Soil factors include concentration of nutrient available in the soil solution, temperature, soil aeration, soil electrical conductivity, soil moisture and soil pH (White and Greenwood, 2013), whereas plant factors include root architecture, plant transpiration and respiration rate, plant age and growth rate, plant genotype and nutrient concentration of plants. Interaction between the nutrients may be either synergistic or antagonistic (Mulder, 1953) i.e. increasing or decreasing the uptake of other nutrient, respectively (Fig. 2.1). Sometimes it is also possible that there is no effect of one nutrient on other nutrient. N interacts positively with P, K, S, B, Fe and Zn and thus a higher level of N enhances availability and uptake of these nutrients. High level of P reduces Zn and also negatively affects the Ca uptake. Increase in level of K reduces Mg and to a lesser extent the uptake of Ca, Fe, Cu, Mn and Zn. High level of Cu can accentuate Mo, Fe, Mn and Zn deficiency. Fe deficiency can be accentuated by liming, low level of K or high level of S, Cu, Mn or Zn. Also Cu, Fe or Zn application is observed to overcome Mn deficiency – especially repeated soil application of Fe. Zn uptake can be decreased by high P level, liming or high level of Cu, Fe or Mn. Zn deficiencies are often associated with Mn deficiencies in some fruit crops. Negative interactions have also been reported between Fe and Zn, Ca and Fe and Zn and Mn (Malvi, 2011). The present study attempts to elucidate the interaction between S and Fe which are not opined earlier with clarity in Mudlers chart of nutrient-nutrient interaction (Mudler, 1953) but have been indicated by several researchers thereafter (Forieri *et al.,* 2013; Sharma *et al.,* 2016; Sharma *et al.,* 2018).

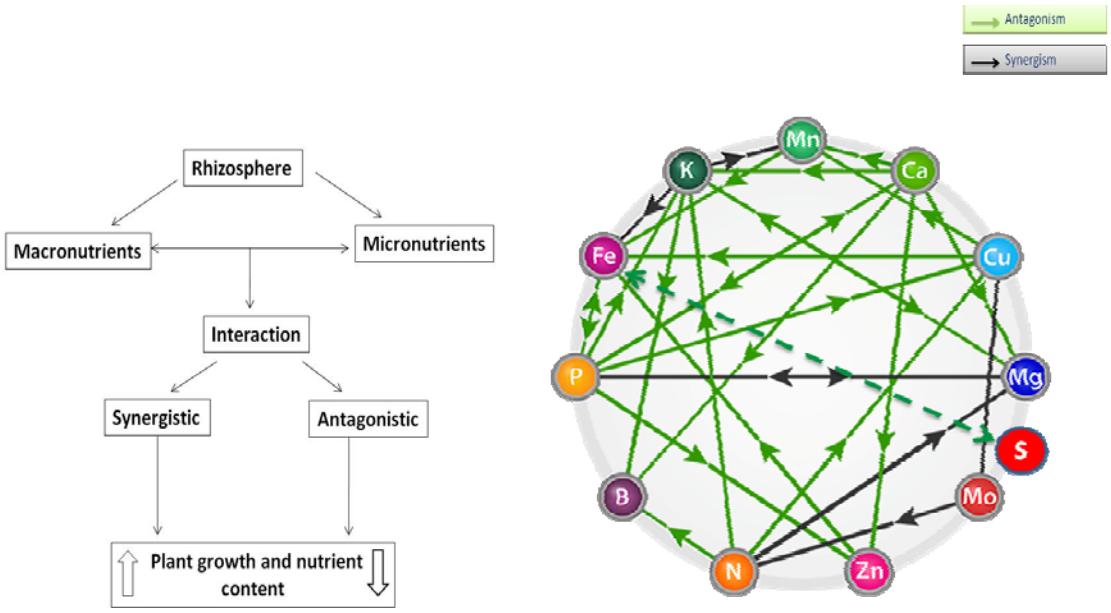

Fig. 2.1. Nature of interaction between macronutrients and micronutrients in the rhizosphere. Dark lines indicate established interactions while dotted line indicates likely interaction between S and Fe (Mudler, 1953; Sharma *et al.,* 2016; Sharma *et al.,* 2018)

2.4. Sulphur in soil and plants

S is present in either organic form i.e. elemental S or other S forms in organic matter or inorganic form i.e. sulphate in the soil. The organic and inorganic forms are inter-convertible by soil microbes through mineralization, oxidation, reduction, mobilization and immobilization processes (Scherer, 2009). Plants absorb S mainly in form of sulphate which are later converted to sulphite by sulphite reductase. Sulphate content in soil depends on soil physical and chemical properties such as pH, electrical conductivity, cation- anion balance, soil type, soil organic matter, SO_4:NH_4 applications, fertilizers applied, etc (Yang *et al.,* 2007; Kotkova *et al.,* 2008). In soils containing low organic matter like sandy soils S deficiency is a common problem and sometimes in soils which are rich in organic matter S deficiency is likely due to slow mineralization process and release of available S for plant uptake. Organic soil containing elemental S and other forms are unavailable for plant uptake and it must be converted to sulphate, the most mobile form of S (Scherer, 2001). Sulphate is negatively charged so its leaching rate is high and it leaches as

about 50% as fast as that of nitrate. S fertilization influences various soil properties such as pH, electrical conductivity and soil nutrients e.g. N, P, Zn, Mn and Fe (Orman and Kaplan, 2011). Elemental S reduces the alkalinity in calcareous soils by generating sulphate anions and this decrease in pH also increases the uptake of Zn, Fe, Cu and Mn by plants (Hilal and Abd-Elfattah, 1987; Attia and El Dosuky, 1996).

S deficiency has been reported in many crops and cause significant reduction in growth and yield e.g. in wheat (Zhao *et al*., 1999), barley (Withers *et al.,* 1995), rice (Lunde *et al.,* 2008) oilseed rape (Ahmad and Abdin, 2000), sugar beet (Hoffmann *et al.,* 2004), soyabean (Chandra and Pandey, 2016) and mustard (Li and Gerendas, 2007). A comparison of crop phenological stages reveals that the reproductive stage of wheat is more sensitive to S supply as compared to the vegetative stage. S requirement of wheat is 15-20 kg ha^{-1} for proper growth and development. Besides, S nutrition is also important for the quality of wheat products (Marschner, 1997; McGrath, 2003; Honermeier and Simioniuc, 2004). S application is necessary to maintain gluten protein composition in wheat (Zörb *et al.,* 2010). S fertilization improves the amino acid content and baking quality of flour of winter wheat (*Triticum aestivum* L.) (Jarvan *et al.*, 2008; Podlesna *et al.*, 2008). S treatment to plant increase the Cu and Zn concentration in shoots and roots by mobilizing these nutrients through soil microorganism (Wang *et al.,* 2008). S-nutrition also affects the yield and grain Fe and Zn nutrition in wheat ((McDonald and Mousavvi, 2009; Orman and Ok, 2012). Additionally S compounds are observed to play an important role in determining plant response to abiotic and biotic stress.

2.4.1. Visual symptoms of sulphur deficiency and toxicity

Soil containing low organic matter (< 2%) such as sandy soils is more prone to S deficient conditions. Sometimes the deficiency symptoms are also observed in high organic matter containing soils, in which the process of mineralization and the breakdown of organic matter are not so rapid to meet the plant need. Plant growing in such soils exhibit intervienal chlorosis and yellowing of mainly younger leaves as S is immobile in plants and does not translocated from older leaves to younger leaves. In wheat, younger leaves are severely affected by chlorosis and at later stages the whole plant becomes pale, whereas in corn intervienal chlorosis is more pronounced. Plants become spindly and small with thin stem under S deficiency. S deficiency reduces growth and metabolic activities of plants which in turn reduce the uptake of

macronutrient such as N, K and Mg (Dong *et al.,* 2017; Courbet *et al.,* 2019). S deficiency also affect the plant at the transcriptomic or metabolic levels such as in Arabidopsis (Forieri *et al.*, 2017), in *Triticum durum* (Ciaffi *et al.*, 2013), and in *Medicago truncatula* (Wipf *et al.*, 2014).

Sulphur toxicity in plants occurs when excess of sulphide forms during S assimilation. Sulphide is the end product of S assimilatory pathway and is important for cysteine synthesis in plants but in excess amount it is toxic as it disrupts the respiratory pathway in mitochondria (Birke *et al.,* 2012; 2013; 2015a; 2015b). Further, the cysteine formed may be converted to hydrogen sulphide (H_2S) through the enzyme L-cysteine desulfhydrase (Álvarez *et al.*, 2010; Guo *et al.*, 2016). This H_2S at low level regulates various physiological process in plants especially formation of adventitious and lateral roots, seed germination, stomatal closure, stress tolerance and flower senescence (Scuffi *et al.,* 2014; Fang *et al.* 2014; Jin and Pei, 2015; Mei *et al.,* 2017; Kou *et al.,* 2018). But at high levels H_2S is toxic to the plants as it induces ROS signaling pathway and inhibition of auxin transport thus alters primary root growth and root system development (Jia *et al.,* 2015). Besides this S toxicity in soil also interferes with the root uptake of other nutrients especially P (Aulakh and Pasricha, 1977).

2.5. Role of sulphur in human nutrition

Humans do not have ability to reduce sulphate to sulphide and can't synthesize amino acids, so they directly require the S containing amino acids from plants and S assimilation pathway in plants is, thus, essential for human sustenance (Tripathy *et al.,* 2010). S in human is essential constituent of amino acids and protein such as keratin and collagen. Sulphur containing amino acids such as cysteine and methionine impart protection against heavy metals and free radicals in human. Glutathione, a S containing compound is an important part of antioxidative system in human. S is also necessary for the production of insulin which plays role in glucose metabolism. Naturally occurring S containing ligands are also useful for detoxification of toxic metal ions and prevent their accumulation in the body (Colovic *et al.,* 2018). Arthritis in human can be cured by soaking the joints in methylsulphonylmethane (MSM), an organic S rich compound and an important 'building block' for healthy bones and joints. It is very useful for your immune system, allergy, pain syndromes and bladder disorders. S compounds such as SAMe, dimethylsulfoxide (DMSO), taurine,

glucosamine and chondrin sulfate have clinical application in the treatment of depression, heart failures, diabetes, cancer, etc. (Parcell, 2002).

2.6. Iron in soil and plants

Fe is prevalent in soil in primary minerals and phyllosilicates mainly in its ferrous state (Fe^{2+}) which is further oxidized in secondary minerals as ferric form (Fe^{3+}) (Torrent and Cabedo, 1986; Adriano, 2001; Stucki *et al.,* 2002). This ferric iron reacts with water and hydroxyl ions to form conjugate bases (Stumm and Furrer, 1987; Cornell *et al.,* 1989; Sposito, 1989). In well drained soils, Fe is normally present in the form of crystalline Fe oxides, geothite and hematite minerals whereas in poorly drained soil it is present as magnetite, ferrihydrite, ferroxite or other noncrystalline forms (Schwertmann, 1985; Cornell and Schwertmann, 2003). Other forms of Fe are soluble and exchangeable form or bound with the organic matter in soluble or insoluble forms (Colombo *et al.,* 2014). Soil pH and redox potential conditions are the main factors determining the Fe availability for plant uptake. Ferrous form of Fe is easily available for plant uptake whereas ferric form remains bound to soil colloids. Under alkaline condition i.e. pH above 7, Fe concentration in soil solution is even lower than the concentration required for the optimal plant growth (Boukhalfa and Crumbliss, 2002). Under this poor availability condition, plants triggers two chief Fe acquisition mechanisms to address the deficiency of Fe (Roemheld and Marschner, 1986; Jolley *et al.,* 1996; Pearson and Rengel, 1997; Ogo *et al.,* 2011) (Fig. 2.2). Strategy I is characteristic of dicots and monocots other than grasses and includes exudation of reducing and chelating compounds in the rhizosphere, modifications in root morphology and histology (forming rhizodermal transfer cells, triggered root growth, more root hair, etc.) and reduction of Fe^{3+} to Fe^{2+} in the root cell membrane. Strategy II used by grasses includes enhanced exudation of phytosiderophores (PS) into the rhizosphere. In response to Fe deficiency, the Fe-efficient genotypes undergo robust metabolic and structural modifications that enable them to grow and produce better dry mass under deficiencies relative to the Fe inefficient genotypes. Takagi (1976) was the pioneer in exploring the PS, mugineic acid family compounds, secreted by the root of Fe deficient graminaceous plants for solubilizing and chelating Fe^{3+} that remain bound to the soil colloids and is otherwise unavailable for plant uptake. Root morphology of a plant may also influence the total release of phytosiderophores (Bernards *et al.,* 2002; Divte *et al.,* 2019) in the rhizosphere.

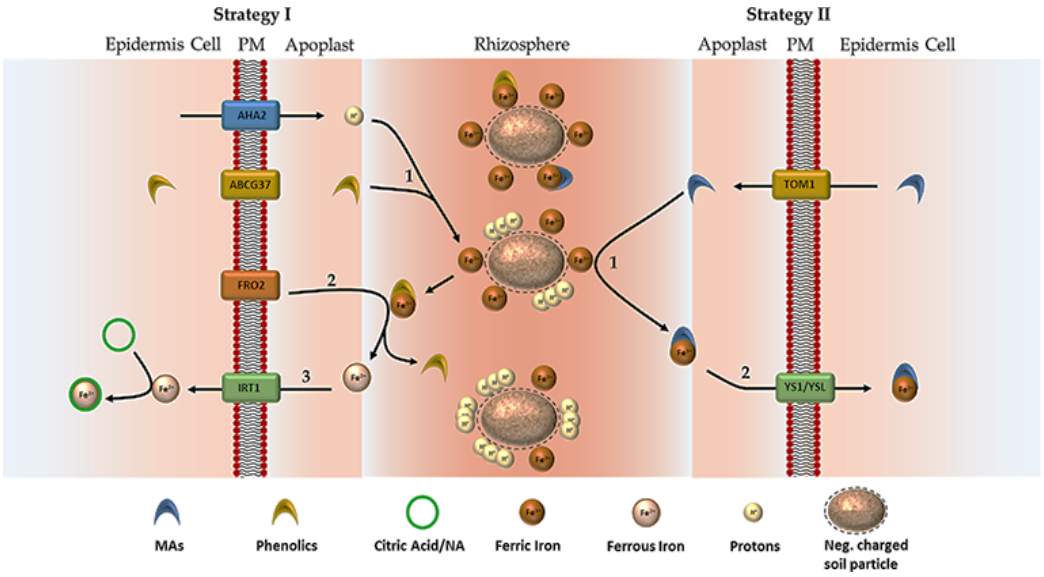

Fig. 2.2: Strategy I and strategy II of iron acquisition in plants. Strategy I plants, exemplified by *Arabidopsis thaliana* and strategy II plants, exemplified by *Zea mays* and rice (Naranjo-Arcos and Bauer, 2016).

Fe is a micronutrient, which means it is required by plants in lesser amount than essential or optional macronutrients. Fe is a significant micronutrient necessary to sustain the metabolism of plants. It performs a major part in chlorophyll, enzyme synthesis, nitrogen fixation and plant growth and development (Briat *et al.,* 2010). Fe is engaged in various metabolic processes such as DNA synthesis, respiration and photosynthesis. It is a constituent of the heme and the Fe-S cluster. Fe is associated with 5-Aminolevulinic acid (ALA) biosynthesis, the precursor of chlorophyll biosynthesis, in crops (Rout and Sahoo, 2015). It is a component of many essential enzymes such as lipoxygenase, ACC oxidase, nitrate reductase and cytochromes of the electron transport chain. Fe deficiency also impacts photosynthetic apparatus structure and function, especially the PS I (Moseley *et al.,* 2002).

2.6.1. Visual symptoms of iron deficiency and toxicity

Fe deficiency in soil is mainly caused by lack of plant available form of Fe. Further, Fe is immobile in plants, so symptoms of deficiency are mainly noted on young leaves (Marschner, 1995). Interveinal chlorosis with leaves turning yellowish to brown in between the green veins is the primary symptom of Fe deficiency owing to a reduction in photosynthetic pigments (Spiller and Terry, 1980; Morales *et al.,*

1998). Fe deficiency chlorosis also known as "lime-induced chlorosis" is a major issue in wheat crop, especially in calcareous soil and causes a significant reduction in yield in arid and semi-arid areas. Severe deficiency results in total yellowing of the leaves, which subsequently turn white, leading to a reduced crop development and productivity (Abadia *et al.,* 2011).

Iron toxicity, on the other hand, is mainly associated with pH and happens where the pH of the soil falls substantially to induce an excess of plant accessible Fe. Fe toxicity may also happen when Zn is deficient or when the soil is in a reduced state due to wet or flooded situation. Excess Fe can lead to dark green to purple leaves (e.g. rice bronzing disease), stunted development of stems and roots. Fe toxicity in soil hinders the uptake of essential nutrients such as P, N or Zn from the soil and also lead to oxidative stress symptoms (Kampfenkel *et al.,* 1995).

2.7. Role of iron in human nutrition

Fe plays a significant part in the synthesis of haemoglobin and O_2 transport in human body. According to the WHO report (2008), around 30% of the world's population is anaemic largely due to Fe deficiency. Fe deficiency anemia can cause fatigue, heart palpitations, pale skin, and breathlessness (Hegde *et al.,* 2006). Fe also helps to preserve many vital functions in the body, including general energy and focus, gastrointestinal processes, the immune system, and the regulation of body temperature. Fe deficiency in females causes autism spectrum disorder in their offspring (Schmidt *et al.,* 2014). Nutritionally, Fe level of ~29 to 73 mg/kg is found in whole grain of wheat (Cakmak *et al.,* 2004) and approximately 75% of this Fe is in the seed, but it is wasted during the processing and milling process (Slavin *et al.,* 2000) and is, therefore, removed from human nutrition. In many countries, wheat products and infant formulas are fortified with Fe to meet the daily dietary recommendation of Fe.

2.8. Sulphur-iron interaction

The study on the interaction of S and Fe has been executed by many researchers (Astolfi *et al*., 2009; Astolfi *et al*., 2011; Sharma, 2015) and the involvement of S in Fe uptake via release of PS has been postulated in different plants (Sharma *et al.,* 2018). S deficiency leads to Fe deficiency due to reduction in formation of S-adenosyl

methionine which regulates the production of PS in Strategy I plants (Fig. 2.3). It has been demonstrated that the Fe deficient plants when re-supplied with S exhibit restoration of their capacity to tolerate Fe deficiency (Astolfi *et al*., 2009; Astolfi *et al*., 2011) since the product of S assimilatory pathway, methionine is directly involved for the synthesis of PS, the Fe chelating agent (Sharma *et al.,* 2018). A general decline in leaf Fe was observed under S deficiency in maize by Astolfi *et al*. (2003). In tomato plants, an increase in the activity of Fe^{3+}-chelate reductase, ^{59}Fe uptake rate and ethylene production in the roots was observed under increasing S availability, which in turn also expresses the LeIRT1 (Fe^{2+} transporter) to facilitate Fe uptake under Fe deficiency (Zuchi *et al.,* 2009). S nutrition alleviated protein damage under Fe deficiency in oilseed rape (Muneer *et al.,* 2013). The effect of combined S and Fe deficiency on major S assimilating enzymes and sulphate transporters was studied by Ciaffi and coworkers (2013) and they suggested that combined S and Fe deprivation induced a complex interplay of enzymes and transporter of S metabolism and uptake at transcriptional, translational and post translational levels. Durum wheat on increasing S supply showed a significant increase in Fe use efficiency and accumulated more Fe in plants under the Fe deficient condition (Zuchi *et al*., 2012).

Fe and S also remain in the form of Fe-S clusters in plant. These are ubiquitous prosthetic groups required in respiration, photosynthesis and have remarkable structural plasticity and versatile chemical feature to participate in electron transfer, substrate binding/activation, Fe/S storage, regulation of gene expression and enzyme activity (Balk and Pilon, 2011). Fe-S clusters are important for the activity of more than 200 types of proteins or enzymes. Structurally, Fe-S cluster has Fe and inorganic sulfide attached to a polypeptide, primarily via a cysteinate Fe ligation. It constitutes one of the most structurally and functionally diverse class of biological prosthetic groups (Johnson and Smith, 2005). The simplest Fe-S clusters are of two types i.e. [2Fe-2S] and [4Fe-4S] types which contain either ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron and sulfide (S^{2-}) ion. This structure is usually integrated into proteins via coordination of the Fe ions by cysteine or histidine residues (Lill, 2009).

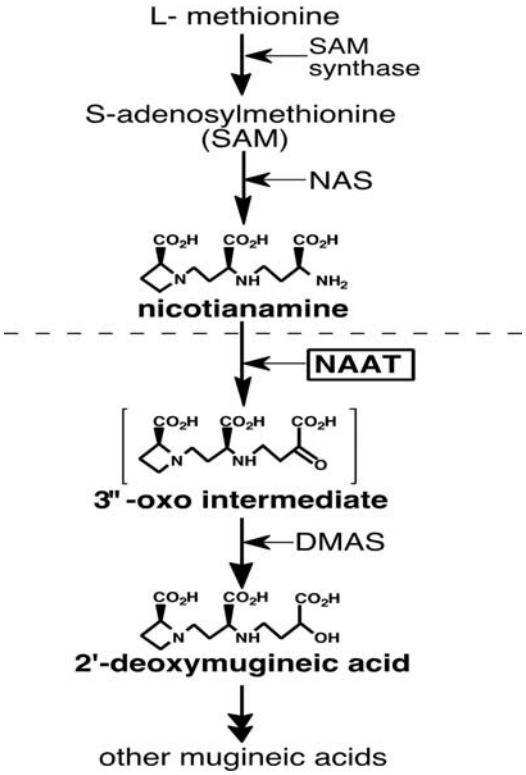

Fig. 2.3: Biosynthesis pathway of phytosiderophores (adapted from Bashir and Nishizawa, 2006).

2.9. Regulation of sulphur and iron uptake in plants

S and Fe uptake in plants is a highly regulated and controlled process which depends on their transport from the rhizosphere in to the plants through the involvement of S and Fe uptake transporters (SULTR and IRT, respectively) followed by their assimilation and translocation from the root to the shoot (Hawkesford and Wray, 2000; De Kok *et al.,* 2002; Morrissey and Guerinot, 2009).

2.9.1. Sulphur transporters

Root uptake of S in the form of sulphate ion from the rhizophere is facilitated by sulphate transporter. Sulphate is then reduced and assimilated in the leaves (Marschner, 1995). Sulphate transport involves two types of transporters i.e. high affinity transporters (HATs) and low affinity transporters (LATs) based on their relative affinity for the substrate (Epstein, 1976). LAT operates at a high substrate concentration (>1mM) than HATs, thus, indicates a low Km value for HATs than LATs. Different HATs for sulphate uptake have specific gene products and overlapping functions (Gigolashvili and Kopriva, 2014). Sulphate transporters

belongs to a small gene family and are divided into five groups (Hawkesford, 2003; Buchner *et al.*, 2004) wherein, Group 1 sulphate transporters are high affinity plasma membrane localized transporters and are predominantly expressed in roots of S deficient plants e.g. SULTR1;1 (Buchner *et al.,* 2010) and SULTR1;3; Group 2 transporters are low affinity sulphate transporters and are present in vascular tissues (Smith *et al.*, 1995; Takahashi *et al.*, 2000) e.g. SULTR2;1 is expressed in all tissues of wheat except grains. Group 3 sulphate transporters are probably involved in modulating SULTR2;1, a Group 2 sulphate transporter (Kataoka *et al.,* 2004a). Group 4 transporters are suggested to involved in vacuolar efflux to cytoplasm (Kataoka *et al.,* 2004b) and Group 5 transporters e.g. SULTR5;2 appears to be involved specifically in Mo accumulation (Tomatsu *et al.,* 2007; Baxter *et al.*, 2008). Sulphate transporters in wheat show tissue specific differential gene expression in response to nutrient levels. The transcript expression of *SULTR1;1* gene was significantly high in durum under S deficiency as compared to S sufficient condition. Fe availability is critical to induce the expression of *SULTR1;1* gene under S deficiency (Sharma *et al.,* 2018).

2.9.2. Iron transporters

Strategy I plants include the expression of Fe regulated transporter (*IRT*), Ferric chelate reductase oxidase (*FRO*) and H^+-ATPase (*HA*) gene whereas in Strategy II plants Yellow stripe 1 (*YS1*)/Yellow stripe 1-like (*YSL*) and recently discovered transporter genes of mugineic acid family phytosiderophores (*TOM1*) are expressed (Kobayashi *et al.*, 2012). In wheat, *YS1* is the first member of YSL family transporter and is the major Fe uptake transporter induced maximally under Fe deficiency. It facilitates the uptake of Fe (III)-PS complexes under Fe deficiency condition (Curie *et al.,* 2001). *YS1* is induced in both root and shoot and is present in both graminaceous and non graminaceous species. In non graminaceous species, it may be involved in uptake of Fe (III)-NA complex. *YS1* (*ZmYS1*) transporter, that belongs to oligopeptide transporter (OPT) super family has been first identified in maize (Curie *et al.,* 2001) and contains 12 putative transmembrane-spanning domains and is highly hydrophobic in nature. Murata and coworkers, (2006) identified *HvYS1*, a specific transporter gene of Fe(III)-PS in barley plants which shows 72.7% homology with *ZmYSI* gene of maize but their expression pattern, diurnal change and

tissue-type specificity of localization are different (Ueno *et al.*, 2009). YSL family members were also discovered in Arabidopsis and rice (Forieri *et al*., 2013). *YS1* gene expression was higher for bread wheat than durum wheat under Fe deficient and S sufficient condition (Sharma *et al.,* 2018). Mutants of *YS1* (ys1) and *YS3* (ys3) showed defect in Fe homeostasis and developed Fe deficiency symptoms i.e. intervienal chlorosis (Nozoye *et al.*, 2011).

2.10. Efficiency for sulphur and iron assimilation regulates their in-plant partitioning

Following root uptake of nutrients, they are subjected to the assimilation process through which they are reduced and then translocated to the shoot. Not all nutrients require reduction however; the efficiency of assimilation is guided by the activity of a few principal reductive enzymes which are discussed separately for S and Fe.

2.10.1. Role of sulphur assimilating enzymes and other regulated metabolic pathways

The activity of S assimilating enzymes is critical for the assimilation of S from the rhizosphere. S is absorbed in the form of sulphate by plants and this sulphate is further metabolized in different components and eventually forms cysteine and methionine amino acids. The enzymes ATP-sulfurylase (ATPS) and adenosine 5’ phosphosulphate reductase (APR) convert sulfate into adenosine phosphosulphate (APS) and sulfite, respectively. This sulfite is reduced by sulfite reductase into sulfide which further forms cysteine on conjunction with O-acetyl serine (OAS) by the enzyme O-acetyl serine thiolyase (OASTL). The formation of OAS takes place by the acetylation of serine in the presence of serine acetyl transferase (SAT) enzyme. APS is either converted to sulfite or phosphorylated to form 3’-phosphoadenosine 5’-phosphosulfate by APS kinase (Leustek and Saito, 1999). S assimilation yields cysteine as the first stable product which then further acts as a precursor for the synthesis of methionine, glutathione and other S containing secondary metabolites. S compounds such as glutathione are known to detoxify the reactive oxygen species and also act as a substrate for the synthesis of phytochelatins, or glucosinolates that aid in defense against herbivores and other pathogens.

2.10.2. Role of iron regulated enzymes and other metabolic pathways

Fe is taken up from the rhizosphere either in ferrous form by strategy I plants or in ferric form by strategy II plants. The key enzyme in strategy I plants, for example in tomato and Arabidopsis, is ferric reduction oxidase 2 (FRO2) and H^+-ATPase (HA). H^+-ATPase present in the root epidermis is induced under Fe deficiency to release protons into the rhizosphere. These protons acidify the soil and make the bound Fe, available to the plants (Schmidt *et al.,* 2003; Santi *et al.,* 2005). FRO 2 reduces the Fe^{3+} to Fe^{2+} and increase plant Fe uptake (Robinson *et al.,* 1999). In strategy II plants the chief enzymes involved in the regulation of Fe uptake include nicotianamine (NA) synthase (NAS) (Higuchi *et al*., 1999), NA aminotransferase (NAAT) (Takahashi *et al*., 1999) and 2′-deoxymugineic acid (DMA) synthase (Bashir *et al*., 2006). NA is synthesized from S-adenosyl methionine through NAS which, later on, is converted into DMA through NAAT and DMA synthase activity (Mori and Nishizawa, 1987; Takahashi *et al.,* 2003; Haydon and Cobbett, 2007). DMA, a mugineic acid family member, is precursor for other PS in cereals. Chelators produced via strategy I or stragety II such as citrate, NA and DMA are all involved in transporting Fe from the roots to other parts of the plant (Jeong and Guerinot, 2009).

2.11. Integration of sulphur assimilation with carbon and nitrogen metabolism

S assimilation pathway may have regulatory linkage with the carbon, nitrate and Fe assimilation pathways (Kim *et al.,* 1999; Kopriva *et al.,* 2002; Mendoza-Cózatl *et al.,* 2019). Cysteine, the first stable product of S-assimilation, is the connecting link between the carbon, N and S metabolic pathway. The carbon backbone of cysteine is synthesized by photosynthetic processes in chloroplast. N is added to this carbon backbone and forms serine which enter into S assimilation pathway and is converted into cysteine with the help of OASTL enzyme. Cysteine is later on converted to methionine and other S containing compounds. Methionine further enters into the Fe metabolic pathway and forms mugineic acid family chelating agents which facilitate the iron uptake. The convergence of these nutrient metabolic pathways is poorly understood and how different metabolic products interact in the enzymic and functional regulatory framework is not yet clear.

N efficiency principally depends upon efficiency for N uptake and its utilization, where uptake is determined by the activity of high-affinity nitrogen

transporter, NRT2.1 or low affinity transporter, NRT1 and its reduction by nitrate reductase (NR), while N utilization is guided by the rubisco, which is responsible for fixing atmospheric carbon dioxide into organic compounds (Andersson and Backlund, 2008). N plays regulatory role in S metabolism by participating in the formation of OAS, which is a product in S assimilation pathway (Kopriva *et. al.,* 2004). The photosynthetic efficiency and performance of the plants depends on the expression and activity of the rubisco enzyme which in turn also required for the synthesis of carbon containing precursors for different metabolic pathways. The carbon backbone for cysteine is also synthesized through the photosynthetic machinery (Mendoza-Cózatl *et al.,* 2019). The rate limiting enzyme of nitrate metabolism i.e., NR is important to regulate the synthesis of amino acids and other N containing compounds. The N part of the S containing amino acids is provided by serine which further converted into cysteine. A large number of research literature is available to decipher the interaction between different plant nutrients viz a viz their role in determining plant growth. N and S show synergistic interaction with each other and together increases the growth and nutrient uptake of maize plants in sandy calcareous soil (Rahman *et al.,* 2011). S supply in legumes is linked to the nodulation, symbiotic nitrogen fixation and N supply to the plants (Becana *et al.*, 2018). S deficiency in mustard reduces the plant N content (Varin *et al.,* 2009; Malliard *et al.,* 2016a). Interaction of S with Fe may be mediated via N as N interacts with Fe by increasing the root release of PS and root uptake of Fe (III)-PS in Fe-deficient wheat plants (Aciksoz *et al.,* 2011). Fe deficiencies lead to decrease in N metabolism in plants (Borlotti *et al.,* 2012), while N fertilization helps in enhanced Zn and Fe uptake and increase yield components of wheat and spring barley (Sedlar *et al.,* 2014).

2.12. Mineral nutrient interactions regulates their uptake and use efficiency

Different nutrient elements available in the growing media are postulated to interact with each other to influence their respective uptake and use efficiency. These interactions can be either synergistic or antagonistic as has been reported by Mudler (1953). S nutrition also regulates the concentration of various other macro and micronutrients such as K, Mg, B, Cu, Zn, Cl, Na, Mo and Mn in plants as illustrated in Fig. 2.4. K and Mg uptake showed negative interaction with the S availability. Mo and Mn deficiency leads to a higher uptake of S (Shinmachi *et al,* 2010; Maillard *et*

al., 2016a; Courbet *et al*., 2019). S deprivation increases the uptake of Cl (Sorin *et al.*, 2015; Etienne *et al.*, 2018) and Cu (Maillard *et al.*, 2016b) through the rhizosphere. The importance of these inter-nutrient interactions still eludes our understanding and is inconclusive in respect of the regulatory attributes governing nutrient uptake and use efficiency.

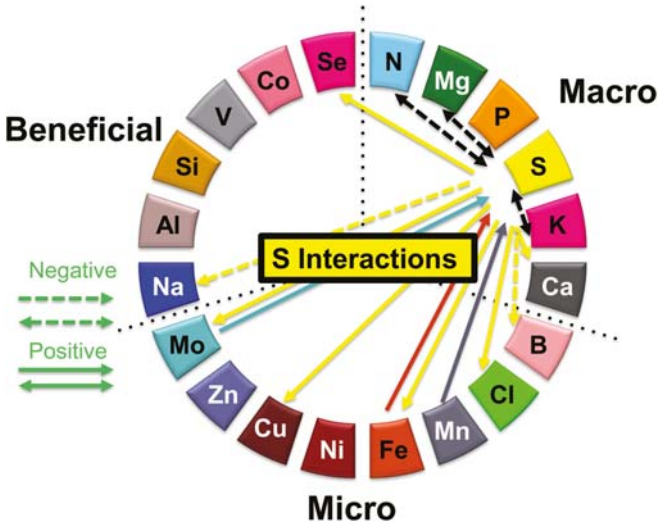

Fig. 2.4. Sulphur crosstalk with other mineral elements. S deficiency may lead to a reduced or increased uptake of other nutrients (dashed and solid lines, respectively) (Maillard *et al.* 2016a; Courbet *et al.*, 2019)

Similarly micronutrients such as Fe, Zn, Mn, Cu, etc have also been shown to play an important role in balanced fertilization of plants. Balanced fertilization is the key to sustainable agriculture which includes efficient utilization of fertilizers based on the demand of the crop. It maintains harmony among all essential nutrients for the growth and yield. It helps to maintain soil fertility, improves quality of environment, increases crop quality and achieves sustainably high yields. Use of micronutrients in fertilizers also increases the plant efficiency to uptake and utilization of macronutrients (Cakmak, 2002; Malakouti, 2007; Cakmak, 2008; Malakouti, 2008).

A critical analysis of the available research reports comprehensively suggest that nutrients in the rhizosphere interact with each other to regulate their availability, uptake and distribution in a soil-plant system, however, the nature of the interactive relationship may depend on the absolute concentrations of the interacting elements. The review also forces us to enlarge our research scene from a few well known and documented interactions such as P-Zn or K-Mg to a whole new scenario which hints

and harps on regulatory interaction between many other nutrients particularly the much lesser known effect of micronutrients on macronutrients. The present study, which blooms from an isolated observation pertaining to Fe availability induced transcript level expression of SULTR1.1 (Sharma *et al*., 2018), was thus taken forward to delve in-depth into the regulatory influence of Fe on S metabolism, likely linkages and underlying mechanisms.

Chapter 3

MATERIALS AND METHODS

The present study **"Physiological and molecular regulation of sulphur uptake and assimilation by iron in wheat"** was conducted in the Division of Plant Physiology and CESCRA, Nuclear Research Laboratory (NRL), ICAR-Indian Agricultural Research Institute (IARI), New Delhi, during 2015-2019. The experimental site is located at geo-coordinates of 28.08°N latitude and 77.12°E longitude and 229 m Above Mean Sea Level (AMSL). The present study involved physiological, biochemical and molecular investigations conducted through pot and solution culture experiments. For the first experiment the bread and durum wheat plants were raised in the pots, at the institute farm while the solution culture experiment was carried out in the growth chamber at NRL, ICAR-IARI.

3.1. Objective 1: To determine the effect of iron on sulphur uptake, assimilation and partitioning under low and sufficient sulphur availability

The objective was realized on Fe- and Fe+ soil with bread and durum wheat through the pot culture experiment.

3.1.1. Experimental set up

Grains of four cultivars of wheat i.e. *Triticum aestivum* L. cv. HD-2967 and HD-2329 and *Triticum durum* L. cv. HI-8713 and HD-4728, procured from the Division of Genetics and Plant Breeding, ICAR-Indian Agricultural Research Institute, New Delhi (India), were used as the experimental materials. Plants were raised in the pots (12 inch diameter) on Fe deficient (~3 ppm) and Fe sufficient soil (~12 ppm) under different S levels viz. 0, 30 and 60 kg S ha^{-1} soil supplied as $CaSO_4.2H_2O$ to study the effect of Fe on the S nutrition of wheat. The other physico-chemical characteristics of the Fe deficient and Fe sufficient soils used in the present experiment are given in the Table 3.1. All treatments were maintained in triplicates and a minimum of five seedlings per pot/replicate were maintained.

3.1.2. Physiological parameters

Plants raised under different Fe and S sufficiency/deficiency treatments were analyzed at 30 (Stage I, tillering); 60 (Stage II, heading) and 120 (Stage III,

physiological maturity) days after sowing (DAS), to determine the following morpho-physiological attributes in triplicate.

Table 3.1: Physico-chemical properties of the Fe sufficient and Fe deficient soil

Properties	Fe sufficient soil	Fe deficient soil
pH	6.5-7.5	7.5-8.5
EC	0.2-0.4	0.2-0.4
Organic carbon	<0.5%	<0.5%
N	<280 kg/ha	<280 kg/ha
P	10-25 kg/ha	10-25 kg/ha
K	120-180 kg/ha	120-180 kg/ha
S	<10 mg/kg	<10 mg/kg
Fe	~12 ppm	~3 ppm

3.1.2.1. Biomass

Wheat shoots were sampled on 30, 60 and 120 DAS for shoot biomass determination. Harvested shoots were oven dried at 80°C for 4 h and then at 60 °C till constant weight was obtained following which the shoots dry mass was recorded and expressed as g dw plant^{-1}.

3.1.2.2. Gas exchange measurements

The light saturated photosynthetic rates (Pn), stomatal conductance (Gs) and transpiration rates (E) of newly expanded leaves (flag leaf) were measured between 10:00 am and 11.30 am at different growth stages (30 and 60 DAS) with an infrared gas analyzer (6400XT, Li-Cor, Lincoln, NE, USA). Leaf temperature during the measurements was maintained at 28 °C and a relative humidity of 50% under a PPFD of 1000 µmol photons m^{-2} s^{-1}. Data were recorded after equilibration to a steady state.

3.1.3. Nutrient analysis of soil and plant sample

3.1.3.1. Sulphur and iron concentration of soil

3.1.3.1.1. Soil sulphur analysis

20 g of soil sample was weighed in a 250 ml conical flask, to which 100 ml of the monocalcium phosphate extracting solution (500mg P L^{-1}) was added and shaken

for one hour. It was then filtered through Whatman No. 42 filter paper and the filtrate was analyzed for sulphate (SO_4^{2-}) following the turbidimetric method (Tabatabai and Bremner, 1970) as described in next section.

3.1.3.1.1.1. Turbidimetric method

20 ml of the clear filtrate was pipetted into a 25 ml volumetric flask and 2 ml of glacial acetic acid along with 1g of barium chloride crystal ($BaCl_2.2H_2O$) was added and was shaken for 10 minutes. After this 1 ml of 0.25% gum acacia solution was added and the volume was made to 25 ml with distilled water and then constant stirring was done for 1 minute. Simultaneously, a solution blank was also prepared as control and the turbidity of control and experimental samples was measured, 10 min after the precipitation of barium sulphate, using UV-Vis spectrophotometer (Specord Bio-200, AnalytikJena, Germany) at 420 nm.

3.1.3.1.1.2. Preparation of standard curve for sulphur

Exactly 0 (blank), 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 10 ml portions of the working standard solution (25 mg S l^{-1}) were pipetted into a series of 25 ml volumetric flask to obtain 0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 mg S l^{-1} concentrations. Turbidity was developed as described above for sample aliquots and volume was made to the 25 ml mark with distilled water and it was shaken for 1 minute. Turbidity intensity was determined after 10 minutes using spectrophotometer at 420 nm wavelength by adjusting 100% transmittance using blank. The standard curve was prepared by plotting absorbance readings of standards against final concentration (0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 mg S l^{-1}).

3.1.3.1.2. Soil iron analysis

The soil samples were sampled, dried and ground. The extraction solution comprised 0.005 M Diethylenetriaminepentaacetic acid (DTPA), 0.01 M calcium chloride dehydrate ($CaCl_2.2H_2O$) and 0.1 M triethanolamine (TEA).1.97 g of DTPA, 1.47 g $CaCl_2.2H_2O$ and 13.3 ml TEA were dissolved separately in distilled water and combined. The pH was adjusted to 7.3 using conc. HCl and volume made upto 1 L with distilled water. 10 g of soil was weighed and 20 ml of the DTPA extraction solution was added. After shaking for 120 minutes, the sample was filtered using filter paper and Fe concentration in samples was determined using Atomic Absorption

Spectrophotometer (AAS, ECIL make, India) using cathode lamp having transmittance at 248.3 nm.

3.1.3.2. Nutrient analysis in plant

S, Fe and N concentration of shoot and grain of the bread and durum wheat were determined as per the following methods at 30, 60 and 120 DAS.

3.1.3.2.1. Shoot sulphur and iron analysis

To measure S and Fe concentration, a known amount of dried shoot and grain tissue was digested in 14 mL diacid mixture containing nitric acid and perchloric acid in 9:4 ratio in digestion tubes and allowed to stand over-night. The tubes were placed in an open air digester maintained at temperature of 80ºC for 1 h and then at 200ºC until white fumes start emanating from the vials indicating an end of the digestion process. After digestion, the solution was cooled to room temperature, filtered and volume was made to 25 mL with double distilled water before analysis of S and Fe. The shoot S level in the acid digest was measured following turbidimetric method (Tabatabai and Bremner, 1970) as described earlier and shoot S concentration was expressed as μg S g^{-1} dry weight and the concentration of Fe were determined against known concentration standard, prepared from stock solution of 1000 mg/l (SRL chemicals, Mumbai, India), on Atomic Absorbance Spectroscopy (AAS, ECIL make, India) and expressed as μg Fe g^{-1} dry weight.

3.1.3.2.2. Shoot Nitrogen

For determination of total nitrogen (N) in plant samples micro Kjeldahl method as per procedure suggested by AOAC (1995) was used. Organic N in the plant samples were converted into NH_4-N by digestion with concentrated H_2SO_4 containing a catalyst mixture (Na_2SO_4 and $CuSO_4$). The NH_4-N in the digest was determined from the amount of NH_3 liberated by distillation of the digest with NaOH. Ammonia absorbed in boric acid containing mixed indicator was determined by titration with a standard sulphuric acid.

The micro Kjeldahl method consisted of three steps i.e. digestion, distillation and titration. For digestion, 0.5 g of prepared plant leaf and grain sample was digested into the digestion tube. 10 ml of concentrated sulphuric acid and 5 g of catalyst mixture were added to the sample. The digestion tubes were loaded in to the digester

and heated the digestion block. The initial block temperature was 100°C till frothing over, and then it was raised to 400°C. The samples turned light green or colorless at the end of the digestion process. After cooling of the digestion tubes, the individual tube was loaded in the distillation unit and on the other side of hose 20 ml of 4% boric acid with mixed indicator was kept in 250 ml conical flask. 40 ml NaOH (40%) was automatically added by distillation unit programme. The digested sample was heated by passing steam at a steady rate and the liberated ammonia was absorbed in 20 ml of 4% boric acid containing mixed indicator solution which turned the pinkish colour solution to green following the absorption of ammonia. Nearly 150 ml of distillate was collected in about 8 minutes. Simultaneously, blank sample was also prepared. The green colour distillate was titrated with 0.02N sulphuric acid and the colour changed to original shade (pinkish colour). The blank and sample titer reading (R) (ml) were noted and the total nitrogen content was calculated in plant samples.

Calculations:

$$\text{Nitrogen content in plant (\%)} = \frac{\text{R (sample titer} - \text{blank titer)} * \text{Normality of acid} * \text{atomic weight of nitrogen} * 100}{\text{Sample weight (g)} * 1000}$$

$$\text{Nitrogen content in plant (\%)} = \frac{\text{R} * 0.1 * 14 * 100}{1 * 1000}$$

$$\text{Nitrogen content in plant (\%)} = \text{R} * 0.14$$

3.1.4. Sulphur, iron and nitrogen use efficiency

S, Fe and N use efficiency were determined at physiological maturity (120 DAS) in terms of the biomass produced per unit of plant S, Fe and N uptake, respectively.

3.1.5. Translocation index of sulphur, iron and nitrogen

Translocation Index (TI) for S, Fe and N were calculated as follows (Rengel and Graham, 1996)

TI (S) = Grain S / Shoot S

TI (Fe) = Grain Fe/ Shoot Fe

TI (N) = Grain N/ Shoot N

3.1.6. S and N assimilating enzymes:

S assimilating enzymes viz. Serine acetyl transferase and O-acetyl serine lyase and N assimilating enzymes viz. Rubisco and Nitrate reductase activity were assayed following the methods Kredich and Tomkins, 1966, Writz *et. al.,* 2004, Singh and Singh, 2001 and Hageman and Hucklesby, 1971, respectively. The enzyme activities were calculated at 30 and 60 DAS in leaf tissue of the bread and durum wheat experimental cultivars.

3.1.6.1. Serine acetyltransferase activity

Serine acetyltransferase activity from the crude plant extracts was determined by the method of Kredich and Tomkins (1966). This enzyme assay is based on the disulfide exchange between CoA liberated by acetyl-CoA during the reaction and dithiobis 2-nitrobenzoic acid (DTNB). Production of thionitrobenzoic acid was followed spectrophotometrically at 412 nm. Freshly harvested wheat tissue samples (200 mg) were ground in a chilled mortar and pestle with 2 ml of ice-cold extraction buffer [100 mM Tris–HCl (pH 8.0), 100 mM KCl, 20 mM $MgCl_2$, 1% 419 Tween 80, 10 mM DTT]. The samples were transferred to microcentrifuge tubes and spun (11,600 g; 10 min; 4ºC). The clear supernatant obtained after centrifugation was used to measure SATase activity. The enzyme reaction mixture contained 0.1 mM acetyl-CoA, 50 mMTris (pH 7.6), 1 mM DTNB, 1 mM EDTA and 1 mM L-serine in 1 ml final volume. Subsequent to reaction initiation by addition of enzyme at room temperature, the initial velocity was estimated by monitoring the increase in absorbance at 412 nm. Rates were calculated using an extinction coefficient for thionitrobenzoic (TNB) acid i.e. 13,600 at 412 nm. The enzyme activity was expressed as nmol tnb min^{-1} g^{-1} fw.

3.1.6.2. O-acetylserine thio lyase (OASTL) activity

Freshly harvested bread and durum wheat leaf samples (200 mg) were ground in a chilled mortar and pestle with 2 ml of ice-cold extraction buffer [100 mM Tris–HCl (pH 8.0), 100 mM KCl, 20 mM $MgCl_2$, 1% 419 Tween 80, 10 mM DTT]. The samples were transferred to microcentrifuge tubes and spinned at 12000 g for 10 min at 4ºC. The clear supernatant obtained after centrifugation was used to measure OAS-TL activity. The enzyme reaction mixture contained 0.1 mM acetyl-CoA, 1 mM L-serine, 3mM sodium sulphide, 10 mM DTT, in 1 ml final volume. Subsequent to

reaction initiation by addition of enzyme at room temperature, the initial velocity was estimated by monitoring the increase in absorbance at 412 nm. The enzyme activity was expressed as nmol cysteine min^{-1} g^{-1} fw.

3.1.6.3. Rubisco activity

Rubisco activity was measured by ribulose-1,5-bisphosphate dependent incorporation of $^{14}CO_2$ in to acid stable product which was estimated by liquid scintillation counter (HIDEX-300 SL, Finland) and expressed as µmol $^{14}CO_2$ fixed g^{-1} fw h^{-1} (Feller *et al.,* 2008; Singh and Singh, 2001). The enzyme extract was prepared by grinding the leaf sample (0.5g) in 5 ml ice cold extraction medium at 4ºC which was then centrifuged for 15 min. at 18,000g at 4ºC. The supernatant was decanted and used as a crude enzyme extract. Reaction mixture contained:100mM Tris–HCl, 40 mM $MgCl_2$, 0.2% BSA (0.25ml), 2.5 mM RuBP (0.10ml), 50 mM $NaH^{14}CO_3$ (0.10ml). The reaction was initiated by adding 0.05 ml of crude enzyme extract to the reaction mixture. This was then incubated for 5 min. at 30ºC and the reaction was terminated after 5 min by adding 0.1 ml of 6 M acetic acid. Background was determined by including zero time reaction samples. The samples were dried directly in vials at 65ºC and 10 ml of scintillation cocktail was added to each of the vials. The radioactivity incorporated in the reaction vials was determined using liquid scintillation counter.

3.1.6.4. Nitrate reductase activity

In vivo leaf NR activity was assayed according to the procedure of Hageman and Hucklesby (1971) with slight modifications. NR was measured in 200 mg of finely chopped leaves that were incubated in a medium containing 5ml of 0.1 M phosphate buffer, 0.02M KNO_3, 5% propanol and two drops of chloramphenicol (0.5mg/ml). The samples were infiltrated in vacuum for 20-30 min till the leaf pieces sink to the bottom of incubation mixture. The reaction was stopped by keeping the tubes in water bath at 70 ^{0}C for 20 min to stop the enzyme activity and complete leaching of the nitrite in the medium. Nitrite was estimated by the method of Evans and Nason (1953). 0.2 ml of the aliquot from reaction mixture was taken and to it 1 ml each of 1.0% sulphanilamide in 1N-HCl and 0.025% N-(1-Napthyl)-ethylene diammonium dichloride (NEDD) were added. The pink colour due to diazotisation was allowed to develop for 30 min after which the volume was made upto 6 ml with

double distilled water. The absorbance was read at 540 nm, using UV-Vis spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). The calibration curve was prepared using sodium nitrite solution. The enzyme activity was expressed as μmol NO_2 g^{-1} fw h^{-1}.

3.1.7. Amino acids concentration

Amino acid composition was determined by UHPLC (Dionex- Thermofisher, USA) using C-18 column 2.2μ, 100 mm column (Thermofisher, USA). Three mg of leaf samples were hydrolysed with 800μL of 6N HCl, 10 μL of phenol and 100 μL of 0.1 N HCl at 150°C for 7 h in vacuum hydrolysis tubes (6ml, 10mm* 150mm, Thermo Scientific, USA). Online derivitization was done by the pre-programmed method using 0.1 M borate buffer, pH 9.0; 75 mM ortho-phthaldialdehyde, 225 mM 3-mercaptopropionic acid in 0.1 M borate buffer, pH 9.0; Fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) reagent (2.5 mg/mL) and injection diluent (50 mL mobile phase A + 1.5 mL phosphoric acid). For the separation condition, the mobile phase A comprised of 10 mM disodium hydrogen phosphate, 10 mM sodium tetraborate and 0.5 mM sodium azide set at pH 7.8. The mobile phase B consisted of methanol: acetonitrile: water in the ratio of 45: 45: 10. The sample injection volume was 10 μL, flow rate of solvent was 0.722 mL/min and the column was kept at 40°C. The DAD detection wavelength was set at 262 and 338 nm and run time was 13 min.

3.2. Objective 2: To investigate the interactive effect of iron and sulphur nutrition on plant root architecture, phytosiderophore synthesis and release in relation to $^{35}SO_4^{2-}$ and ^{59}Fe uptake and partitioning

The objective was realized through the hydroponic culture experiment performed with bread and durum wheat cultivars.

3.2.1. Experimental set up

Seeds of four cultivars of wheat (*Triticum aestivum* L. cv. HD-2967 and HD-2329 and *Triticum durum* L. cv. HI-8713 and HD-4728), procured from the Division of Genetics and Plant Breeding of this institute, were surface sterilized by rinsing for 3 min in 70% ethanol followed by 10 min in 15% hydrogen peroxide solution and finally in distilled water and were sown on autoclaved sand in plastic trays. Trays

were kept in a seed germinator in dark at 25°C and were watered as and when necessary. After three days of germination, the trays with emerging seedlings were moved to light to prevent etiolation. Five days old healthy seedlings were gently removed from sand and transferred to the nutrient solution culture (Plate 3.1). The roots were washed off the sand particles with deionized water prior to transfer (Zhang *et al.,* 1991) to the Fe and S deficient and sufficient solutions i.e., 1 and 100 µM Fe^{3+} as Fe^{3+}-EDTA (Khobra *et al.,* 2014) and 0, 1.2 and 2.5 mM SO_4^{-2} as K_2SO_4 (Zuchi *et al.,* 2012), in glass tanks (10 liter capacity) with darkened sides, to prevent algal growth and, under continuous aeration. Plants were grown in a climate chamber under 300 µmol m^{-2} s^{-1} PAR at leaf level and 14 h/10 h day/night regime (temperature 27ºC diurnal; 20ºC nocturnal; relative humidity 70-80%). The S-deficient nutrient solution was prepared by replacing sulphate salts with appropriate amount of chloride salts of K^+, Mn^{2+}, Zn^{2+} and Cu^{2+}. Concentration of other nutrients in the solution culture were as follows: $CaNO_3$; 2 mM, KH_2PO_4; 0.25 mM $MgCl_2$;1 mM, KCl; 0.10 mM, H_3BO_3; 1 mM, $MnSO_4$;0.50 mM, $CuCl_2$; 0.20 mM, $(NH_4)_2Mo_7O_{24}$;0.02 mM and $ZnCl_2$;0.001 mM. All the chemicals used for preparation of nutrient solution were of AR grade. The nutrient solution was changed every three days to maintain the pH of 5.6 to 5.8 throughout the experimental duration.

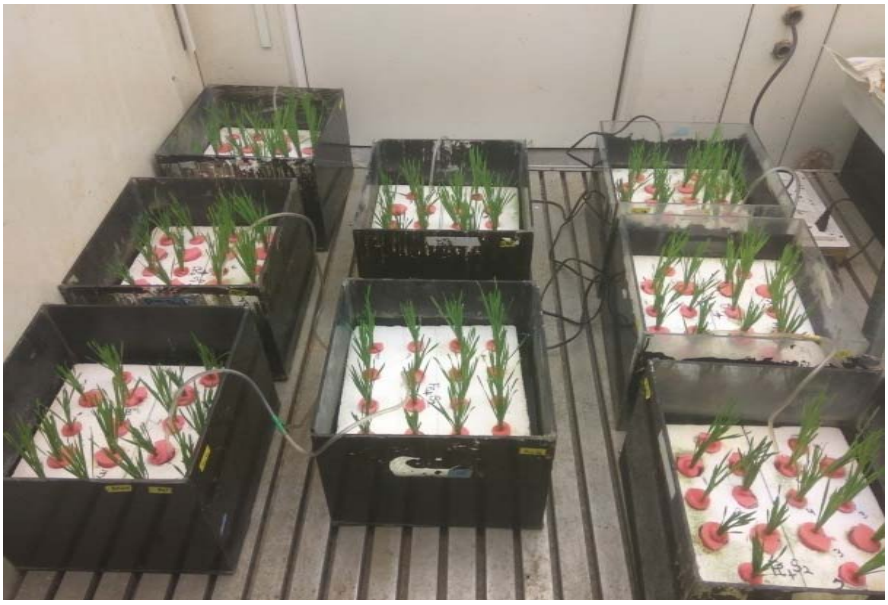

Plate 3.1: Experimental set up of bread and durum wheat varieties in the nutrient solution culture

3.2.2. Physiological parameters

Plants raised under different Fe/S sufficiency/deficiency treatments were analyzed at 8, 11 and 14 days after transfer (DAT) to determine the following morpho-physiological attributes in triplicate with 10 seedlings per replicate per treatment, if not stated otherwise.

3.2.2.1. Plant biomass and chlorophyll content

Wheat shoots and roots were sampled on 8, 11 and 14 days after transfer of plants to the nutrient solution for shoot and root biomass determination. Plants were oven dried at 80°C for 4 h and then at 60 °C till constant weight was obtained and mass values were expressed as g dw plant^{-10}.

Leaf chlorophyll was measured at 11 DAT as per the dimethyl sulphoxide (DMSO) method (Hiscox and Israelstam, 1979). Freshly sampled, uppermost fully expanded leaves were cut into small pieces and 25 mg sample were put in test tubes containing 5 ml of dimethyl sulphoxide (DMSO). The test tubes were then kept in oven at 65ºC for 4h to facilitate the extraction of chlorophyll into the solution. The absorbance was measured at 645 and 663 nm using UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). Chlorophyll ‘a’, chlorophyll ‘b’ and total chlorophyll were calculated according to Arnon (1949) and expressed as mg g^{-1} dw.

Ratio of Chlorophyll a/b and total chlorophyll/carotenoids was also calculated.

Chlorophyll ‘a’ = [(12.7 x OD_{663}) (2.69 x OD_{645})]. Vx1/1000xW

Chlorophyll ‘b’ = [(22.9 x OD_{645}) (4.68 x OD_{663})]. Vx1/1000xW

Total Chlorophyll = [(22.2 x OD_{645}) + (8.02 x OD_{663})]. Vx1/1000xW

Where,

OD_{663} = Absorbance values at 663 nm

OD_{645} = Absorbance values at 645 nm

W = Weight of the sample in mg

V=Volume of the solvent in ml

3.2.2.2. Root characteristics

Root morphological traits were measured at 8, 11 and 14 DAT by using the Win-RHIZO, Regent Instruments Inc. Fresh roots were used for root scanning and three representative plants were taken for each replication and scanning was done in triplicates for each treatment. Root scanning data were retrieved to calculate total root length, total root volume and total root surface area.

3.2.3. Root release of phytosiderophore and its content in the root tips

Root release of PS, diurnal pattern of PS release and PS content of roots were determined at 8, 11 and 14 DAT under Fe and S deficient and sufficient treatments and their combinations, in bread and durum wheat cultivars.

3.2.3.1. Collection of root exudates and determination of phytosiderophore release

PS release from wheat plants was analyzed by determining PS content in root washings. A subset of 10 plants was removed from the respective treatments at 2 h after the onset of the light period and the roots were washed two times for 1 min in deionised water. Root systems were then submerged into 20 ml deionised water for 4 h with continuous aeration. Thereafter, micropur (10 mg l^{-1}) (Roth, Karlsruhe, Germany) was added to prevent microbial degradation of PS. PS content in root washings were determined using the Fe-mobilization assay (modified from Takagi, 1976; Gries and Runge, 1995).

3.2.3.2. Iron-mobilization assay

In the modified Fe-mobilization assay, 2 ml of freshly precipitated Fe $(OH)_3$ and 0.5 ml of 0.5 M Na acetate buffer (pH 5.6) was added to 8 ml of the collection solution, shaken for 2 h and filtered through Whatman # 1 filter paper into 0.2 ml of 6N HCl. Ferric iron was reduced by addition of 0.5 ml of 8% hydroxylamine-hydrochloride and heating to 60°C for 20 min. The concentration of ferrous iron was then determined spectrophotometrically by measuring absorbance at 562 nm after adding 0.2 ml of 0.25% ferrozine and 1 ml of 2 M Na-acetate buffer (pH 4.7).

3.2.3.3. Determination of phytosiderophore content in the root tips

Wheat seedlings were removed from the respective nutrient solution treatments at 2 hours after the onset of light and their root tips (about 3mm) were collected and homogenised to a fine powder with liquid nitrogen. Distilled water at

100ºC was added to aliquots of the powdered tissue (500 µl mg^{-1} FW) and homogenates were incubated for 10 min at 80ºC. Insoluble material was removed by 10 min centrifugation at 12,000 rpm and the pellet was then re-extracted with 500 µl of boiling water as described above. After a further centrifugation step, the supernatant so obtained was used for determination of PS content in root tips using the Fe-mobilization assay (modified from Takagi, 1976; Gries and Runge, 1995). Amount of PS was calculated using the following formula and expressed as nmol Fe equivalent g^{-1} root fw.

$$\text{PS} = \frac{\text{A} \times \text{final volume of the collected root exudates}}{2.15 \times \text{sample volume used in the assay} \times \text{TM}}$$

Where, A = Absorbance values at 562 nm

TM = Tissue mass

3.2.4. Plant nutrient content

Tissue Fe, S, Zn and Mn concentration were measured by taking a known amount of dried shoot and root tissue and digested the same in 10 mL of diacid mixture i.e., HNO_3:$HClO_4$ in 9:4 ratio for 24 h at room temperature (33 ºC) and then at 250 ºC on a hot plate. The samples were filtered and volume was made to 25 mL with double distilled water before analysis of S and other micronutrients. The S level was measured following turbidimetric method (Tabatabai and Bremner, 1970) as described earlier in this chapter and the concentration of micronutrients were determined against known concentration standard, prepared from stock solution of 1000 mg/l (SRL chemicals, Mumbai, India), on Atomic Absorbance Spectroscopy (ECIL make, Hyderabad, India) at wavelengths of 248.3 nm, 213.9nm and 279.5nm for Fe, Zn and Mn, respectively.

3.2.5. Application and radiochemical determination of ^{59}Fe and ^{35}S tracer

Radiotracers of ^{59}Fe and ^{35}S as $FeCl_3$ (specific activity 666.0 MBq/ mmole) and ^{35}S as -sulphuric acid (specific activity 740 MBq/ mmole), procured from the Board of Radiation and Isotope Technology (BRIT), Mumbai, India and were used to study the interactive effect of Fe and S in bread and durum wheat cultivars raised on Fe and S deficient and sufficient i.e., 1 and 100 µM Fe^{+3} and 0, 1.2 and 2.5 mM SO_4^{-2} nutrient solution culture, either individually and in combination treatment.

Two separate short term ^{59}Fe and ^{35}S uptake experiments were conducted, for which 250 ml volume of the respective Fe and S treatment solutions were taken in 300ml conical flasks which were painted black on the exterior surface to limit the light interference on the root growth. Radiolabeled Fe and S were then added to the experimental flasks, maintaining a minimum initial ^{59}Fe and ^{35}S activity of 2000 Bq per ml. Eleven days old seedlings raised under different S and Fe availability conditions were then transferred to the ^{59}Fe and ^{35}S tagged nutrient solutions under aeration and ~200 µmol light intensity for both ^{59}Fe and ^{35}S in a fume hood for an hour of incubation (25±2ºC) at room temperature. ^{59}Fe uptake by plants under variable Fe and S availability condition was determined as per the radiolabel depletion method and was expressed as Bq g^{-1} plant dry weight (dw). ^{35}S uptake on the other hand was measured by determining the relative accumulation of ^{35}S in the root and shoot and was expressed in terms of both concentration and uptake respectively as Bq g^{-1} shoot or root dw and Bq per plant shoot or root and the total plant ^{35}S uptake.

^{59}Fe remaining in the incubation medium and shoot and root uptake of ^{35}S were determined by drying the respective extract/ tissue in an oven maintained at 80°C for 4 h for extracts until few mL (A) and at 60°C until complete dryness for the plant tissue. A known quantity of the plant tissues were ashed, dissolved in 5 mL of 1% HCl (w/v) and concentrated in an oven at 60°C to a few mL (B). Finally the concentrated extracts of ^{59}Fe (A) and ^{35}S (B) were taken in 10 mL of scintillation cocktail and the incorporated radioactivity was assayed using the liquid scintillation counter (HIDEX-300 SL, Finland). Translocation index of ^{35}S was measured by dividing shoot ^{35}S to total plant ^{35}S uptake. Further, the ratio of ^{35}S to ^{59}Fe was also calculated to determine interactive relationship and competition if any, for the uptake between Fe and S.

3.3. Objective 3: To determine the transcript level expression of sulphate transporters *SULTR1;1* and *SULTR 2;1*, Fe-PS transporter *YS1* and nitrogen transporter *NRT2.1* in relation to root and shoot S, N and Fe.

Transcript expression profile of *SULTR1;1*(high affinity sulphate transporter) , *SULTR2;1* (low affinity sulphate transporter), *YS1* (Fe-PS complex transporter) and

NRT2.1 (high affinity nitrate transporter) gene were studied in the root tissues of bread and durum wheat seedlings raised for 11 days on Fe and S deficiency treatment combination as detailed at 3.2.1 using the following protocol.

3.3.1. RNA isolation- Trizol method

100 mg of root tissue was ground in liquid nitrogen.1 ml of trizol was added to it and incubated for 5 min at room temperature in mortar itself. The contents were then transferred to a 1.5 ml eppendorf and 200µl chloroform was added with thorough mixing. It was followed by 15 min incubation at room temperature and centrifuged at 13,000 rpm for 15 min at 4ºC. Aqueous phase was transferred to fresh tubes and add 0.5 ml of isopropanol was added, stored at room temperature for 15 min and again centrifuged at 13,000 rpm for 15 min at 4ºC. Supernatant was discarded and the pellet was washed in 500µl of 70% chilled ethanol and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatant was again discarded and the pellet was allowed to dry for 10-15 min in incubator at 37°C and eluted in 50 µl DEPC treated H_2O and incubated at 60ºC for 10 min. The RNA so obtained was stored at -80ºC until further investigations.

3.3.2. Validation through Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

3.3.2.1. cDNA synthesis

cDNA synthesis was carried out by using Revert Aid H Minus First Strand cDNA synthesis kit (Thermo scientific, USA) as per the instructions of the manufacturer's protocol.

3.3.2.2. Primer synthesis:

Forward and reverse primers of the target genes *SULTR1;1 , SULTR2;1, YS1* and *NRT2.1* were synthesized using Primer3 software (Table 3.2).

3.3.2.3. Quantitative real-time PCR

Quantitative RT-PCR analysis was carried out by using KAPA SYBR Green qPCR mix on a Bio-Rad CFX96 machine. Actin gene of wheat was used as an internal control gene. Reaction mixture (20 µL) volumes contained various components (Table 3.3). Reactions were run in Bio-rad qRT-PCR CFX 96 machine

using the standard cycling program (Table 3.4). Relative expression levels of target genes were quantified by using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

3.3.2.4. Relative quantification and expression analysis of *SULTR1;1, SULTR2;1, YS1* and *NRT2.1* genes

Gene quantification was achieved using the comparative CT (Cycle Threshold) method and is expressed as ‘‘n-fold up or down regulation of transcription’’ in relation to a calibrator which is represented by the smallest signal detectable for that specific gene. For relative quantification, values are expressed relative to a reference sample, called the calibrator (0 h). The expression of selected genes was calibrated by that of the reference gene, actin at each time point and was converted to the relative expression ratio (fold of expression)

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

Where,

$\Delta\Delta CT$ = Average ΔCT of target - Average ΔCT of calibrator

ΔCT = Average CT of target - Average CT of endogenous control

CT values were analyzed by CFX software v3.0 designed to perform relative quantification using comparative CT ($\Delta\Delta CT$) method by Bio-rad qRT-PCR CFX 96 machine for relative gene expression of target genes. Algorithm of this model is one of the features of the CFX 3.0 software provided by Bio-rad qRT-PCR CFX 96 machine, thus this bioinformatics tool was used for relative quantification.

3.4. Statistical analysis

Samples were analyzed in triplicate and mean values were used in comparisons analysis. Means were compared among treatments by the Duncan’s multiple range test at $P \leq 0.05$ considered to indicate statistical significance using the SPSS Statistics 17.0 (IBM, Armonk, NY, USA) and graphs were plotted using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California, USA). CD values were calculated by three way ANOVA using OPSTAT.

Table 3.2: List of primer sequences used in our study

S. No.	Gene specific Primers	Primer Sequence
1	***SULTR1;1***	Forward 5'CAGCCTGGTGCACCTCTTCC3'
		Reverse 5'CAGAGGCTGGCGATGGTGAG3'
2	***SULTR2;1***	Forward 5'CCGGATCTCTATCCTCGTGCTA3'
		Reverse 5'GATGAAAGTCGCGTTGATGAAGC3'
3	***YS1***	Forward 5'TGGGTTCGGGTCAACCTTGC3'
		Reverse 5'CCAGCCTATCCCTGGCTCCT3'
4	***NRT2.1***	Forward 5'GCATGGCGTATTGCCTACTT3'
		Reverse 5'TAGCCGTAGAGGAGGACGAA3'
3	***ACTIN***	Forward 5'TCGGTGAAGGGGACTTACAAAGG3'
		Reverse 5'CGTACCACACAATGTCGCTTAGG3'

Table 3.3: Components of reaction mixture used for the Quantitative RT-PCR analysis

S. No.	Component	Volume for 1 reaction
1.	2X KAPA SYBR Green qPCR mix	10.0 µl
2.	Gene specific forward primer (200 nM final)	0.4 µl
3.	Gene specific reverse primer (200 nM final)	0.4 µl
4.	cDNA (100 ng)	1.0µl
5.	DEPC-treated water	8.2 µl
Total volume		20.0 µl

Table 3.4: Standard cycling program for reactions used for Quantative RT-PCR analysis

S. No.	Step	Temperature (°C)	Duration
1.	Initial Denaturation	95	3 min
2.	Denaturation	95	10 sec
3.	Annealing	60	30 sec
		Plate read and Repeat step 2 to 3 for 39more cycles	
4.	Melt curve	55-95	1 min
5.	Hold	4	_

Chapter 4

RESULTS

Sulphur (S) and iron (Fe) nutrition is critical for growth and development of plants since these mineral nutrients play a pivotal role in ensuring an optimized efficiency of various metabolic processes such as photosynthesis, respiration, protein synthesis, etc. Further, these nutrients have been studied widely but individually and there is a dearth of information and consensus on their interactive influences on respective elemental uptake by plant and effect on plant growth. The present study entitled **"Physiological and molecular regulation of sulphur uptake and assimilation by iron in wheat"** was thus, aimed at studying the interaction of Fe and S metabolism in bread and durum wheat, since these cultivars are known to differ in their Fe deficiency response.

In the first objective (4.1), the effect of Fe sufficient and deficient condition and varying S availability studied in terms of growth and gas exchange attributes, nutrient (S, Fe and N) uptake, assimilatory enzymes, physiological use efficiency and TI of nutrients, etc., under soil culture experiment, while in the second objective (4.2) studied the interactive regulation of Fe and S on their uptake and use under the nutrient solution culture. This experiment was conducted with variable availabilities of Fe and S. The role of root system architecture and phytosiderophores (PS) in determining the uptake of nutrients was also investigated. Further, in the third objective (4.3) the transcript level expression of S, Fe and N transporter genes viz. *SULTR1;1, SULTR2;1, YS1* and *NRT2.1* respectively, were studied to decipher the Fe and S interaction at the transcriptional level. Further, a short term experiment using elemental radiotracers ^{59}Fe and ^{35}S was also conducted to determine the interactive regulation of S by Fe in wheat. Results obtained for all the above experiments are detailed, objective wise, under the following heads.

4.1. To determine the effect of Fe on S uptake, assimilation and partitioning under low and sufficient S availability.

4.1.1. Effect of iron and sulphur availability on plant growth characteristics under soil culture

4.1.1.1. Shoot biomass

Shoot biomass was observed under variable Fe and S level at three growth stages (30 (stage I), 60 (stage II) and 120 (stage III) days after sowing) for the two

experimental years (2016 and 2017) and is presented in Table 4.1, Fig. 4.1. Shoot biomass across the wheat cultivars was in general higher under the Fe and S sufficient treatment (Fe+S2) than Fe and S deficient (Fe-S0) treatment. There was a significant increase in the shoot biomass with S supply at stage II and stage III of the plant growth in both the experimental years i.e. 2016 and 2017. Under Fe deficient (Fe-) as well as Fe sufficient (Fe+) condition, an increasing S supply increased the shoot biomass across all the experimental wheat cultivars. Durum wheat as compared to bread wheat, in general, showed more average shoot biomass at all the growth stages. The significant effect of increasing Fe and S availability on shoot biomass was observed more clearly at stage II than at stage I and stage III of the plant growth. The shoot biomass showed a growth related increase from stage I to stage III i.e. from tillering to maturity stage, irrespective of the nutrient availability.

4.1.1.2. Gas exchange attributes

Changes in gas exchange attributes i.e. photosynthesis (Pn), stomatal conductance (Gs) and transpiration (E) under Fe deficient and sufficient condition and varying S levels were observed at 30 (stage I) and 60 (stage II) days after sowing over two experimental seasons (2016 and 2017).

4.1.1.2.1. Photosynthetic rate

A significant increase in photosynthesis rate was observed with increasing S supply under both Fe sufficient (Fe+) and Fe deficient (Fe-) condition. Fe availability also increased photosynthetic rate in both bread and durum wheat. The durum wheat cultivars showed a higher photosynthetic rate as compared to the bread wheat cultivars and were more responsive to nutrient availability in terms of photosynthetic rate. The observation on high photosynthetic rate at stage II than stage I was recorded over both experimental seasons (Table 4.2, Fig. 4.2). A marked increase in photosynthetic rate with increasing Fe and S availability (Fe+S2) were observed across the bread and durum wheat.

4.1.1.2.2. Stomatal conductance

Stomatal conductance, in general, increased with plant growth from stage I to stage II for both experimental years. Cultivars level differences for stomatal conductance between the two crop years might have been caused by a variation in pot soil moisture content in 2016 and 2017. Stomatal conductance increased significantly in HD-4728 cultivar of bread wheat at stage II whereas for other cultivars it was not

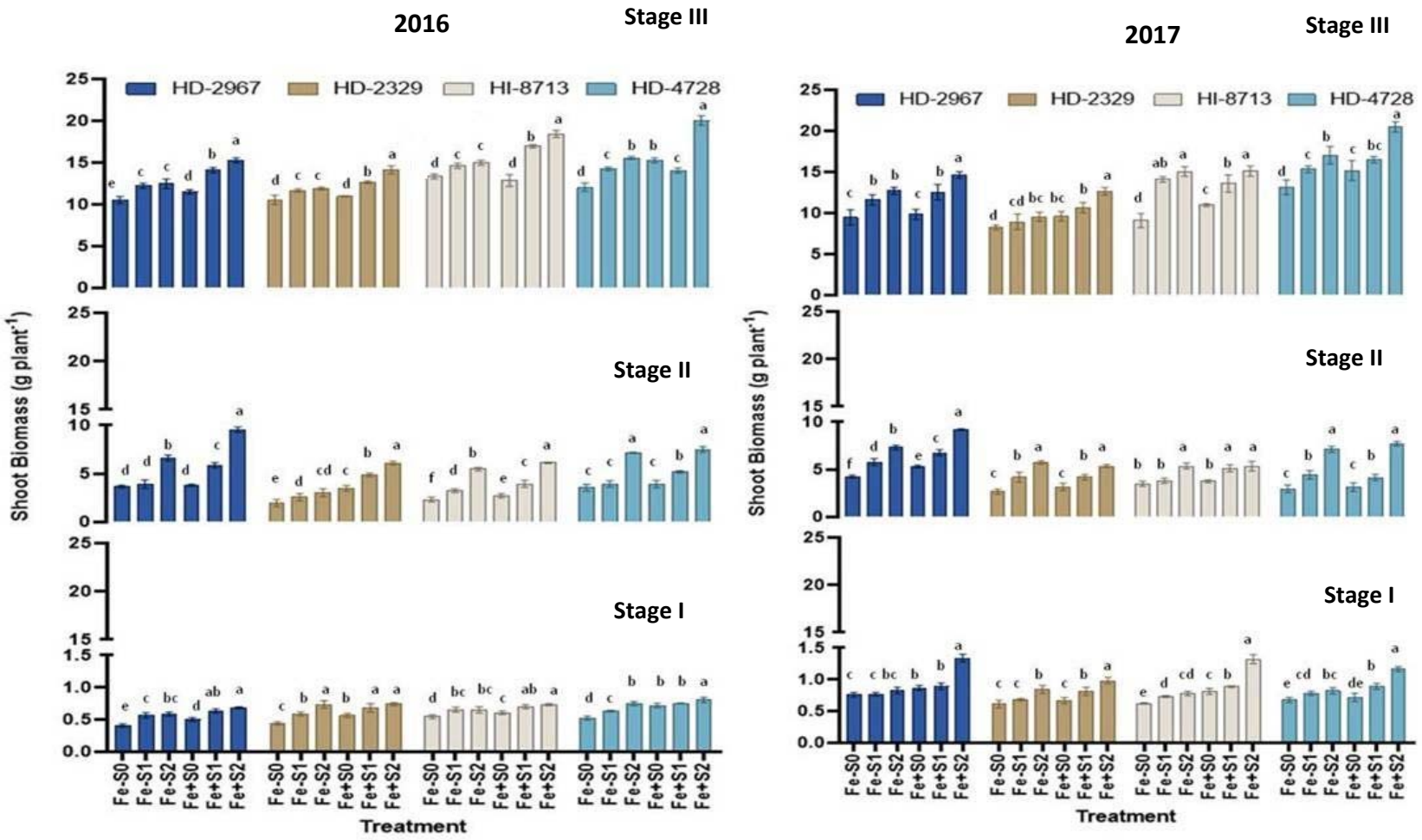

Fig.4.1: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on shoot biomass of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.1: Interactive effect of Fe and S availabilities on shoot biomass of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS), stage II (60 DAS), stage III (120 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Shoot Biomass (g plant^{-1})					
			2016			2017		
	Iron (Fe)	Sulphur (S)	Stage (D)					
			Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	0.41	3.68	10.5	0.76	4.27	9.5
		S30	0.57	3.92	12.3	0.77	5.80	11.6
		S60	0.59	6.62	12.5	0.83	7.32	12.7
		Mean	0.52	4.74	11.7	0.8	5.79	11.3
	Fe+	S0	0.50	3.82	11.5	0.87	5.32	9.8
		S30	0.64	5.87	14.1	0.89	6.77	12.6
		S60	0.68	9.55	15.3	1.34	9.21	14.6
		Mean	0.61	6.41	13.6	1.03	7.10	12.3
HD-2329	Fe-	S0	0.44	1.97	10.6	0.61	2.69	8.2
		S30	0.59	2.58	11.7	0.69	4.18	8.9
		S60	0.74	3.04	11.9	0.84	5.75	9.5
		Mean	0.59	2.53	11.4	0.7	4.21	8.9
	Fe+	S0	0.56	3.49	11.0	0.66	3.17	9.6
		S30	0.68	4.86	12.7	0.82	4.20	10.7
		S60	0.74	6.12	14.1	0.98	5.37	12.7
		Mean	0.66	4.82	12.6	0.82	4.24	11.0
HI-8713	Fe-	S0	0.55	2.34	12.9	0.62	3.48	9.1
		S30	0.66	3.25	17.0	0.73	3.82	14.1
		S60	0.65	5.48	18.4	0.78	5.37	15.1
		Mean	0.62	3.69	16.1	0.7	4.22	12.8
	Fe+	S0	0.61	2.74	13.4	0.81	3.77	11.0
		S30	0.70	3.94	14.6	0.89	5.13	13.6
		S60	0.74	6.14	15.0	1.32	5.37	15.1
		Mean	0.68	4.27	14.3	1.01	4.75	13.2
HD-4728	Fe-	S0	0.53	3.54	12.1	0.68	2.97	13.1
		S30	0.63	3.94	14.2	0.78	4.47	15.3
		S60	0.75	7.17	15.6	0.82	7.13	17.1
		Mean	0.64	4.88	14.0	0.80	4.86	15.2
	Fe+	S0	0.72	3.93	15.2	0.72	3.17	15.2
		S30	0.75	5.18	14.0	0.89	4.17	16.5
		S60	0.81	7.53	20.0	1.16	7.72	20.5
		Mean	0.76	5.54	16.4	0.92	5.02	17.4
CD at 5%		**C**		0.10			0.18	
		T		0.13			0.21	
		D		0.09			0.15	
		C X T		0.25			0.42	
		C X D		0.18			0.29	
		T X D		0.22			0.36	
		C X T X D		0.43			0.72	

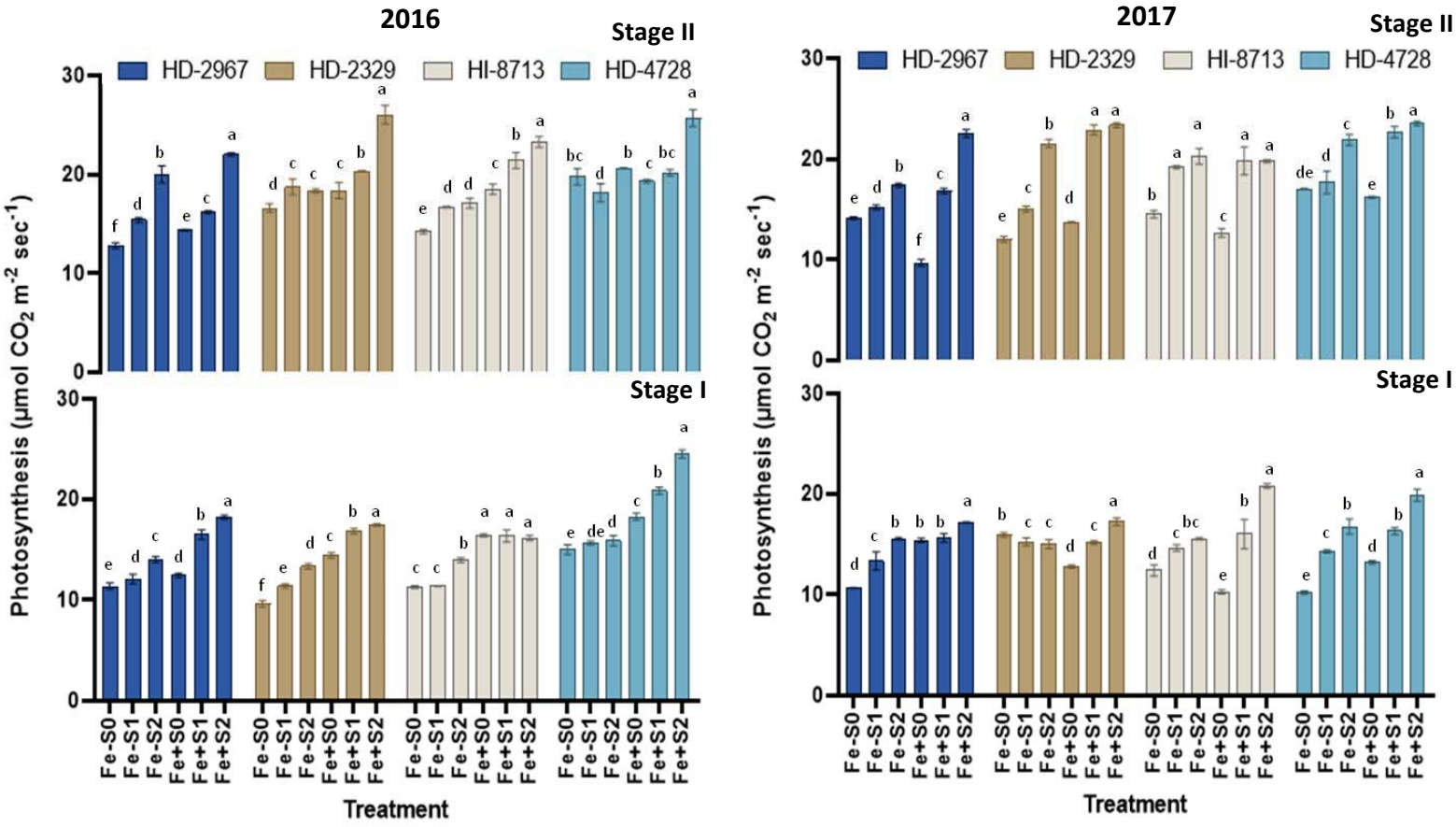

Fig. 4.2: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on photosynthesis of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at two growth stages (I and II) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

Table 4.2: Interactive effect of Fe and S availabilities on photosynthesis of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Photosynthesis (µmol CO_2 m^{-2} sec^{-1})			
			2016		2017	
	Iron (Fe)	Sulphur (S)	Stage (D)			
			Stage I	Stage II	Stage I	Stage II
HD-2967	Fe-	S0	11.3	12.8	10.6	14.1
		S30	12.1	15.4	13.3	15.2
		S60	14.0	20.0	15.5	17.4
		Mean	12.5	16.1	13.2	15.6
	Fe+	S0	12.4	14.4	15.4	9.7
		S30	16.5	16.2	15.6	16.8
		S60	18.2	22.0	17.1	22.5
		Mean	15.7	17.5	16.0	16.3
HD-2329	Fe-	S0	9.6	16.6	15.9	12.0
		S30	11.4	18.7	15.2	15.0
		S60	13.3	18.3	15.0	21.5
		Mean	11.4	17.9	15.4	16.2
	Fe+	S0	14.4	18.4	12.8	13.7
		S30	16.8	20.3	15.2	22.9
		S60	17.4	26.0	17.2	23.4
		Mean	16.2	21.6	15.1	20.0
HI-8713	Fe-	S0	11.3	14.2	12.4	14.5
		S30	11.4	16.7	14.6	19.2
		S60	14.0	17.1	15.5	20.3
		Mean	12.2	16.0	14.2	18.0
	Fe+	S0	16.4	18.5	10.3	12.7
		S30	16.3	21.4	16.0	19.8
		S60	16.1	22.6	20.8	19.8
		Mean	16.3	20.8	15.7	17.4
HD-4728	Fe-	S0	15.0	19.8	10.2	17.0
		S30	15.6	18.1	14.3	17.7
		S60	15.9	20.6	16.7	21.9
		Mean	15.5	19.5	13.7	18.9
	Fe+	S0	18.2	19.3	13.2	16.2
		S30	20.8	20.2	16.3	22.7
		S60	24.4	25.7	19.9	23.5
		Mean	21.2	21.7	16.5	20.8
CD at 5%	**C**		0.21		0.23	
	T		0.26		0.28	
	D		0.15		0.16	
	C X T		0.52		0.56	
	C X D		0.30		0.32	
	T X D		0.37		0.39	
	C X T X D		0.74		0.79	

much affected by availability of Fe and S at both stage I and stage II during both the crop seasons. Mean stomatal conductance when averaged over the bread and durum wheat cultivars for a crop season was significantly higher in bread than durum class of wheat (Table 4.3, Fig. 4.3).

4.1.1.2.3. Transpiration rate

Transpiration rate was relatively higher in durum than the bread wheat at both stage I and stage II for both the crop seasons. A dose dependent increase in the transpiration rate of bread wheat with increasing S supplies was observed both under Fe sufficient (Fe+) and deficient (Fe-) condition at all the stages of growth during both seasons with few exceptions. Under Fe and S deficiency (Fe-S0) the rates of transpiration was significantly reduced in both durum and bread wheat cultivars. An increase in transpiration rate under Fe+S1 over Fe-S1 was clearly evident and suggested possible role of Fe in influencing the gas exchange attributes of wheat under optimum S availability (Table 4.4, Fig. 4.4).

4.1.2. Effect of iron and sulphur availability on plant nutrient content under soil culture 4.1.2.1. Shoot sulphur

Shoot S was measured in bread and durum wheat cultivars under Fe deficient (Fe-) and sufficient (Fe+) condition and variable S levels at three stages viz. stage I, stage II and stage III of plant growth during two crop seasons (2016 and 2017) is presented in Table 4.5 , Fig. 4.5. Shoot S was much lower under S deficient than S sufficient condition of growth and further its uptake also depended on the Fe availability. Under Fe availability, the shoot S content was more as compared to Fe deficient condition. Combined deficiency of Fe and S (Fe-S0) markedly reduced the shoot S level at all the stages of observation in both the bread and the durum wheat. Mean average shoot S over different Fe and S treatments, in general, was higher in the durum wheat than the bread wheat cultivars at stage I and stage II of growth.

4.1.2.2. Shoot iron

Variations in shoot Fe content for bread and durum wheat cultivars under Fe deficient (Fe-) and sufficient (Fe+) condition and variable S levels at stage I, stage II and stage III of plant growth during two years is presented in Table 4.6, Fig. 4.6. Under combined Fe and S deficiency (Fe-S0) the shoot Fe content is around three times lower than the sufficient (Fe+S2) condition. A significant increase in shoot Fe

content on increasing the S supply under both Fe+ and Fe- condition was observed over the experimental cultivars at all growth stages. The Fe content in bread wheat in general, was found to be higher than the durum wheat when averaged over the treatment at all the stages during both seasons. Highest shoot Fe content was measured under Fe+S2 treatment for bread and durum wheat cultivars and also the shoot Fe was higher under Fe-S2 condition than Fe-S0 condition which shows the positive effect of S supply on shoot Fe under Fe deficient (Fe-) condition.

4.1.2.3. Shoot nitrogen

Variation in shoot N was determined in bread and durum wheat cultivars under differential Fe and S availabilities at three crop growth stages only for the year 2017 (Fig. 4.7). There was an increase in shoot N with increasing S supply in both bread and durum wheat both with (Fe+) and without (Fe-) at all the three growth stages. Differences among the cultivars were not much significant for shoot N at all the stages. A general increase in shoot N under Fe+S0 than Fe-S0 was observed at stage I. However, at stage II the above pattern was reversed and a higher shoot N was observed under Fe-S0 than Fe+S0 which might be attributed to the dilution effect.

4.1.2.4. Grain sulphur

Grain S content also showed similar trend as shoot S and increased with increasing S supply (S0<S1<S2). More grain S content was measured in Fe sufficient (Fe+) treatment as compared to Fe deficient (Fe-) treatment in both bread and durum wheat cultivars. The availability of Fe under sufficient S condition (Fe+S1/Fe+S2) significantly improved the grain S as compared to Fe deficient and S sufficient (Fe-S1/Fe-S2) in both the bread and durum wheat cultivars over both the experimental years (Fig. 4.8).

4.1.2.5. Grain iron

Grain Fe was almost two fold more in Fe and S sufficient (Fe+S2) condition as compared to Fe and S deficient (Fe-S0) condition (Fig. 4.8). A dose dependent significant increase in grain Fe content was observed under Fe+ condition with increasing S supply. There was an increase grain Fe content in Fe-S0 treatment than Fe+S0 treatment, which was due to the sufficient availability of Fe in the soil. An increase was also observed under Fe-S0 treatment as compared to Fe-S1/S2 treatment. This shows that S supply under Fe deficiency also play crucial role in Fe uptake.

Table 4.3: Interactive effect of Fe and S availabilities on stomatal conductance of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Stomatal conductance (mol H_2O m^{-2} sec^{-1})			
			2016		2017	
	Iron (Fe)	Sulphur (S)	Stage (D)			
			Stage I	Stage II	Stage I	Stage II
HD-2967	Fe-	S0	0.060	0.151	0.107	0.200
		S30	0.067	0.222	0.044	0.151
		S60	0.059	0.251	0.090	0.177
		Mean	0.062	0.208	0.080	0.176
	Fe+	S0	0.033	0.230	0.047	0.180
		S30	0.114	0.222	0.082	0.235
		S60	0.097	0.292	0.177	0.323
		Mean	0.082	0.248	0.102	0.246
HD-2329	Fe-	S0	0.068	0.128	0.092	0.101
		S30	0.050	0.158	0.210	0.226
		S60	0.127	0.184	0.124	0.243
		Mean	0.082	0.157	0.142	0.190
	Fe+	S0	0.084	0.158	0.020	0.172
		S30	0.068	0.182	0.098	0.152
		S60	0.077	0.220	0.064	0.180
		Mean	0.076	0.187	0.060	0.168
HI-8713	Fe-	S0	0.075	0.136	0.067	0.211
		S30	0.074	0.145	0.125	0.280
		S60	0.070	0.172	0.097	0.214
		Mean	0.073	0.151	0.096	0.235
	Fe+	S0	0.070	0.155	0.064	0.162
		S30	0.110	0.182	0.140	0.238
		S60	0.053	0.185	0.048	0.159
		Mean	0.078	0.174	0.084	0.186
HD-4728	Fe-	S0	0.103	0.128	0.195	0.247
		S30	0.117	0.210	0.139	0.132
		S60	0.108	0.275	0.093	0.169
		Mean	0.109	0.204	0.142	0.183
	Fe+	S0	0.107	0.144	0.072	0.282
		S30	0.122	0.331	0.183	0.288
		S60	0.083	0.378	0.103	0.205
		Mean	0.104	0.284	0.119	0.258
CD at 5%	**C**		0.002		0.004	
	T		0.002		0.004	
	D		0.001		0.003	
	C X T		0.004		0.009	
	C X D		0.002		0.005	
	T X D		0.003		0.006	
	C X T X D		0.006		0.012	

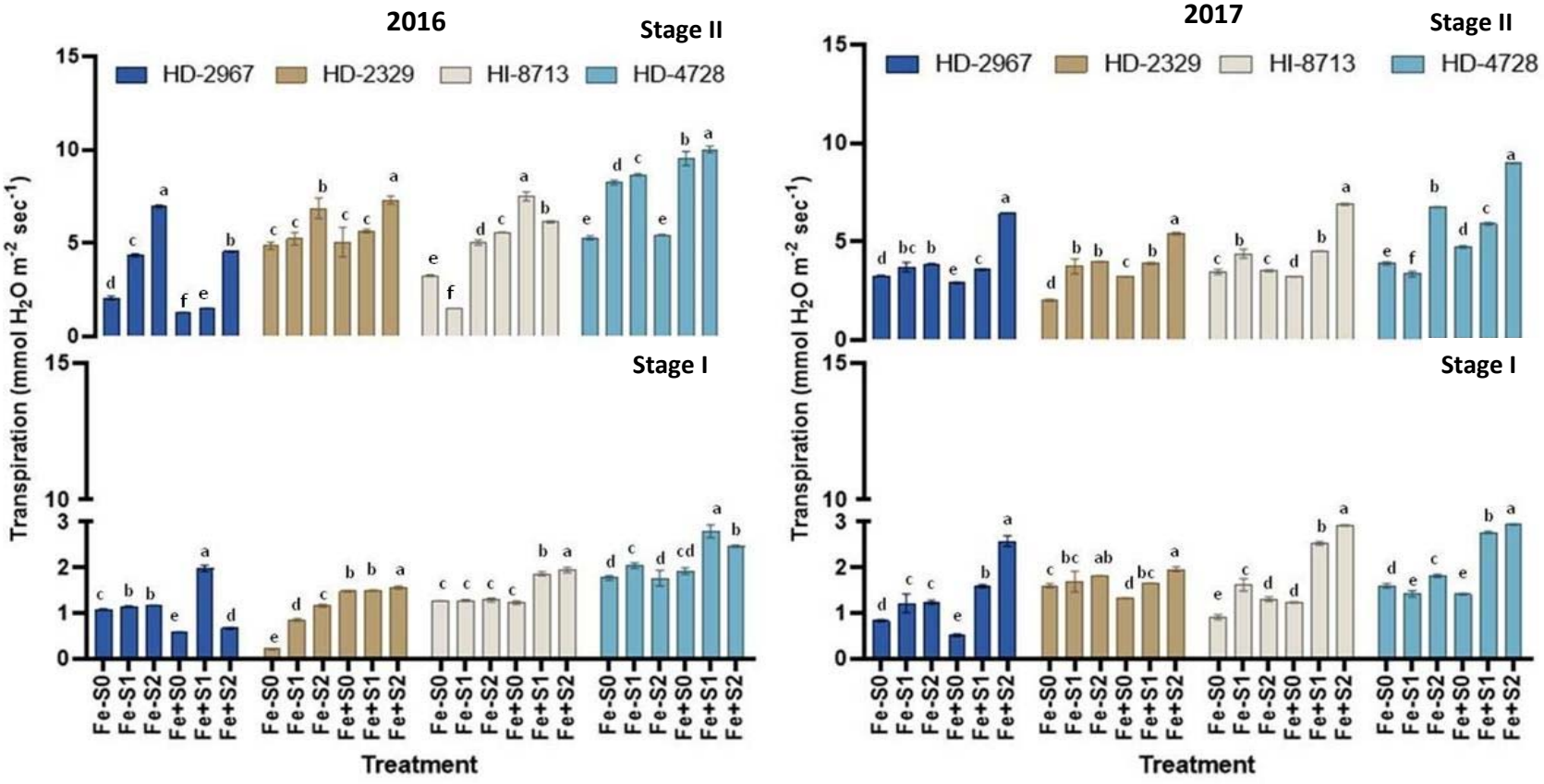

Fig.4.4: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on transpiration of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at two growth stages (I and II) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.4: Interactive effect of Fe and S availabilities on transpiration of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Transpiration (mmol H_2O m^{-2} sec^{-1})			
			2016		2017	
	Iron (Fe)	Sulphur (S)	Stage (D)			
			Stage I	Stage II	Stage I	Stage II
HD-2967	Fe-	S0	1.08	2.07	0.84	3.26
		S30	1.15	4.34	1.22	3.71
		S60	1.18	6.96	1.24	3.87
		Mean	1.13	4.46	1.10	3.61
	Fe+	S0	0.58	1.30	0.52	2.92
		S30	1.98	1.52	1.60	3.58
		S60	0.67	4.56	2.58	6.44
		Mean	1.08	2.46	1.57	4.31
HD-2329	Fe-	S0	0.22	4.86	1.60	2.02
		S30	0.86	5.24	1.70	3.74
		S60	1.17	3.21	1.82	3.98
		Mean	0.75	4.23	1.71	3.25
	Fe+	S0	1.48	7.39	1.33	3.22
		S30	1.50	5.65	1.66	3.89
		S60	1.57	7.31	1.97	5.42
		Mean	1.52	6.78	1.65	4.18
HI-8713	Fe-	S0	1.27	3.26	0.91	3.47
		S30	1.28	1.50	1.63	4.39
		S60	1.29	5.03	1.31	3.52
		Mean	1.28	3.26	1.28	3.79
	Fe+	S0	1.23	5.58	1.24	3.21
		S30	1.87	7.51	2.54	4.52
		S60	1.95	6.14	2.93	6.90
		Mean	1.68	6.83	2.23	4.88
HD-4728	Fe-	S0	1.78	5.28	1.60	3.89
		S30	2.04	8.24	1.42	3.36
		S60	1.77	8.67	1.82	6.76
		Mean	1.86	7.40	1.61	4.67
	Fe+	S0	1.93	5.44	1.42	4.73
		S30	2.80	9.53	2.77	5.91
		S60	2.47	10.02	2.95	8.98
		Mean	2.40	8.33	2.38	6.54
CD at 5%	**C**		0.05		0.05	
	T		0.07		0.06	
	D		0.04		0.03	
	C X T		0.13		0.11	
	C X D		0.08		0.06	
	T X D		0.09		0.08	
	C X T X D		0.19		0.16	

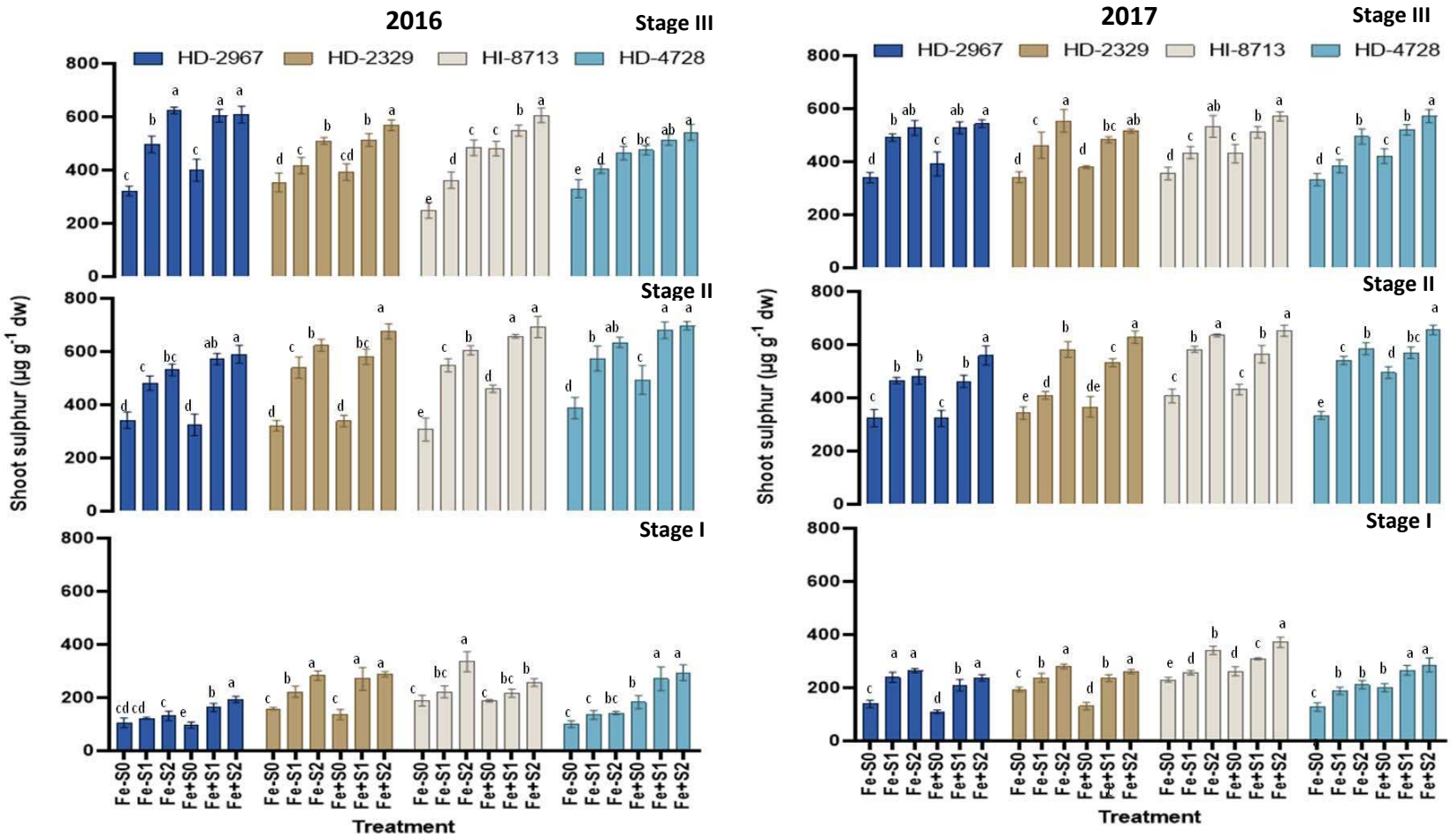

Fig.4.5: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on shoot sulphur of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.5: Interactive effect of Fe and S availabilities on shoot sulphur of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS), stage II (60 DAS), stage III (120 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Shoot sulphur (µg g^{-1} dw)					
			2016			2017		
	Iron (Fe)	Sulphur (S)	Stage (D)					
			Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	105.3	342.2	321.2	142.8	323.9	339.9
		S30	122.5	481.3	495.8	239.3	465.0	491.5
		S60	131.0	531.3	623.4	264.1	479.4	527.0
		Mean	119.6	451.6	480.1	215.4	422.7	452.8
	Fe+	S0	97.0	324.8	399.6	110.1	322.9	391.6
		S30	162.7	571.8	604.3	208.9	462.4	527.8
		S60	193.8	590.0	608.7	237.3	559.3	543.4
		Mean	151.2	495.5	537.5	185.4	448.2	487.6
HD-2329	Fe-	S0	158.5	321.5	353.9	193.6	342.7	341.8
		S30	222.3	540.0	416.9	238.0	409.8	462.3
		S60	282.9	623.6	509.8	280.7	581.7	554.2
		Mean	221.2	495.0	426.8	237.4	444.8	452.8
	Fe+	S0	135.9	338.9	392.3	131.1	366.6	380.0
		S30	270.7	580.4	513.8	236.7	532.4	483.2
		S60	288.2	675.7	568.3	261.5	628.4	515.6
		Mean	231.6	531.7	491.5	209.8	509.1	459.6
HI-8713	Fe-	S0	189.1	307.1	248.9	230.4	407.5	355.6
		S30	221.7	548.9	362.4	256.8	582.6	434.3
		S60	335.3	604.9	484.2	341.0	635.0	533.4
		Mean	248.7	487.0	365.1	276.1	541.7	441.1
	Fe+	S0	188.1	460.2	480.8	262.3	431.3	430.8
		S30	217.0	657.3	548.0	309.2	564.3	511.1
		S60	257.1	692.6	605.9	371.1	652.1	571.0
		Mean	220.7	603.4	544.9	314.2	549.2	504.3
HD-4728	Fe-	S0	100.3	387.2	330.2	127.3	334.6	332.6
		S30	134.9	574.5	405.4	188.3	540.9	383.3
		S60	142.5	634.6	464.2	211.2	584.7	494.7
		Mean	125.9	532.1	399.9	175.6	486.7	403.5
	Fe+	S0	182.8	493.3	475.0	200.4	495.2	421.4
		S30	281.0	680.6	514.1	265.9	570.0	520.1
		S60	293.7	697.0	541.8	285.9	655.3	572.1
		Mean	252.5	623.6	510.3	250.7	573.5	504.5
CD at 5%		C		10.1			8.6	
		T		12.3			10.5	
		D		8.7			7.4	
		C X T		24.6			21.0	
		C X D		17.4			14.9	
		T X D		12.3			18.2	
		C X T X D		42.7			36.4	

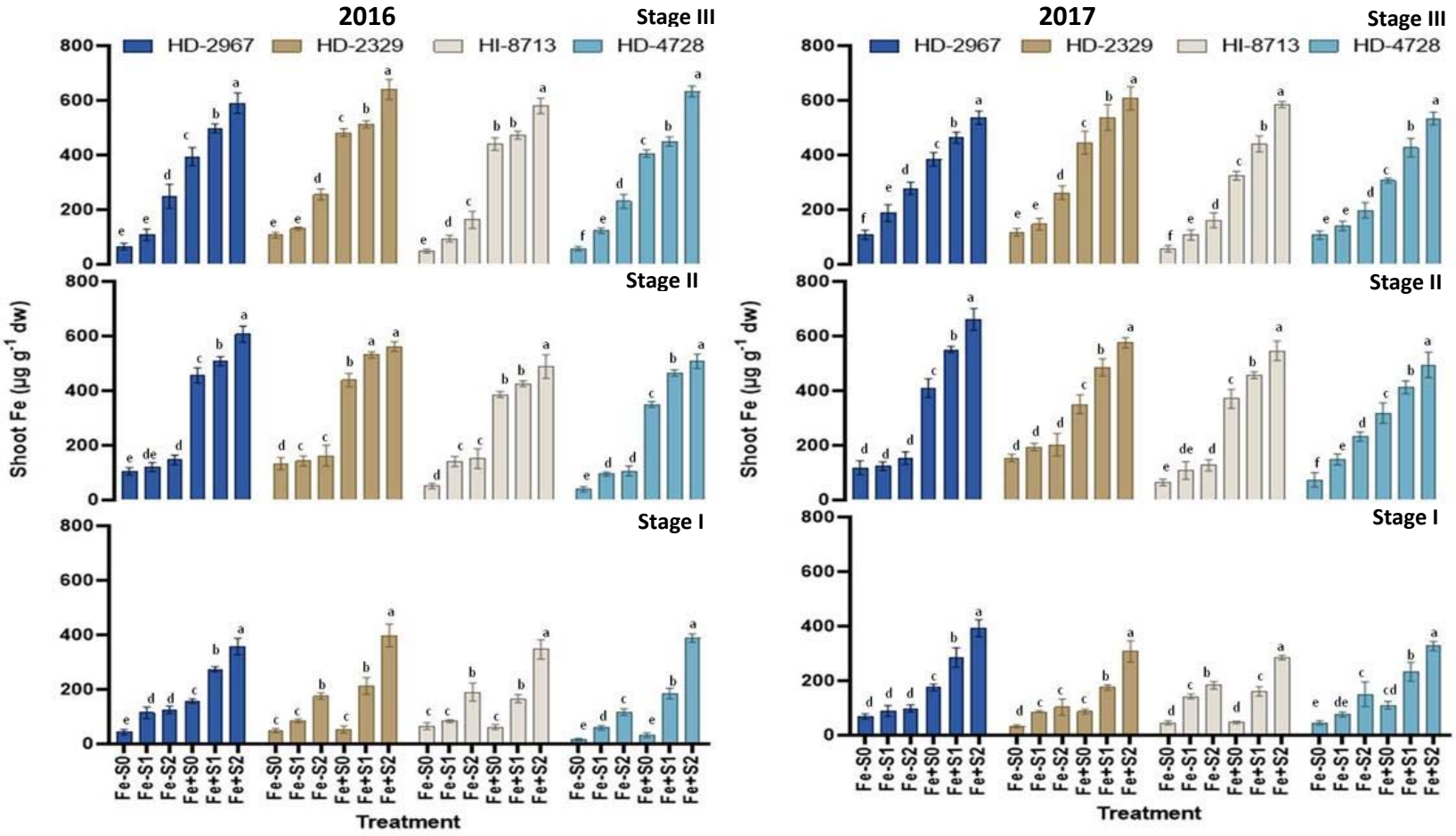

Fig. 4.6: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on shoot iron (Fe) of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

Table 4.6: Interactive effect of Fe and S availabilities on shoot iron of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS), stage II (60 DAS), stage III (120 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Shoot iron (µg g^{-1} dw)					
			2016			2017		
	Iron (Fe)	Sulphur (S)	Stage (D)					
			Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	43.7	104.8	65.6	68.8	118.6	108.1
		S30	114.7	120.7	108.6	89.4	123.8	187.6
		S60	125.1	147.1	249.0	98.2	153.2	277.8
		Mean	94.5	124.2	141.1	85.5	131.9	191.2
	Fe+	S0	157.0	455.9	394.4	175.4	409.3	384.4
		S30	274.0	507.9	498.0	284.7	550.5	463.2
		S60	357.0	607.0	589.8	392.7	661.7	536.5
		Mean	262.7	523.6	494.0	284.3	540.5	461.4
HD-2329	Fe-	S0	48.9	133.1	107.0	32.6	153.7	117.8
		S30	84.7	142.8	131.0	86.6	193.7	146.9
		S60	174.9	161.8	255.7	103.0	202.5	262.3
		Mean	102.8	145.9	164.6	74.1	183.3	175.6
	Fe+	S0	53.3	439.2	482.4	86.7	350.4	445.7
		S30	212.9	530.9	512.6	175.0	485.2	537.2
		S60	397.8	561.4	640.4	307.5	575.2	607.7
		Mean	221.3	510.5	545.2	189.7	470.3	530.2
HI-8713	Fe-	S0	66.0	51.6	48.1	45.9	64.2	57.0
		S30	84.1	140.4	94.3	142.2	108.0	107.4
		S60	190.1	151.4	162.7	183.3	126.8	161.3
		Mean	113.4	114.5	101.7	123.8	99.6	108.6
	Fe+	S0	61.9	386.4	440.2	46.9	370.7	324.4
		S30	166.1	425.8	472.7	161.1	456.9	440.8
		S60	347.0	488.5	580.1	284.6	546.3	584.9
		Mean	191.7	433.6	497.6	164.2	458.0	450.1
HD-4728	Fe-	S0	16.4	39.9	57.0	45.7	74.0	106.8
		S30	59.4	95.0	123.4	76.5	148.5	141.0
		S60	117.4	106.1	230.8	150.5	232.5	197.8
		Mean	64.4	80.3	137.1	90.9	151.7	148.6
	Fe+	S0	31.7	349.8	405.6	110.3	318.1	306.9
		S30	184.8	464.6	449.1	232.6	412.5	426.8
		S60	388.5	507.6	632.9	327.0	494.4	533.8
		Mean	201.7	440.6	495.9	223.3	408.3	422.5
CD at 5%		C		8.0			9.5	
		T		9.8			11.6	
		D		6.9			8.2	
		C X T		19.6			23.3	
		C X D		13.9			16.4	
		T X D		16.9			20.1	
		C X T X D		33.9			40.3	

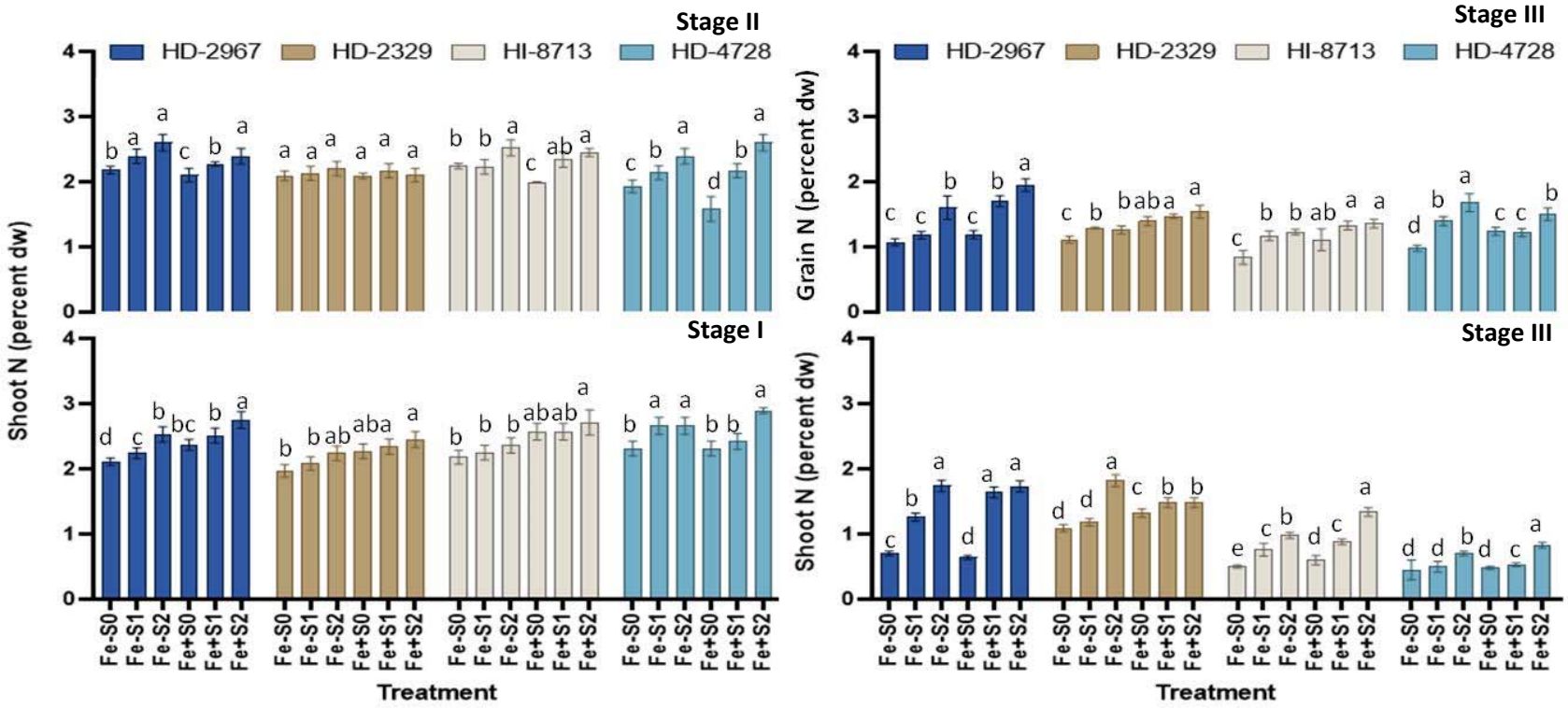

Fig.4.7: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on shoot and grain nitrogen (N) content of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at threesulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

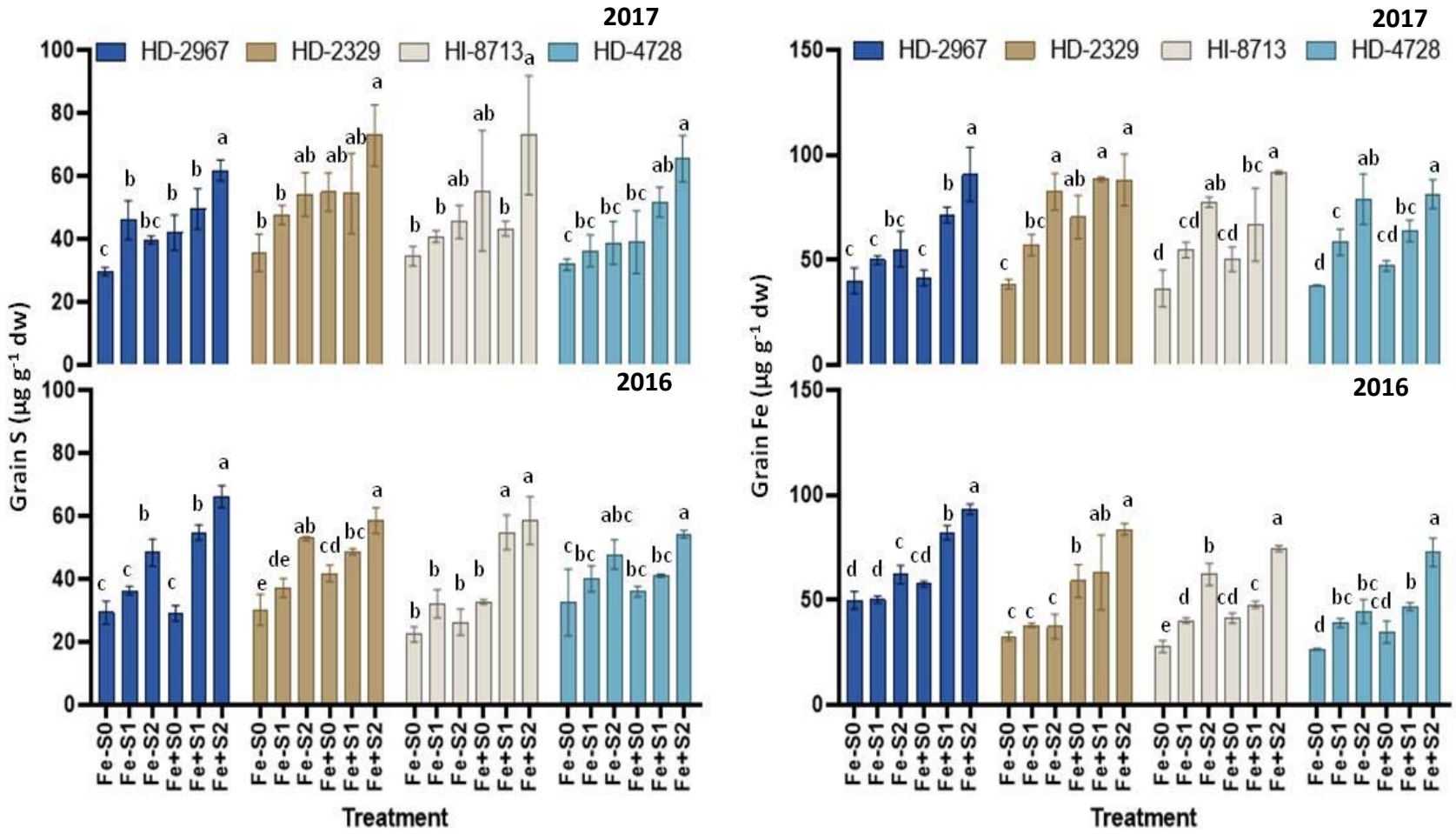

Fig. 4.8: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on grain sulphur (S)and grain iron(Fe) of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at harvest (stage III) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

4.1.2.6. Grain nitrogen

Grain N in both bread and durum wheat cultivars under varying Fe and S availabilities was observed in the year 2017 (Fig. 4.7). The synergistic effect of S on N was observed in both bread and durum wheat and the grain N was higher in Fe+S2 treatment as compared to Fe-S0 and there was a dose dependent increase with increasing S supply. The effect of Fe was not so clear on the N content of the grain and variations among the treatments were not significant.

4.1.3. Effect of iron and sulphur availability on sulphur, iron and nitrogen use efficiency under soil culture

S, Fe and N use efficiency were observed for bread and durum wheat in response to different Fe and S availability at physiological maturity (stage III) for two experimental years (2016 and 2017). In general, higher nutrient availability is known to reduce the nutrient use efficiency while reverse is true under nutrient deficient treatment. More or less an identical pattern of variation in response of Fe and S supply on sulphur use efficiency (SUE) was observed at both the experimental years (2016 and 2017). A decline in SUE was evident in the presence of Fe over the Fe- treatment. Application of S irrespective of dose (S_{30} and S_{60}) caused a decline in SUE on both Fe+ and Fe- soil, however the magnitude of decline was greater under Fe+ than Fe- treatment (Table 4.7, Fig. 4.9). Iron use efficiency (IUE) in bread and durum wheat cultivars greatly reduced under Fe sufficient (Fe+) treatment as compared to Fe deficient (Fe-) treatment. Significant reduction in IUE with increasing S supply was observed under Fe deficient condition but under Fe sufficient treatment there is not much difference in both bread and durum wheat cultivars in both cropping year. Highest reduction in IUE was observed under Fe+S2 condition as compared to Fe-S0 treatment (Table 4.7, Fig. 4.9). Nitrogen use efficiency (NUE) showed significant reduction with the availability of Fe and S. Fe availability did not significantly altered the IUE as no variations were observed in NUE values when Fe-S0/S1/S2 was compared with Fe+S0/S1/S2. A dose dependent decrease was observed with increasing S in NUE for both bread and durum wheat cultivars except HD-2329 (Fig. 4.11). Durum wheat cultivars showed higher NUE than the bread wheat. Lowest NUE was observed in nutrient sufficient (Fe+S2) condition and highest under nutrient deficient (Fe-S0) condition in both the bread and durum wheat cultivars.

4.1.4. Effect of iron and sulphur availability on shoot to grain translocation index of S, Fe and N under soil culture

Shoot to grain translocation of S, Fe and N was measured in terms of translocation index (TI) under differential Fe and S availabilities in both bread and durum wheat at physiological maturity (stage III) and seasonal variations in TI is presented in Fig. 4.10 and Fig. 4.11. TI for S did not change significantly at different levels of Fe and S supply, whereas for TI for Fe was significantly reduced under Fe+ than Fe- condition. S supply under both Fe sufficient (Fe+) and deficient (Fe-) treatments reduced the TI for Fe in 2016 but the effect was insignificant in the year 2017. TI for N showed a slight decrease with increasing S supply but there was no effect of Fe on TI of N (Fig. 4.11). Highest shoot to grain TI for N was observed in Fe+S0 condition in all the cultivars except HD-4728 when compared with the Fe-S0 condition. Application of S either at 30 (S1) or 60 (S2) kg S ha^{-1} dose to Fe+ or Fe- treatments caused a significant reduction in shoot to grain TI for N across all bread and durum wheat cultivars. Durum wheat, in general, showed a higher mean TI for N than the bread wheat cultivars when averaged over the Fe and S treatments.

4.1.5. Effect of iron and sulphur availability on activities of key enzymes involved in sulphate and nitrate/carbon assimilation under soil culture

4.1.5.1. Activity of sulphate assimilating enzymes

Changes in the activity of key enzymes of sulphur assimilation pathway i.e. serine acetyl transferase (SAT) and O-acetylserine thiolyase activity (OASTL) were studied under variable Fe and S availability conditions during 2016 and 2017 experimental years. Both these enzymes are important for the formation of cysteine, first product of sulphate assimilation.

4.1.5.1.1. Serine acetyl transferase activity

The SAT activity was observed under variable Fe and S levels at stage I and stage II for both bread and durum wheat (Table 4.8, Fig. 4.12). Fe deficiency either with or without S induced the SAT activity more significantly over the Fe sufficient treatment at S1 and S2 in bread wheat. SAT activity showed a general increase under conditions of S supply either with S1 and S2. SAT activity appears to be much more induced under Fe deficiency than Fe sufficiency condition in soil low in available S at stage I of growth particularly in bread wheat cultivars. Durum wheat showed higher

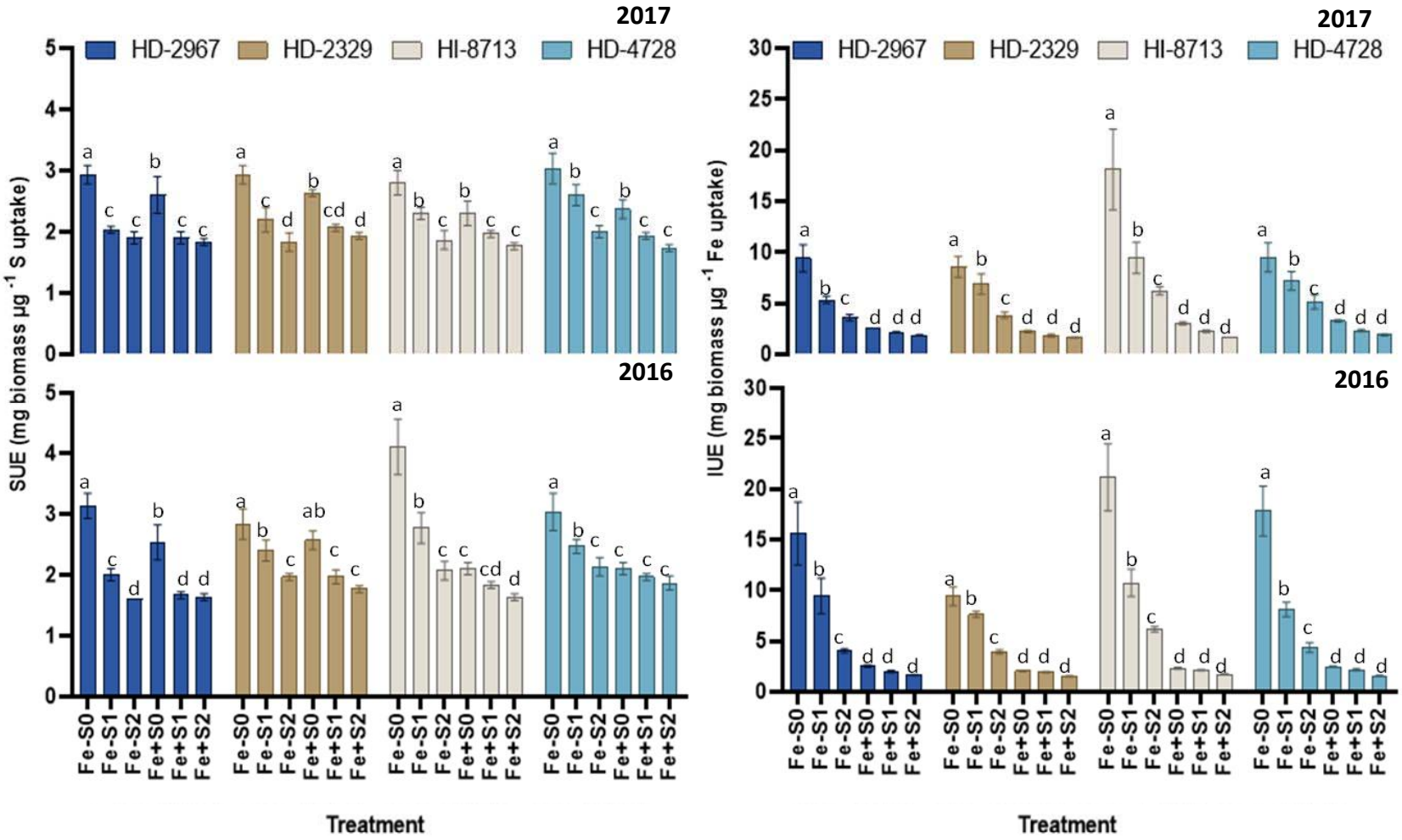

Fig.4.9: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on sulphur use efficiency (SUE) and iron use efficiency (IUE) of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at physiological maturity at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.7: Interactive effect of Fe and S availabilities on sulphur use efficiency and iron use efficiency of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS), stage II (60 DAS), stage III (120 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Sulphur use efficiency (µg g^{-1} dw)		Iron use efficiency (µg g^{-1} dw)	
	Iron (Fe)	Sulphur (S)	2016	2017	2016	2017
HD-2967	Fe-	S0	3.1	2.9	15.6	9.4
		S30	2.0	2.0	9.4	5.3
		S60	1.6	1.9	4.1	3.6
		Mean	2.2	2.3	9.7	6.1
	Fe+	S0	2.5	2.6	2.5	2.6
		S30	1.7	1.9	2.0	2.2
		S60	1.6	1.8	1.7	1.9
		Mean	1.9	2.1	2.1	2.2
HD-2329	Fe-	S0	2.8	2.9	9.4	8.6
		S30	2.4	2.2	7.6	6.9
		S60	2.0	1.8	3.9	3.8
		Mean	2.4	2.3	7.0	6.4
	Fe+	S0	2.6	2.6	2.1	2.2
		S30	1.9	2.1	2.0	1.9
		S60	1.8	1.9	1.6	1.6
		Mean	2.1	2.2	1.9	1.9
HI-8713	Fe-	S0	4.1	2.8	21.1	18.1
		S30	2.8	2.3	10.7	9.5
		S60	2.1	1.9	6.3	6.2
		Mean	3.0	2.3	12.7	11.3
	Fe+	S0	2.1	2.3	2.3	3.1
		S30	1.8	2.0	2.1	2.3
		S60	1.7	1.8	1.7	1.7
		Mean	1.9	2.0	2.0	2.4
HD-4728	Fe-	S0	3.0	3.0	17.8	9.5
		S30	2.5	2.6	8.1	7.2
		S60	2.2	2.0	4.4	5.1
		Mean	2.6	2.6	10.1	7.3
	Fe+	S0	2.1	2.4	2.5	3.3
		S30	1.9	1.9	2.2	2.3
		S60	1.8	1.8	1.6	1.9
		Mean	2.0	2.0	2.1	2.5
CD at 5%	**C**		0.12	0.82	NS	0.70
	T		0.15	1.00	0.12	0.86
	C X T		0.29	2.01	0.24	1.71

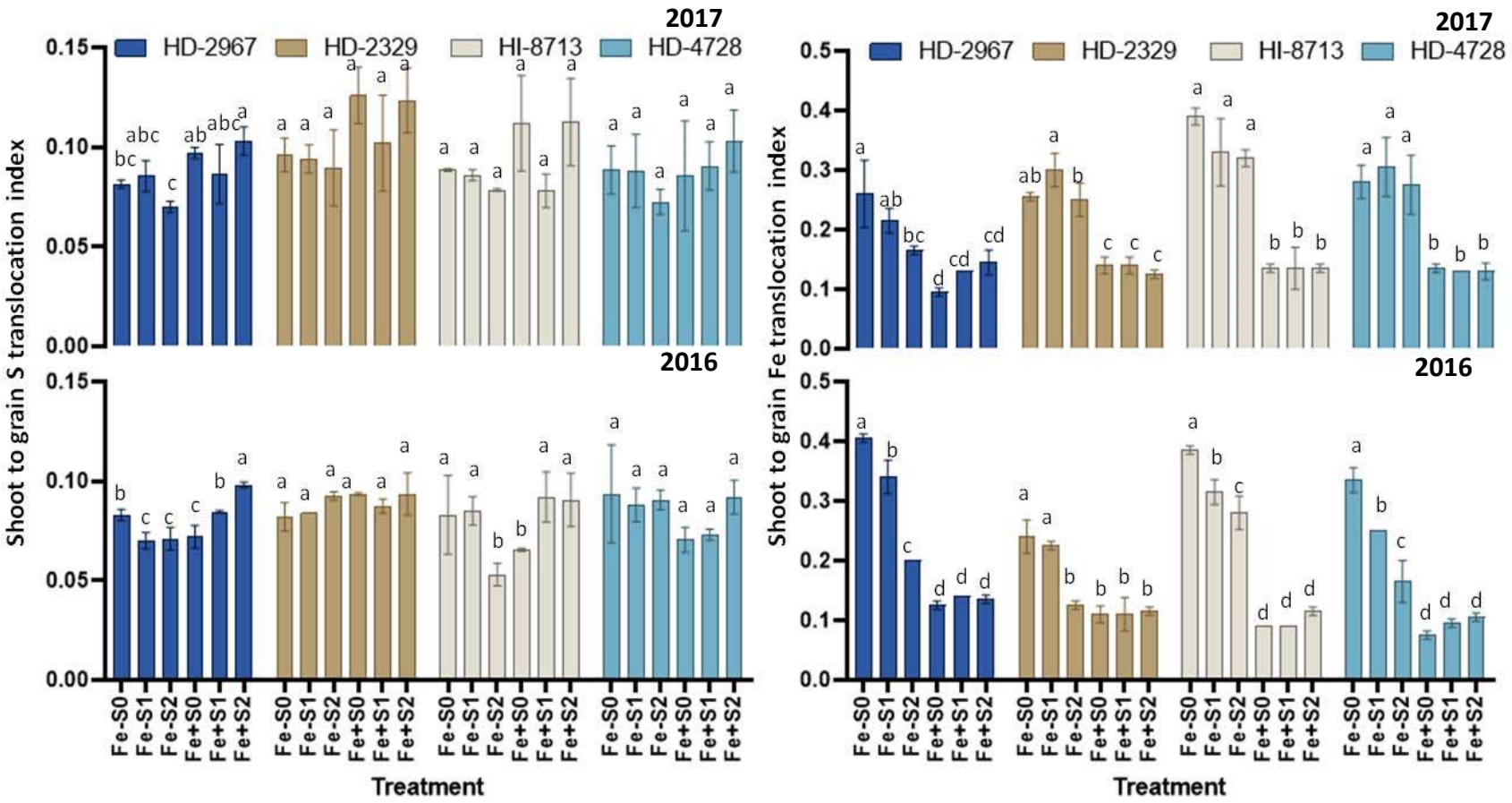

Fig.4.10: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on shoot to grain S and Fe translocation index of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at physiological maturity at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

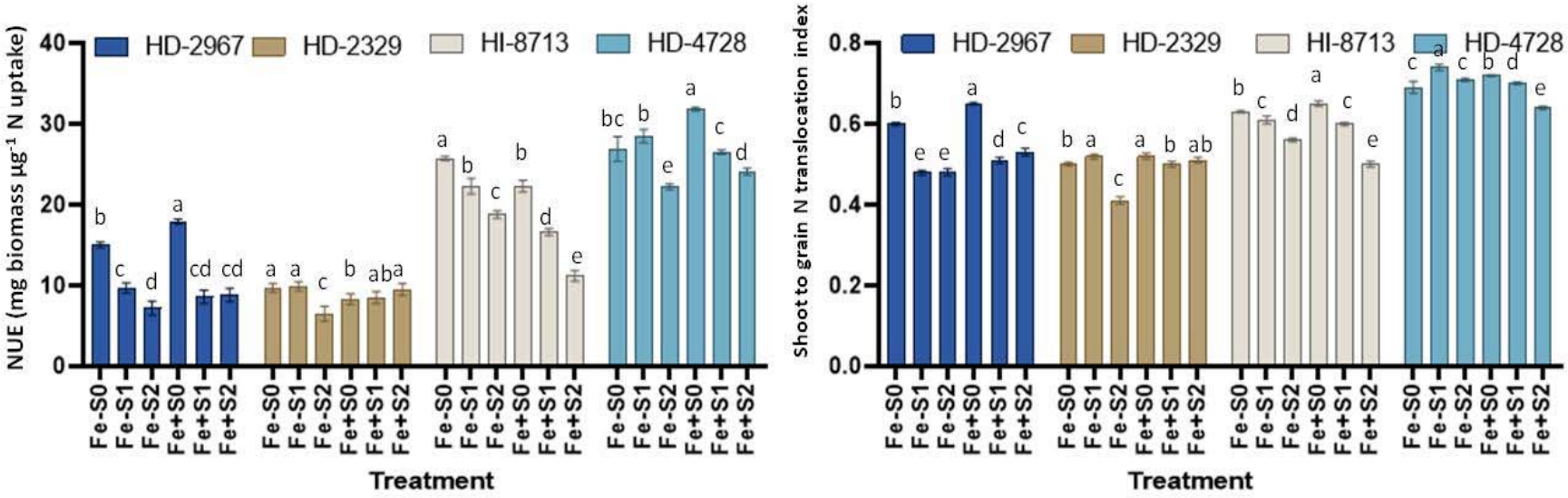

Fig.4.11: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on nitrogen use efficiency (NUE) and shoot to grain N translocation index of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at physiological maturity at threesulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3).Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

SAT activity under Fe and S sufficient than the Fe and S deficient condition and in general showed a significant higher increase in SAT activity than bread wheat under Fe+S2 than Fe+S0 or Fe-S2 condition.

4.1.5.1.2. O-acetylserine thiolyase activity

OASTL activity of bread and durum wheat cultivars as affected by Fe and S supply at two growth stages is presented in Table 4.9, Fig. 4.13. S supply modulated the enzyme activity in a dose dependent manner and an increase in the OASTL activity was observed in both the bread and durum wheat cultivars at both stage I and stage II under Fe+ and Fe- condition in the presence of S (S0 < S30 < S60). Fe deficiency when compared with Fe sufficient condition of growth, irrespective of S availability, significantly reduced the OASTL activity to almost 50%, especially in durum wheat. Fe supply modulated the enzyme activity with a dose dependent increase in the activity in both the wheat cultivars at both stage I and stage II. A higher mean OASTL activity was observed at stage II than at stage I. Bread and durum wheat cultivar irrespective of stage of sampling and year of observation did not differ significantly for OASTL activity between themselves.

4.1.5.2. Activity of carbon and nitrate assimilating enzymes

Changes in the activity of key enzymes of nitrate/carbon assimilation pathway i.e. nitrate reductase (NR) and rubisco were studied under Fe and S availability conditions during the experimental year 2017 and is presented in Table 4.10, Fig. 4.14.

4.1.5.2.1. Rubisco activity

Carbon assimilation via photosynthesis machinery is an important mechanism responsible for providing carbon skeleton for the synthesis of amino acids. The effect of Fe and S availability on the activity of key carbon assimilation enzyme rubisco was thus measured. Fe and S deprivation led to a significant reduction in the rubisco activity as compared to the Fe and S sufficient condition (Table 4.10, Fig. 4.14). Presence of S increased the activity of rubisco both under Fe- and Fe+ conditions in a dose dependent manner. The variations within the cultivars and treatments were also found to be significant, with a highest activity observed under Fe+S2 treatment in durum wheat.

4.1.5.2.2. Nitrate reductase activity

Availability and uptake of both N and S are critical for the synthesis of amino acids. Treatments with different combination of Fe and S significantly enhanced the activity of NR with a highest activity measured under Fe+S2 combination treatment (Table 4.10, Fig. 4.14). The combined treatment effect of Fe and S supply on NR activity was more significant as compared to individual nutrient (Fe or S) availability. Application of S under both Fe+ and Fe- condition improves the NR activity however the induction in NR activity was significantly higher under the Fe+ than the Fe- condition.

4.1.6. Effect of Fe and S availability on amino acids concentration under soil culture

Amino acid biosynthesis is modulated by both Fe and S supply, however their interactive effect on amino acid biosynthesis is rarely and poorly documented. The results on quantification of amino acid as influenced by Fe sufficient and Fe deficient condition under deficient to optimum availability of S across the bread and durum wheat cultivars at Stage II i.e. 60 DAS are presented in Table 4.11. Genetic variability in terms of Fe and S supply response on amino acids profiling of experimental wheat cultivars was observed. In general, the mean average amino acid levels declined under Fe+ than Fe- condition when averaged over the S treatments with a few exceptions. Level of the most of the amino acids in general, were reduced in bread wheat under increasing S availability irrespective of the Fe condition of the soil when averaged over the cultivars within the group, while an increase in amino acid content were observed in the durum wheat. Durum wheat appeared to respond to S application and this response was independent of soil Fe availability. In general, a relatively higher accumulation of threonine (Thr), tyrosine (Tyr), proline (Pro) and aspartate (Asp) were observed when compared to other amino acids when averaged over the Fe and S treatment. While lowest values were recorded for alanine (Ala), valine (Val) and lysine (Lys).

A significantly higher level of mean proline content was measured under Fe- than Fe+ treatment which was also found to increase with an S application. The other amino acid which were predominantly induced under the Fe- than the Fe+ condition were Asp, glutamine (Glu), histidine (His), Thr, Tyr and phenyl alanine (Phe). A

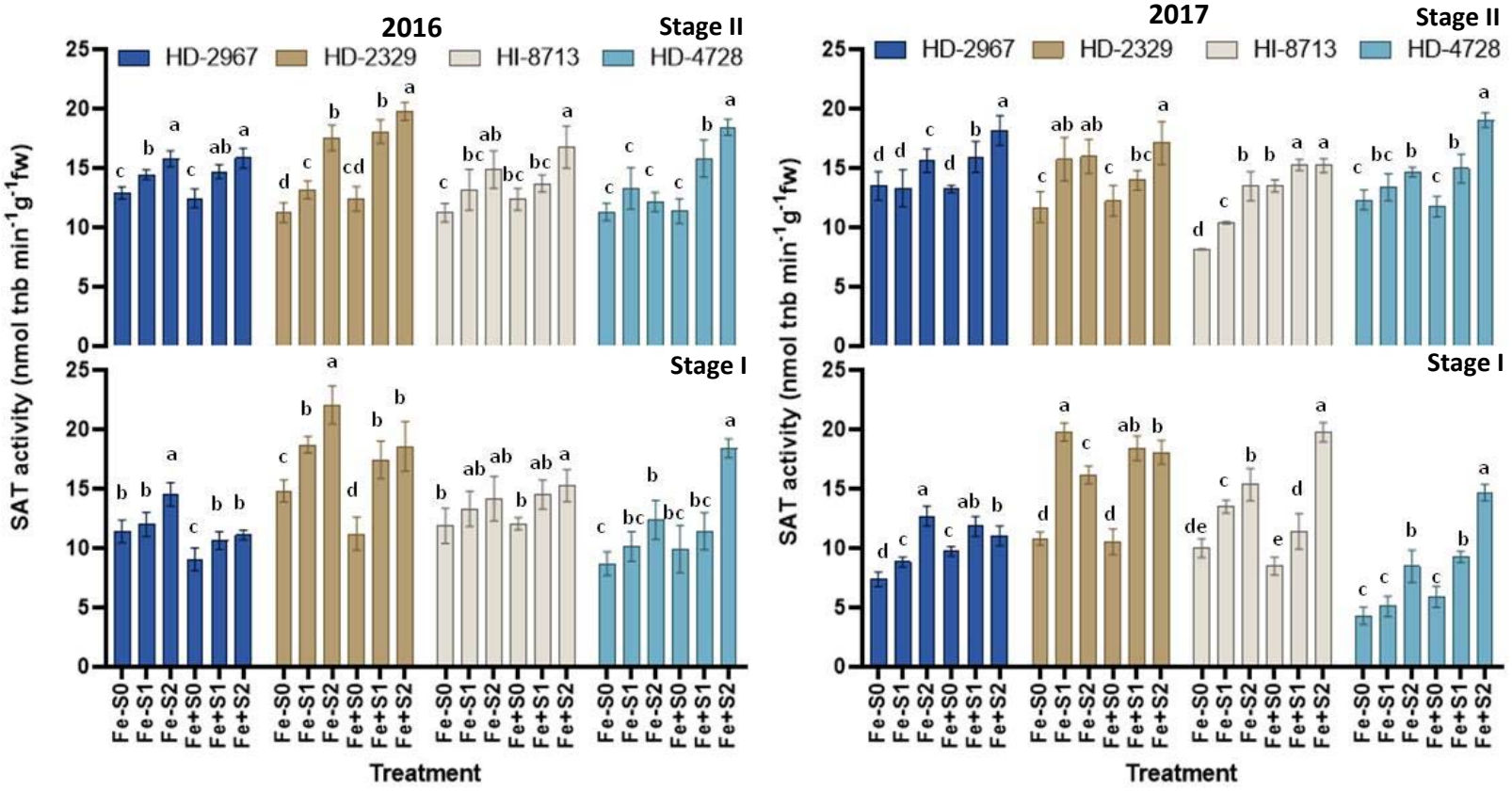

Fig. 4.12: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on serine acetyl transferase (SAT) enzyme activity of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at two growth stages (I and II) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.8: Interactive effect of Fe and S availabilities on serine acetyl transferase activity of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		Serine acetyl transferase (nmol tnb min^{-1} g^{-1} fw)			
			2016		2017	
	Iron (Fe)	Sulphur (S)	Stage (D)			
			Stage I	Stage II	Stage I	Stage II
HD-2967	Fe-	S0	11.4	12.9	7.4	13.5
		S30	12.0	14.4	8.8	13.3
		S60	14.6	15.8	12.7	15.6
		Mean	12.7	14.4	9.6	14.1
	Fe+	S0	9.0	12.4	9.7	13.2
		S30	10.6	14.7	11.8	15.9
		S60	11.1	15.8	11.1	18.2
		Mean	10.3	14.3	10.9	15.8
HD-2329	Fe-	S0	14.8	11.3	10.8	11.7
		S30	18.7	13.2	19.8	15.7
		S60	22.1	17.5	16.2	16.0
		Mean	18.5	14.0	15.6	14.5
	Fe+	S0	11.2	12.4	10.5	12.2
		S30	17.4	18.1	18.4	14.0
		S60	18.6	19.8	18.1	17.1
		Mean	15.7	16.8	15.7	14.4
HI-8713	Fe-	S0	11.9	11.2	10.0	8.1
		S30	13.3	13.2	13.2	10.4
		S60	14.2	14.9	15.3	13.5
		Mean	13.1	13.1	12.8	10.7
	Fe+	S0	12.1	12.4	8.5	13.5
		S30	14.5	13.7	11.4	15.3
		S60	15.2	16.8	19.8	15.2
		Mean	13.9	14.3	13.2	14.7
HD-4728	Fe-	S0	8.7	11.3	4.3	12.3
		S30	10.1	13.3	5.1	13.4
		S60	12.4	12.1	8.4	14.7
		Mean	10.4	12.2	5.9	13.4
	Fe+	S0	9.9	11.4	5.9	11.7
		S30	11.4	15.8	9.3	15.0
		S60	18.4	18.5	14.7	19.0
		Mean	13.2	15.2	10.0	15.2
CD at 5%	**C**		0.6		0.5	
	T		0.7		0.6	
	D		0.4		0.3	
	C X T		1.4		1.1	
	C X D		0.8		0.7	
	T X D		0.9		0.8	
	C X T X D		1.9		1.6	

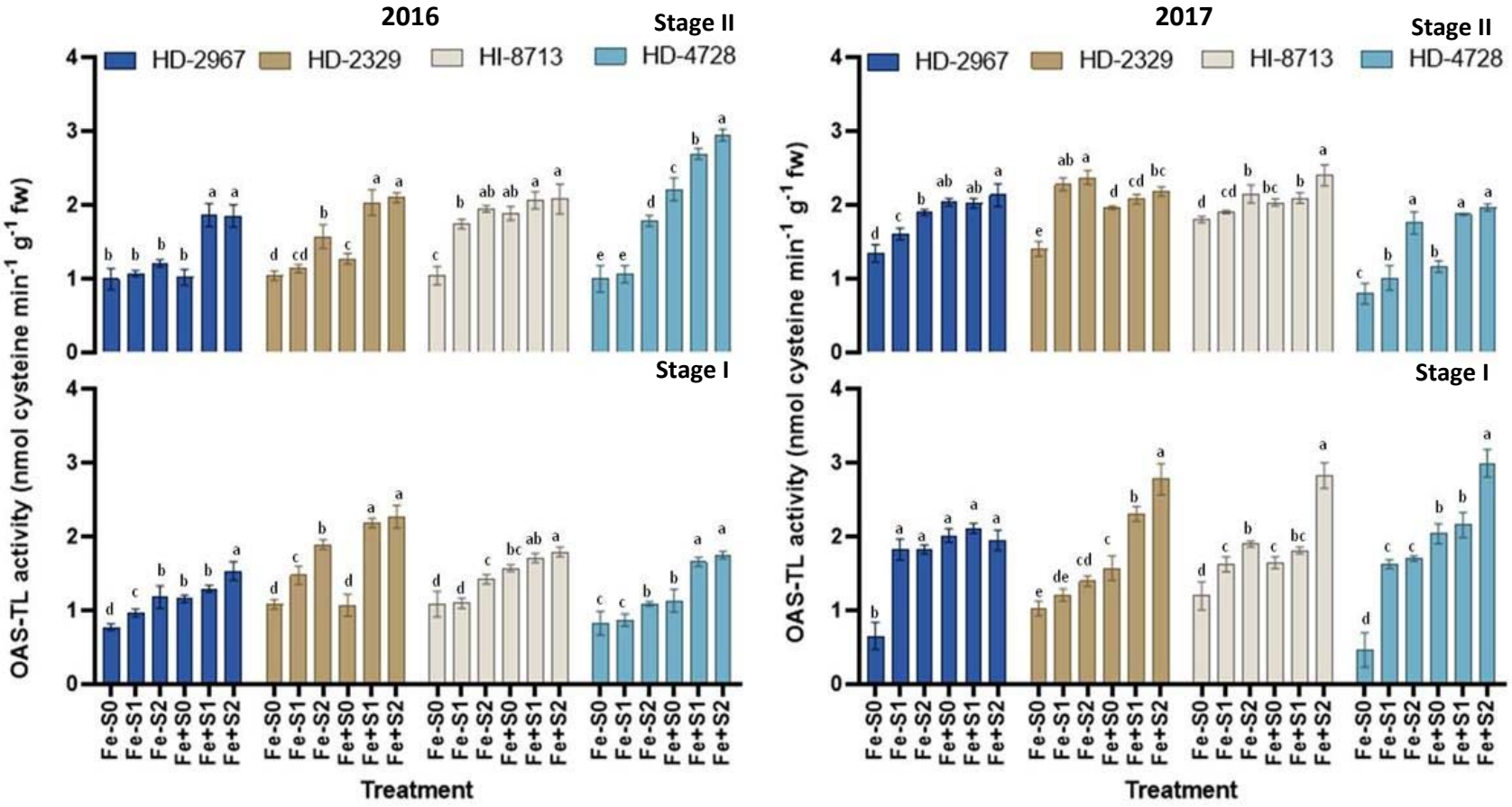

Fig.4.13: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on O-acetyl serine lyase (OASTL) enzyme activity of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at two growth stages (I and II) at three sulphur levels (S0, S1 and S2) recorded over two crop seasons (2016 and 2017). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.9: Interactive effect of Fe and S availabilities on O-acetyl serine thiolyase activity of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)) raised over two crop seasons (2016-2017)

Cultivars (C)	Treatments (T)		O-acetyl serine thiolyase (nmol cysteine min^{-1} g^{-1} fw)			
	Iron (Fe)	Sulphur (S)	2016		2017	
			Stage (D)			
			Stage I	Stage II	Stage I	Stage III
HD-2967	Fe-	S0	0.8	1.0	0.7	1.3
		S30	1.0	1.1	1.8	1.6
		S60	1.2	1.2	1.8	1.9
		Mean	1.0	1.1	1.4	1.6
	Fe+	S0	1.2	1.0	2.0	2.0
		S30	1.3	1.9	2.1	2.0
		S60	1.5	1.9	2.0	2.1
		Mean	1.3	1.6	2.0	2.1
HD-2329	Fe-	S0	1.1	1.0	1.0	1.4
		S30	1.5	1.1	1.2	2.3
		S60	1.9	1.6	1.4	2.4
		Mean	1.5	1.3	1.2	2.0
	Fe+	S0	1.1	1.3	1.6	2.0
		S30	2.2	2.0	2.3	2.1
		S60	2.3	2.1	2.8	2.2
		Mean	1.8	1.8	2.2	2.1
HI-8713	Fe-	S0	1.1	1.0	1.2	1.8
		S30	1.1	1.7	1.6	1.9
		S60	1.4	1.9	1.9	2.2
		Mean	1.2	1.6	1.6	2.0
	Fe+	S0	1.6	1.9	1.6	2.0
		S30	1.7	2.1	1.8	2.1
		S60	1.8	2.1	2.8	2.4
		Mean	1.7	2.0	2.1	2.2
HD-4728	Fe-	S0	0.8	1.0	0.5	0.8
		S30	0.9	1.1	1.6	1.0
		S60	1.1	1.8	1.7	1.8
		Mean	0.9	1.3	1.3	1.2
	Fe+	S0	1.1	2.2	2.0	1.2
		S30	1.7	2.7	2.2	1.9
		S60	1.8	2.9	3.0	2.0
		Mean	1.5	2.6	2.4	1.7
CD at 5%		**C**	0.05		0.05	
		T	0.06		0.07	
		D	0.04		0.04	
		C X T	0.12		0.13	
		C X D	0.07		0.08	
		T X D	0.09		0.09	
		C X T X D	0.18		0.19	

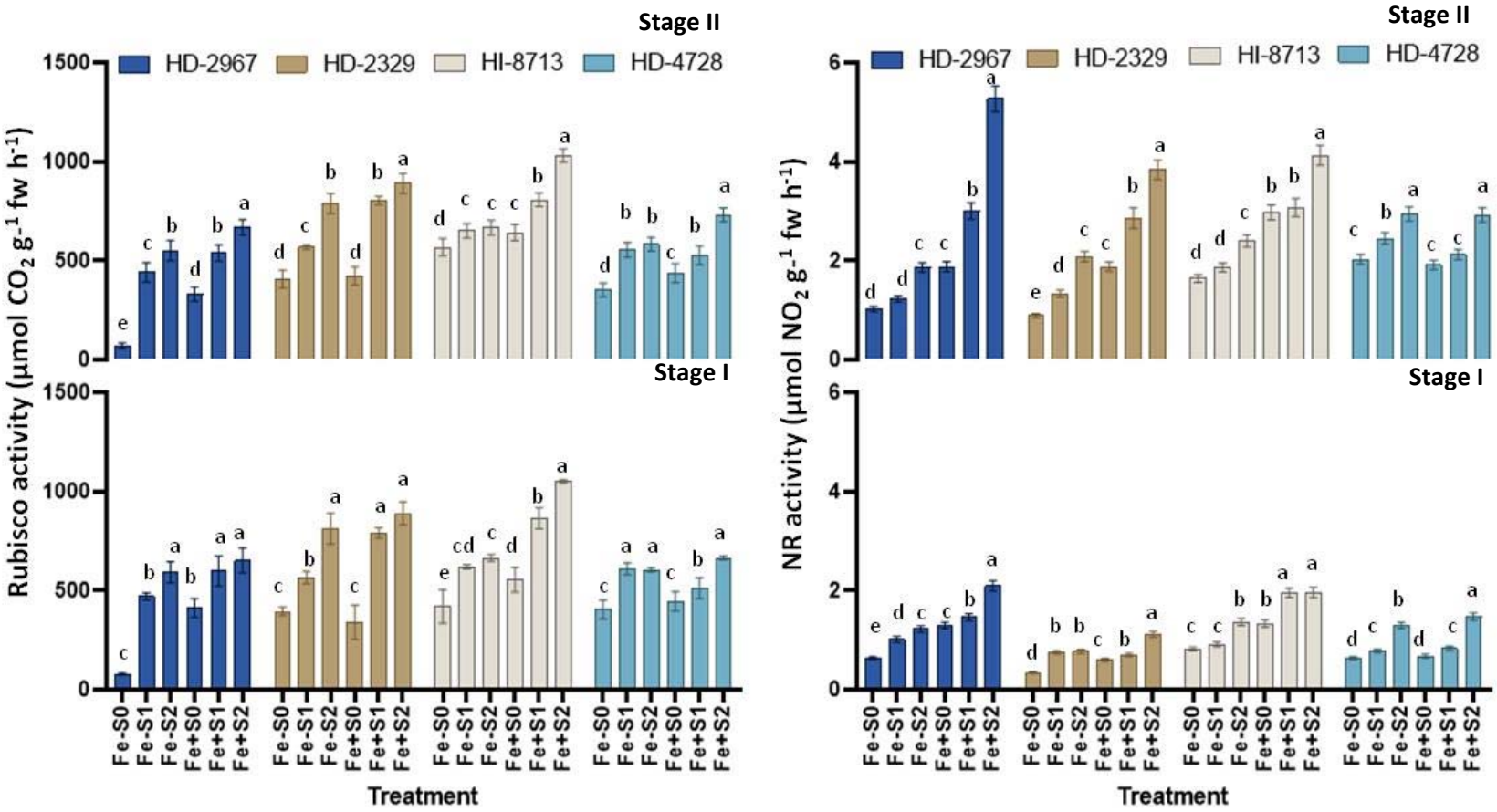

Fig.4.14: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on rubisco and nitrate reductase activity of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at two growth stages (I and II) at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.10: Interactive effect of Fe and S availabilities on rubisco and nitrate reductase activity of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (30 DAS) and stage II (60 DAS)

Cultivars (C)	Treatments (T)		Rubisco (µmol CO_2 g^{-1} fw h^{-1})		Nitrate reductase (µmol NO_2 g^{-1} fw h^{-1})	
	Iron (Fe)	Sulphur (S)	2017			
			Stage (D)			
			Stage I	Stage II	Stage I	Stage III
HD-2967	Fe-	S0	79.9	70.2	0.64	1.02
		S30	470.1	442.0	1.01	1.23
		S60	591.9	550.5	1.22	1.86
		Mean	380.6	354.2	0.96	1.37
	Fe+	S0	412.2	331.1	1.30	1.88
		S30	598.6	539.0	1.46	3.00
		S60	651.5	668.5	2.10	5.27
		Mean	554.1	512.9	1.62	3.38
HD-2329	Fe-	S0	394.8	408.4	0.34	0.89
		S30	566.3	569.8	0.75	1.33
		S60	812.1	788.4	0.77	2.08
		Mean	591.1	588.8	0.62	1.43
	Fe+	S0	339.2	422.9	0.60	1.88
		S30	790.5	802.6	0.70	2.86
		S60	888.6	890.1	1.12	3.84
		Mean	672.7	705.2	0.81	2.86
HI-8713	Fe-	S0	419.6	567.4	0.82	1.64
		S30	619.2	650.0	0.91	1.86
		S60	663.7	667.2	1.36	2.40
		Mean	567.5	628.2	1.03	1.97
	Fe+	S0	554.3	641.9	1.33	2.97
		S30	864.7	807.1	1.96	3.07
		S60	1051.3	1030.6	1.96	4.13
		Mean	823.4	826.5	1.75	3.39
HD-4728	Fe-	S0	404.5	351.6	0.64	2.03
		S30	610.5	553.5	0.78	2.45
		S60	604.1	582.8	1.30	2.94
		Mean	539.7	496.0	0.90	2.47
	Fe+	S0	444.9	436.2	0.68	1.91
		S30	512.1	526.8	0.83	2.12
		S60	664.3	730.8	1.47	2.92
		Mean	540.4	564.6	0.99	2.32
CD at 5%	**C**		20.8		0.05	
	T		25.5		0.06	
	D		NS		0.04	
	C X T		50.9		0.12	
	C X D		29.4		0.07	
	T X D		NS		0.09	
	C X T X D		72.0		0.17	

reverse order was observed for serine (Ser), glycine (Gly), arginine (Arg), Ala, Val, leucine (Leu) and Lys amino acids which showed a higher content under the Fe+ than the Fe- treatment when averaged over the S treatment and wheat groups. Effect of S supply in increasing the level of amino acids was more pronounced under the Fe+ than the Fe- treatment more so for the durum wheat than the bread wheat groups when averaged over the cultivars.

Changes in the content of S containing amino acids i.e. methionine and cysteine of bread and durum wheat under variable Fe and S condition is presented in Fig. 4.15. Methionine content in general was higher in the bread wheat than the durum wheat cultivars when averaged over Fe and S treatment. Further, an increase in the methionine level was recorded under Fe- than the Fe+ treatment without S supply in bread wheat cultivars. The effect of Fe availability on methionine content was S dependent and increased with an increasing S supply (S0 < S1 < S2). The effect of Fe and S interaction on methionine levels of durum wheat cultivars were insignificant. Highest methionine levels were measured in bread wheat cultivars HD-2329 followed distantly by HD-2967. More or less a similar pattern of variation in response to Fe and S supply was observed for cysteine which was also higher in the two bread wheat than the durum wheat cultivars. Cysteine content in general was significantly higher under Fe+ than Fe- conditions of growth without the S supply across both the experimental wheat species. Further, S application positively affected the cysteine content of the leaves in a dose dependent manner with an exception of the bread wheat cultivar HD-2967 which showed a marginal decline in cysteine levels with an increasing S supply under Fe deficiency.

4.2. To investigate the interactive effect of Fe and S nutrition on plant root architecture, phytosiderophore synthesis and release in relation to $^{35}SO_4^{2-}$ and ^{59}Fe uptake and partitioning.

4.2.1. Effect of Fe and S availability treatments on plant growth attributes under solution culture

4.2.1.1. Shoot and root biomass and total chlorophyll

Shoot and root biomass of the investigated wheat cultivars was, in general, higher under Fe and S sufficient treatments (Fe+S1 and Fe+S2) when compared with the respective tissue biomass under nutrient deficient condition in solution culture. Fe

and S deficiency, either alone or in combination significantly reduced the plant growth attributes across the experimental cultivars at all the stages i.e. stage I (8 Days after transfer (DAT)), stage II (11 DAT) and stage III (14 DAT) (Table 4.12, Fig. 4.16). The reduction in shoot biomass under the Fe and S deficient than the Fe and S sufficient treatments was significantly higher in durum than the bread wheat. The chlorophyll content of the nutrient sufficient and deficient plants was also measured for the bread and durum wheat cultivars at stage II (11 DAT) (Fig. 4.17). The reduction in chlorophyll content was higher under the combined deficiency (Fe-S0) and individual deficiency (Fe-S+ or Fe+S-) treatment than observed over the nutrient sufficient control (Fe+S2) significantly and more drastically over the individual deficiency treatments than that of the nutrient sufficient control (Fe+S2). Durum wheat showed a greater reduction in the leaf chlorophyll than the bread wheat under the combined deficiency of Fe and S (Fe-S0).

4.2.1.2. Root characteristics

Root characteristics were observed in terms of root volume, root surface area and root length. Mean root volume was not affected under individual or combined deficiency of Fe and S. Root volume increased from stage I to stage III for both the bread and durum wheat cultivars. Durum wheat cultivars, in general, exhibited higher root volume than that of bread wheat cultivars at different stages of observation under both Fe- and Fe+ condition. Fe deficiency in bread wheat showed a marginal but insignificant increase in root volume at stage III (Table, 4.13 Fig. 4.18).

Root surface area represents the extent of the coverage of the roots in the rhizosphere and it was higher under the individual or combined deficiency of Fe and S as compared to the nutrient sufficient treatment (Table 4.13, Fig. 4.18). Fe and S supply in general, decreased the root surface area in a dose dependent manner. Mean root surface area increased with the growth of the wheat cultivars and was maximal at stage III. The combined deficiency of Fe and S (Fe-S0) increased the root surface area significantly in bread wheat at all the stages, whereas in durum wheat the affect was insignificant at the later stage of growth.

Length of root which indicates the depth of soil exploration and nutrient exploitation was significantly higher under Fe- than Fe+ treatment (Table 4.14, Fig. 4.19). Average root length was increased with plant growth and was maximum under

Table 4.11: Interactive effect of Fe and S availabilities on amino acid content of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at growth stage II at three sulphur levels (S0, S1 and S2).

Cultivars (C)	Treatments (T)		Amino acids (µmol g^{-1} dw)													
	Iron (Fe)	Sulphur (S)	ASP	GLU	SER	HIS	GLY	THR	ARG	ALA	TYR	VAL	PHE	ILE	LEU	LYS	PRO
HD-2967	**Fe-**	**S0**	0.88	0.69	0.21	0.21	0.16	1.33	0.63	0.07	1.92	0.03	0.19	0.49	0.16	0.03	2.15
		S30	0.81	0.58	0.14	0.14	0.11	1.12	0.34	0.06	1.39	0.02	0.15	0.34	0.10	0.02	1.94
		S60	0.74	0.54	0.13	0.10	0.11	0.90	0.20	0.03	1.85	0.01	0.15	0.33	0.06	0.03	2.39
		Mean	0.81	0.60	0.16	0.15	0.13	1.12	0.39	0.05	1.72	0.02	0.16	0.39	0.11	0.03	2.16
	Fe+	**S0**	0.74	0.52	0.39	0.23	0.21	0.54	5.72	0.03	3.86	0.06	0.13	0.31	0.50	0.31	2.48
		S30	0.65	0.41	0.22	0.18	0.20	0.33	1.18	0.04	3.72	0.10	0.26	0.26	0.32	0.09	2.27
		S60	0.35	0.25	0.17	0.16	0.14	0.07	0.53	0.02	0.45	0.17	1.02	0.17	0.27	0.00	1.16
		Mean	0.58	0.40	0.26	0.19	0.18	0.31	2.48	0.03	2.68	0.11	0.47	0.25	0.36	0.13	1.97
HD-2329	**Fe-**	**S0**	1.49	1.04	0.39	0.34	0.35	2.72	1.13	0.11	3.27	0.06	1.42	0.09	0.28	0.10	2.41
		S30	0.97	0.64	0.23	0.09	0.17	1.22	0.53	0.06	1.54	0.03	0.70	0.05	0.12	0.05	2.20
		S60	0.40	0.27	0.00	0.05	0.08	0.09	0.01	0.00	1.00	0.02	0.48	0.08	0.05	0.01	3.73
		Mean	0.96	0.65	0.21	0.16	0.20	1.35	0.55	0.06	1.94	0.03	0.86	0.07	0.15	0.05	2.78
	Fe+	**S0**	0.43	0.47	0.24	0.01	0.49	1.29	5.53	0.02	1.20	0.05	0.10	0.13	0.06	0.23	1.93
		S30	2.31	0.38	0.14	0.11	0.20	1.07	1.07	0.05	0.86	0.13	0.12	0.09	0.02	0.18	1.33
		S60	0.51	0.18	0.13	0.10	0.10	0.24	0.70	0.08	0.17	0.45	0.13	0.06	0.01	0.12	1.29
		Mean	1.08	0.34	0.17	0.07	0.26	0.87	2.43	0.05	0.75	0.21	0.11	0.09	0.03	0.18	1.51
HI-8713	**Fe-**	**S0**	1.03	0.62	0.18	0.08	0.09	0.56	0.68	0.07	0.53	0.04	0.97	0.19	0.13	0.09	1.30
		S30	0.94	0.58	0.12	0.12	0.13	0.93	0.36	0.05	1.07	0.04	0.75	0.20	0.12	0.08	2.27
		S60	1.33	0.47	0.05	0.12	0.16	0.78	0.17	0.04	1.24	0.04	0.64	0.31	0.09	0.06	2.74
		Mean	1.10	0.55	0.12	0.11	0.13	0.76	0.40	0.05	0.95	0.04	0.79	0.23	0.11	0.08	2.10
	Fe+	**S0**	2.08	0.58	0.28	0.10	0.12	0.43	0.36	0.07	0.03	0.06	0.14	0.36	0.15	0.19	0.13
		S30	0.25	0.51	0.24	0.14	0.15	0.89	0.37	0.08	0.19	0.29	0.17	0.57	0.16	0.21	0.19
		S60	0.23	0.42	0.15	0.09	0.14	0.76	1.01	0.09	0.24	0.36	0.30	0.71	0.29	0.28	0.21
		Mean	0.85	0.50	0.22	0.11	0.13	0.69	0.58	0.08	0.15	0.24	0.20	0.55	0.20	0.23	0.17
HD-4728	**Fe-**	**S0**	0.86	0.55	0.11	0.05	0.10	0.67	0.48	0.03	0.87	0.03	0.41	0.15	0.09	0.04	2.13
		S30	0.93	0.64	0.19	0.14	0.14	0.98	0.41	0.04	1.13	0.04	0.60	0.23	0.02	0.06	1.84
		S60	0.33	0.25	0.17	0.11	0.17	0.84	0.37	0.04	1.21	0.03	0.12	0.59	0.16	0.04	0.37
		Mean	0.70	0.48	0.15	0.10	0.14	0.83	0.42	0.04	1.07	0.03	0.38	0.32	0.09	0.05	1.44
	Fe+	**S0**	0.31	0.48	0.21	0.15	0.14	1.06	0.46	0.13	0.12	0.32	0.21	0.16	0.43	0.26	0.20
		S30	0.58	0.77	0.38	0.03	0.60	1.30	0.52	0.16	0.46	0.37	0.22	0.22	0.46	0.37	1.78
		S60	0.41	0.38	0.50	0.02	0.62	1.12	0.64	0.17	0.52	0.39	0.28	0.22	0.52	0.40	1.95
		Mean	0.43	0.55	0.36	0.07	0.45	1.16	0.54	0.15	0.37	0.36	0.24	0.20	0.47	0.34	1.31

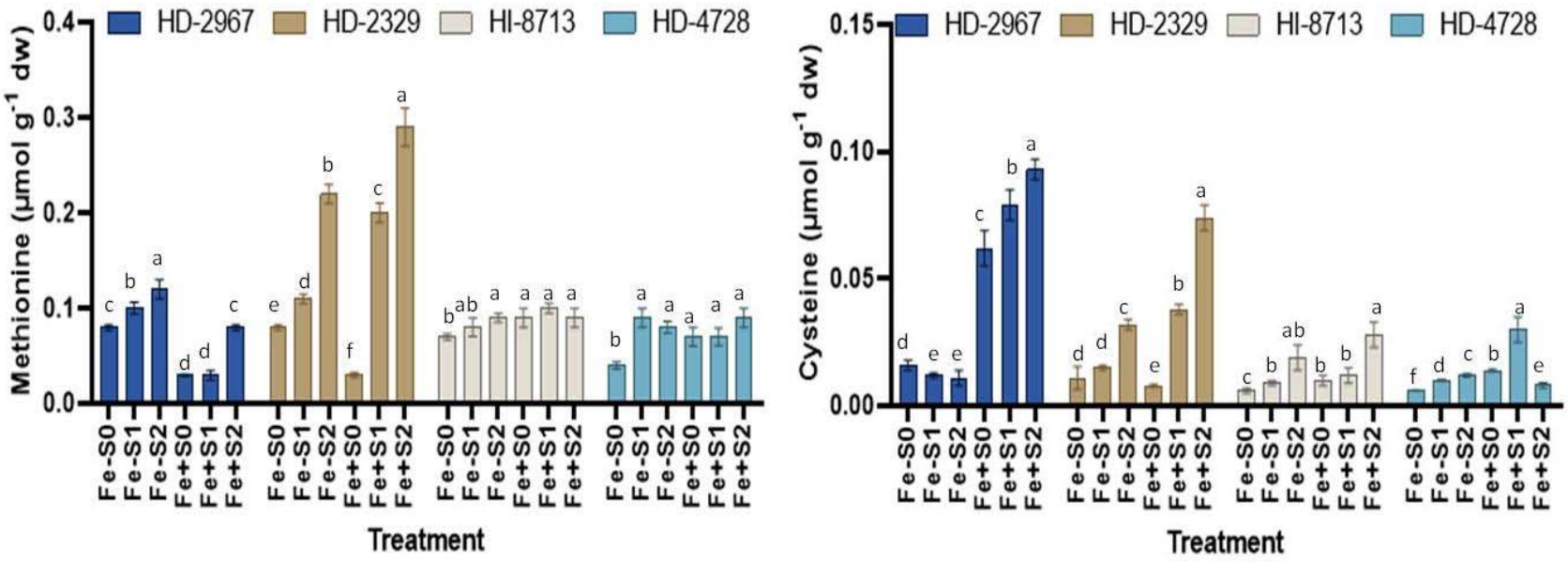

Fig.4.15: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) soil condition on cysteine and methionine content of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at growth stage II at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

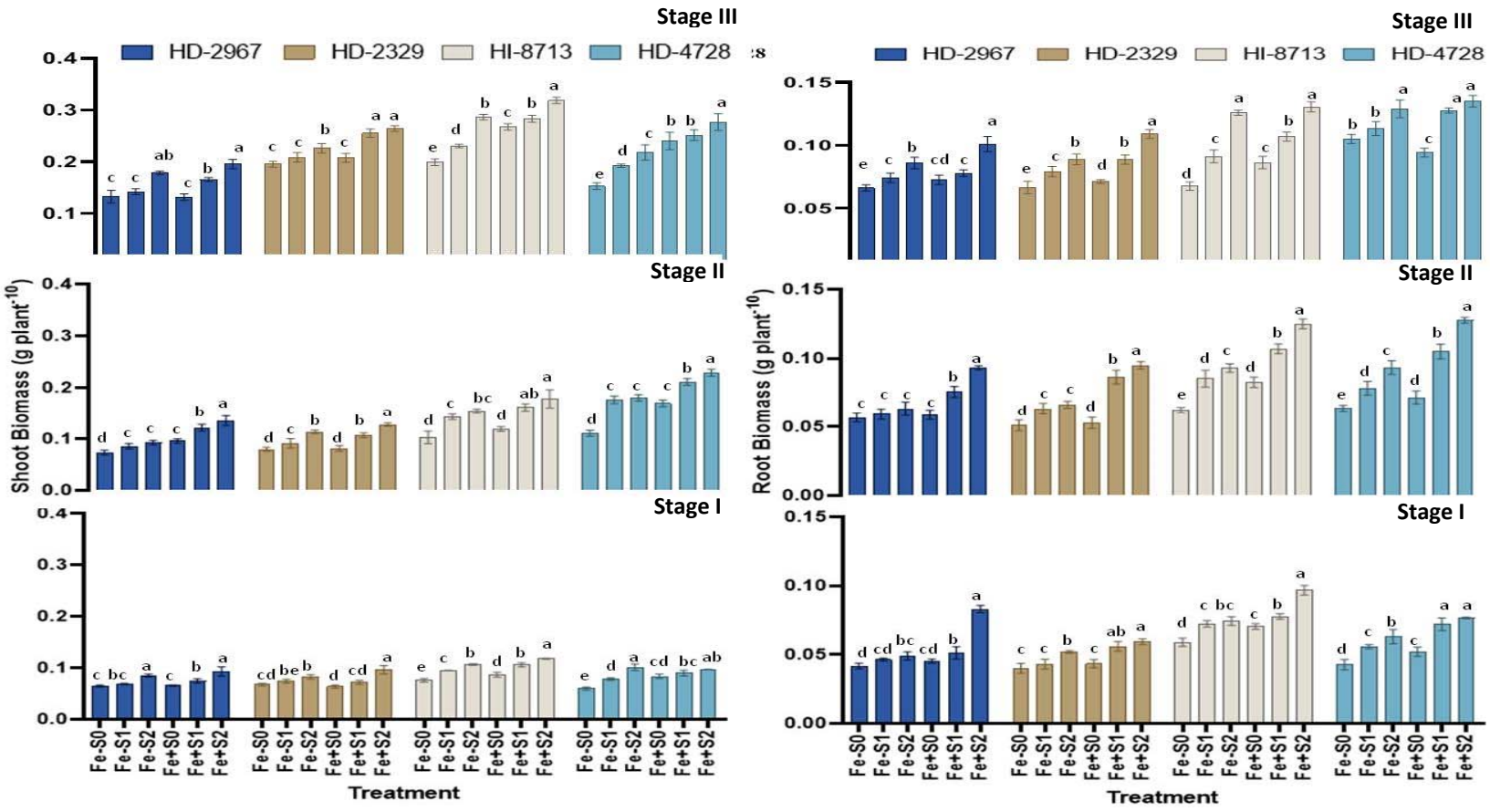

Fig.4.16: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatment on shoot and root biomass of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.12: Interactive effect of Fe and S availabilities on shoot and root biomass of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (7 DAS), stage II (11 DAS), stage III (14 DAS)) under solution culture

Cultivars(C)	Treatments (T)		Shoot Biomass (g plant^{-10})			Root Biomass (g plant^{-10})		
			Stage (D)					
	Iron (Fe)	Sulphur (S)	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	0.065	0.073	0.133	0.042	0.057	0.066
		S30	0.068	0.085	0.142	0.046	0.059	0.074
		S60	0.085	0.092	0.179	0.049	0.063	0.086
		Mean	0.073	0.084	0.151	0.046	0.060	0.076
	Fe+	S0	0.066	0.095	0.132	0.046	0.059	0.073
		S30	0.075	0.121	0.166	0.051	0.076	0.078
		S60	0.093	0.135	0.196	0.083	0.093	0.101
		Mean	0.078	0.117	0.165	0.060	0.076	0.084
HD-2329	Fe-	S0	0.067	0.079	0.195	0.040	0.051	0.067
		S30	0.074	0.091	0.209	0.043	0.063	0.079
		S60	0.082	0.113	0.226	0.052	0.066	0.089
		Mean	0.074	0.094	0.210	0.045	0.060	0.078
	Fe+	S0	0.063	0.081	0.248	0.043	0.053	0.071
		S30	0.072	0.106	0.255	0.056	0.086	0.089
		S60	0.096	0.127	0.264	0.060	0.095	0.109
		Mean	0.077	0.105	0.256	0.053	0.078	0.090
HI-8713	Fe-	S0	0.076	0.102	0.199	0.059	0.062	0.068
		S30	0.095	0.142	0.231	0.072	0.085	0.091
		S60	0.107	0.154	0.286	0.075	0.093	0.126
		Mean	0.092	0.133	0.239	0.069	0.080	0.095
	Fe+	S0	0.086	0.119	0.268	0.070	0.083	0.086
		S30	0.106	0.162	0.283	0.078	0.107	0.107
		S60	0.118	0.178	0.319	0.097	0.125	0.131
		Mean	0.103	0.153	0.276	0.082	0.105	0.108
HD-4728	Fe-	S0	0.060	0.110	0.153	0.043	0.064	0.105
		S30	0.078	0.176	0.192	0.055	0.078	0.113
		S60	0.101	0.180	0.218	0.063	0.093	0.129
		Mean	0.080	0.156	0.188	0.054	0.078	0.116
	Fe+	S0	0.083	0.170	0.240	0.052	0.072	0.094
		S30	0.090	0.211	0.252	0.072	0.105	0.128
		S60	0.097	0.229	0.277	0.077	0.128	0.135
		Mean	0.090	0.203	0.256	0.067	0.101	0.119
CD at 5%		C		0.003			0.001	
		T		0.003			0.002	
		D		0.002			0.001	
		C X T		0.006			0.003	
		C X D		0..005			0.002	
		T X D		0.006			0.003	
		C X T X D		0.011			0.006	

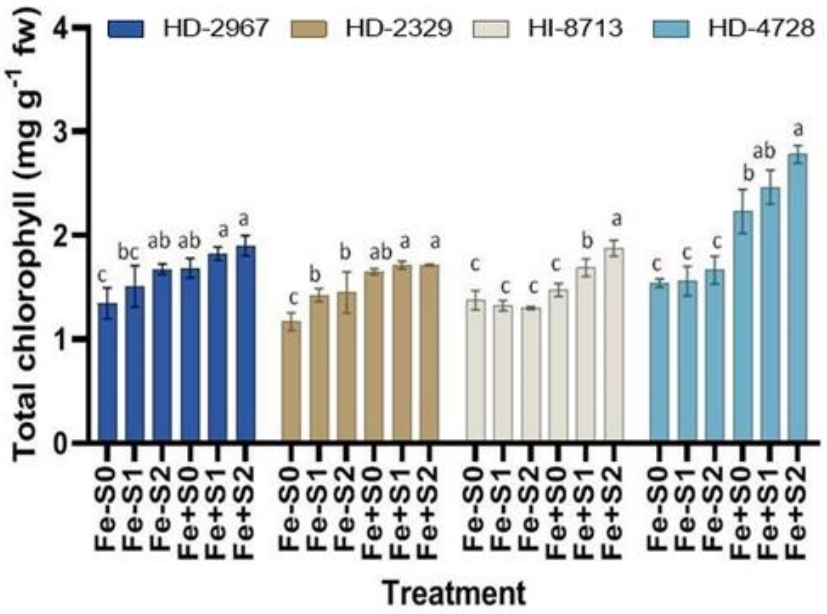

Fig.4.17: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatment on total chlorophyll of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

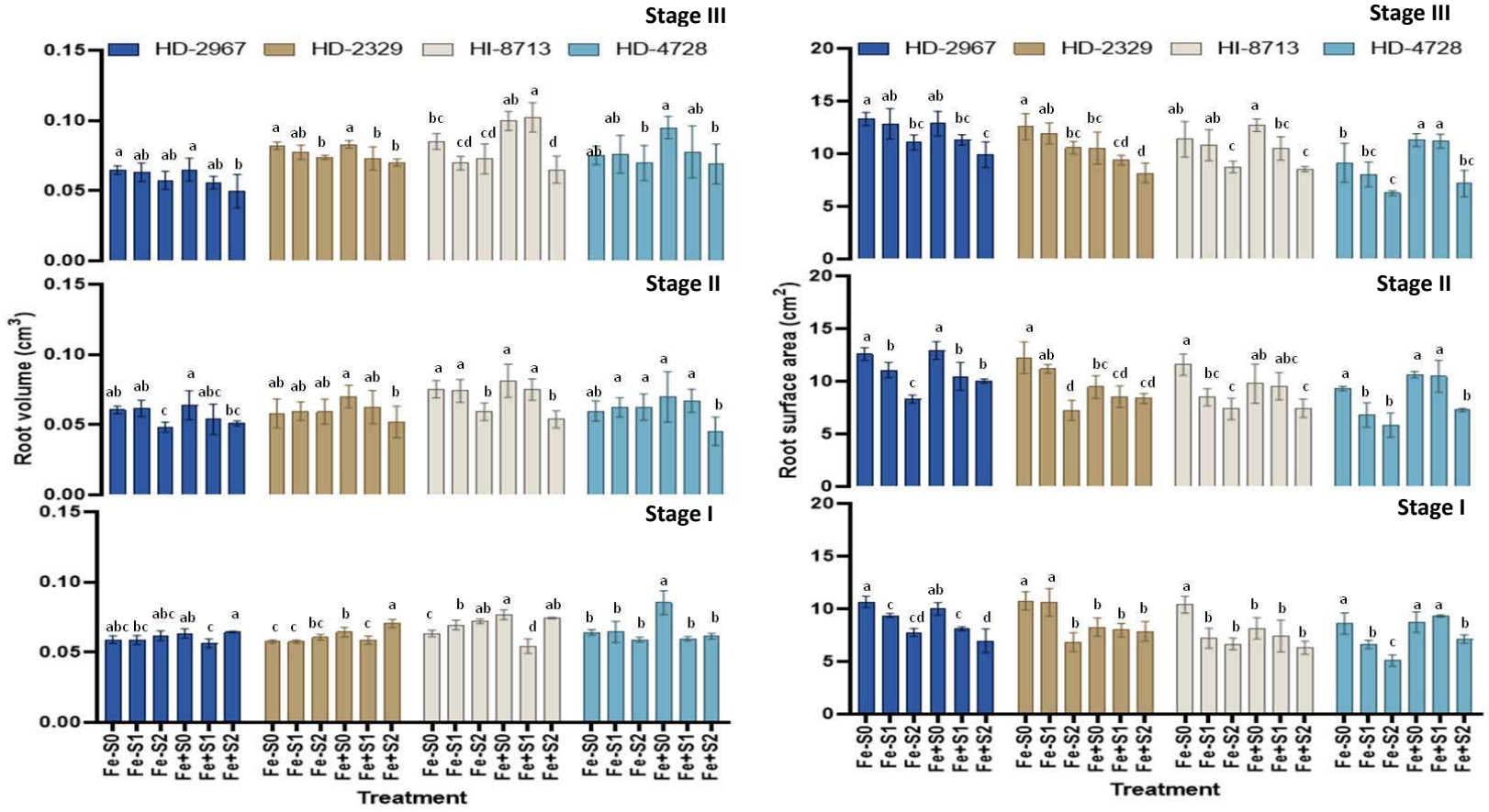

Fig.4.18: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton root characteristics of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three stages (I, II and III) at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.13: Interactive effect of Fe and S availabilities on root volume and surface area of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (7 DAT), stage II (11 DAT), stage III (14 DAT))

Cultivars(C)	Treatments (T)		Root volume (cm^3)			Root surface area (cm^2)		
			Stage (D)					
	Iron (Fe)	Sulphur (S)	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	0.059	0.061	0.065	10.7	12.6	13.3
		S30	0.059	0.062	0.063	9.4	11.1	12.8
		S60	0.062	0.048	0.057	7.8	8.3	11.1
		Mean	0.060	0.057	0.062	9.3	10.6	12.4
	Fe+	S0	0.063	0.064	0.065	10.0	12.9	12.9
		S30	0.057	0.057	0.056	8.1	10.5	11.3
		S60	0.064	0.051	0.050	7.0	10.0	9.9
		Mean	0.061	0.057	0.057	8.3	11.1	11.4
HD-2329	Fe-	S0	0.057	0.058	0.082	10.8	12.2	12.6
		S30	0.058	0.060	0.077	10.6	11.2	11.9
		S60	0.061	0.063	0.074	6.8	7.2	10.6
		Mean	0.059	0.061	0.078	9.4	10.2	11.7
	Fe+	S0	0.064	0.071	0.083	8.3	9.5	10.5
		S30	0.059	0.063	0.073	8.0	8.5	9.4
		S60	0.070	0.072	0.070	7.9	8.4	8.2
		Mean	0.064	0.069	0.075	8.0	8.8	9.4
HI-8713	Fe-	S0	0.063	0.077	0.085	10.4	11.5	11.4
		S30	0.069	0.075	0.070	7.2	8.4	10.8
		S60	0.072	0.072	0.073	6.7	7.4	8.7
		Mean	0.068	0.075	0.076	8.1	9.1	10.3
	Fe+	S0	0.077	0.080	0.100	8.2	9.8	12.7
		S30	0.054	0.075	0.102	7.4	9.5	10.5
		S60	0.074	0.056	0.065	6.3	7.4	8.6
		Mean	0.068	0.070	0.089	7.3	8.9	10.6
HD-4728	Fe-	S0	0.064	0.066	0.075	8.6	9.3	9.1
		S30	0.064	0.065	0.076	6.6	6.8	8.0
		S60	0.059	0.067	0.070	5.1	5.8	6.3
		Mean	0.063	0.066	0.074	6.8	7.3	7.8
	Fe+	S0	0.085	0.086	0.095	8.7	10.6	11.3
		S30	0.059	0.064	0.078	9.3	10.5	11.2
		S60	0.061	0.065	0.069	7.1	7.3	7.1
		Mean	0.069	0.072	0.081	8.4	9.5	9.9
CD at 5%		**C**		0.003			0.4	
		T		0.003			0.4	
		D		0.002			0.3	
		C X T		0.007			0.9	
		C X D		0.005			0.6	
		T X D		0.006			0.8	
		C X T X D		0.012			NS	

the Fe-S0 treatment but decreased with the availability of Fe and S. Mean root length averaged separately for the bread and durum wheat cultivars was higher for the bread than the durum species of wheat under any given treatment at all the observed growth stages.

4.2.2. Effect of Fe and S availability treatments on phytosiderophores content and release by roots under solution culture

4.2.2.1. Phytosiderophore content in root tips

Availability of phytosiderophores (PS) content in root tips is expected to determine and control the quantum release of PS in the rhizosphere to eventually regulate the uptake of Fe by the bread and durum wheat cultivars at different growth stages (Fig. 4.20). There was almost two to three fold increases in PS content in root tips under the Fe deficient than the Fe sufficient condition for the bread wheat cultivars while the durum species continue to possess a significantly lower root PS at all the stages of observation under Fe- condition. A ten folds higher root PS content in both the bread and durum wheat was observed than the root release of PS under the Fe deficient condition. Average PS content in the root tips was maximal at the stage II than the stage I and stage III. Bread wheat exhibited a higher PS content than the durum wheat cultivars. Both wheat cultivars did not reveal any significant PS content in root tips under the Fe sufficient condition.

4.2.2.2. Phytosiderophore release by roots

Root release of PS was significantly higher under the Fe deficient than the Fe sufficient condition in both bread and durum species at different growth stages (Fig. 4.20). S supply under the Fe deficient condition significantly induced the PS release. Maximum release of PS was observed under Fe-S2 when compared to all other investigated Fe and S deficient and sufficient nutrient treatment. Bread wheat cultivars showed a significantly higher release of PS than the durum wheat cultivars under Fe deficiency. Durum wheat cultivars showed a marginal increase in PS release at stage III than the stage I and stage II but even at this stage the PS release by durum were lower than the bread wheat. Further, a higher release of PS was also observed at stage III as compared to stage I and stage II of the plant growth in the bread wheat cultivars. Negligible or insignificant release of PS was recorded under the Fe sufficient nutrient condition in both the experimental wheat groups.

4.2.3. Effect of Fe and S availability treatments on plant nutrient content under solution culture

The variation in shoot and root nutrients (S, Fe, Zn and Mn) were observed under variable Fe and S supply in bread and durum wheat cultivars at stage II i.e. 11 DAT.

4.2.3.1. Shoot and root sulphur

Shoot S content was, in general, higher than the root S content across all the experimental wheat cultivars with an exception of HD-4728 under Fe+S2. Mean S accumulation in the shoot and the root tissue, when averaged over S level, was significantly higher under Fe+ than Fe- treatment. Pattern of variations for shoot S under individual deficiency of Fe and S was more or less similar in bread and durum wheat. Fe and S sufficient treatments (Fe+S0, Fe+S1 and Fe+S2) showed significant increase in S content as compared to Fe-S0 treatments. Increasing the S supply increased the root S content and highest root S was observed in Fe+S2 treatment. Durum wheat, in general, accumulated more S in roots than the bread wheat (Fig. 4.21).

4.2.3.2. Shoot and root iron

Shoot Fe, irrespective of the experimental cultivars and Fe and S treatments, was significantly lower than the root Fe. Fe content in shoots of Fe deficient plants was about half of the Fe sufficient plants across the wheat cultivars. Under different Fe and S sufficient (Fe+S2) treatments, the shoot Fe concentration showed significant increase over the Fe-S0 condition. Increase in S availability (S1 and S2) in the growing medium, under Fe deficient as well as Fe sufficient treatments, caused a greater accumulation of shoot Fe particularly in the bread wheat. In general, the bread wheat cultivars showed a greater shoot Fe accumulation than the durum wheat both under Fe deficient and Fe sufficient treatments irrespective of S supply. Root Fe, in general, was significantly higher under Fe+ than Fe- treatment when averaged over the experimental cultivars and S levels. Further, the root Fe content of the bread wheat was distinctly higher than the durum wheat cultivars under Fe deficiency, both with or without S. A distinctly reduced Fe accumulation both in the shoot and the root of the bread and the durum wheat cultivars, under lower S availability condition (S0 and S1) when compared with high S availability (S2) under both Fe+ and Fe- treatment suggests a clear regulatory role of S on Fe uptake in wheat. (Fig. 4.21).

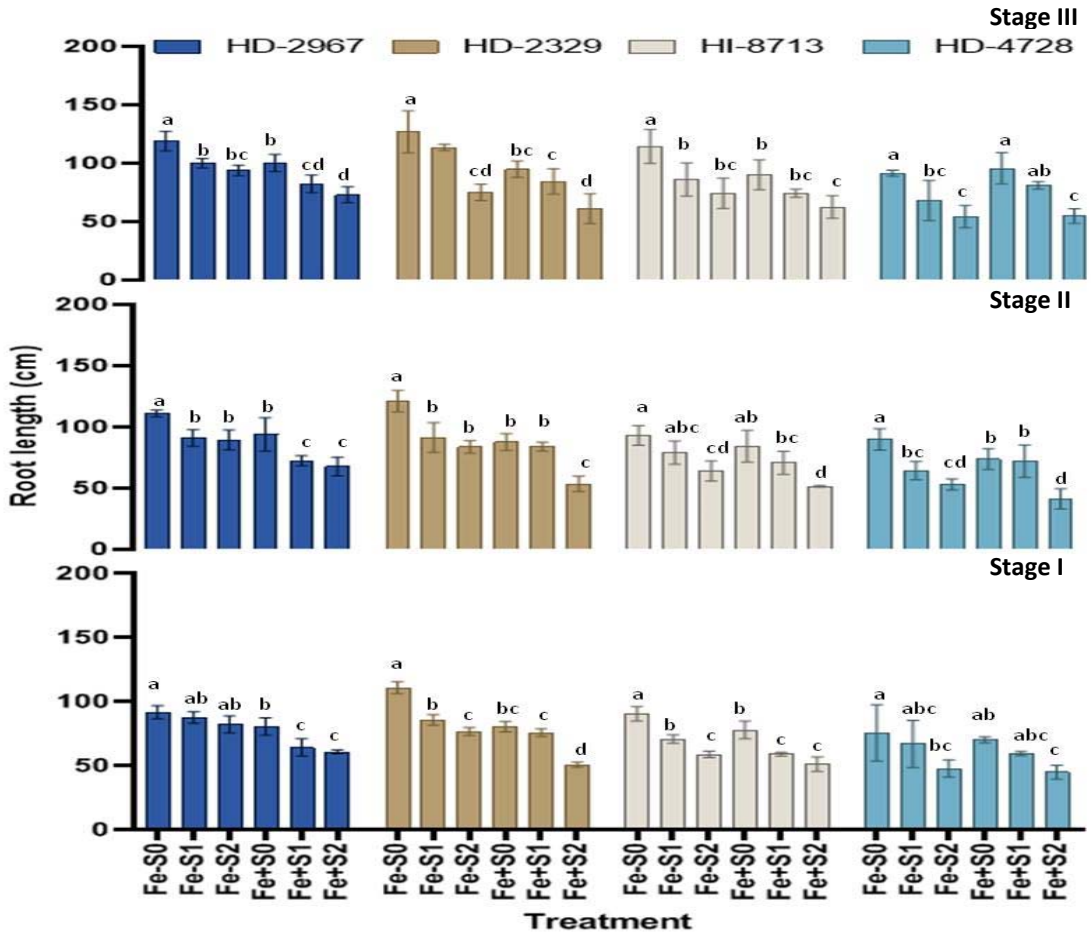

Fig.4.19: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton root length of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three stages (I, II and III) at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

Table 4.14: Interactive effect of Fe and S availabilities on root length of bread (cv. HD-2967, HD-2329) and durum (cv. HI-8713, HD-4728) wheat at different stages (stage I (7 DAS), stage II (11 DAS), stage III (14 DAS)) under solution culture

Cultivars(C)	Treatments (T)		Root length (cm)		
			Stage (D)		
	Iron (Fe)	Sulphur (S)	Stage I	Stage II	Stage III
HD-2967	Fe-	S0	91.5	111.1	119.1
		S30	87.2	91.4	99.9
		S60	82.0	89.5	93.7
		Mean	86.9	97.3	104.2
	Fe+	S0	80.3	94.0	100.2
		S30	63.9	72.5	82.4
		S60	60.6	67.7	73.0
		Mean	68.3	78.1	85.2
HD-2329	Fe-	S0	110.6	121.2	126.8
		S30	85.4	91.5	113.6
		S60	76.5	83.7	75.1
		Mean	90.8	98.8	105.2
	Fe+	S0	80.2	87.6	94.9
		S30	75.6	84.1	84.4
		S60	50.6	53.7	61.1
		Mean	68.8	75.1	80.1
HI-8713	Fe-	S0	90.2	93.1	114.4
		S30	70.6	79.1	86.1
		S60	58.6	64.0	74.2
		Mean	73.1	78.7	91.6
	Fe+	S0	77.6	84.4	90.2
		S30	58.8	70.6	74.2
		S60	50.8	51.6	62.6
		Mean	62.4	68.9	75.7
HD-4728	Fe-	S0	75.4	89.9	91.3
		S30	66.6	64.4	68.2
		S60	47.6	53.0	54.2
		Mean	63.2	69.1	71.3
	Fe+	S0	70.1	73.8	95.7
		S30	59.1	72.1	81.0
		S60	44.7	41.4	54.8
		Mean	58.0	62.4	77.1
CD at 5%		C		3.3	
		T		4.0	
		D		2.9	
		C X T		8.1	
		C X D		NS	
		T X D		NS	
		C X T X D		NS	

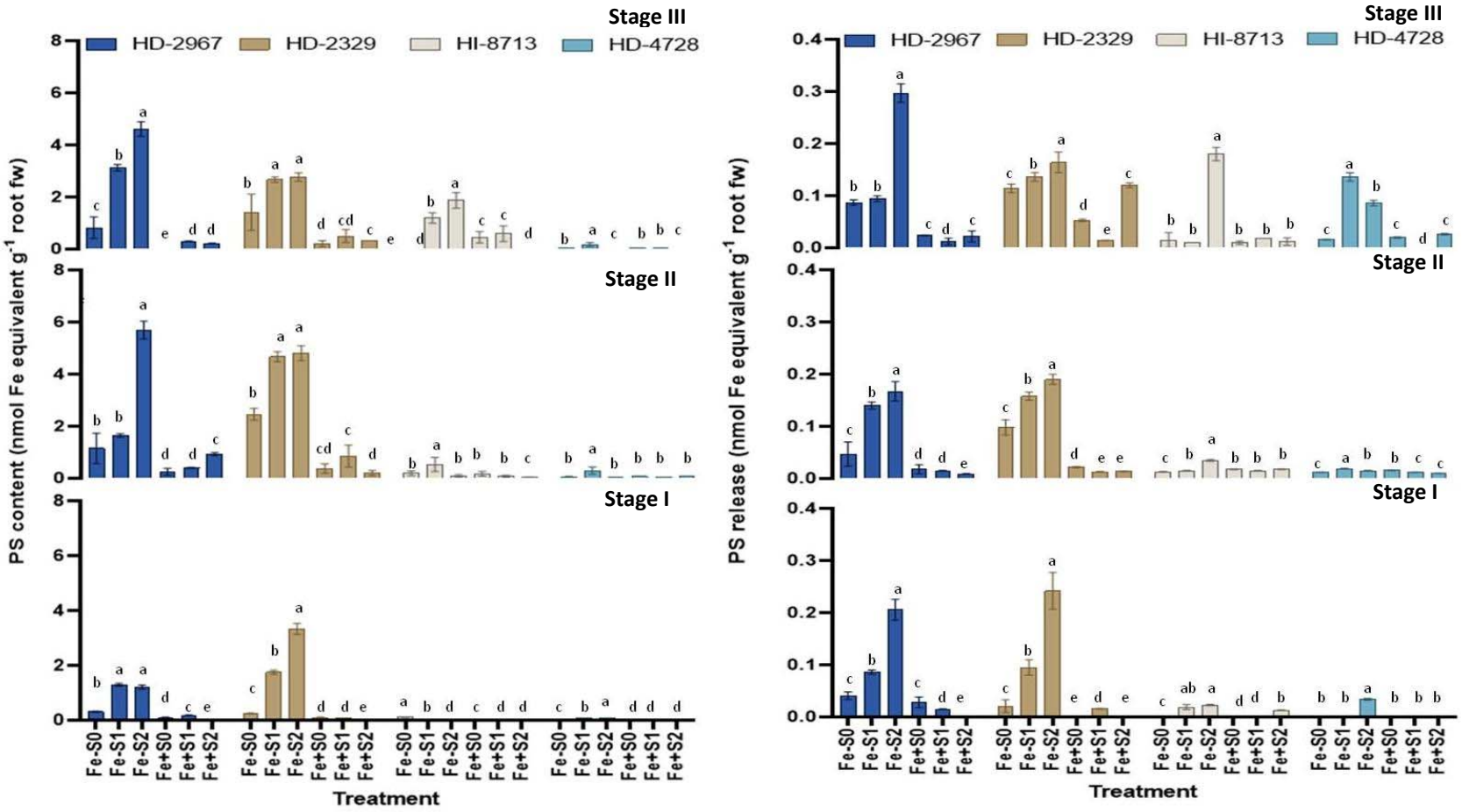

Fig.4.20: Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton phytosiderophore (PS) content and release by root tips of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three growth stages (I, II and III) at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

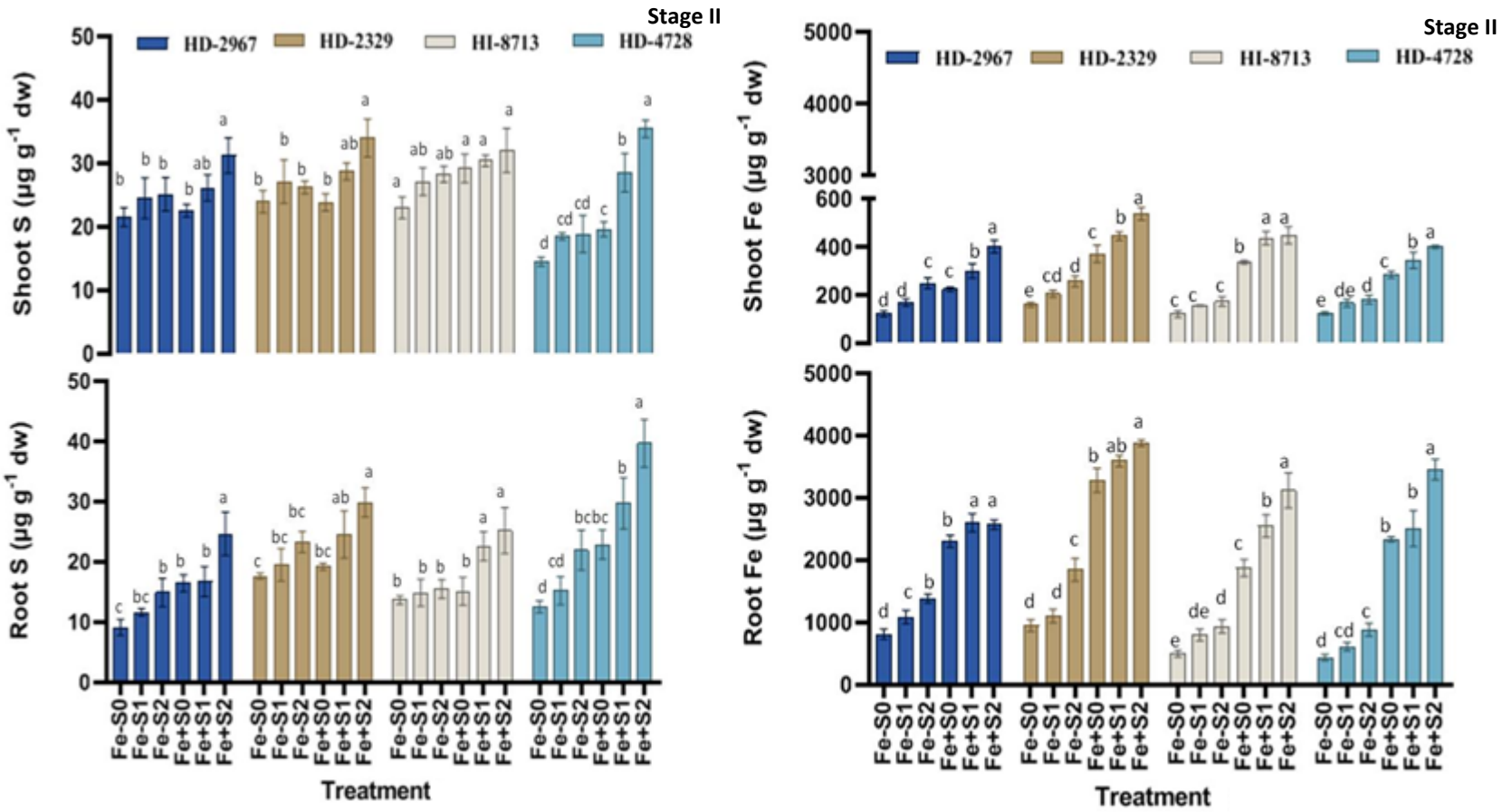

Fig. 4.21:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton shoot and rootsulphur (S) andiron (Fe) of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

4.2.3.3. Shoot and root zinc

Wheat roots, in general, accumulated a higher Zn content than the shoot across the cultivars. Availability of Fe in the growing medium had a negative effect on Zn uptake by the root and its translocation to the shoot as evident from a relatively lower mean Zn content of both shoot and root under Fe+ than under Fe- treatment. The observed pattern of variation in Zn content of root and shoot tissue was more or less similar for the bread and the durum wheat cultivars with a few exceptions. High S supply under Fe- condition significantly increased the shoot Zn content when compared with Fe-S0 treatment. However, the variation in root Zn for the similar set of treatments was relatively insignificant and hints at a shift in the pattern of root to shoot translocation of Zn in response to S availability. Zn concentration of both shoot and root, in general was higher in durum than bread wheat cultivars (Fig. 4.22).

4.2.3.4. Shoot and root manganese

Shoot of both bread and durum wheat cultivars, in general, accumulated a lower Mn than the root. Fe deficiency, either with or without S availability, caused an increase in root uptake of Mn and its translocation to the shoot. Similarly an increase in S availability, either with or without Fe, caused a decline in Mn concentration of both shoot and root across the bread and the durum wheat cultivars when compared with the S0 treatment (Fig. 4.22).

4.2.4. Radiotracer studies (^{59}Fe and ^{35}S) to determine interactive regulation of S by Fe

Effects of Fe on S uptake and vice versa were evidenced using elemental radiotracers ^{59}Fe and ^{35}S in both bread and durum wheat cultivars at stage II i.e. 11 DAT. Mean ^{35}S concentration of both shoot and root when averaged over the S treatments; in general, was significantly lower under Fe- than Fe+ condition (Fig. 4.23). However, between the S availability treatments, a dose dependent decline in ^{35}S concentration in shoot and root was measured with increasing S supply when compared to the S0 control under both either with or without Fe across the bread and the durum wheat cultivars. This decline in ^{35}S concentration under S2 than S0 or S1 condition was related to the S nutrition or perception of hunger for sulphur which was higher under S0 than S1 and S2 in descending order. HD-2967 showed highest shoot ^{35}S accumulation under both Fe sufficient and deficient treatment and was followed

by HD-4728, a durum wheat cultivar. Root ^{35}S concentration of HD-2967 on the other hand was lowest under both Fe- and Fe+ treatments when compared with other experimental wheat cultivars. Data on ^{35}S uptake expressed as Bq per shoot, per root and per plant basis is presented in Fig. 4.24. Higher shoot ^{35}S uptake than the root ^{35}S uptake both under Fe+ and Fe- condition was measured in bread and durum wheat cultivars. ^{35}S uptake is in following order in wheat cultivars HD-2967 > HD-4728 > HD-2329 > HI-8713. Reduction in shoot ^{35}S uptake was evident under increasing S supply irrespective of the Fe availability conditions. More or less a similar trend was measured for root and plant ^{35}S uptake. Bread and durum wheat cultivars exhibited not only a high uptake of ^{35}S but also a higher ^{35}S translocation under Fe sufficient than Fe deficient treatments (Fig. 4.25). The root to shoot ^{35}S translocation index declined with increase in S availability under both Fe+ and Fe- treatments particularly in HD-2329 and HI-8713 wheat cultivars. Bread wheat cultivars HD-2329 showed the highest translocation index under Fe sufficient than Fe deficient condition irrespective of S availability across the experimental wheat cultivars.

In contrast, a reverse pattern of variation to ^{35}S concentration was measured for the plant ^{59}Fe concentration under different Fe/S treatment combinations (Fig. 4.26). In general, the Fe deficient plants showed a higher ^{59}Fe concentration than Fe sufficient plants and bread wheat cultivars showed a greater accumulation of ^{59}Fe than the durum wheat. S availability in the growing medium, both in the presence or absence of Fe, improved the ^{59}Fe uptake across all the experimental wheat cultivars. Highest ^{59}Fe activities were measured in S2 treatment both with Fe- and Fe+ treatment however, the quantum activity accumulation was significantly higher under Fe- treatment.

Efficiency for ^{35}S uptake in relation to ^{59}Fe uptake is presented in Fig. 4.26 and clearly reflects a positive effect of Fe availability on S uptake particularly under conditions of limited S availability for plant growth. ^{35}S to ^{59}Fe ratio was highest along the experimental cultivar under Fe+S0 condition but declined with S supply. On the other hand, even Fe-S0 treatment showed a higher ^{35}S to ^{59}Fe ratio when compared to other S availability treatment within the group but the ^{35}S to ^{59}Fe ratio under Fe- treatment was significantly lower than the Fe+ treatment with highest values being observed for HD-2967 > HD-4728 > HD-2329 > HI-8713.

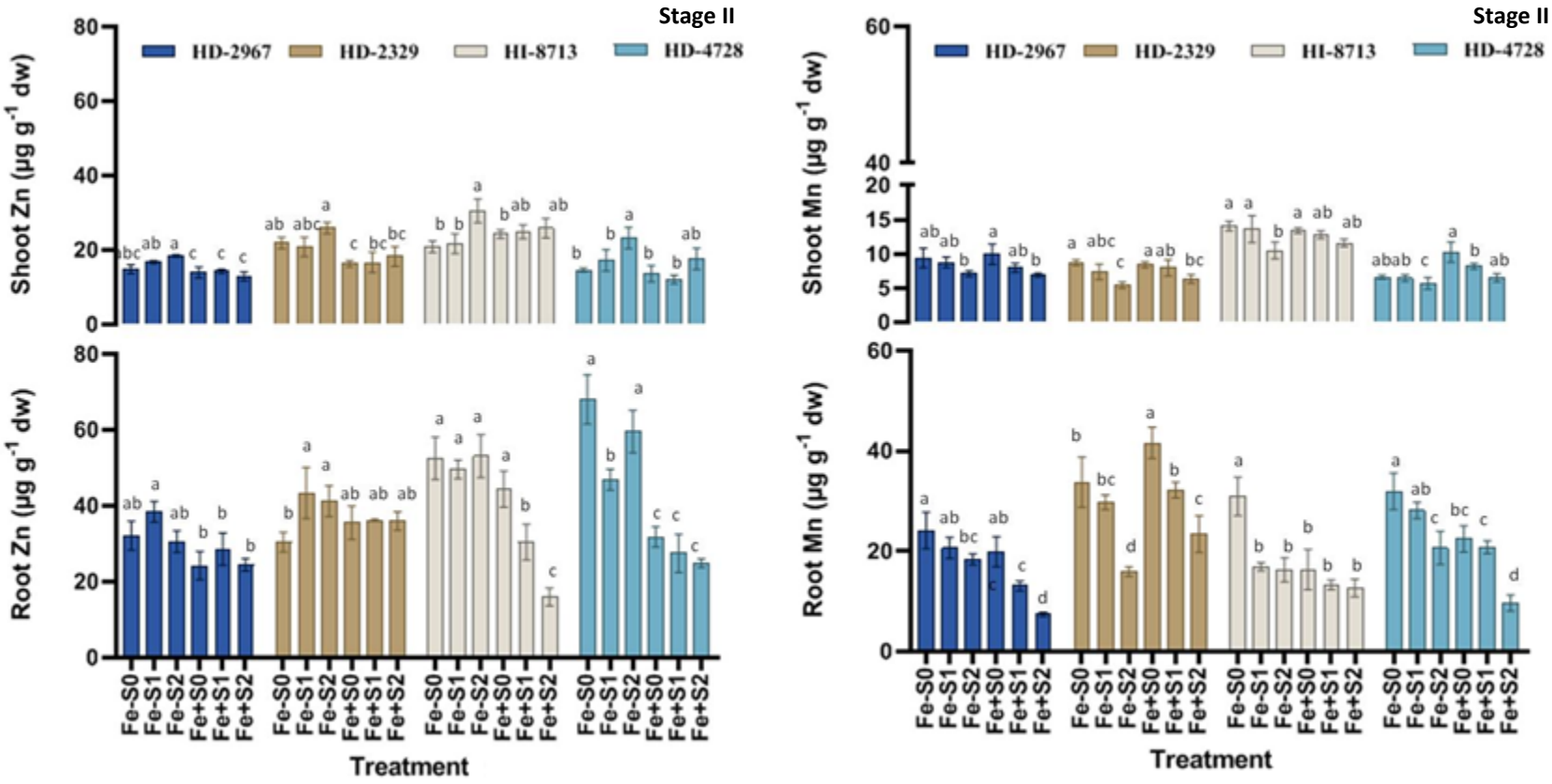

Fig. 4.22:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton shoot and root zinc (Zn) and manganese (Mn) of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

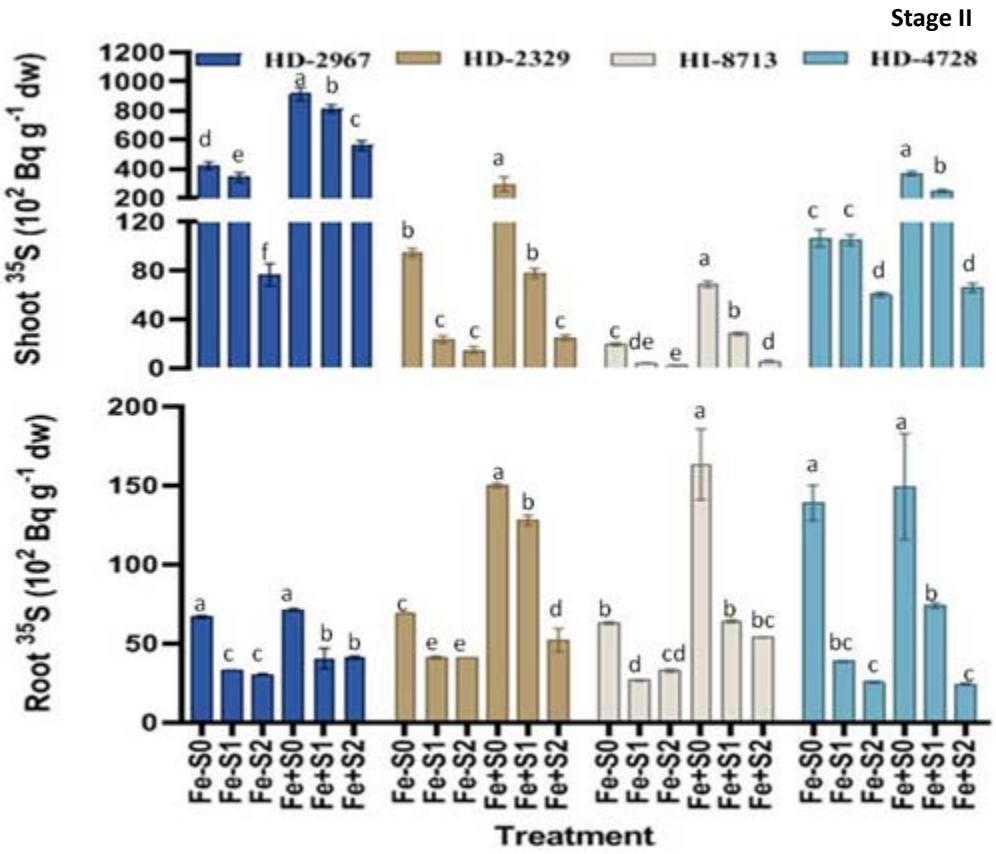

Fig. 4.23:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton shoot and root ^{35}S concentration of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

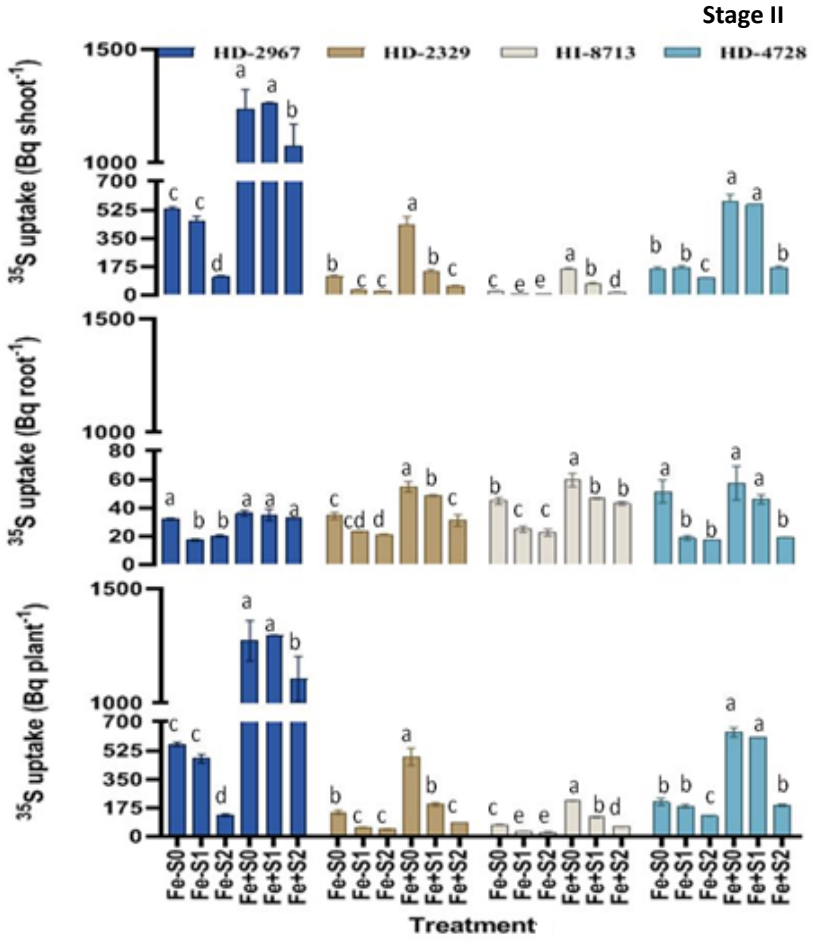

Fig. 4.24:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton shoot and root ^{35}S uptake of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

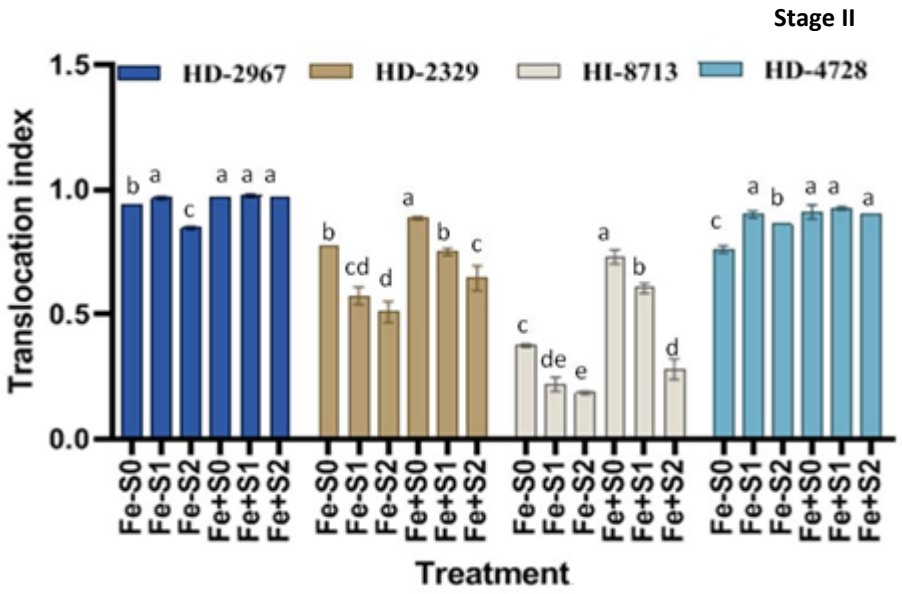

Fig. 4.25:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatment on ^{35}S translocation index of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different ($P < 0.05$) as analyzed by Duncan's multiple range test.

4.3. To determine the transcript level expression of sulphate transporters *SULTR1;1* and *SULTR2;1*, Fe-PS transporter *YS1* and nitrogen transporter *NRT2.1* in relation to root and shoot S, N and Fe.

The effect of Fe and S availability on bread and durum wheat cultivars was measured in terms of transcript level expression of S, Fe and N transporters i.e. *SULTR1;1, SULTR2;1, YS1* and *NRT2.1* in roots under different combinations of Fe and S availability in the nutrient solution culture (Fig. 4.27). *SULTR1;1*, a high affinity sulphate transporter was significantly induced under the sulphur deficient condition either with or without Fe both in bread and durum wheat cultivars and decreased with an increase in S supply in a dose dependent manner. Presence of Fe in the nutrient solution culture induced the expression of *SULTR1;1* genes and maximum expression was observed under Fe+S0 treatment when compared to the other nutrient treatment combinations for all the investigated wheat cultivars. Highest expression of *SULTR1;1* gene was recorded in HD-2967 and HD-4728 cultivars under Fe+S0 condition. An inverse pattern of expression was exhibited by *SULTR2;1*, a low affinity sulphate transporter. S sufficient treatment induced the expression of *SULTR2;1* in both the bread and the durum wheat cultivars. S deprivation led to an almost three fold reduction in the expression of *SULTR2;1* as compared to S sufficient condition especially under Fe sufficient (Fe+S1 and Fe+S2) treatment. HD-2329 cultivars showed a least expression of *SULTR2;1* when compared with the other cultivars while a relatively higher expression of the gene was observed in the durum than the bread wheat cultivars. An increase in the S supply under the Fe sufficient condition caused a significant increase in the expression of *SULTR2;1* while insignificant treatment differences were observed under the Fe deficient condition.

YS1, a Fe-PS complex transporter in the root was significantly induced under the Fe deficiency and its expression was dependent on S availability. Higher S supply significantly increased the expression levels of YS1 in both bread and durum wheat cultivars more so in a dose dependent manner. Bread wheat cultivars exhibited a higher transcript expression of *YS1* gene as compared to durum wheat cultivars. Maximum *YS1* gene expression was observed under Fe-S2 condition while insignificant treatment variations were observed in the expression of *YS1* under varying S supply condition under the Fe sufficient treatment.

NRT2.1, a high affinity nitrate transporter showed a higher expression under the Fe deficient than Fe sufficient condition across the wheat cultivars. *NRT2.1* expression was maximum for cultivars HD-2967 and HD-4728, while the other bread and the durum wheat cultivars (HD-2329 and HI-8713, respectively) showed a significantly lower transcript expression of *NRT2.1* chiefly under the Fe deficient than the Fe sufficient conditions of growth. Further, the transcript level expression of *NRT2.1* showed a significant increase with S supply but only under Fe- condition for the cultivars HD-2967 and HD-4728 while no distinct relationship between the Fe and S supply and the expression of *NRT2.1* could be established for the remaining experimental cultivars i.e. HD-2329 and HI-8713. The present results highlighted the interactive influence of Fe and S supply on growth and development of wheat species at the morphological physiological, biochemical and molecular level and are discussed critically in light of the available literature in the followed chapter.

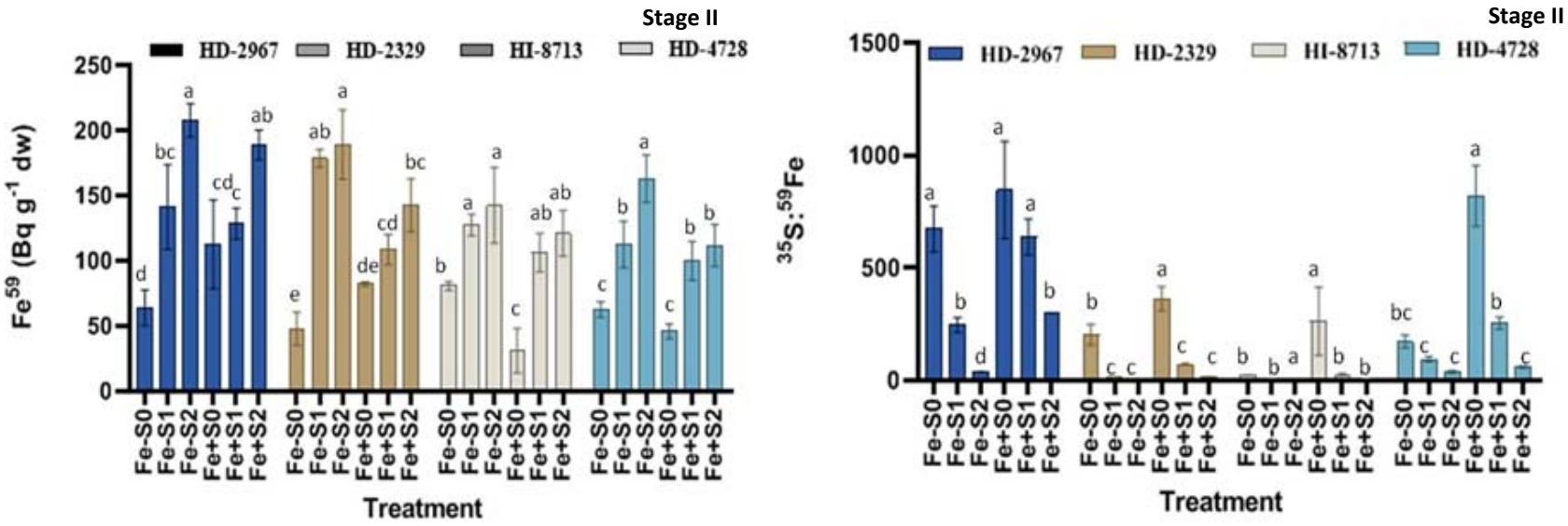

Fig. 4.26:Effect of Fe deficient (Fe-) and Fe sufficient (Fe+) treatmenton ^{35}S to ^{59}Fe ratio of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat cultivars at three sulphur levels (S0, S1 and S2). Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

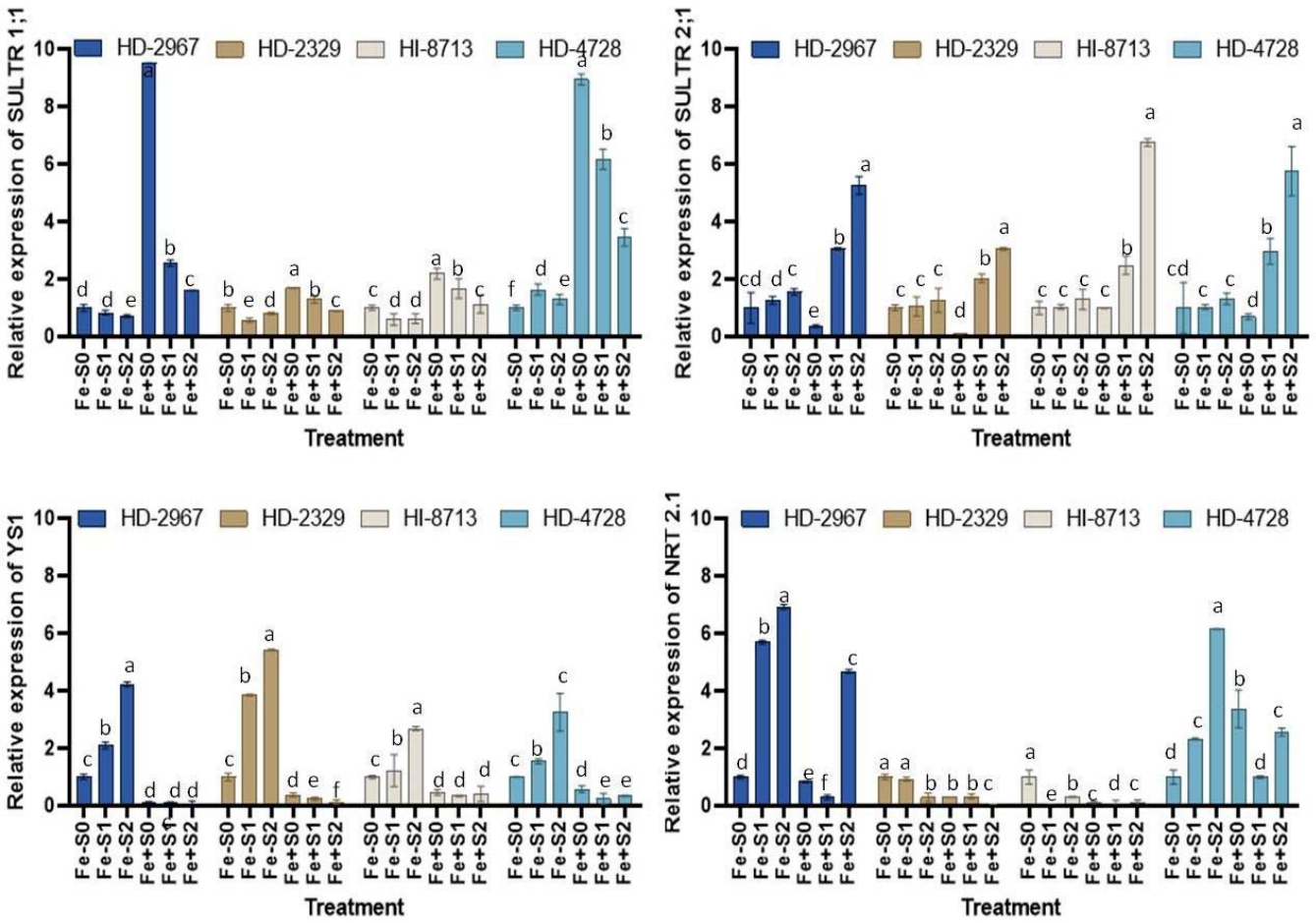

Fig. 4.27: Relative transcript abundance of *SULTR1;1, SULTR2;1,YS1* and *NRT2.1* in the roots of bread (HD-2967 and HD-2329) and durum (HI-8713 and HD-4728) wheat grown under different iron and sulphate supply.Presented values are mean ± SD (n=3). Data bar with different alphabet, within a cultivar, are significantly different (P < 0.05) as analyzed by Duncan's multiple range test.

Chapter 5

DISCUSSION

Plants synthesize necessary molecules from minerals, water, and light to complete their life cycle. However, the production of these cellular metabolites is not uniform and changes with environmental conditions and under abiotic and biotic stress. Further under these conditions, plants also resort to dynamic allocation of resources into the metabolic processes to regulate growth, development and nutrient assimilation. The efficient resource utilization is particularly important when resources are scarce (i.e. nutrient limitation) or when resources need to be diverted to support other critical processes such as defense (Yang *et al.*, 2012). Plants have evolved mechanisms to sense resource availability and regulate growth and development accordingly. The present study delves into the physiological, biochemical and molecular basis of interactive regulation of growth and development of bread and durum wheat by iron (Fe) and sulphur (S) availability in soil and nutrient solution culture.

Literature is rampant with research that talks about the individual regulatory roles of Fe and S, but only a few of them speak on the possible cross talk between these two very important essential mineral nutrients (Mendoza-Cózatl *et al.,* 2019). Role of S nutrition in ameliorating the effect of Fe shortage by regulating the production and release of the chelating compounds that mobilize unavailable form of Fe (Fe^{3+}) in the rhizosphere is amply documented (Foreiri *et al*., 2013; Muneer *et al*., 2013). However, a reversal of regulatory relationship is also postulated (Astolfi *et al*., 2004) and an absolute requirement of Fe for induction of HATS has been reported (Sharma *et al*., 2018). Iron–sulfur (Fe–S) clusters play a central role in regulating the carbohydrate, protein and the nucleic acid metabolism. Fe–S clusters, owing to their main components i.e., Fe^{2+} and S^{2-}, are highly reactive. It is thus important to decipher and understand the mechanisms that regulate the uptake, assimilation, translocation and utilization of Fe and S. Further, these mechanisms may be differentially regulated depending on the dynamics of crosstalk between Fe and S networks. Recent researches reveal that there is an active crosstalk between regulatory networks controlling the uptake and use of nutrients (Forieri *et al.*, 2013; Zuchi *et al.*, 2015; Hantzis *et al*., 2018). This section surmises the dynamics of Fe and S

interaction viz. a viz. the regulatory control of Fe and S uptake and their utilization with emphasis on Fe deficiency responses and their interaction with S metabolism.

Availability of nutrients is considered to be a major requirement for determining plant growth and productivity. Wheat crop requires ~15-30 kg S ha^{-1} for optimum growth and development and the demand for S is particularly higher at the reproductive than the vegetative stage of growth. In the conducted experiment, the combined Fe and S availabilities resulted in significant increase in shoot biomass in bread and durum wheat at all stages of crop growth i.e., Stage I, II and III. These results are in agreement with the previous studies which reported an increase in the shoot biomass in wheat (Salvagiotti and Miralles, 2008) and barley (De Bona *et al.,* 2011) under condition of optimum S-availability. A similar affect of S-supply was observed by Ali *et al*. (2012) who reported an increase in shoot biomass and yield attributes at an increasing S-supply from 0 to 75 kg S ha^{-1} in wheat, with maximum yield at 50 kg S ha^{-1}. Different other groups also demonstrated similar results citing an appreciation in shoot biomass under optimum S-application (Eriksen and Mortensen, 2002; Kumar *et al*., 2014; Singh *et al*., 2014). Further, an absolute requirement of Fe for sustaining wheat growth and productivity has been reported (Oburger *et al.,* 2014; Divte *et al*., 2019; Kaur *et al*., 2019). They showed that there was significant reduction in shoot biomass of bread and durum wheat cultivars in response Fe deficiency. A combined optimum application of Fe and S in soil is thus critical to derive favourable growth characteristics (Ciaffi *et al*., 2013).

Availability of mineral nutrients is known to, either directly or indirectly, impact various physiological processes such as increased cell division and expansion, enzymatic activities and photosynthesis (Buresh *et al*., 1993; Singh and Chatterjee, 1980; Shanmugam and Veeraputhran, 2000; Meena *et al*., 2013). In the present study, a significant decline in photosynthetic rate observed under combined deficiency of Fe and S (Fe-S0) over the Fe and S sufficient (Fe+S1 and Fe+S2) treatment, might be related to the observed reduction in chlorophyll content under Fe deficiency, Fe being an essential component of the photosynthetic process. Various findings showed that Fe deficiency reduces the chlorophyll content, diminish the activity of stromal enzymes and disrupts the structure of thylakoid membrane, to adversely affect the net photosynthetic rates (Hurley *et al*., 1986; Morales *et al*., 1994; Bertamini *et al*., 2001; Chouliaras *et al*., 2004). A reduction in net photosynthetic rate (Pn) in leaves of pea

due to lime induced Fe deficiency was also observed by Nenova, (2009). Besides Fe, S deficiency, in the present study, also adversely affected the photosynthetic efficiency and a combined deficiency of Fe and S showed a much more drastic reduction in the Pn as compared to their individual deficiency of Fe and S. Burke *et al.* (1986) have also reported a significant reduction in the chlorophyll content of individual chloroplasts and chlorophyll content per unit leaf area and thus the net photosynthetic rate under S deficiency. The observed decrease in photosynthesis under individual or combined deficiency of Fe and S, may be attributed to the reduction in shoot biomass. Durum wheat which possessed a more robust photosynthetic machinery even under nutrient deficient condition of growth showed a relatively higher shoot mass than the bread wheat cultivars which showed a relatively lower Pn particularly at stage II (60 DAS). Further iron deficiency has also been suggested to impair transpiration rates by influencing the stomatal conductance in peach (Eichert *et al*., 2010). A similar pattern of variation under Fe deficiency was observed in case of the transpiration rate (E) in the present study while the affect on the stomatal conductance (Gs) was not discreet.

Shoot S concentration significantly increased with an increasing S supply to the Fe sufficient (Fe+) as well as Fe deficient (Fe-) soil. The Fe sufficient soil showed a relatively higher amount of shoot S concentration than the Fe deficient soil. Ercoli *et al.* (2012) while working with durum wheat observed that an application of fertilizer sulphur significantly increased the S content throughout the plant, with a highest increase at S_{60}. The accumulation of S also increased with the plant growth in both the experimental wheat species, i.e., the bread and the durum wheat, which might be related to the higher demand for S at the reproductive stage of plant growth. The combined effect of Fe and S application on shoot S accumulation has been reported in durum wheat and revealed a higher accumulation of S under the Fe deficiency, which is in contrast to our findings (Astolfi *et al*., 2003; Zuchi *et al.,* 2012). On the other hand, work of Kumavat *et al*. (2006) which revealed an increase in S uptake under Fe+ condition, clearly strengthens the findings of the present investigation.

Sulphur has also been shown to contribute to the nutritional status and quality parameters of the grains in winter wheat (Gyori, 2005). An increase in grain S content under increasing Fe and S availability was measured. This result is in agreement with previous studies that showed the significant effect of sulphur fertilizer on the yield

and yield components of wheat (Inal *et al*., 2003; Khan *et al*., 2003; Girma *et al*., 2005). An increase in shoot and grain Fe under an increasing S-supply (S0 < S1 < S2) was observed for the investigated bread and durum wheat cultivars in the present study while a significant decline in the shoot Fe was quantified under Fe deficiency. These results reveal synergy with the findings in the literature which showed an increased Fe concentration in the shoots of wheat and several other crops such as barley, tomato, etc under an increasing S-supply (Astolfi *et al*., 2003; 2004; 2006a; b; 2009; Zuchi *et al*., 2012; Wu *et al*., 2014) and indicate that the sulphur availability helps the plant to cope with the Fe deficiency. The interactive effect of Fe and S are species specific and, in general, cereals such as wheat, barley, rice and some of the other crops such as tomato, cucumber, etc. exhibit crosstalk between Fe and S networks to regulate their uptake (Li *et al*. 2007; Ciaffi *et al*., 2013; Paolacci *et al*., 2014; Forieri *et al*., 2017; Garnica *et al*., 2018).

Sulphur deprivation caused a decrease in shoot N content, in the present study. A maximum shoot N was observed under Fe+S2 condition particularly at Stage I (30DAS) while at other growth stages the shoot N was influenced principally by S than the Fe availability. Synergistic relationship between S and N uptake has been reported in wheat and other crops (Salvagiotti, 2009; Rossini *et al.,* 2018; Dhillon *et al*., 2019). An insignificant affect of Fe on N uptake, as observed in the present study, is amply supported by Abbas *et al*. (2012) who also observed an insignificant effect of Fe on not only N but also on P and K content of wheat crop.

Another important plant attribute of consequence to improving the grain quality is the shoot to grain nutrient translocation. The shoot to grain translocation of S was not significantly affected by the S-supply, while the Fe availability increased the translocation of S, although marginally. On the other side, translocation of Fe from shoot to grain was significantly affected by both Fe and S supply and there was a reduction in TI of Fe under increasing availability of Fe and S. Shoot to grain TI of N showed a significant decline under the increasing level of S, while it was unaffected by the Fe availability. A synergistic effect of N on the uptake, translocation and accumulation of Zn in grains of wheat has been reported (Erenoglu *et al*., 2011). SUE, IUE and NUE were found to decrease under the nutrient sufficient (Fe+S2) as compared to the nutrient deficient condition (Fe-S0). The observed decline in sulphate uptake and translocation under Fe deficiency, with a few exceptions, may be

explained on the basis of the low sulphur demand for protein synthesis (De Kok *et al.*, 2002; Anderson and Fitzgerald, 2003).

OASTL/SAT the two key enzymes of sulphate assimilation are responsible for catalyzing the final step for the biosynthesis of cysteine, which is a major connecting link between sulphate, nitrate and carbon assimilation (Haas *et al*., 2008; Koprivova and Kopriva, 2014). The carbon skeleton and the nitrogen of cysteine are shown to originate from serine (Ho and Saito, 2001), where the nitrogen in serine is a product of nitrogen reduction and assimilation (Kopriva and Rennenberg, 2004). SAT plays an important role in the regulation of cysteine biosynthesis, which might be limited by the availability of OAS (Wirtz *et al*., 2004) and a relatively higher activity of OASTL than SAT is desirable for efficient sulphate assimilation (Heeg *et al*., 2008). In light of the significance of the above two key enzymes in S-assimilation and their suggested regulation by carbon and nitrogen, the affect of Fe and S nutrition on the activity of SAT and OASTL were determined in the present study. An increase in the SAT and OASTL activities were determined under the increasing S supply both under Fe deficient and sufficient conditions of growth. Further, the activity of both the enzymes depended on Fe nutrition, as evident from a significantly higher SAT activity observed at stage II and a significantly higher OASTL activity at both stage I and stage II for all the investigated bread and durum wheat cultivars under the Fe sufficient (Fe+) than the Fe deficient (Fe-) treatment. Ciaffi *et al.* (2013) studied the effect of Fe and S deprivation on sulphate uptake and assimilation pathways in terms of activity and transcript level expression of genes coding for enzymes involved in sulphate assimilation and reduction in durum wheat. Their results showed an insignificant effect of Fe and S nutrition on the OASTL enzyme activity and the expression of *TdSAT2* but reported a higher expression of *TdOASTL1* in the shoots of the durum wheat under the Fe+S0 than the Fe-S0 treatment.

Activity of the key enzyme of nitrogen assimilation pathway i.e., nitrate reductase (NR) was also assessed under variable availabilities of Fe and S in the bread and the durum wheat cultivars grown on field soil. In general, an increase in NR activity was observed under optimum availability of both Fe and S. In line with the present results, a similar decline in the plant NR activity and an accumulation of amino acids was reported under S deficiency by several researchers (Reuveny *et al*., 1980; Migge *et al*., 2000; Prosser *et al*., 2001). However, the reduction of NR activity

and mRNA levels occur later during the process of plant adaptation to sulfur-limiting conditions (Prosser *et al*., 2001). Castignetti and Smarrelli, (1986) while working out the the NR activity in squash cotyledon hypothesized that NR activity in higher plants was also involved in iron acquisition via phytosiderophores. Further, the Fe deficiency, Fe being an integral component of the NR, caused an expected reduction in NR activity while it was boosted under the Fe sufficient condition. Borlotti *et al.* (2012) reported a Fe deficiency induced decline only for the nitrate reductase activity (both at the root and leaf level) but not on the glutamine synthetase and glutamate synthase activity, which in fact increased significantly under the Fe- than the Fe+ treatment in cucumber. Activity of RuBPCase, or rubisco, an enzyme involved in the first major step of carbon fixation, showed a significant increase under the $Fe+S+^{(1,2)}$ than the Fe-S0 treatment in the present study. Muneer *et al*. (2013) also observed a similar decline in the activity not only for rubisco but also for sucrose synthase and net photosynthesis in response to Fe-deficiency in the absence of S i.e., Fe-S0. The present and other findings in the literature clearly indicate that S nutrition plays a significant role in alleviating chloroplast damage caused by Fe-deficiency and that sulphur deficiency affected CO_2 assimilation rates, rubisco enzyme activities and protein abundance (Chandra and Pandey, 2014). Gilbert *et al*. (1997) showed that the sulphate deprivation has no latency for effects on the ribulose-1,5-bisphosphate carboxylase/oxygenase activity and photosynthesis in young leaves of wheat.

S-availability has been amply documented to influence the synthesis of S containing amino acids, methionine and cysteine. However, our recent understanding that there are large number of proteins which require a metallic co-factor for their function, Fe availability is also being linked lately with the regulation of amino acids which occurs mainly through the formation of Fe-S clusters (Rocha and Dancis, 2016; Tauraine *et al*., 2019). However, there are insufficient reports available on the interactive regulation of amino acid biosynthesis under the individual or combined deficiency of Fe and S in crop plants. Availability of serine is a prerequisite for optimum synthesis of cysteine via methionine in the sulphate assimilation pathway (Hell and Wirtz, 2011). Serine is also known to regulate the biosynthesis of phytosiderophore (PS) under Fe deficiency, where the synthesis is casually related to the Fe deficiency tolerance of crops particularly the graminaceous species. Further, the nitrogen in the serine is a product of nitrogen reduction and assimilation (Kopriva

and Rennenberg, 2004). The above linkages suggest that serine may be the most potent regulator determining interactive response of S, F e and N in plants. The present results showed a higher accumulation of serine, methionine and cysteine in the leaves of the bread than the durum wheat. A higher content of serine under the Fe+S0 than the Fe-S0 in both the bread and the durum wheat species was observed. Further, an increasing S-supply reduced the serine content of the leaves (S0 < S30 < S60) although the affect was more under Fe- than under Fe+ condition. The decline in serine content coincided with an increase in the methionine and the cysteine content at higher availability of S. An increase in S-containing and free amino acids in the presence of optimum S- supply has been reported in several crops (Zhao *et al*., 1999; Jarvan *et al*., 2008; Veliz *et al*., 2017). A look at the amino acid biosynthesis pathway clearly shows that any availability of serine in excess of its requirement for the cysteine synthesis is channelled for the synthesis of glycine (Hildebrandt *et al*., 2015). The glycine content was higher under the Fe+ than the Fe- condition under S deficiency, which showed a further decline with S-supply (S0 > S30 > S60) in the bread wheat but not the durum wheat. The observed lower glycine content under the Fe- treatment and its decline with S-supply, which was more under the Fe- than the Fe+ treatment, only for the bread wheat may be related to a relatively lower allocation of the serine for the glycine biosynthesis, owing to the formers higher allocation for the synthesis of the methionine, which is a substrate both for the cysteine and the phytosiderophore biosynthesis. The explanation draws support from the observed higher content of the methionine and the cysteine in the bread wheat than durum wheat, under the Fe- treatment with an increasing S-supply. A significantly higher biosynthesis and release of PS under optimum availability of S has been reported in the bread than the durum wheat (Sharma *et al*., 2018). The results also open new vistas to probe the role of N nutrition in influencing the serine content, which may be playing a balancing role in interactive regulation of Fe and S in plants.

Regulatory influence of Fe on S response in plants was also elucidated under the nutrient solution culture conducted with the bread and the durum wheat cultivars under variable level of Fe and S i.e., Fe and S deficient and sufficient solutions i.e., 1 and 100 μM Fe^{3+} and 0, 1.2 and 2.5 mM SO_4^{-2}. Individual or combined deficiency of Fe and S caused a significant reduction in root and shoot biomass across the experimental wheat cultivars while the application of S with or without Fe bettered

the respective tissue dry matter accumulation more so in a dose dependent manner. A similar decline in root shoot mass under Fe deficiency either with or without S has been reported in barley (Astolfi *et al*., 2009; 2011), tomato (Zuchi *et al*., 2015), wheat (Zuchi *et al*., 2012) and rice (Wu *et al*., 2014). However, the severity of the nutrient deficiency response was more pronounced in shoot than the root tissue (Zuchi *et al*., 2009; 2012; Astolfi *et al*., 2009). Reduction in root and shoot mass followed the order Fe+S2,1 > Fe-S2,1 > Fe+S0 > Fe-S0. A pattern similar to that observed above was recorded by Astolfi *et al*. (2011). The individual or combined deficiency of Fe and S in general, improved the root shoot ratio when compared with Fe and S supplemented plants which may be attributed to relatively lower partitioning of photosynthates for root growth and development under nutrient sufficient condition. While supply of S to Fe deficient plants improves their sensitivity to Fe deficiency and promotes root to shoot ratio. However, a clear trend for this trait between bread and durum wheat could not be established. An increase in root: shoot ratio under Fe deficiency has been reported across crops (Chen *et al*., 2018). A higher root to shoot ratio of the durum than the bread wheat under Fe deficiency suggests that the durum wheat cultivars are relatively more sensitive to Fe deficiency than the bread wheat.

Root growth characteristics such as root volume, root surface area and root length are important to extract sufficient amount of nutrient from the soil solution under the condition of nutrient deficiency. Root volume, in the present study, did not differ significantly while a significant increase in the root surface area was observed for both the bread and the durum wheat cultivars under the individual and the combined deficiency of Fe and S. A similar result on root attributes was obtained in palm tree though only under Fe deficiency by Nurmalasari *et al.* (2016). Root length was significantly increased under the combined deficiency of Fe and S as compared to Fe and S sufficient condition in both bread and durum wheat cultivars at all the three growth stages. S deficient plants also showed an increase in the root length. Increase in root length and surface area under the condition of limited nutrient availability, is regarded as an important mechanism which helps the plant to adapt under nutrient stress and to achieve a sustained uptake of nutrients under these condition by exploiting a larger soil volumes (Gruber *et al*., 2013). A similar trend for the root length has also been reported under N and phosphorus deficiency (Schippers and Olff, 2000; Trubat *et al*., 2006).

A negative correlation between plant growth attributes and chlorosis was measured in Trifolium cultivar (Wei *et al*., 1994). More or less a similar effect of Fe deficiency on leaf chlorophyll was measured for bread and durum wheat cultivars in the present study and that the affect was independent of S availability when compared with the Fe sufficient treatment. Individual or combined deficiency of Fe and S was also shown to cause Fe chlorosis of sugarcane, tomato and soybean (Mathur *et al*., 1994; Zuchi *et al*., 2009; Raj *et al*., 2019). Regulatory role of Fe and S on chlorophyll biosynthesis was reported by Garai and Trupathy (2018), who overexpressed uroporphyrinogen methyltransferase 1 (UPM1) to understand the interactive influence of Fe on N and S assimilation in Arabidopsis and suggested that siroheme, an iron containing tetrapyrole and a prosthetic group of NiR and SiR, synthesized from uroporphyrinogen III, a chlorophyll biosynthesis intermediate, is required for the N and S assimilation.

Role of phytosiderophores in regulating Fe deficiency tolerance is well known (Banakar *et al*., 2017; Masuda *et al*., 2017; Divte *et al*., 2019). Biosynthesis and release of PS by the roots is reported to occur in gramineae species only under Fe deficiency. Under Fe deficiency, an increase in the synthesis of S containing amino acid methionine and which are converted to S-adenosyl methionine is reported to control the biosynthesis of PS (Mori and Nishizawa, 1987). Khobra and Singh, (2018) and Divte *et al.* (2019) showed that Fe availability determines the PS production by regulating the partitioning of SAM between the ethylene and the PS biosynthesis pathways and that the synthesis of PS is also induced under deficiency of other metal micronutrients. Sharma *et al.* (2018), on the other hand, provided evidence for the role of S availability on Fe deficiency induced PS biosynthesis. The present findings were in league with the above results and showed a higher production of PS under the Fe-S+ than the Fe+S- treatment, more so in the bread than the durum wheat. The synthesis and release of PS was observed to be maximum under the Fe-S2 treatment in the bread wheat. A decrease in sulphur availability caused a reduction in PS release and thus the ability of the plant to cope with the Fe deficiency (Astolfi *et al*., 2006; Astolfi *et al*., 2011, Ciaffi *et al*., 2013, Sharma *et al*., 2018). Durum wheat, on the other hand, showed a significantly lesser root content and release of PS than the bread wheat and hence was more sensitive to the Fe deficiency (Cakmak *et al*., 1998; Sharma *et al*., 2018).

The shoot and root Fe concentration across the experimental wheat cultivars depended principally on Fe nutrition with Fe sufficient plants showing significantly higher Fe accumulation than the Fe deficient treatment when averaged over the S availability condition. However, between Fe deficient and Fe sufficient treatments the accumulation of Fe in shoot and root tissues varied significantly with S availability and increased in a dose dependent manner. also measured an increase in Fe uptake in the shoot of with increasing S supply. A similar effect of S on Fe uptake was also measured in durum wheat, barley and in rice (Zuchi *et al*., 2012; Astolfi *et al*., 2011; Wu *et al*., 2014). Mean average shoot and root S level irrespective of S supply were lower under Fe- than Fe+ condition across the experimental wheat cultivars. A significant increase in both root and shoot S was measured under S deficiency in the presence than absence of Fe. An increase in S supply irrespective of Fe availability condition caused an increase in both root and shoot S concentration moreso in a dose dependent manner. A similar increase in grain, shoot and root S concentration with increasing S supply was measured in rice (Wu *et al*., 2014). A positive effect of Fe supply on shoot and root S concentration was also measured in durum wheat (Zuchi *et al*., 2012), tomato (Astolfi *et al*., 2011) and safflower (Ravi *et al*., 2008). However, Zuchi *et al*. (2009) reported a reduction in S uptake at higher S supply under Fe sufficient than Fe deficient condition. In the present study the positive effect of Fe availability on root and shoot S uptake was uniformly measured across the bread and durum wheat cultivars under not only in S deficient (S0) condition but also at medium (S1) and high (S2) supply. The presence of Fe appears to induce the uptake of S by the root more prominently than its translocation to the shoot under both S deficient and sufficient supply (Zuchi *et al*., 2009).

A higher shoot and root Zn concentration of bread and durum wheat observed under Fe defciency with increasing S availability than the Fe sufficient condition points towards competitive inhibition of Fe on Zn uptake and translocation (Pahlavan-Rad and Pessarakli, 2009; Orman and Ok, 2012). An increase in shoot and grain Zn concentration with increasing S availability was also observed across the bread and durum wheat types (McDonald and Mousavvi, 2009). S application has been reported to aid biofortification of wheat grain with Fe, Zn, Cu and Mn (Shivay *et al*., 2016). Durum wheat types were observed to maintain a higher root Zn and exhibited a lower efficiency for root to shoot translocation of Zn than the bread wheat

types. A higher root to shoot translocation of Zn is pre requisite to ensure a higher transport of foliage Zn to the grain (Niyigaba *et al*., 2019). Shoot and root Mn, on the other hand, was not significantly affected by Fe availability and decreased with increasing S supply in the nutrient solution. Shoot Mn concentrations were two-three times lower than the root Mn concentration. S appears to be a negative regulator of Mn uptake and translocation (Wu *et al*., 2014). Insignificant affect of Fe availability on grain Mn was also reported in wheat (Pahlavan-Rad and Pessarakli, 2009). High S was also observed to reduce root to shoot translocation of Mn in polish wheat (Sheng *et al*., 2016). A reduced Mn uptake with increasing Fe availability was also measured in wheat grown on calcareous soil (Ghasemi-Fasaei and Ronaghi, 2008).

The regulatory interaction between Fe and S was further confirmed through short term radiotracers studies involving ^{59}Fe and ^{35}S. Use of radiotracers to study the short term uptake of ^{59}Fe under variable availabilities of Fe and S across bread and durum wheat cultivars showed a higher mean average ^{59}Fe uptake by bread wheat than durum wheat cultivars under Fe deficient than Fe sufficient condition of growth. A similar difference in ^{59}Fe uptake between bread and durum wheat cultivars was reported by Parveen *et al.* (2019). ^{35}S accumulation in the shoot was higher under Fe sufficient than Fe deficient treatments and was inversely related to the S status of the plant. Contrary to our observation of ^{35}S uptake and ^{35}S translocation under Fe+S0 than Fe-S0 treatment, Astolfi *et al*. (2004) reported maximum ^{35}S uptake and its translocation under Fe and S deprivation (Fe-S0) than the Fe sufficient and S deprived (Fe+S0) condition. However, their experimental plants were raised first under sufficient Fe condition (80 µM Fe^{III}-EDTA) for 10 days period before these were transferred to Fe limited condition (0.1µM Fe^{III}-EDTA) for 24 h Fe deprivation period. Hence the ^{35}S uptake and translocation measured by them in maize represents the effect of only 24 h of deprivation in contrast to our reported study which represented the actual Fe deprivation condition since wheat plants were never exposed to Fe+ condition until 11 days of growth, the stage at which ^{35}S uptake and translocation was studied across the bread and durum wheat cultivars. A higher ^{35}S to ^{59}Fe ratio measured in S0 treatment under Fe+> Fe- in comparison to S1 and S2 treatments indicates the role of high affinity sulphate transporter (SULTR1;1) which appears to have been greatly induced under Fe+ than Fe- condition. Criticality of Fe availability for induction of SULTR1;1 has been reported in wheat by Sharma *et al.* (2018).

Further, the present study also elucidated the variation in the induction of S (SULTR1;1, SULTR2;1), Fe (YS1) and N (NRT2.1) transporters under Fe and S sufficient and deficient condition in bread and durum wheat cultivars. The expression of the *SULTR1;1*, a high affinity sulphate transporter, in wheat was found to be tissue and species specific with highest expression under the S deficient condition (Howarth *et al*., 2003; Buchner *et al*., 2004; Hopkins *et al*., 2005). Results of the present study clearly showed that the transcript expression of *SULTR1;1* was highest under the Fe+S0 condition as compared to the other Fe and S sufficient and deficient treatment combinations in both bread and durum wheat cultivars. The numerous studies conducted on *SULTR1;1* also reported a similar result in respect of the post transcriptional regulation under Fe and S treatments (Takahashi *et al*., 2011; Hindt and Guerinot, 2012). The expression of *SULTR1;1* was also influenced by Fe availability and an induction was observed under the Fe sufficient than the Fe deficient condition. These results find support from our earlier findings on *SULTR1;1* expression under the Fe+S0 (Sharma *et al*., 2018). On the other hand, the expression pattern of *SULTR2;1,* a low affinity sulphate transporter showed a reverse pattern to the expression of *SULTR1;1* and was highly expressed under the Fe and S sufficient condition. A dose dependent decrease in the expression of *SULTR2;1* was observed with the reduction in the Fe and S supply. *SULTR2;1* is specific to wheat and is reported to be involved in the long distance sulphate transport (Buchner *et al.,* 2010). However, in *Arabidopsis*, a differential expression of two group 2 genes viz., *SULTR2;1* and *2;2* in the root xylem parenchyma and phloem as well as in relation to sulphur nutrition in shoots and roots has been reported (Gigolashvili and Kopriva, 2014).

Expression of Yellow Stripe 1 (*YS1*) transporters, involved in the uptake of the Fe(III)–MAs complexes from the rhizosphere at the plasma membrane (Curie *et al*., 2009), as affected by Fe and S-supply was significantly induced under the Fe deficient than the Fe sufficient treatment, more so for the bread than the durum wheat. A higher expression of *YS1* has been amply documented in cereal species under Fe deficiency (Nozoye *et al*., 2015; Kim and Guerinot, 2007). S-supply further improved the *YS1* expression in a dose dependant manner (S0 < S1 < S2). Favourable regulation of *YS1* transporter activity under the Fe-S+ than Fe-S- treatment has also been reported earlier in wheat from our laboratory (Sharma *et al*., 2018). Close dependence of *YS1*

expression on S-supply in conjunction with the positive influence of S-supply on the PS biosynthesis and release suggests that an induced expression of *YS1* might be caused by a higher availability of PS/ PS-metal complex in the rhizosphere (Sharma *et al*., 2018), rather than a genetic control.

In order to decipher the interactive influence of N in determining Fe and S availability response in plants, the transcript expression of *NRT2.1*, which is a high affinity NO3 transporter and encodes a main component of the root NO_3^- uptake system (Krouk *et al*., 2006) was also studied. *NRT2.1* expression showed species and cultivar dependence along with a significant induction in expression under Fe deficiency with an increasing S- supply. Fan *et al*. (2017) showed that the expression of *NRT2.1* was highly regulated by the expression of *NRT1.1*. It is likely that the bread and the durum wheat cultivars used in the present study differed in their expression of *NRT1.1* to differentially alter the expression of the *NRT2.1*. Favourable regulation of other NO_3^- transporter viz., *OsNRT1.1*b and *OsNRT2.3*b have been reported to improve not only the nitrogen nutrition but also the uptake of iron and phosphate to consequently increase grain yield and NUE in rice under field conditions (Fan *et al*., 2016; Fan *et al*., 2017).

It is thus amply clear that iron availability condition does influence the S availability response as evident from a higher uptake, assimilation and translocation of S under the Fe+S0 than the Fe-S0 treatment. The research journey which started from a mere indication on the role of Fe in altering the expression of *SULTR1.1* proved to be highly rewarding as reflected in the present study, where through a series of experiments, executed at the physiological, biochemical and molecular level, the critical role of Fe in improving the S nutrition of wheat particularly under S-deficiency was evidenced. Further, the study also projects a possible role of N-nutrition in regulating the Fe-S interaction in plants.

Chapter 6

SUMMARY AND CONCLUSION

Sulphur (S), an essential macronutrient and constituent of amino acids such as cysteine and methionine, is also known to regulate many other key plant metabolic processes such as photosynthesis, respiration, etc. S deficiency in cultivable soil and crop plants is often regarded as a major nutritional and production constraint. Deficiency of S in agricultural soil is quite widespread (>41%) and can be attributed to a complete neglect of S and an overemphasis on NPK application on farms post green revolution, which has led to a shift in the nutrient balance and the dynamics of interactive equation between the mineral elements. Crop plants exhibit inter and intra species variation in S deficiency tolerance and that the existing genotypic variability for the trait is waiting to be explored and exploited. It, however, requires a more in depth elucidation and understanding of the mechanisms regulating S deficiency tolerance which may operate at the physiological/ biochemical and/or molecular level. The dynamics of S deficiency tolerance may further change completely with the status of the interacting mineral elements in the rhizosphere. A high or low level of the interacting element may stimulate or impede not only the uptake of S but also its translocation and distribution within the plant. The present study titled "**Physiological and molecular regulation of sulphur uptake and assimilation by iron in wheat**" hypothesizes a role of soil iron (Fe) in determining plant S. It is likely that the soil Fe regulates the plant S nutrition by influencing the genes governing the S-uptake (HATS and LATS) and/or the activity of key enzymes participating in sulfate assimilation (SAT and OASTL). The present study, thus, deciphered the regulatory role of Fe availability on plant S nutrition of bread and durum wheat cultivars viz., HD-2967, HD-2329 and HI-8713, HD-4728 respectively. The choice of bread and durum wheat species as experimental material was supported by known distinct variation in their Fe deficiency tolerance, which favored the bread wheat. The studies were performed over two well conceived experiments employing soil and nutrient solution as the growth supporting medium. The pot culture experiment was conducted with field soil having low and sufficient Fe (~3 and ~12 ppm Fe, respectively) at three levels of S i.e., 0 (S_0), 30 (S_{30}) and 60 (S_{60}) kg S ha^{-1}) and variation in response of the bread and the durum wheat cultivars in respect of the plant biomass, gas exchange

attributes (Pn, Gs and E), S and Fe uptake and partitioning, S and Fe use efficiency and translocation index were measured. Since, the assimilation of sulphate-S not only depends on the activity of SAT and OASTL but also on the availability of serine, which is a connecting link between the S, N and C metabolism, it is postulated that the Fe–S interaction may also affect the plant N nutrition. Further, a nutrient culture experiment was conceived to study the molecular basis of Fe-S interaction in wheat raised under Fe deficient (1 µM, Fe-) and sufficient (100 µM, Fe+) solutions with three S levels viz., 0 (S0), 1.25 mM (S1) and 2.5 mM (S2), which were equivalent to those used in the soil culture experiment. Observations pertaining to plant biomass, leaf chlorophyll, root characteristics, phytosiderophore (PS) content in root and its release, shoot S, Fe, Mn and Zn, transcript expression of sulphate-S, metal-PS and nitrate transporters viz., *SULTR1;1* which encodes SULTR1;1, a high affinity S transporter expressed in the root, *SULTR2;1* encodes a low affinity sulphate transporter SULTR2;1; *YSI* which encodes for Fe-PS complex uptake transporter and *NRT2.1*, a high affinity nitrate uptake transporter were recorded under variable Fe and S availability combination treatments. Another short term uptake experiment was also conducted using radiotracers of Fe and S (^{59}Fe and ^{35}S) to bring forth compelling and conclusive evidence on the critical role of Fe in regulating S-nutrition of crops. Plants raised under variable Fe-S combination treatments were exposed to radioactivity in solution culture @2000 Bq/ml and were monitored for the uptake of the respective radiotracers, measured using liquid scintillation counter. The salient findings of the experiments are summarized as follows:

- Combined deficiency of Fe and S significantly reduced shoot growth, while S supply bettered it under both Fe- and Fe+ condition.
- Gas exchange attributes particularly, photosynthesis was increased with increasing Fe and S supply in a dose dependent condition in both bread and durum wheat cultivars, while there was no significant difference in stomatal conductance and transpiration rates.
- Shoot S was lower under S deficient than S sufficient condition of growth and further its uptake also increased with the Fe availability.
- Combined Fe and S deficiency decreased the shoot Fe content than the sufficient condition. Shoot Fe content also increased significantly with S supply.

- Shoot N was increased with increasing S supply but unaffected with the presence of Fe.
- Higher nutrient availability decreased the SUE, IUE and NUE in both the bread and durum wheat cultivars.
- Fe sufficiency either with or without S induced the S assimilating enzymes i.e. SAT and OASTL activity more significantly over the Fe deficient treatment.
- A higher induction of nitrate/carbon assimilating enzymes was measured under Fe- or Fe+ treatments with S availability when compared with individual or combined deficiency of Fe and S.
- Cysteine, a sulphur containing amino acid than methionine, was more significantly affected by Fe availability. A combined deficiency of Fe and S or Fe deficiency with variable S showed significantly lower level of cysteine when compared to individual S deficiency or S supply under Fe+ condition.
- Shoot and root biomass was significantly increased under Fe and S sufficient than the Fe and S deficient treatment under nutrient solution culture.
- PS synthesis and release was markedly improved under Fe- than Fe+ condition with S supply particularly in bread wheat cultivars.
- S and Fe availability also influenced the uptake of Zn and a competitive inhibition of Zn uptake by Fe was recorded.
- S supply decreased plant Mn content, whereas there was insignificant effect of Fe on Mn uptake by plants.
- Radiotracer evidence of ^{59}Fe and ^{35}S in short term uptake solution culture experiment supports the regulatory role of Fe on S uptake.
- Mean ^{35}S concentration of both shoot and root when averaged over the S treatments, in general, was significantly lower under Fe- than Fe+ condition.
- The root to shoot ^{35}S translocation index declined with increase in S availability under both Fe+ and Fe- treatments particularly in HD-2329 and HI-8713 wheat cultivars.
- S availability in the growing medium, both in the presence or absence of Fe, improved the ^{59}Fe uptake.

- HATS *SULTR1;1* expression was more significantly altered than LATS *SULTR2;1* under S deficiency but with Fe+ than Fe- condition.
- LATS *SULTR2;1* expression was highest in Fe^{+} condition and increased with S supply.
- *NRT2.1* expression was significantly induced under Fe deficiency with S supply and indicates a regulatory interaction between the macro and micro nutrients.
- *YS1* was induced under Fe- than Fe+ with S supply, more so in dose dependent manner.

It is, thus, amply clear that iron availability condition does influence the S availability response as evident from a higher uptake, assimilation and translocation of S under the Fe+S0 than the Fe-S0 treatment. The research journey which started from a mere indication on the role of Fe in altering the expression of *SULTR1;1* proved to be highly rewarding as is reflected in the present study, where through a series of experiments executed at the physiological, biochemical and molecular level, the critical role of Fe in improving the S nutrition of wheat particularly under S-deficiency was evidenced. Further, the study also projects a possible role of N-nutrition in regulating the Fe-S interaction in plants, which can be the subject of an altogether new investigation.

PHYSIOLOGICAL AND MOLECULAR REGULATION OF SULPHUR UPTAKE AND ASSIMILATION BY IRON IN WHEAT

ABSTRACT

The present study hypothesizes the role of iron (Fe) availability in determining S uptake and distribution in bread (cv. HD-2967 and HD-2329) and durum (cv. HI-8713 and HD-4728) wheat, which are known to differ distinctly for their Fe deficiency tolerance response. In the first experiment, the wheat cultivars were grown on Fe deficient (~ 3ppm) and Fe sufficient (~12 ppm) soil under different S levels viz. 0 (S0), 30 (S1) and 60 (S2) kg S ha^{-1}. A higher shoot mass, leaf chlorophyll and gas exchange attributes particularly the rate of photosynthesis were recorded under the Fe+S2 than the Fe-S0 treatment. The shoot S was significantly increased under Fe+ than Fe- treatment with an increasing S-supply. Fe availability was also observed to positively induce the activity of key enzymes of sulfate assimilation viz., SAT and OASTL. A higher content of S-containing amino acid, cysteine was observed under Fe+S0 than Fe-S0. Further, an increasing S-supply caused a dose dependant increase in cysteine content under both Fe+ and Fe- treatments. Possible role of plant N-nutrition in mediating the Fe-S interaction was also examined. S-supply was observed to positively regulate the N uptake, however, the affect was independent of Fe availability. The second experiment performed under Fe and S deficient and sufficient (Fe: 1 µM (Fe-) and 100 µM (Fe+); S: 0 (S0), 1.25 mM (S1) and 2.5 mM (S2)) conditions in nutrient solution showed relatively higher biomass accumulation and synthesis and release of phytosiderophore in the bread than the durum wheat under Fe deficiency. An increase in shoot Fe with an increasing S-supply and vice-versa was observed under both Fe+ and Fe- treatments. The study indicates a critical role of Fe in determining S availability and uptake in wheat, which was further confirmed through the use of radiotracers of Fe and S (^{59}Fe and ^{35}S). The results showed a higher uptake of ^{35}S under Fe+S0 than Fe-S0 condition. Under Fe deficiency, the ^{59}Fe uptake was higher under S2 than S1 and S0 condition, which may be attributed to a favorable regulation of phytosiderophores (PS) biosynthesis by S. An increase in *SULTR1;1* and *SULTR2;1* transcript expressions were observed in the presence of Fe. These results suggest that Fe and S synergistically interact and may regulate their respective uptake by inducing *SULTR1;1*, high affinity sulphate transporters, which is of consequence under the limited S-availability.

Keywords : Bread wheat, Durum wheat, Phytosiderophores, Radiotracers

गेहूं में लोहे द्वारा सल्फर उदग्रहण और आत्मसात करने का कार्यिकी और आणविक विनियमन

सार

वर्तमान अध्ययन, ब्रेड (cv. एच डी -2967 और एच डी- 2329) और ड्यूरम (cv. एच आई-8713 और एच डी-4728) गेहूं, जिनकी लोहे (Fe) की कमी में सहनशीलता भिन्न - भिन्न है, में सल्फर (S) उदग्रहण और वितरण का निर्धारण करने में लोहे की उपलब्धता की भूमिका की परिकल्पना करता है। पहले प्रयोग में, गेहूँ की खेती को विभिन्न सल्फर स्तरों 0 (S0), 30 (S1) और 60 (S2) किलो S हैक्टर$^{-1}$ के तहत कम लोहे (~ 3ppm) और पर्याप्त लोहे (~ 12 ppm) वाली मिट्टी पर उगाया गया था। पौधे के प्ररोह भार, पत्ती क्लोरोफिल और गैस विनिमय विशेषताएँ विशेष रूप से प्रकाश संश्लेषण में, Fe-S0 उपचार की तुलना में Fe+S2 के तहत ज्यादा दर दर्ज की गई थी। बढ़ती S आपूर्ति के साथ प्ररोह S, Fe- उपचार से Fe+ के अंतर्गत काफी बढ़ा हुआ पाया गया। लोहे की उपलब्धता को सल्फेट आत्मसात करने के महत्वपूर्ण एंजाइमों जैसे SAT और OASTL की गतिविधि को सकारात्मक रूप से प्रेरित करने के लिए भी देखा गया। सल्फर युक्त एमिनो एसिड, सिस्टीन की उच्च मात्रा Fe-S0 की तुलना में Fe+S0 के तहत देखी गई थी। इसके अलावा, बढ़ती सल्फर-आपूर्ति ने Fe+ और Fe- दोनों उपचारों के तहत सिस्टीन की मात्रा में वृद्धि की। Fe-S परस्पर क्रिया में संयंत्र नाइट्रोजन-पोषण की संभावित भूमिका की भी जांच की गई। सल्फर-आपूर्ति ने नाइट्रोजन अपटेक को सकारात्मक रूप से विनियमित किया, हालांकि, प्रभाव लोहे की उपलब्धता से स्वतंत्र था। पोषक तत्वों के घोल में लोहा और सल्फर की कमी और पर्याप्तता (Fe: 1 (M (Fe-) और 100)M (Fe +); S: 0 (S0), 1.25 mM (S1) और 2.5 mM (S2) के तहत किये गए दूसरे प्रयोग में, लोहे की कमी के तहत ड्यूरम गेहूं की तुलना में ब्रेड गेहूं में अपेक्षाकृत ज्यादा भार संचय और फाइटोसिडरोफोर संश्लेषण और स्राव पाया गया। Fe+ और Fe- दोनों उपचारों के तहत बढ़ती सल्फर-आपूर्ति के साथ प्ररोह Fe में वृद्धि और इसके विपरीत बढ़ती Fe-आपूर्ति के साथ प्ररोह S में वृद्धि देखी गयी। यह अध्ययन गेहूं में सल्फर की उपलब्धता और उदग्रहण में लोहे की एक महत्वपूर्ण भूमिका को इंगित करता है, जिसकी Fe और S (^{59}Fe और ^{35}S) के रेडियोअनुरेखक के उपयोग के माध्यम से पुष्टि की गई। परिणामों ने Fe-S0 की स्थिति की तुलना में Fe+S0 के तहत ^{35}S का उच्च उदग्रहण दिखाया। Fe की कमी के तहत, S1 और S0 की स्थिति की तुलना में S2 के तहत ^{59}Fe का उदग्रहण अधिक था, जिसे S द्वारा फाइटोसाइडरोफोरस (PS) जैवसंश्लेषण के अनुकूल विनियमन के लिए जिम्मेदार ठहराया जा सकता है। लोहे की उपस्थिति में *SULTR1;1* और *SULTR2;1* अभिव्यक्ति में वृद्धि देखी गई। ये परिणाम बताते हैं कि Fe और S सहक्रियात्मक रूप से परस्पर प्रभाव करते हैं और *SULTR1;1*, उच्च आत्मीयता सल्फेट ट्रांसपोर्टरों, जो कि सीमित S-उपलब्धता में कार्य करते है, को प्रेरित करके अपने संबंधित उत्थान को विनियमित कर सकते है।

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