Biofortification of cereals with iron by manipulating soil pH in calcareous soil

By

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2016
To,

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-----------------------------

Pia Muhammad Adnan Ramzani
DEDICATED TO MY DEAR PARENTS

THEIR EAGERNESS FOR MY HIGHER EDUCATION

HAS TRULY BRIGHTENED MY LIFE
ACKNOWLEDGEMENTS

In the name of Almighty ALLAH, the most Gracious and Merciful. To Almighty Allah we pray that He may guide us the right path, crown our endeavors with success, and bless our lives with abundant prosperity. Countless Darood-o-Salam upon the Lovingly Holy Prophet MUHAMMAD (Peace Be Upon Him), the fountains of knowledge, who has guided his “Ummah” to seek knowledge from cradle to grave.

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May ALLAH bless all these people with long, happy and peaceful lives (Ameen)

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Iron (Fe) deficiency is a prevalent nutritional deficiency throughout the world, affecting an estimated 3.7 billion people. Increasing Fe concentration in food crops is an important global challenge due to the high incidence of Fe deficiency in human populations. Cereals grown on calcareous soil are low in Fe. High pH, high temperature, low organic matter, and poorly managed soil with respect to fertility are factors that cause low Fe availability to cereal crops in calcareous soil. Iron fertilization in calcareous soil is not effective due to its rapid conversion into unavailable forms and the poor mobility of Fe in phloem. Iron-organic compounds in manure are effective in maintaining Fe availability to plants. Hence, there is a need for effective strategies to overcome Fe availability to plants. Biofortification of food crops with Fe to combat iron deficiency problems in humans, is a cost-effective and sustainable agricultural strategy to alleviate malnutrition. We hypothesized that Fe nutrition management in calcareous soil can increase growth, yield, and Fe bioavailability from cereals. To explore the role of Fe in alleviating Fe deficiency in cereals and to eliminate anemia in humans a project was proposed with six studies. Two lab studies were conducted to lower soil pH and to determine the soil acidification effect on Fe bioavailability from Fe fertilizer and from organic amendments. Greenhouse and field studies were conducted on the basis of the lab studies for Fe biofortification in cereals and to enhance iron bioavailability from cereal grain. Iron application with organic amendments significantly improved growth, yield, photosynthetic parameters, and nutritional value of cereal grain in sulfur treated low pH calcareous soil. Iron biofortified rice grain increased haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration in anemic and non-anemic rats. Moreover, high Fe bioavailability (ferritin) in grain had a beneficial influence on the re-creation of ferritin reserves in liver and blood serum, and also did not induce negative alterations in general growth parameters of animals. As a whole, the project showed that Fe biofortification in cereals in pH manipulated calcareous soil significantly improved Fe bioavailability from cereal grains.
Chapter 1

Introduction

The world’s population continues to increase with time. Thus, food demand is also increasing. However, natural resources are limited (United Nations, 2012). In addition, malnutrition is becoming a serious threat to the people of poor communities, especially in developing countries (Sperotto et al., 2012a). In developing countries, a total of 805 million people are not leading a healthy life and suffer from hunger. State of Food Insecurity in the World (FAO, 2014) states that about 13.5 % of the total population lacks enough food for their daily intake of calories. According to one estimate, every 3rd person in the world suffers from hidden hunger due to essential nutrients and vitamin deficiency resulting in poor health conditions (Kennedy et al., 2003).

Iron (Fe) deficiency causes 0.8 million deaths annually and is ranked third among the risk factors of micronutrient deficiency (WHO, 2007). Iron malnutrition leads to anemia in humans, especially pregnant women and preschool children (WHO, 2010). Iron deficiency anemia is the most severe type of iron deficiency (Lozoff and Georgieff, 2006). It can result in a low resistance to infection, impaired psychomotor development, impaired cognitive function in children, poor academic performance, fatigue, fetal resorption, low productivity, and increased risk of maternal mortality (Bothwell and MacPhail, 2004; Murray-Kolb and Beard, 2009).

The main cause of micronutrient malnutrition is consumption of monotonous food that is poor in microelements (Bouis et al., 2011). In developing countries the majority of the population depends on cereal-based foods that are low in bioavailable Fe (Gibson et al., 2010). Thus, low Fe contents from staple foods result in Fe deficiency in humans, especially in developing countries (Sperotto et al., 2012a).

Fe is a micronutrient required by all organisms, and in plants it plays an important role in metabolic pathways including photosynthesis, respiration, chlorophyll formation, and several redox reactions (Briat et al., 1995; Briat and Lobréaux, 1997). Iron deficient crops show interveinal chlorosis, stunted growth, and reduced yield (Kabir et al., 2013). Iron plays an
important role in functioning of cell’s power house, which is impaired under Fe deficiency resultanting poor plant health (Bashir et al., 2013a).

Iron deficiency is a common problem for crops growing in calcareous soil (Laird et al., 2010). High pH and high HCO$_3^-$ contents are two most important factors that limit Fe availability in calcareous soil (Bloom and Inskeep, 1988; Alcantara et al., 2002). Soil pH, complexing ligands, and redox potential affect the availability of Fe for crops (Beckwith et al., 1975). Iron exists mainly as oxidized Fe$^{3+}$ in mineral soil under aerobic environments and is slightly soluble if soil pH is more than 7 (Marschner, 1995). Soils with pH above 5.5-6.5 cannot maintain inorganic Fe$^{3+}$ oxides to the level of soluble Fe. Due to high pH and calcareousness, Pakistani soils promote the precipitation of Fe$^{3+}$ oxides, which are insoluble and not available to plants.

Phytic acid and polyphenols in food act as anti-nutrients and are considered to be the major inhibitors of Fe bioavailability while ascorbic acid, inulin, garlic, and onion have been reported to be the major enhancers of Fe bioavailability (Fairweather-Tait et al., 2005; Scholz-Ahrens and Schrezenmeir, 2007; Gautam et al., 2010). Iron bioavailability inhibitors such as polyphenols and phytate inhibit Fe absorption, but it was concluded that their inhibitory effect on Fe absorption can be limited by increasing Fe content (Carlson et al., 2012; Tako et al., 2011, 2013).

Ferritin, an Fe storage protein, can deposit thousands of Fe atoms in non-toxic form and ferritin Fe is bioavailable to humans as ferrous sulphate (Lonnerdal, 2007; Arosio et al., 2009). Attempts have been made to biofortify rice crop s by introducing the ferritin gene, in the last decade (Drakakaki et al., 2005; Masuda et al, 2013).

Improving concentration and bioavailability of Fe in cereal grains is, therefore, an important challenge and a high-priority research area (Bouis and Welch, 2010; Cakmak et al. 2010a). Various approaches are being used, including supplementation (nutrient as clinical treatment), fortification (adding a particular nutrient in food items), food modification/diversification (cooking and processing of food on nutritional value in mind), and biofortification to combat Fe deficiency in humans (Frossard et al., 2000; Winichagoon, 2002; Bouis et al., 2011)
Many problems have been reported with supplementation as Fe tablets due to adverse side effects in humans (Winichagoon, 2002; Mimura et al., 2008). Fortification of common foods with Fe, is also not effective because of poor Fe bioavailability from the Fe-fortified foods, and also changes in product taste, which may cause resistance by consumers to the fortified products (Frossard et al., 2000; Powell et al. 2013). Iron deficiency could also be mitigated by enhancing grain Fe concentration and bioavailability of Fe in grains such as wheat and rice (Bouis and Welch, 2010). In this regard, biofortification, which is a process of enhancing the bioavailable nutrient in the edible portion of crops, is considered the most suitable approach (Mayer et al., 2008; Bouis et al., 2011).

Approaches for Fe biofortification of crops such as genetic engineering, agronomic, transgenic and plant breeding have been developed (Cakmak et al., 2010a; Wei et al., 2012). It is reported by Bashir et al. (2013) that plant breeding has failed so far in developing Fe biofortified polished rice. On the other hand, transgenic varieties sometimes may provide more nutrients than genotype selection, but many countries have strict regulations in commercialization of these transgenic varieties (Saltzman et al., 2013). Biofortification of food crops with Fe through agronomic approaches is a widely applied strategy (Cakmak et al., 2010a). Agronomic biofortification of food crops is considered the most sustainable approach.

In high pH, calcareous soil, agronomic biofortification will be ineffective unless one lowers the soil pH. The major issue in calcareous soil is quick transformation of soluble Fe compounds to less soluble oxides and hydroxides. Hence, to reduce Fe’s rapid transformation and to increase its availability, soil pH manipulation using acidifying materials could be a useful approach (Malakouti and Gheibi, 1988). Different studies have reported that microbial oxidation of elemental sulfur decreases soil pH and leads to mineral solubilization that increases mineral availability to crops (Kaplan et al., 1998; Iqbal et al., 2012).

Organic matter is an important source of mineral nutrients. But Pakistani soils are deficient in organic matter and essential micronutrients (Niazmi and Khan, 1989). Due to harsh climatic conditions, organic matter mineralizes soon after its application. Application of micronutrients mixed with organic matter can enhance its effectiveness. Thus, we need more recalcitrant sources of organic matter that persist.
Soil physical properties, i.e. soil aggregation, are representative of soil quality (Vrdoljak and Sposito, 2002) that can be corrected by application of manure, biosolids, and other kinds of organic matter. Application of manure to soil (mainly poultry manure) not only provides a solution to waste disposal, but also improves soil physical properties with time when applied in combination with fertilizers (Hati et al., 2006; Bandyopadhyay et al., 2010). Mixing inorganic salts of micronutrients with different organic materials can enhance the efficacy of micronutrients. Thus, biochar is among the most sustainable sources of organic carbon. Biochar plays an important role in nutrient dynamics and improves soil fertility and crop productivity (Liu et al., 2013). Biochar affects mineral forms of Fe by acting as an electron shuttle in redox-mediated reactions (Kappler et al., 2014). Graber et al. (2014) reported that redox catalytic activity associated to biochar solubilized Fe by decreasing soil pH. Most recently, carboneous product has been used for Zn biofortification of crops (Gartler et al., 2013).

Little or no work about soil pH manipulation with elemental sulphur in calcareous soil (as in Pakistan) for Fe biofortification in cereals has been reported in the primary literature. Research, therefore, is needed to better understand how soil acidification will act in calcareous soil as well as influence crop growth, yield, and Fe bioavailability.

In Pakistan, Fe biofortification with integrated use of Fe fertilizer and organic amendments in pH-manipulated calcareous soil is new and has not been evaluated in the context of Pakistani agriculture. Therefore, the main objectives of this integrated research project were to:

- Define the rate of elemental sulfur for lowering soil pH
- Determine soil acidification effects on Fe bioavailability in unamended and organic amended calcareous soil
- Estimate Fe bioavailability in cereals as affected by Fe fertilizer and organic amendments in unamended and pH manipulated calcareous soil
- Investigate the effect of Fe biofortified rice grain with regard to Fe deficiency anemia elimination by a using normal and iron deficient rat study
Chapter 2

Review of Literature

There is a dire need to form new agriculture practices policies that not only meet the food requirements of the growing world but also fulfill nutritional requirements (Welch and Graham, 2004). Cereals grown in calcareous soil are poor in essential mineral contents i.e. Fe and Zn causing severe threat to population that depends on such mineral deficient food. In this chapter I discuss the status of soil Fe, its significance in plants and humans, and strategies to combat Fe deficiency in soil, plants, and ultimately in humans.

2.1 Status and forms of Fe in soil: an overview

The predominant forms of Fe in soil are hematite, goethite, leidocrocite, ageetite, maghemite, and ferrihydrite (Schwertmann and Taylor, 1989). Each of these oxides contains the \( \text{Fe}^{3+} \) form of Fe except magnetite, which contains Fe (II) as well as Fe (III). These forms exist predominately in the clay-size fraction of soil, but magnetite can exist in the silt and sand size fraction of soil. In arid and semi-arid regions goethite and hematite are the dominant minerals; in slightly weathered or reduce soil magnetite is the dominant form of Fe. In soils with changing redox condition, ferrihydrite is likely to exist in most of the soils as a small fraction but as a major component. All these mineral oxides are found in well crystalline structure, but ferrihydrite shows a small size of crystal structure with particle size of \( \leq 10 \) nm. Particle size and surface reactivity of mineral oxides are the major factors that influence their solubility and availability. Schwertmann, (1991) gave the stability order of different mineral oxides as follows: hematite = goethite > lepidocrocite = magnetite > ferrihydrite.

Iron is an important component of layer silicates in soil (20-50 g kg\(^{-1}\)) but is not available for plant growth. In acid soil (pH <4.5) some fraction of Fe is present as exchangeable ions. As primary or secondary minerals Fe exists as pyrite, amphibolite, pyroxenes, and siderite. These are mostly present in reduced or relatively less weathered soils.

Yields of Fe deficient crops can, in principle, be increased through application of Fe to soils. However, as the uptake of Fe from soils is highly complex, improving crop yields through fertilization with Fe has been shown to be difficult (Schulte and Kelling, 2004). For example,
application of iron to soils in the form of ferrous sulfate (FeSO$_4$) has generally resulted in, at most, limited effects on crop yields (Frossard et al., 2000). Other forms in which Fe might be added to soils (e.g. as chelates) are possibly more effective, but also expensive, and generally too costly for use on low value staple crops (Akinrinde, 2006).

2.2 Iron deficiency in calcareous soil

Soil pH is most important factor that determines nutrient availability. The most suitable pH for crops is around 6.0 to 6.5. Only a few crops can tolerate high pH. Most of the nutrients are available at 6.5 to 7.5 pH. High pH soil makes most micronutrients unavailable, thus micronutrient deficiency is widespread in high pH calcareous soils. In calcareous soil HCO$_3$ and CO$_3$ make various complexes with Fe, P, and Zn, thus reducing availability of mineral nutrients. Calcium carbonate provides a reactive surface that acts as a sink for protons during acid/base reactions involving dissolved Fe species in the soil solution (Loeppert, 1988; and Abd El-Haleem, 1996). Singh and Dahiya (1975) found that chemically available Fe decreased with increasing CaCO$_3$ and time of incubation. They also reported that increasing levels of CaCO$_3$ in soil resulted in a decrease in some forms of exchangeable and Available Fe. The decrease in exchangeable Fe was probably from the release of Ca$^{2+}$ from hydrolysis of CaCO$_3$. The decrease in the other forms of Fe might be due to oxidation of soluble-native and added Fe through direct reaction with CaCO$_3$. Singh and Dahiya (1976) found that excessive amounts of CaCO$_3$ decreased the availability of Fe to plants and it is often necessary to supply additional Fe to the soil to complete life cycle of plant. Soil deficiency of nutrient leads to nutrient deficient plants (Cifuentes et al., 1993). Iron is 4$^{th}$ most abundant element present in the Earth’s crust. But its availability is limited in most soils. Iron changes its oxidation state very quickly. Celik and Katkat (2007) reported that at high pH, Fe(II) ion converts into insoluble Fe (III) ion. High lime contents in calcareous soil also decrease Fe uptake. Redox conditions are also detrimental to Fe availability.

Mengel and Kirkby, (1987) reported that Fe solubility and availability is pH dependent. It is also reported that Fe (III) solubility and activity decease 1000 time with every one unit increase in pH (Latimer 1952).

Iron makes complexes with organic matter (i.e. humic substances) that are influenced by redox condition and pH. At low pH, Fe (III) complexes with organic matter are present
(Goodman, 1987) but at high pH values greater than 6, humic substances are less significant because of hydrolysis of Fe and precipitation of Fe oxides. In calcareous soil where pH is high, Fe$^{+3}$ is not retained by soil humates against hydrolysis. In a pH range of 7.5-8.5 the solution Fe$^{+3}$ concentrations remaining are minimal (Lindsay, 1979). In calcareous aerated soil as values of redox potential and soil pH are more except under condition of flooding or saturation of soil pores for longer period of time, therefore, total Fe concentration is insufficient to meet the requirement of plants.

2.3 Strategies to overcome Fe deficiency in soil

In cultivated calcareous soil, the potential value of applied Fe depends on the solubility of the applied compound and the capacity of plant roots to assimilate Fe from these compounds (Garacia-Mina et al., 2003). Therefore, different strategies are adopted to maintain the available Fe status of soil.

2.3.1 Sulfur effect in lowering of soil pH and Fe solubilization

Decreasing soil pH is an effective way to deal with the stabilization of nutrients in calcareous and alkaline soil. Phosphorus, Fe and Zn become unavailable in calcareous and alkaline soil due to high pH and high concentration of calcium ions (Deluca et al., 1989; Tisdale et al., 1993; Kaplan and Orman, 1998). Common methods for dealing with these deficiencies, are the use of chemical fertilizers that, in addition to the high cost and low efficiency, also have the risk of environmental pollution (Malakouti et al., 1991).

Different chemical fertilizers have been used to lower the soil pH. Any fertilizer that contains ammonium or produces ammonium can reduce the pH (i.e. ammonium sulfate, ammonium phosphate) (Extension Education Center 423 Griffing Avenue, Suite 100 Riverhead, New York 11901-307). Along with these fertilizers elemental S is considered a cost effective approach to lowering of soil pH. Because these chemical fertilizers have some constraints, (e.g ammonium sulfate is more expensive, also the amount of ammonium sulfate needed to achieve the same decrease in pH is six times the amount of elemental S required; and ammonium sulfate is toxic to crops like blueberries Potassium sulfate can also be used to lower pH but it is documented that sulfate – S containing fertilizers are not very effective in decreasing soil pH. Ferrous sulfate can also be used but it is not cost effective and the total amount of ferrous sulfate is eight times more than the amount of elemental S needed. Thus,
elemental S is the most common and cost effective acidify matter (Tisdale, 1993). After oxidation each mole produces two moles of hydrogen ions (H+) in the soil and reducing soil pH leads to dissolution of nutrients in the root (Modaihsh et al., 1989; Kaplan and Orman, 1998; Besharati and Salehrastin, 1999). The oxidation of elemental S has a significant positive correlation with soil pH as with the oxidation of S soil pH decreases (Lawrence and Germida, 1988; Kaplan and Omran, 1998).

2.3.2 Water soluble, exchangeable and DTPA extractable Fe

 Extraction of soil with neutral (i.e. CaCl₂, MgCl₂, or KCl) or buffered (e.g. NH₄C₂H₃O₂) salt solution will displace solution and exchangeable Fe. Acid buffered extraction may not be suitable for calcareous soil as it may result in considerable release of carbonates and oxides along with release of Fe from mineral structure. The extractability of Fe can be increased by using reducing agents (reductive dissolution). However, these extractants are not that much used in practice.

2.3.3 Use of synthetic Fe chelates

 Use of synthetic chelates may help in combating Fe deficiency in plants (Pestana et al., 2003). Iron chelators increase Fe availability in soil by increasing Fe’s concentration in soil solution thus increasing its diffusion and rapid replenishment of depleted zones and by providing ease to uptake by roots (Lindsay, 1995). Chelates are very effective in improving Fe status of alkaline and calcareous soil, but due to high stability constants root may impair high energy to take up available Fe (Rodrıguez-Lucena et al., 2010). Another constraint in applying chelates to soil is that applied chelates may leach when frequent irrigation is applied. The most frequently used iron chelates are ethylenediaminetetraacetic acid (ETDA), and diethylinetriaminepentaacetic acid (DTPA) and are characterized by low stability in soil while ethylenediamine-di(o-hydroxy-p-sulphoxyphenylacetic) acid (EDDSHA), ethylenediamine-di(o-hydroxy-p-methylphenylacetic) acid (EDDHMA), ethylenediamine-di(o-hydroxy-phenylacetic) acid (EDDA) can be used in soil applications as these have high stability constant. But Fe chelates are expensive and out of reach for most of the farmers in developing countries (Akinrinde, 2006).

2.3.4 Vivianite
Soil application of synthetic Fe-phosphate Fe₃(PO₄)₂·8H₂O has shown significant results in supplying of Fe with time. Iron (II)-phosphate is analogous to the mineral vivianite. Vivianite is a compound that can be easily made by mixing ferrous sulphate and di-ammonium phosphate or mono-ammonium phosphate and vigorous shaking. Del Campillo et al. (1998) and Rosado et al. (2000) found vivianite to be an effective compound to combat Fe deficiency in plants that were grown in calcareous soil. Farmer hasitat to use this due to the lack of its commercial production and high cost.

2.4 Significance of Fe for plants

Iron plays a vital role in various physiological and metabolic functions not only in plants but also in the human body. Iron is a transitional metal and changes its oxidation state very rapidly, thus it can be used as a cofactor for many important processes like oxidative phosphorylation, electron transfer, and DNA synthesis (Hass et al., 2005). Iron acts as a catalyst of chlorophyll formation, an important component of cytochromes, is involved in nitrogen fixation, and a component of ferridoxin. Ferridoxin (iron-sulfur protein) acts as an electron transmitter in many basic metabolic processes (Marschner, 1995). Iron also plays its role in some degradation processes (i.e. reactions of peroxides). Being a part of the heme protein, Fe plays the key role in hemoglobin formation and oxygen transport via hemoglobin. Iron is also important for binding of oxygen to red blood cells (RBC), formulation of cytochrome and myoglobin, brain development and function, and contraction and relaxation of muscles (Başar, 2005).

2.5 Severity of Fe deficiency in crops

Iron is a key component of the chlorophyll ring structure. Any change in Fe availability leads to major alteration in overall plant metabolism. Iron deficiency causes yellowing of young leaves. The most common visible Fe deficiency symptom is leaf chlorosis and soil calcareousness favors this (Tagliavini and Rambola, 2001). Plant Fe deficiency is common in many regions (Wiersma, 2005). In severe conditions interveinal chlorosis causes serious damage to crops. Leaf yellowing results in poor photosynthetic activity. In Fe limitation, crop growth will be reduced. The plant life cycle slows, leading to reduced yield. Susceptibility to disease also increases in Fe limiting conditions (Rashid and Ryan, 2004; Chatterjee et al., 2006).
Severe yield losses also occur in Fe deficient conditions. It is estimated that soybean yield loss in the USA is about 300,000 tons a year (Hansen et al., 2004). Peach production was reduced up to 20-30% due to interveinal chlorosis (Başar, 2003a). Thirty percent Pakistani soils are Fe deficient (Jaskani, 2012).

2.6 Strategies to overcome Fe deficiency in plants

2.6.1 Soil pH manipulation and Fe bioavailability

Nutrients like Fe, Zn, and P become deficient at high pH soil. Soil acidity favors the solubilization of mineral cations in soil with high calcium carbonate contents. Malakouti and Gheibi (1988) reported that consumption of S in calcareous soil and with neutralizing lime increased solubility and availability of iron.

Wu et al. (2014) conducted an experiment to determine if S influenced Fe accumulation using different levels of S. Concentration of Fe increased in rice and then decreased with increasing S concentration. This study suggested that S application may improve Fe contents in rice when cultivated in low S content soils, while Fe content may decrease in rice with S inputs (fertilizers, atmospheric deposition) in high-sulfur soils. Similar studies were conducted by Heydarnezhad et al. (2012) and Kavamura et al. (2013) to investigate nutrient (i.e. Fe, Zn and P) concentration in calcareous soils. Sulfur application increased the concentration of Fe, Zn and P in soil. This study suggested that S application not only increased enhance solubility of Fe and Zn but also increased bioavailability of Fe and Zn in calcareous soil. The acidity produced during S oxidation increases the availability of nutrients such as P, Fe, Mg, Mn, Ca, and SO₄ in soils (Lindemann et al., 1991).

Experiments have demonstrated that S in soil affects Fe uptake in rice because S can regulate the formation of Fe plaque on the root surface of rice (Hu et al., 2007; Gao et al., 2010; Fan et al., 2010), influence Fe uptake by rice (Liu and Zhu, 2005), and influence the formation of phytosiderophore, which is closely linked with Fe uptake by plants (Cao et al., 2002; Jin et al., 2005). Sulfur can increase the Fe transport in plants xylems (Hu and Xu, 2002; Na and Salt, 2011) and phloem, as well as accelerate the activation of deposited Fe in the apoplast by acidifying apoplast pH (Holden et al., 1991; Toulon et al., 1992). Previous studies showed that S supply can increase the accumulation of Fe in rice seedlings (Min et al., 2007). Hassan
and Olsen (1966) suggested that applied S directly increased the amount of Fe and Mn removed from the neutral and calcareous soils by production and consumption of sulfides (e.g. FeS and MnS formation).

2.6.2 Injection of ion salts

Iron salts in liquid form (FeSO₄ and ammonium citrate) have been injected into plant xylem vessels and has significant results in reducing Fe induced chlorosis in fruits like pear, kiwifruit, peach, olives, and apple (Wallace and Wallace, 1986; Wallace, 1991). Application of Fe as bullets into trunks by making holes is also an effective and long lasting (2-3 years) cure for Fe chlorosis (Wallace, 1991). However, it may cause phytotoxicity when iron concentration injection time and plant growing times are wrongly chosen. Because of quick transformation of Fe from available (Fe²⁺) to unavailable form (Fe³⁺).

2.6.3 Blood meal

Use of blood meal is considered an effective approach to combat Fe chlorosis in trees (Taglivaini et al., 2000). Blood meal is a byproduct of the slaughter house and an excellent source of Fe for plants (Kalbasi and Sharimatmadari, 1993). Blood meal can contain Fe ranging from 20-30 g kg⁻¹. In blood meal, Fe is found as ferrous sulphate and in complex with the heme group of hemoglobin.

2.6.4 Foliar application of Fe

Under field conditions acidic solution sprays (e.g. citric, ascorbic, and H₂SO₄) have proved effective at re-greening Fe chlorotic leaves without applying exogenous iron (Aly and Soliman, 1998; Tagliavini et al., 1995). There was a decrease in apoplastic pH and re-greening of Fe chlorotic leaves by applying citric and sulfuric acid (Kosegarten et al., 2004). But there are some constrains in adopting this techniques as studied by Tagliavini et al. (2000). More than one sprays are needed to correct Fe deficiency and much care is needed because high doses of spray solution causes leaf burn.

2.7 Organic amendments and nutrient availability

Organic matter supplies organic chemicals to the soil solution that can serve as chelates and increase metal availability to plants, providing metal chelates, and increasing the solubility of nutrients in soil solution (Du Laing et al., 2009; McCauley et al., 2009).
2.7.1 Animal manure

Animal manure has been used for many years alone and in combination with chemical fertilizers. Its effectiveness is enhanced when applied with mineral fertilizers. Animal manure has the ability to dissolve soil insoluble organic compounds. Incubation of Fe salts with organic manure can improve efficiency of Fe sources before application (Tagliavini et al., 2000).

The chemical composition of poultry manure reveals that it has a high concentration of nitrogen compared to various other organic amendments (Bujoczek et al., 2000) and high nitrogen contents change the dynamics of Fe contents in wheat as the activity of YS1 protein needed for Fe transport is promoted in the root cell membrane (Murata et al., 2008; Curie et al., 2009).

With increasing trend of people towards poultry industry disposal of poultry waste is becoming an issue of great concern due to its high nutritional value for soil and plant. Poultry manure is used as a source of organic fertilizer as it contains many nutrients (Moore et al., 1995). It also contains many secondary elements, micronutrients, and some heavy metals (Gupta and Gardner, 2005). The fraction of plant available nutrients can be changed by applying manure because manure can change the soil biota and physical properties of the soil (Demir et al., 2010).

2.7.2 Compost

A sustainable approach to manage municipal waste is the use of compost made from municipal solid waste (Aggelides and Londra, 2000; Soumare et al., 2003a). Physicochemical properties of soil and plant nutrient status can be improved by use of compost because mature compost contains plant nutrients. Application of compost improved readily available Fe, Zn, and Cu contents soil (Soumare et al., 2007). Application of compost influences the nutrient dynamics due to changes in physicochemical conditions of soil nutrient mobility and bioavailability; availability of a few nutrients can increase or vice versa (Gardiner et al., 1995). Upon decomposition, release of mineral elements (Fe, Zn) from organic complexes also increases availability of mineral nutrients such as Fe and Zn (Dudley et al., 1986; Nyamangara, 1998). However, complete knowledge of minerals (Fe, Zn)
behavior should be understood before application of compost to maintain a sustainable and reliable agro-ecosystem.

### 2.7.3 Biochar

Biochar affects mineral forms of Fe by acting as an electron shuttle in redox-mediated reactions (Kappler et al., 2014). Recently, Graber et al. (2014) noted how the redox catalytic activity associated with biochar solubilized Fe from a sandy soil, increasing the metal release with decreasing pH. One of the main mechanisms proposed to justify the benefits of biochar is its positive impact on the availability of soil nutrients (Xu et al., 2013). Direct effects of biochar on soil fertility have been mainly related to the presence of nutrients in mineral form on the biochar surface (Kimetu et al., 2008). However, indirect effects of biochar on soil fertility are to change soil physico-chemical and biological properties (such as pH, redox conditions, porosity, water retention capacity, and biotic interactions at the rhizosphere), leading to nutrient mobilization (Lorenz and Lal, 2014; Ngo et al., 2014; Jeffery et al., 2015).

### 2.8 Iron for human health

It is recognized that micronutrient deficiency causes harmful impacts to public health (Black, et al., 2008; Stein, 2010). Iron scarcity causes fatigue, poor work performance, reduced immunity, deficient oxygen supply to RBCs, and death. In most part of the world, Fe deficiency particularly affects preschool children and women (Benoist et al., 2008). Mortality rate and overall burden of disease has increased due to micronutrient deficiency. Common periods of high Fe demand include pregnancy, periods of blood loss during surgery, or Fe demand due to insufficient Fe absorption (Trost et al., 2006).

Rice is usually very low in Fe contents compared to recommended dietary allowances. The daily dietary dose is about 0.06 g day\(^{-1}\) for adult women with a low bioavailability of Fe (5%) and 0.02 g day\(^{-1}\) with high bioavailable Fe, in developing countries (WHO, 2004). According to UNSSCN (2010) about 88% of all pregnant women of 16 years and 63% of - and 14-year-old children are considered to be anemic in South Asia.

### 2.9 Strategies to combat iron deficiency in humans

To improve Fe supply, three strategies namely food diversification, supplementation, and food fortification have been practiced. These three aim to improve Fe supply and
bioavailability in food (Bothwell, et al., 2004; McDonagh et al., 2015). Diversification of food increases intake and provision of an Fe-rich diet that is bioavailable to humans. On the other hand, supply of Fe in the form of medicine and pills is supplementation. While either addition of bioavailable Fe or reduction of the inhibitory effect of different compounds to the most frequently consumed dietary products are categorized as food fortification. In the long run, biofortification of plant based food is the most recent strategy to improve bioavailable Fe contents. All these approaches need certain conditions to be fulfilled.

2.9.1 Food diversification

Food diversification aims to increase Fe contents of frequently consumed daily diet. Diversification can improve Fe bioavailability in the variety of foods i.e. fruits, vegetables and meat. But this has some limitations too: meat and fish that are rich in haem iron are quite expensive, while fruits and vegetables rich in vitamin C are seasonal and available for short periods of time only. Thus, making it difficult to enhance their intake.

2.9.2 Supplementation

A therapeutic approach that is utilized either to treat or to prevent severe micro nutrient deficiency is supplementation (Imdad and Bhutta, 2012). In certain countries significant results have been shown by supplementation programs organized by the health department. Vitamin A supplementation for the cure of night blindness and newborn mortality has shown remarkable success. Fe-folate supplementation to pregnant women is also a feasible approach and has been shown to have a positive impact on anemia. But in developing countries the target of daily compliance is hard to achieve, also, lack of proper infrastructure, disruption of stocks, lack of proper contact with the target population and official health care department make it difficult to provide a timely supply of micronutrients such as Fe and Zn (Bothwell et al., 2004).

Another limitation to the supplementation approach is not addressing the root cause of malnutrition. It is a short term solution to malnutrition and nutrient deficiencies. Supplemented food shows a variety of physiological and absorption responses of nutrients compared to nutrients found in food (i.e. zinc, Fe and folic acid) (Bailey et al., 2015). Iron supplemented food may not be the solution for Fe malnutrition. Different trials on supplementation and screening for Fe-deficiency anemia (IDA) in young children reported
that no clear benefits of supplementation were observed (McDonagh et al., 2015). It is also reported that supplemental Fe in the human diet with higher doses may cause serious health hazards (i.e. gastric problems, gastric upset, vomiting and nausea, faintness, abdominal pain or constipation) (Murray-Kolbe and Beard, 2009; Aggett et al., 2012). Oxidative stress that leads to damage of cellular components due to lack of supply of some antioxidants also results due to Fe introduction to the diet (Ibrahim et al., 1997).

2.9.3 Fortification

A more long term strategy to overcome Fe malnutrition is fortification of food items. Fortification is meant for large numbers of population while supplementation exposes a certain group of individuals. Fortification is more beneficial where micro nutrient deficiency is widespread. However, it needs more time for implementation than supplementation. Fortification programs needs some industrial engagement and policy decisions. The food vehicle and amount of fortificant added is equally critical. Many food products are sensitive to color or flavor changes and oxidative damage of some nutrients (Hurrel, 2002). Recently published data from Powell et al. (2013) has reported that dietary fortified iron intake is negatively associated with quality of life in patients, probably as a result of low bioavailability as well as an antagonistic mechanism with other metals. The toxic effect of high doses of Fe is also known (Golub et al., 2009).

2.9.4 Biofortification

Biofortification with Fe in staples provides an economical tool to reduce Fe malnutrition (Jeong and Guerinot, 2008; Nagesh et al., 2012). Enrichment of crops with micronutrients before harvest is known as biofortification. Biofortification enhance micronutrients, thus it is an important tool to overcome micronutrient malnutrition (Brinch-Pedersen et al., 2007).

Biofortification is considered a long term solution to combat Fe malnutrition (Zimmermann and Hurrell, 2002). Biofortification is only a one time investment of money for fertilizers. For a healthy body, a dose of 2-6 ppm is enough to improve Fe level (Haas et al., 2005). Successful biofortification needs the acceptance from consumers, adaptation by farmers, and cost effectiveness.

2.10 Approaches for iron biofortification
Biofortified staple foods may not deliver equally high levels of minerals and vitamins per day, but they can increase micronutrient intake for the resource-poor people who consume them daily, and therefore complement existing approaches (Bouis et al., 2011). Cereals are rich in anti-nutrients, the plant genome along with their growth conditions determine the level of these inhibitors (Hunt, 2003). For Fe biofortification of crops, genetic engineering, agronomic, transgenic, and plant breeding approaches have been developed (Bouis et al., 2011; Sperotto et al., 2012a)

2.10.1 Breeding and genetic approaches

Breeding and genetic approaches have been used for many years to obtain genotypes that are rich in micronutrients. Aung et al. (2013) and Masuda et al. (2012, 2013) studied three combined approaches to biofortify rice grain. A combination of genes is involved in Fe homeostasis that can be used to enrich rice grain with Fe. A successful Fe biofortified and vitamin A-fortified rice named “Golden Rice” was introduced by Goto et al. (1999) and Ye et al. (2000). Conventional and modern plant breeding and biotechnological approaches suggested that Fe contents in rice are a genotypic character that is significantly different for different genotypes hence, new Fe-enriched varieties can be screened or bred (White and Broadley, 2005; Wen et al., 2005). These approaches are not always very successful either because of environmental and genotypic interactions or there may be a lack of a target genome (Palmgren et al., 2008; Zhao, 2010). Source and sink strategy were prioritized to biofortify rice grain with Fe and Zn (Wirth et al., 2009; Masuda et al., 2013). Traditional breeding efforts to biofortify polished rice have not proven very effective as there are limited variations in Fe contents. Over 20,000 rice accessions from Latin America, Asia, and the Caribbean were evaluated for Fe and Zn contents. It revealed that maximum concentration was only 8 mg kg\(^{-1}\) in polished grains (Graham, 2003; Martínez et al., 2010). It is reported by Bashir et al. (2013a) that plant breeding has failed so far in developing Fe biofortified polished rice.

2.10.2 Transgenic approaches

Goto et al. (1999) was the first to explore transgenic approaches to enrich Fe in endosperm over a decade ago. Since then, countless efforts have been made to improve grain Fe contents by Fe homeostasis gene expression that either increase the Fe uptake from soil,
ultimately accelerating Fe translocation from roots and shoots to grains, or by improving efficacy of Fe storage protein (Kobayashi and Nishizawa, 2012; Lee et al., 2012). Studies also suggested that stability of selected trait over number of plant generations is nevertheless still a challenging task. Oliva et al. (2014) introduced an *indica* variety with phytoferritin over expresser events without selectable marker genes; however, the level of Fe was not sufficient to reach the target. Transgenic varieties may sometimes provide more nutrients than genotype selection but many countries don’t allow commercialization of these transgenic varieties (Saltzman et al., 2013).

### 2.10.3 Soil and crop management

Rice grain Fe contents are regulated by soil and other environmental factors (Barikmoa et al., 2007; Zuo and Zhang, 2011). Several sources of micronutrients can be used such as inorganic salts, natural organic polymers, and synthetic chelators. Foliar application of micronutrients is considered very effective as it requires fewer amounts of fertilizers and a quick response crop response compared to soil application (Mortvedt, 2000).

Synthetic Fe chelates are also considered an effective approach to biofortify crops with Fe and are used both in soil and foliar application. Their initial cost may be prohibitive, but these have proved cost effective for high value crops (Fageria et al., 2002). Studies suggested that foliar application of Fe was effective in increasing Fe contents of wheat in arid climates (Habib, 2009; Pahlavan-Rad et al., 2009), but foliar application remained ineffective in humid areas. Pahlavan-Rad (2009) and Habib (2009) evaluated the effectiveness of complex micronutrient application as foliar sprays and suggested that complex micronutrient foliar application is superior to single application as wheat grain concentration of Fe and Zn were improved by complex micronutrient foliar application.

Application of organic amendments, such as farmyard manure, increases nutrients concentration, improves nutritional quality, and enhances nutrient balance of crops (Graham et al., 2001). On decomposition of organic matter, different organic acids (i.e. oxalic, phenolic, citric, and malic) are released. These organic acids form complexes with Fe, hence enhance its mobility and bioavailability (Lindsay, 1995). The most recent approach is to enhance bioavailable Fe contents while reducing phytate contents and to increase total Fe content, but these are not very practical at this time (Raboy et al., 2000; Hurrell et al., 2003).
Most Fe biofortification studies are conducted under favorable glasshouse conditions, with only limited studies performed under field conditions (Masuda et al., 2008, 2012).

2.11 Nutritional factors affecting Fe bioavailability

Apart from high pH and elevated lime contents there are some other factors that affect Fe bioavailability. Among these factors, phytic acid and poly-phenolics are most important.

2.11.1 Phytic acid (Phytate)

Phytate is a stored form of seed phosphorus deposited during seed development (Doria et al., 2009). Phytic acid acts as a binding agent in the intestinal tract of human as it makes strong bonding with Ca, Zn, Fe, and other essential mineral elements during digestion (Garcia et al., 1999). As an anti-nutrient, phytate reduces bioavailability of important nutrients and causes micronutrient malnutrition (Welch, 2002). The main challenge is to reduce phytate contents to assure maximum ferritin concentration. It is the only way we can enrich crops with micronutrients like Fe. Total Fe contents are of no meaning unless we decrease phytic acid concentration that limits its bioavailability.

2.11.2 Polyphenol

Like phytate, polyphenol is also considered an antinutrient that interacts with essential mineral contents of food and makes them unavailable for absorption (Idris et al., 2006; Abd El Rahaman et al., 2007). Sharma and Kapoor (1997) showed that nutrient absorption by human body was significantly influenced by polyphenols and phytate present in pearl millet. Studies also suggested that polyphenols act as chelating agents that affect Fe bioavailability by forming insoluble complexes (Hurrell and Egli, 2010).

Many cereals contain high contents of polyphenols i.e. maize. Liyana-Pathirana and Shahidi (2005) reported that food digestion enhances the antioxidant capacity of cereals and cereal based food. The solubility and functionality of polyphenols present in cereals increases in stomach and duodenum. In vitro digestion studies showed that the amount of antioxidants released by the array of cereals in the human gut may be higher than expected (Perez-Jimenez and Saura-Calixto, 2005). In the plant cell wall, lignin is present that is known to have polyphenolic properties. About 30% of plant biomass and 3-7% of bran is made up of
lignin. Lignin compounds are considered to be inert during digestion but their polyphenolic structure gives them antioxidant properties (Fardet et al., 2008)

Del Pozo-Insfran et al. (2006) evaluated varietal difference in antioxidant fraction. He demonstrated that Mexican purple maize showed a significantly higher antioxidant capacity than American purple and white varieties. However, these were attributed to the specific anthocyanins and/or the composition of polyphenols in the plants.

Tough, fermentation, malting, sprouting, soaking, and cooking have long been documented by many researchers to lower down anti-nutrient concentration (Lewu et al., 2010; Osman, 2011) such information still needs more investigation.

2.11.3 Ferritin

Ferritin is a stable Fe storage protein consisting of a 24-subunit shell around a 4500-atom iron core (Theil et al., 2004). It is reported that Ferritin doesn’t form complexes with other cations, thus increasing Fe availability to humans. Ferritin is a stable protein and doesn’t denature in the human elementary tract (Theil et al., 2001; Murray-Kolb et al., 2003).

In most seeds ferritin content ranges from 8-80 μg/g of seed. Several studies suggested that rice, wheat, and corn have low bioavailable Fe contents (May et al., 1980) while nodule forming crops are rich in ferritin (Ambe et al., 1987). Ferritin-Fe contents are bioavailable to humans and a source of Fe for completion of the human life cycle (Lonnerdal et al., 2007). Iron stored in ferritin is completely bioavailable (Goto and Yoshihara, 2001).

Ferritin is present in all crops, but differing in concentration. Biofortification also aims to enhance ferritin concentration of crops. Ferritin binds to free radicals that are damaging to cells. Ferritin also acts as a temporary storage form of Fe that is available in Fe limiting conditions (Briat et al., 2010a). It is reported that ferritin is the key component in alleviating oxidative stress (Mata et al., 2001). The main function of ferritin in seeds is protection against free Fe damage through Fenton oxidation (Briat et al., 2010b).

2.12 Models used to determine Fe bioavailability

The bioavailability and Fe absorption from the daily diet are influenced by the type and quantity of Fe present in food and by the presence of inhibitors and promoters of Fe
absorption in the diet and the individual’s Fe status (Duque et al., 2014). The urgency of addressing Fe deficiency stems from its implication in a number of health conditions, some serious or even fatal. Rat models are most frequently used for testing the effects of agents that are toxic or potentially hazardous to humans. This also refers to the toxicity of metals and a possible preventive and therapeutic effect (Brzóska et al., 2012; Al-Rejaie et al., 2013).

It was observed by Zielińska-Dawidziak et al. (2012) that in Fe deficient rats, decreased level of hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), serum and liver ferritin were increased to normal values or better after feeding ferritin isolate. Expression of soybean ferritin in rice resulted in Fe bioavailability similar to that of ferrous sulfate fortified rice when evaluated in a rat hemoglobin repletion model (Kolb et al., 2002) and human lactoferrin produced in rice had bioavailability similar to that of ferrous sulfate in young women (Lonnerdal et al., 2006). In mice, Fe biofortified rice feeding test and Caco-2 cell models confirmed that metal bioavailability in rats and humans increases with the increased level of metals in rice grains (Zheng et al., 2010; Lee et al., 2012b). Recently it was shown that biofortification of rice with Zn significantly increases Zn uptake in Caco-2 cell models as well as in rat pups, and is suggested to have a similar effect in human populations (Jou et al., 2012).
Chapter 3

Iron biofortification of wheat grain through integrated use of organic and chemical fertilizers in pH manipulated calcareous soil

Abstract

High incidence of iron (Fe) deficiency in human populations is an emerging global challenge for researchers attempting to alleviate Fe deficiency by increasing Fe concentration in food crops. With the aim to increase Fe bioavailability in wheat, this study was conducted to evaluate the potential of iron sulphate combined with biochar and poultry manure for Fe biofortification of wheat grain in a pH manipulated calcareous soil. In two incubation studies the rates of sulfur (S) and Fe combined with various organic amendments for lowering pH and Fe availability in calcareous soil were optimized. In a pot experiment, optimized rate of Fe along with biochar (BC) and poultry manure (PM) was evaluated for Fe biofortification of wheat in normal and S treated low pH calcareous soil. Iron applied with BC in S applied low pH soil (Fe + BC + S), provided the maximum increase in root-shoot biomass and photosynthesis up to 79, 53 and 67%, respectively, as compared to control. Grain Fe and ferritin concentration increased up to 140% and 120%, respectively while phytate and polyphenol decreased 35 and 44%, respectively in treatment when Fe was applied with BC and S compared to the control. Combined use of Fe and BC could be an effective approach for enhancing growth and Fe biofortification of wheat in pH manipulated calcareous soil.

Keywords: biochar, sulfur, ferritin, phytate, polyphenol
3.1 Introduction

Dietary deficiency of essential micronutrients such as iron (Fe) affect more than two billion people worldwide (White and Broadley, 2009; WHO, 2011). Countries where people depend on cereal based food have high incidence of micronutrient deficiencies (Cakmak et al., 2010a; Bouis et al., 2011). The World Health Organization estimates that approximately 25% of the world’s population suffers from anemia (WHO, 2010) and that Fe-deficiency anemia led to the loss of over 46,000 disability adjusted life years in 2010 alone (Murray and Lopez, 2013). Many possible strategies like dietary diversification, mineral supplementation, and post-harvest food fortification are used to improve micronutrient intake in the human diet. Biofortification circumvents these problems by increasing the micronutrient contents in edible part of crops and enhancing their bioavailability and absorption in human body during digestion (Bouis et al., 2011).

Low chemical solubility and low bioavailability of Fe in calcareous soil is a serious problem for crops (Laird et al., 2010; Seher et al., 2011). Iron is not deficient in mineral soil but high pH (Alcantara et al., 2002) and high HCO$_3$ contents in calcareous soil (Bloom and Inskeep, 1988) are two important factors that limit Fe availability. Iron fertilization in calcareous soil is not effective due to Fe’s rapid conversion into unavailable forms and poor mobility of Fe in phloem (Rengel et al., 1999; Cakmak et al., 2010a). Phytic acid and polyphenols are important anti-nutrients, which inhibit Fe bioavailability (Gautam et al., 2010). It was reported that acidifying calcareous soil with sulfur (S) and neutralizing lime increased phytoavailability of Fe (Lippman, 1916; Malakouti et al., 1988). Elemental S decreases the soil pH (Iqbal et al., 2012) and affects the Fe availability because Fe in soil reacts with S to form iron sulfide (Dent, 1986), sulfide can reduce ferric to ferrous (Murase and Kimura, 1997). Sulfur influences the formation of phytosiderophores, which are closely linked with Fe uptake by plants (Cao et al., 2002; Jin et al., 2005). Sulfur can increase the Fe transport in plants xylem (Hu and Xu, 2002; Na and Salt, 2011) and phloem, as well as accelerate the activation of deposited Fe in the apoplast (Holden et al., 1991; Toulon et al., 1992) and increase the accumulation of Fe in plants (Li et al., 2007).
Organic matter releases organic chemicals that serve as chelators and increase metal (Fe in this case) availability to plants by solubilizing the nutrients in soil solution (Du Laing et al., 2009; McCauley et al., 2009). Poultry manure has high nutrient composition and nitrogen (N) contents; high N improve Fe concentration in wheat by increasing Fe activity and abundance of Fe transporter proteins such as yellow stripe 1 (YS1) in the root cell membrane (Bujoczek et al., 2000; Curie et al., 2009). Biochar (BC) affects soil fertility by changing soil physico-chemical and biological properties leading to nutrient mobilization (Xu et al., 2013; Jeffery et al., 2015). In redox mediated reaction BC affects mineral forms of Fe by acting as an electron shuttle (Kappler et al., 2014). Biochar solubilizes Fe by decreasing soil pH due to its redox catalytic activity (Graber et al., 2014). In addition, soil properties changed by BC application may increase nutrient mobilization and uptake in the rhizosphere via increasing the exploratory capacity of the root density and modifying nutrient solubility (Lehmann et al., 2011).

Chemical fertilizers (soil and foliar applied) and organic amendments are used to increase plant growth and Fe biofortify cereals (White and Broadley, 2009; Cakmak et al., 2010a; Alburquerque et al., 2015). However, little is known about the combined use of iron sulphate with BC and poultry manure for Fe biofortification of wheat in pH altered calcareous soil. The objectives of this study were: 1) to define the time and rate of elemental S for lowering soil pH; 2) to find out optimum level of S and Fe for calcareous soil acidification and Fe biofortification; 3) to investigate if Fe fertilizer, BC, and PM alone as much affective as affective in pH altered calcareous soil.

3.2 Materials and Methods

3.2.1 Experimental soil

Soil was collected from a field area of Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan. Soil was collected from 0-15 cm depth and uniformly mixed. Soil was air dried, passed through a 2-mm sieve to remove pieces of stones and homogenized. A sunsample was collected for various physico-chemical properties of soil. Properties of experimental soil and organic amendments are shown in Table 3.1. The
soil was alkaline calcareous in nature and deficient in plant available Fe and Zn and also had a low proportion of organic matter.

Table 3.1 Physico-chemical properties of soil and organic amendments used in the experiment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Values</th>
<th>Physic-chemical properties for wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>%</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Silt</td>
<td>%</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Clay</td>
<td>%</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>–</td>
<td>7.9</td>
<td>5.5-6.5</td>
</tr>
<tr>
<td>Organic matter</td>
<td>%</td>
<td>0.7</td>
<td>≥1%</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>g kg⁻¹</td>
<td>21.9</td>
<td>≤1%</td>
</tr>
<tr>
<td>HCO₃</td>
<td>mmol L⁻¹</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>DTPA-Fe</td>
<td>ppm</td>
<td>4.21</td>
<td>4.5-5.5 ppm</td>
</tr>
<tr>
<td>DTPA-Zn</td>
<td>ppm</td>
<td>0.59</td>
<td>1 ppm</td>
</tr>
<tr>
<td>N</td>
<td>mg kg⁻¹</td>
<td>120</td>
<td>150 mg kg⁻¹</td>
</tr>
<tr>
<td>P</td>
<td>mg kg⁻¹</td>
<td>7.9</td>
<td>9.5 mg kg⁻¹</td>
</tr>
<tr>
<td>K</td>
<td>mg kg⁻¹</td>
<td>108</td>
<td>125 mg kg⁻¹</td>
</tr>
</tbody>
</table>

Characteristic of organic amendments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Farm yard manure</th>
<th>Biochar</th>
<th>Compost</th>
<th>Poultry manure</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.71</td>
<td>7.64</td>
<td>7.75</td>
<td>7.31</td>
</tr>
<tr>
<td>EC</td>
<td>dS m⁻¹</td>
<td>4.9</td>
<td>1.96</td>
<td>4.17</td>
</tr>
<tr>
<td>Organic matter</td>
<td>%</td>
<td>51.05</td>
<td>59.1</td>
<td>55.2</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>27</td>
<td>51</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td>Fe</td>
<td>mg kg⁻¹</td>
<td>312.3</td>
<td>308.9</td>
<td>292.8</td>
</tr>
<tr>
<td>Zn</td>
<td>mg kg⁻¹</td>
<td>351.9</td>
<td>281.8</td>
<td>155.5</td>
</tr>
<tr>
<td>Mn</td>
<td>mg kg⁻¹</td>
<td>316.7</td>
<td>161.4</td>
<td>187.6</td>
</tr>
<tr>
<td>Cu</td>
<td>mg kg⁻¹</td>
<td>266.6</td>
<td>177.7</td>
<td>269.2</td>
</tr>
</tbody>
</table>

3.2.2 Incubation study 1

3.2.2.1 Manipulation of soil pH

A preliminary incubation experiment was conducted to lower the soil pH. For slow and steady soil acidification, elemental S was used. Sulfur was manually crushed with a plastic spatula to obtain a homogeneous powder, sieved (<200 μm) and weighed. Different rates of S (1.5, 2, 2.5, 3 g kg⁻¹ soil) were used for calculation of S needed. Sulfur was used as a single
treatment and with combination of four different organic amendments (poultry manure, farmyard manure, compost, and biochar). All are used at the rate of 1% (w/w). Properties of organic amendments are given in Table 3.1. Acidity is produced in aerobic conditions by microbial oxidation of S to sulfuric acid.

$$S^0 + 1.5O_2 + H_2O \rightarrow H_2SO_4$$

Soil (300 g) with 60% moisture content was mixed with four rates of S and was incubated in a completely randomized design at 25°C for 18 weeks. Soil pH was measured by taking 2 g soil from each pot and with soil:solution ratio of 1:2.5. Data was calculated on a weekly basis by pH meter (JENCO pH meter, 671 P model). This data was used to calculate the amount of S to achieve a pH value of 6.5 in each soil.

### 3.2.3 Incubation study 2

#### 3.2.3.1 Calculation and selection of Fe rates

A second incubation study was conducted in a completely randomized design to select the rate of FeSO₄·7H₂O for Fe biofortification of wheat. Hydrated ferrous sulphate (FeSO₄·7H₂O) was used as Fe source. Different rates of Fe i.e. 0.0075, 0.0125, 0.175 g kg⁻¹ were used as a single treatment and in combination with two organic amendments (biochar and poultry manure, 1% w/w) selected from the 1st incubation study. These treatments were applied under control and pH manipulated soil. Sulfur rate (2.5 g S kg⁻¹ soil) as selected from study 1 was applied for manipulation of soil pH. Iron rate was selected on the basis of DTPA extractable Fe. Soil (500 g) with 60% moisture content was used in this experiment and was incubated at 25°C for 130 days. Data was calculated after each 10 days interval. This data was used to calculate the amount of Fe needed to obtain a DTPA extractable Fe value up to 9 ppm in each soil as and 5 g soil extracted by 0.005 MDTPA (Lindsay and Norvell 1978) and measured by atomic absorption spectrophotometer (Perkin Elmer, AAAnalyst 100, Waltham, USA).

### 3.2.4 Plant experiment

#### 3.2.4.1 Experimental plants growth and conditions
A pot experiment was conducted in the wire house of Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan to evaluate the potential of Fe$_2$SO$_4$.7H$_2$O combined with organic amendments for Fe biofortification of wheat in pH manipulated calcareous soil. Pots were filled with 8 kg sieved soil and two organic amendments (biochar and poultry manure @ 1% w/w) in each pot according the treatment plan (see Table 3.2). Prior to seed sowing into the pots, soil pH was lowered by S (rate of S as selected in study 1), and S added 4 weeks behor sowing wheat seed. Poultry manure was collected from the poultry farm of University of Agriculture Faisalabad. Biochar was made from Eucalyptus feedstock at 400°C temperature. After making biochar from feedstock, it was ground and thoroughly mixed into pots. Iron was applied as determined in the 2nd incubation study (0.0075 g kg$^{-1}$) at the sowing time of wheat. Recommended doses of N (urea), phosphorus (P) (single super phosphate) and potassium (K) (sulphate of potash) i.e. 0.06, 0.05, 0.03 g kg$^{-1}$ equivalent, respectively were applied. Full doses of P and K were applied at the time of sowing while N dose was applied in two splits. The first sub-dose was applied at sowing and the remaining applied 30 days after sowing.

Six seeds of wheat variety (Glaxy-2013) were sown in each pot at a depth of 2 cm and 3 seedlings per pot were maintained after emergence. Throughout the experimental period, de-ionized water was used to maintain soil moisture as needed. Fifty days after sowing, plant physiological parameters such as photosynthetic rate (A), transpiration (E) rate, stomatal conductance (gs), and substomatal conducts (Ci) were measured by using a CIRAS-3 (PP System, Amesbury, MN, USA). Plant were harvested at maturity and manually threshed to separate the grain. After shade drying, samples were kept in a forced-air-driven oven at 60°C until constant weight. Dry weight of root, shoot and grains yield were recorded after oven drying.

**3.2.4.2 Grain analysis**

Whole grain samples were ground in a mill (IKA Werke, MF 10 Basic, Staufen, Germany) to a mesh size of 0.5-mm. Sample was placed in muffle furnace at 550°C for complete oxidation of organic matter until the appearances of gray white ash (AOAC, 2003). Ground subsamples of known weight were digested in a di-acid mixture (HNO$_3$:HClO$_4$ ratio of 2:1)
Zinc and Fe concentrations in the digest were measured by atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Abbreviations</th>
<th>Iron solubilizing agent (Elemental Sulfur)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfur g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Control</td>
<td>C</td>
<td>–</td>
</tr>
<tr>
<td>Biochar</td>
<td>BC</td>
<td>–</td>
</tr>
<tr>
<td>Poultry Manure</td>
<td>PM</td>
<td>–</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Biochar</td>
<td>Fe + BC</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Poultry Manure</td>
<td>Fe + PM</td>
<td>–</td>
</tr>
<tr>
<td>Sulfur</td>
<td>S</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Sulfur + Biochar</td>
<td>S + BC</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Sulfur + Poultry Manure</td>
<td>S + PM</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Sulfur + Iron</td>
<td>S + Fe</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Sulfur + Iron + Biochar</td>
<td>S + Fe + BC</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
<tr>
<td>Sulfur + Iron + Poultry Manure</td>
<td>S + Fe + PM</td>
<td>2.5 g kg(^{-1}) soil</td>
</tr>
</tbody>
</table>

Total protein concentration in grain was determined with the Bradford colorimetric method (Bradford, 1976). Fat was determined by ether extract method while using Soxhlet apparatus (AOAC, 2003). Crude fiber was determined according to the procedure described in AOAC (2003). A moisture free and ether extracted sample, made of cellulose was first digested with dilute H\(_2\)SO\(_4\) and then with dilute KOH solution. The undigested residue collected after digestion was ignited and loss in weight after ignition was registered as crude fiber. Phytate was determined by an indirect method as described by Haug and Lantzsch (1983). A 60 mg whole grain ground sample was extracted with 10 mL of 0.2 N HCl at room temperature for 2 h and final reading was taken on a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan). From prepared grain sample, polyphenol was measured by using the Folin–Ciocalteau method (Aguilar-Garcia et al., 2007). Absorbance was taken by spectrophotometer at 760 nm.
wavelength via the calibration curve of gallic acid and expressed as gallic acid equivalent. 

For ferritin quantification, a 5 g grain sample was prepared as seed method described by Lukac et al. (2009) with slight modifications. Ferritin concentration was measured by direct ELISA (enzyme linked immunosorbent assay) using anti-body (Rabbit anti-ferritin) coated microtiter wells and mouse monoclonal anti-ferritin antibody as antibody enzyme (Horseradish peroxidase) conjugate solution and absorbance was taken at 450 nm wavelength (Catalog Number: BC-1025). All devices used for chemical and biochemical analysis were soaked in diluted HNO₃ (pro analysis quality, Merck) and washed with deionized water.

3.2.5 Statistical analysis

The data of incubation studies were analyzed using MS Excel 2010. The data of pot experiments was subjected to one-way analysis of variance (ANOVA) using Statistix 8.1 software (Analytical Software, USA, 2005). Significant difference of treatment means were separated by post hoc Tukey’s test at P < 0.05.

3.3 Results

3.3.1 Time course of pH manipulation in non-planted soil

Soil pH value decreased gradually in the pots receiving different doses of elemental S and was stable after 4 weeks of incubation. Among the four organic amendments, application of S in soil with BC and PM [Fig. 3.1 (b, c)] lowered soil pH constantly compared to soil with compost (CO) and farmyard manure (FYM) [Fig. 3.1 (d, e)]. Addition of S decreased soil pH by about 1-1.9 units in BC and PM amended soil compared to the control. This effect was more pronounced in the 18th week in the pots amended with BC and PM because of their lower initial pH. Elemental S applied with out any organic amendments show similar trend as with biochar and poultry manure. Without S, a slight increase in soil pH was observed by the application of CO and FYM, compared to the control (Fig. 3.1). This increase in soil pH was due to their slightly alkaline nature.
Soil pH

**Sulfur**

- Control
- S_a
- S_b
- S_c
- S_d

**Biochar**

- Control
- BC
- BC+S_a
- BC+S_b
- BC+S_c
- BC+S_d

**Compost**

- Control
- CO
- CO+S_a
- CO+S_b
- CO+S_c
- CO+S_d
Fig 3.1 a-e: Soil pH manipulation with elemental S and different organic amendments. Error bars represent standard errors of the mean (n=3)

S: sulfur, S a= 1.5 g elemental sulfur kg⁻¹ soil, S b= 2 g elemental sulfur kg⁻¹ soil, S c= 2.5 g elemental sulfur kg⁻¹ soil, S d=3 g elemental S kg⁻¹ soil

BC: 1% biochar, PM: 1% poultry manure, CO: 1% (w/w) compost, FYM: 1% (w/w) farm yard manure
3.3.2 Integrated effect of chemical and organic amendments on DTPA-extractable Fe in pH manipulated calcareous soil

Data in Fig. 3.2a (treatment vs soil pH) revealed that DTPA-extractable Fe concentration increased in BC and PM amended soil along with different rates of Fe fertilizer in low pH soil compared to the control. The S addition at 2.5 g kg\(^{-1}\) soil along with 0.0175 g Fe kg ha\(^{-1}\) gave the highest increase in DTPA-extractable Fe concentration (i.e. 12.1 and 12.5 ppm by PM and BC.)
Fig 3.2 a-c: Relationship of DTPA-extractable Fe with, treatment vs pH (a), pH vs days (b), days vs treatments (c). Error bars represent standard errors of the mean (n=3).

PM: poultry manure (1% w/w), BC: biochar (1% w/w), Fe a=0.0075 g kg⁻¹, Fe b=0.0125 g kg⁻¹, Fe c=0.0175 g kg⁻¹ amended soil, respectively. The DTPA-extractable Fe without S was 9.5 and 7.9 ppm in BC and PM, respectively. Iron application at 0.0175 g kg⁻¹ gave 7.8 and 11.1 ppm Fe in unamended and low pH soil, respectively. The data of pH vs days (Fig. 3.2b) showed that DTPA-extractable Fe decreased slowly from 10 to 110 days after incubation at low pH, however, DTPA-extractable Fe was decreased sharply at the unamended pH. Maximum DTPA-extractable Fe (i.e. 10.8 and 8.4 ppm) was observed at 30 and 10 days after incubation, respectively in pH manipulated and unamended soil. Data in Fig. 3.2c (days vs treatments) illustrated that DTPA-extractable Fe was 12.7 and 13.7 ppm in PM and BC amended soil, respectively at 20 days after incubation and it remained 7.5 and 8.6 ppm at 130 days after incubation. Iron application alone at 0.0175 g kg⁻¹ was 12.7 ppm at 20 days after incubation and remained 7.1 ppm at 130 days after incubation.

Application of Fe fertilizer (0.0075 g kg⁻¹) combined with PM and BC, was selected for the pot trial which gave DTPA-extractable Fe of 9.9 and 10.3 ppm, respectively, double the amount required by plants to complete their life cycle (Fig. 3.2a).
3.3.3 Pot experiment

3.3.3.1 Growth and physiological parameters

Separate application of organic amendments (BC and PM) and Fe without S increased growth and physiological parameters non-significantly compared to the control. However, with the addition of S, significant increase in growth and physiology were observed compared to the control (Table 3.3). Maximum increase in shoot dry weight 53% was observed by combined application of BC and Fe followed by combined application of PM and Fe (47%) in S treated low pH soil over the control. Similarly, combined application of Fe with BC and PM significantly increased grain yield 27 and 23%, respectively compared to the control in S amended soil (Table 3.3). A significant increase of 58 and 79% in root dry weight of wheat was recorded by combined application of Fe with PM and BC in S treated low pH soil compared to the control. Photosynthetic rate in all treatments increased significantly where Fe was applied with BC and PM with or without S application, compared to the control (Table 3.3). Maximum increase in photosynthetic rate of 67% was observed by application of Fe + BC followed by 54% with Fe + PM in S-treated low pH soil with respect to the control. Significant increase in transpiration rate was observed where Fe was applied with BC and PM in unamended and S amended soil with respect to the control (Table 3.3). Maximum increase of 93 and 79% in transpiration rate was observed by Fe application with BC and PM in S treated soil, respectively compared to the control. There was a significant increase in stomatal conductance in all treatments where Fe was applied with organic amendments. The highest increase, 46 and 44% in stomatal conductance, was observed in Fe application with BC and PM in S amended soil with respect to the control. Likewise, sub-stomatal conductance of wheat decreased in all treatments where Fe was applied with organic amendments, but the maximum decrease of 17% in sub-stomatal conductance was recorded when Fe was applied with BC compared to the control.
### Table 3.3 Effect of Fe application along with BC and PM on growth and physiological parameters of wheat in normal and S treated low pH soil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot DW (g pot⁻¹)</th>
<th>Root DW (g pot⁻¹)</th>
<th>Grain Yield (g pot⁻¹)</th>
<th>A (μmol CO₂ m⁻² s⁻¹)</th>
<th>E (μmol H₂O m⁻² s⁻¹)</th>
<th>Gs (μmol m⁻² s⁻¹)</th>
<th>Ci (μmol mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>11.7 f</td>
<td>1.9 f</td>
<td>15.1 g</td>
<td>11.9 g</td>
<td>2.8 f</td>
<td>247.7 f</td>
<td>162 a</td>
</tr>
<tr>
<td>BC</td>
<td>13.3 d-f</td>
<td>2.3 c-f</td>
<td>16.2 e-g</td>
<td>13 fg</td>
<td>3.1 d-f</td>
<td>263.3 d-f</td>
<td>147 a-c</td>
</tr>
<tr>
<td>PM</td>
<td>12.7 ef</td>
<td>2.1 d-f</td>
<td>16.5 d-g</td>
<td>12.6 fg</td>
<td>2.9 ef</td>
<td>255 d-f</td>
<td>157 ab</td>
</tr>
<tr>
<td>Fe</td>
<td>13.4 d-f</td>
<td>1.9 f</td>
<td>16.9 c-f</td>
<td>14 d-f</td>
<td>3.7 c-f</td>
<td>285.3 c-f</td>
<td>139 a-c</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>16.6 a-c</td>
<td>2.9 a-c</td>
<td>17.8 b-d</td>
<td>15.3 cd</td>
<td>4.4 a-c</td>
<td>295 cd</td>
<td>134 c</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>15.9 a-d</td>
<td>2.6 b-e</td>
<td>17.4 b-e</td>
<td>15 c-e</td>
<td>6 b-e</td>
<td>291.7 c-e</td>
<td>136 bc</td>
</tr>
<tr>
<td>S</td>
<td>12.7 ef</td>
<td>2.1 ef</td>
<td>15.8 fg</td>
<td>12.6 fg</td>
<td>3.2 c-f</td>
<td>254 ef</td>
<td>152.3 a-c</td>
</tr>
<tr>
<td>S + BC</td>
<td>15.1 b-e</td>
<td>2.7 b-d</td>
<td>16.6 d-f</td>
<td>14 d-f</td>
<td>3.8 b-f</td>
<td>273 d-f</td>
<td>141.3 a-c</td>
</tr>
<tr>
<td>S + PM</td>
<td>14.5 c-e</td>
<td>2.6 b-e</td>
<td>16.2 e-g</td>
<td>13.4 e-g</td>
<td>3.3 c-f</td>
<td>269 d-f</td>
<td>145.3 a-c</td>
</tr>
<tr>
<td>S + Fe</td>
<td>16.2 a-c</td>
<td>2.8 bc</td>
<td>18.2 a-c</td>
<td>16.6 bc</td>
<td>4.3 a-d</td>
<td>318 bc</td>
<td>134 c</td>
</tr>
<tr>
<td>S + Fe + BC</td>
<td>17.9 a</td>
<td>3.4 a</td>
<td>19.3 a</td>
<td>19.9 a</td>
<td>5.4 a</td>
<td>361 a</td>
<td>131.3 c</td>
</tr>
<tr>
<td>S + Fe + PM</td>
<td>17.2 ab</td>
<td>3 ab</td>
<td>18.7 ab</td>
<td>18.3 ab</td>
<td>5 ab</td>
<td>357.7 ab</td>
<td>132.3 c</td>
</tr>
</tbody>
</table>

(Tukey’s test HSD₀.₀₅: Shoot DW 2.57, Root DW 0.57, Grain yield 1.4, A 1.8, E 1.2, gs 40, Ci 24.6)

Photosynthesis rate (A), transpiration rate (E), stomatal conductance (Gs) and sub-stomatal conductance (Ci) measured in each experimental pots as influenced by Fe fertilizer and organic amendments (BC and PM) in both normal and S treated pH manipulated soil. Quantities sharing similar letters are statistically similar to each other at p ≤ 0.05

#### 3.3.3.2 Iron and zinc concentration

Iron application alone increased Fe content in maize grain up to 67% while applied with S-amended soil, increased up to 109%, over control (table 3.4). Biocar applied as a single treatment decreased Fe contents up to 8% and BC applied with Fe increased 96% and BC applied with Fe in S-amended soil increase grain Fe contents up to 142% as compare to control. Iron applied with PM in creased grain Fe contents up to 86%, while Fe applied with
PM in S amended soil increased grain Fe contents up to 132% over control. However, results revealed that Fe applied with organic amendments in S amended low pH soil is more effective as compared to without S amended soil. Soil amended with S and PM showed a significant increase of 24% in Zn concentration of wheat grain compared to the control (Table 3.4). The maximum increase of 35% in Zn concentration of wheat grain was recorded by applying Fe + PM in S treated low pH soil over control.

3.3.3.3 [Phytate]:[Fe] and [Phytate]:[Zn] ratio

Iron applied with BC and PM significantly decreased the [phytate]:[Fe] ratio in unamended and S amended low pH soil compared to the control (Table 3.4). A non-significant effect on [phytate]:[Fe] ratio was observed when BC was applied alone compared to the control. Among organic amendments, BC applied with Fe showed the lowest value of [phytate]:[Fe] ratio (i.e. 60% less than the untreated control). The lowest values of [phytate]:[Fe] ratio

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fe (mg kg⁻¹)</th>
<th>Zn (mg kg⁻¹)</th>
<th>Phytate/Fe</th>
<th>Phytate/Zn</th>
<th>Ash (%)</th>
<th>Fat (%)</th>
<th>Fiber (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>49.7 fg</td>
<td>20.5 c-e</td>
<td>20.5 ab</td>
<td>58.1 ab</td>
<td>1.3 c</td>
<td>1.9 c</td>
<td>1.1 c</td>
</tr>
<tr>
<td>BC</td>
<td>45.7 g</td>
<td>19.2 de</td>
<td>22.1 a</td>
<td>61.4 a</td>
<td>1.3 c</td>
<td>1.9 c</td>
<td>1.1 c</td>
</tr>
<tr>
<td>PM</td>
<td>57.3 fg</td>
<td>22.2 b-e</td>
<td>17 bc</td>
<td>51.2 c</td>
<td>1.4 c</td>
<td>2 bc</td>
<td>1.2 bc</td>
</tr>
<tr>
<td>Fe</td>
<td>84.1 de</td>
<td>18.9 e</td>
<td>10.2 ef</td>
<td>53 bc</td>
<td>1.6 bc</td>
<td>2.3 a-c</td>
<td>1.4 a-c</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>97.5 b-d</td>
<td>19.1 de</td>
<td>8.2 fg</td>
<td>49.1 c</td>
<td>1.7 ab</td>
<td>2.3 a-c</td>
<td>1.5 a-c</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>92.9 cd</td>
<td>23.2 a-d</td>
<td>8.8 fg</td>
<td>41.1 d</td>
<td>1.7 ab</td>
<td>2.3 a-c</td>
<td>1.4 a-c</td>
</tr>
<tr>
<td>S</td>
<td>58 fg</td>
<td>21.8 b-e</td>
<td>13.4 de</td>
<td>52.8 bc</td>
<td>1.4 c</td>
<td>1.9 c</td>
<td>1.2 bc</td>
</tr>
<tr>
<td>S + BC</td>
<td>63.7 fg</td>
<td>22.7 a-e</td>
<td>14.8 cd</td>
<td>48.3 c</td>
<td>1.4 c</td>
<td>2.1 a-c</td>
<td>1.3 a-c</td>
</tr>
<tr>
<td>S + PM</td>
<td>66.6 ef</td>
<td>25.4 ab</td>
<td>13.4 de</td>
<td>41 d</td>
<td>1.4 c</td>
<td>2.1 a-c</td>
<td>1.2 bc</td>
</tr>
<tr>
<td>S + Fe</td>
<td>99.1 bc</td>
<td>21.7 b-e</td>
<td>7.2 fg</td>
<td>38.7 d</td>
<td>1.7 ab</td>
<td>2.3 a-c</td>
<td>1.5 a-c</td>
</tr>
<tr>
<td>S + Fe +</td>
<td>120.3 a</td>
<td>23.8 ab</td>
<td>5.4 g</td>
<td>31.9 e</td>
<td>1.9 a</td>
<td>2.5 a</td>
<td>1.7 a</td>
</tr>
<tr>
<td>BC</td>
<td>115.8 ab</td>
<td>27.7 a</td>
<td>5.9 g</td>
<td>28.8 e</td>
<td>1.8 ab</td>
<td>2.4 ab</td>
<td>1.6 ab</td>
</tr>
</tbody>
</table>

(Tukey’s test HSD₀.₀₅ = Fe 18, Zn 4.2, Phytate/Fe molar ratio 3.5, Phytate/Zn molar ratio 5.8, Ash 0.26, Fat 0.59, Fiber 0.46)

Quantities sharing similar letters are statistically similar to each other at p ≤ 0.05
occurred in Fe + BC and Fe + PM in S treated low pH soil and were a 74 and 71% decrease, respectively, compared to the control. A decrease in [phytate]:[Zn] ratio of 29% occurred when Fe was applied with PM compared to the control. Similarly, a significant decrease in [phytate]:[Zn] molar ratio was observed when Fe and organic amendments were used along with S compared to the control. The maximum decrease in [phytate]:[Zn] ratio occurred in Fe + BC + S and Fe + PM + S treatments and were 33 and 45% less, respectively, compared to the control.

3.3.3.4 Physico-chemical analysis (Ash, Fat, Fiber)

Ash contents in wheat grain increased significantly with Fe application separately and with BC and PM compared to the control in unamended and S amended low pH soil (Table 3.4). Iron application as a single treatment and Fe + S increased ash content up to 23 and 31%, respectively compared to the control. The maximum increase in ash contents (46 and 39%) was observed where combined application of Fe with BC and PM was used, respectively in S treated low pH soil compared to the control. A significant increase in fat content of wheat grain was observed by application of Fe + BC + S and Fe + PM + S compared to the control (Table 3.4). The fat contents increased up to 32 and 26% where Fe was applied with BC and PM in S treated soil, respectively compared to the control. Iron, BC, and PM applied separately and in combination showed non-significant effect on fiber contents in wheat grain compared to the control. Iron applied with BC and PM increased fiber content up to 55% and 46%, respectively in pH manipulated soil compared to the control.

3.3.3.5 Phytate and polyphenol concentration

There was a significant decrease in phytate and polyphenol concentrations in wheat grain when Fe was applied with organic amendments compared to the control [Figs. 3.3 (a, b)]. The decrease in phytate and polyphenol concentrations (i.e. 20 and 19%, respectively) were observed when Fe was added with BC compared to the control. Similarly, phytate and polyphenol concentration decreased significantly when Fe was added with BC and PM in S treated low pH soil [Figs. 3.3 (a, b)]. Minimum concentrations (7.7 and 3.9 mg g⁻¹ of phytate and polyphenol, respectively) were measured when Fe + BC + S treatment was used compared to the control. There was a decrease of 35 and 34% in phytate and 44 and 41%
polyphenol concentration s, respectively, in treatments where Fe + BC and Fe + PM were added in S treated low pH soil compared to the control.

HSD value= Phytate 1.4, Grain polyphenol 1.4

Fig 3.3 a, b: Phytate and polyphenol concentration in wheat grain measured in various Fe treatments under unamended and pH manipulated calcareous soil. Values are the means and letters are significantly different from each other at p<0.05 (n=3).

3.3.3.6 Protein and ferritin concentration
Protein concentration in wheat grain significantly increased with Fe application alone, and Fe + BC and Fe + PM along with S compared to the control [Fig. 3.4 (a)]. Iron applied with PM and S increased protein concentration up to 22 and 24%, respectively compared to the control. The highest increase in protein concentration 29 and 27% was observed by combined

![Graph (a)](image)

![Graph (b)](image)

HSD value= Grain ferritin 0.33, Grain protein 2.1

Fig. 3.4 a, b: Protein and ferritin concentration in wheat grain measured in various Fe treatments under unamended and pH manipulated calcareous soil. Values are the means and letters are significantly different from each other at p<0.05 (n=3).
application of Fe + BC + S and Fe + PM + S, respectively, compared to the control. Iron applied alone and combined with BC and PM in both unamended and S treated soil, significantly increased grain ferritin concentration compared to the control [Fig. 3.4 (b)]. Combined application of Fe with BC and PM increased ferritin concentration in grain up to 66 and 60%, respectively compared to the control. Similarly, significant increase in ferritin concentration was determined when Fe, BC and PM were applied with S compared to the control. The maximum value of ferritin concentration of wheat grain (1.49 and 1.46 µg g\(^{-1}\)) occurred in the combined application of Fe + BC + S and Fe + PM + S, respectively and was 1.2- and 1.1- fold higher compared to the control.

3.4 Discussion

Lowering soil pH with elemental S has been tested successfully by many researchers (Erika et al., 2012; Iqbal et al., 2012). Oxidation of elemental S produces sulfate and sulfate complexes of Fe, enhancing Fe solubility and its bioavailability. Iron solubility in S amended (low pH) soil along with organic amendments increases the processes in addition to the action of protons [Fig. 3.2 (a)]. Organic amendments such as BC and poultry manure have potential to improve soil physico-chemical and biological properties leading to increase Fe mobilization and enhance its bioavailability (Du Laing et al., 2009; Xu et al., 2013). Iron is not absent in mineral soil but due to high pH (Sabbagh et al., 2012) and calcareousness, its availability decreases to plants (del Campillo and Torrent, 1992).

Quick transformation of Fe from soluble to relatively insoluble oxides and hydroxides in calcareous soil, some adsorbing material was used followed by mineralization of these adsorbed Fe compounds using pH manipulating agent like elemental S. Photosynthesis and respiration greatly depend on availability of Fe; it is the key component of chlorophyll and respiratory enzymes. Iron deficiency causes physiological alterations of leaf morphology, xylem vessel morphology, stomatal conductance, leaf hydraulic conductance, and leaf water potential in chlorotic leaves (Eichert et al., 2010). In the current study, it was observed that Fe application along with BC in pH manipulated soil improved physiological parameters like photosynthetic rate, transpiration rate and stomatal conductance. The mechanism behind this might be solubilization of Fe by using S that reduced soil pH and increases nutrient
availability as reported by Wang et al. (2006a). Furthermore, it is a well-established fact that biochar has high cation exchange capacity that provides essential nutrients to plants via exchange processes (Grabert et al. 2014; Kappler et al., 2014)

In the current study, plant growth was significantly improved by using Fe with BC in unamended and acidified soil (Table 3.3). Several mechanisms might be operating behind the growth improvement of wheat. Rhizospheric pH manipulation by H\(^+\) released in soil desorbs Fe from the BC surface and increases its mobility to plants. Along with Fe, several other essential nutrients also release from soil and biochar surfaces (Kimetu et al., 2008) that are important in plant growth. Agronomic parameters like shoot and root dry weight and grain yield were significantly improved in treatments where Fe along with BC were applied. My results are in line with Alburquerque et al. (2015) who reported that BC application along with fertilizer enhanced plant growth and yield more as compare to alone biochar. Biochar has additional indirect mechanisms that are also important from the plant growth point of view, i.e. nutrient supply by reduced leaching, changes in redox condition of soil, high water holding capacity, and microbial interaction thus increasing microbial biomass (Lehmann et al., 2011). Yang et al. (2003) found that use of organic amendments improved micronutrient accumulation in plants, which also corresponds to my results.

The microelement (i.e. Fe and Zn) concentration of wheat grain was significantly improved using Fe and BC in acidified soil. The driving force behind this might be lower soil pH as is evident from my incubation study (see Fig. 3.2). Mühlbachová et al. (2005) and Zhao et al. (2010) showed that soil pH played the most important role in determining nutrient speciation, solubility from mineral surfaces, movement, and eventually bioavailability of the essential micronutrients. My findings are in line with Iqbal et al. (2012) in which elemental S draws nutrients from natural and anthropogenic pools by solubilizing and their uptake by plants is increased. Kingery et al. (1993) found elevated level of Zn in soils heavily fertilized with poultry manure. According to Uprety et al. (2009) application of poultry manure (and other manures) can considerably increase the micronutrient content in soil because these nutrients are given as supplements in poultry feeds, and are present in the manure. Demir et al. (2010) in this instance is inconsistent with my results, because he found that poultry manure could
be a good medium of growth as it increased leaf and fruit Zn level but concentration of Fe remained unaffected.

Apart from soil factors, many nutritional factors are important in reducing Fe bioavailability in cereal grains. Among these factors, phytic acid is most important. Phytic acid is the primary storage form of phosphorus and forms complexes with important minerals like Fe and Zn, thus inhibiting their absorption (Jin et al., 2009). Reddy et al. (2000) found that grain phytic acid correlated negatively with Fe absorption, and in my study, phytate concentration decreased in wheat grain when soil pH was decreased with S. I found that as bioavailable Fe concentration increased, phenolic compounds decreased significantly by using Fe with BC in acidified soil. Tako and Glahn (2010) showed that increased Fe concentration can limit the polyphenolic inhibitory effect on Fe absorption provided that polyphenolic content remains constant as mineral (Fe, Zn) contents are not deficient.

Modern agriculture emphasizes not only the quantity of produce but the quality as among its top priorities. Neutral soil pH promotes the precipitation of poorly ordered Fe minerals (ferrihydrite), whereas acid and submerged conditions enhance the mobilization of Fe minerals (Claudio et al., 2014). Along with H⁺ released from acidity that is produced by S, roots excrete organic acids in the rhizosphere that further improve Fe availability. Organic acids stimulate long-distance transport of the metal in Fe deficient plants (López-Millán et al., 2001).

Previously, carbonaceous products have been used for Zn biofortification of crops (Gartler et al., 2013). To the best of my knowledge, BC is being reported for the first time for Fe biofortification of wheat in acidified calcareous soil. Regarding other quality parameters, I observed that wheat fiber and ash contents were significantly improved by using BC along with Fe in acidified soil (Table 3.4).

To increase fiber contents of food some supplementation practices have been used. Traditionally, fiber supplementation has been focused on the use of milling by-products of wheat and corn (Matz, 1991). Although food supplementations and fortification efforts are successful in developed countries in developing countries their success remains limited (Bouis et al., 2011). The protein content of wheat grain may vary between 10-18% of the
total dry matter (Zuzana et al., 2009). In the present study, protein contents of wheat grain increased by using Fe along with BC + S. Ferritin, an iron storage protein, binds Fe as a non-toxic form and ferritin iron is bioavailable to humans as ferrous sulphate (Arosio et al., 2009). In the last decade, attempts have been made for biofortification of staple food crops by introducing the ferritin gene (Drakakaki, 2005; Masuda et al., 2013). Ferritin content increased in wheat grain proving that agronomic biofortification of wheat with Fe by reducing anti-nutrition factors can be an effective strategy to combat Fe deficiency in humans. Ferritin is not only a bioavailable Fe source for humans but it also provides Fe when its supply is scarce and protects plants from oxidative stress (Briat et al., 2010a, b).

3.5 Conclusions

In conclusion, soil acidification with S improved Fe solubilization and its bioavailability. Iron application along with organic amendments (BC and PM) was an effective strategy to enhance Fe biofortification of wheat in acidified soil. Ferritin concentration in wheat grain grown in calcareous soil could be enhanced by optimizing Fe bioavailability through acidification and its translocation in plant body could serve as a valuable approach to fight the malnutrition problem in the world. Among the organic amendments (BC and PM), BC applied with Fe in acidified calcareous soil remain a best treatment with regards to Fe bioavailability.
Exploiting iron bioavailability and nutritional value of maize grain through combined use of ferrous sulphate with biochar and poultry manure in sulfur treated low pH calcareous soil

Abstract

Iron (Fe) deficiency has affects half of the world population and is considered one of the most widespread nutritional problems. Factors such as pH, calcium carbonate, and bicarbonate in calcareous soil reduce iron availability to crops and limit its concentration in grain. In the present study, we evaluated the potential of Fe fertilizer combined with organic amendments like biochar (BC) and poultry manure (PM) for Fe biofortification of maize grain in sulfur (S) treated low pH calcareous soil. Elemental sulfur (S) was used to lower soil pH and solubilize Fe. Application of Fe combined with amendment (BC) and S significantly increased root (69%) and shoot (86%) dry weight, grain weight (28%), photosynthetic rate (74%), transpiration rate (57%), and stomatal conductance (33%), respectively compared to the control. Similarly, combined application of Fe + BC + S increased starch (34%), protein (64%) and fat (100%) while the anti-nutrient phytate and polyphenol contents decreased up to 29 and 40%, respectively over the control. Application of Fe with BC + S gave maximum increase of Fe and ferritin (120 and 170%, respectively), while S + PM increased Zn and Mn up to 40 and 60%, respectively over the control. This study indicated that applying Fe with BC + S is beneficial for crop growth, enhances the nutrient status of grains and reduces the phytate and polyphenol contents in maize grain.

Key words: biofortification, zinc, manganese, ferritin, protein, phytate
4.1 Introduction

Iron deficiency is a serious problem for crops grown on calcareous soils (Laird et al., 2010). High pH and high carbonates in calcareous soil limit Fe availability (Bloom and Inskeep, 1988; Fanrong et al., 2011). Iron fertilization in calcareous soil is not effective because Fe$^{+2}$ rapidly converts into unavailable Fe$^{+3}$ forms, which causes poor mobility of Fe in soil (Rengel et al., 1999; Cakmak et al., 2010a).

Among the cereals crops maize (*Zea mays* L.) is the third most important cereal crop (Sleper and Poehlman, 2006). Maize-based diets are deficient of necessary micronutrients such as Fe, Zn, provitamin A, and the people who depend on such diets have stunted growth, high incidence of anemia, less physical activity, and infant morbidity (Saltzman et al., 2013). In the developing world the majority of the population depends on cereal-based foods containing low concentrations of bioavailable Fe (Cakmak et al. 2010a; Bouis et al. 2011). In maize, phytic acid and polyphenols are considered to be the major inhibitors of iron bioavailability because they interact with food constituents such as Fe and make them unavailable, but the inhibitory effect on Fe absorption can be limited by increasing Fe bioavailability (Tako et al., 2013).

Iron biofortification is a sustainable and cost-effective agricultural strategy to alleviate malnutrition, particularly of rural families that do not have enough money and lack healthcare facilities (White and Broadley, 2009; Bouis and Welch, 2010). With Fe biofortification, the main target is to increase the bioavailable of Fe in cereals especially maize. Ferritin is a non-heme protein that binds Fe in a non-toxic form and ferritin Fe is bioavailable in humans as ferrous sulphate (Lonnerdal, 2007). Ferritin in seeds thus appears not only to be an Fe storage mechanism, but also protects plants from oxidation (Briat et al., 2010b). Numerous attempts have been made to biofortify staple food crops by introducing ferritin gene in the last decade (Masuda et al., 2013).

Different approaches have been used to enhance Fe bioavailability in maize by manipulating soil factors that reduce Fe bioavailability. Elemental S decreases the soil pH (Iqbal et al., 2012) and enhances the phytosiderophore formation process, which is closely
linked with Fe uptake by plants (Cao et al., 2002; Jin et al., 2005). Interestingly, S is also involved in Fe transport in xylem and increased accumulation of Fe in plants (Na and Salt, 2011).

Similarly, organic chemicals released from organic matter serve as chelaters and enhance solubility of nutrients and increase metal availability to plants (McCauley et al., 2009). Among the different forms of organic amendments, biochar (BC) and poultry manure (PM) are considered the best because of their long lasting effect on soil health and nutrient availability (de Cesare Barbosa et al., 2015). High nitrogen content in PM enhances Fe concentration in plants by increasing the efficiency of Fe transporter proteins such as yellow stripe 1 (YS1) in the root cell membrane (Bujoczek et al., 2000; Curie et al., 2009). Biochar acts as an electron shuttle in redox mediated reactions and affects mineral forms of Fe (Kappler et al., 2014). Redox catalytic activity is associated with biochar solubilized Fe with decreasing soil pH (Graber et al., 2014). Biochar affects soil fertility by changing soil physico-chemical and biological properties leading to nutrient mobilization (Jeffery et al., 2015).

Studies have been conducted on Fe biofortification with organic amendments in unaltered and problem soils but to the best of my knowledge, no such study was yet reported on Fe biofortification in maize with different organic amendments in pH manipulated (i.e. acidified) calcareous soil. The main objective of this study was to: 1) lower soil pH in calcareous soil with elemental S; and 2) determine how Fe fertilizers and organic amendments in unmended and low pH soil affect plant growth and Fe bioavailability.

4.2 Materials and Methods

4.2.1 Soil acidification

Experimental soil was acidified using elemental sulfur (S) as previously described in chapter 3.

4.2.2 Pot experiment
Soil used in experiment was obtained from a field area of Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan. Before conducting the experiment, all pieces of stone and straw were removed by passage through a 2-mm sieve and analyzed for physico-chemical properties. Soil texture was clay loam (sand 39%, silt 29%, clay 32%) as determined by hydrometer method (Gee and Bauder, 1986). Saturated soil paste had a pH value 7.81. Organic matter, 0.68%, was determined by Walkley-Black method (Jackson, 1962). Calcium carbonate (CaCO₃) was 23.9 g kg⁻¹ soil as determined by acid dissolution (Allison and Moodie, 1965). Plant available Fe and Zn was 4.1 and 0.6 ppm, respectively, as extracted by 0.005 M DTPA (Lindsay and Norvell, 1978). Phosphorus extracted by Olsen method (Watanabe and Olsen, 1965) was 7.7 mg kg⁻¹, nitrogen 115 mg kg⁻¹ (Bremner and Mulvaney, 1982) and extractable potassium 102 mg kg⁻¹ (Richards, 1954).

Each experimental pot was filled with 12 kg sieved soil. Two organic amendments biochar (BC) and poultry manure (PM) were applied at rates of 1% w/w. Biochar was made by using Eucalyptus as a feed stock at 400°C temperature. Biochar used in this experiment had the following properties: pH 7.64, EC 1.96, organic matter 59.1%, Fe 308 mg kg⁻¹, Zn 282 mg kg⁻¹. Poultry manure was collected from a poultry farm at the University of Agriculture Faisalabad with following properties: pH 7.31, EC 5.1, organic matter 53.3%, Fe 382 mg kg⁻¹, Zn 501 mg kg⁻¹.

Prior to seed germination in the pots, soil pH was lowered by elemental S (the rate of S was selected from the incubation study). Iron was applied at a rate of 0.0075 g kg⁻¹ by using FeSO₄·7H₂O source at the time of sowing the maize crop. Recommended doses of nitrogen (N), phosphorus (P), and potassium (K) were applied at rates of 0.11, 0.08, and 0.06 g kg⁻¹, respectively as urea, single super phosphate, and sulfate of potash. Full doses of P and K were applied at the time of sowing while N was applied in 2 split doses. Half the dose of N was applied at sowing and the remaining half was applied 30 days later. Four seeds of maize cultivar “DK 6714” were sown in each pot at a depth of 2 cm and one seedling per pot was maintained after emergence. Deionized water was used to irrigate the pots when required throughout the crop season. Treatments comprised in this experiment are detailed in Table 4.1.
Forty-five days after sowing gaseous exchange parameters such as photosynthetic rate (A), transpiration rate (E), stomatal conductance (gₛ) and sub-stomatal conductance (Cᵢ) were measured by using CIRAS-3 (PP System Amesbury, MN, USA). At maturity plants were harvested and grain was separated by manual threshing. After washing with deionized water and shade drying, samples were kept in a forced-air-driven oven (Tokyo Rikakikai, Eyela WFO-600 ND, Tokyo, Japan) at 60°C until constant weight was obtained, then the dry weight of root, shoot, and grain was recorded.

4.2.3 Grain analysis

For chemical and biochemical analysis, whole grain samples were ground in a mill (IKA Werke, MF 10 Basic, Staufen, Germany) and passed through a 0.5-mm sieve. For metal (Fe, Zn, Mn) determination, known weight of ground subsamples of whole grain were digested in a di-acid mixture (HNO₃:HClO₄ ratio of 2:1) (Jones and Case, 1990). In the digested samples, concentration of Fe, Zn, and Mn were measured by atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA).

Protein concentration in whole grain sample was determined using the Bradford colorimetric method (Bradford, 1976). Fat was determined by dry extraction method using a Soxhlet apparatus (AOAC, 2003). For starch estimation in grain the iodine test was performed by using glucose as standard in the procedure described by Sullivan (1935). The concentration was measured at 660 nm on a spectrophotometer. Phytate was determined by the method described by Haug and Lantzsch (1983). After grinding of whole grain sample, 60 mg of each sample was extracted with 10 mL of 0.2 N HCl at room temperature for 2 h and the concentration was measured on a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan). Whole grain sample for total polyphenol determination was prepared following the method described by Gómez-Alonso et al. (2007). Polyphenol from prepared grain sample was measured by using the Folin–Ciocalteau method (Aguilar-Garcia et al., 2007). Absorbance was measured by spectrophotometer at 760 nm via the calibration curve of gallic acid and expressed as gallic acid equivalent. For ferritin quantification, 5 g grain sample was prepared as seed in the method described by Lukac et al. (2009) with some modifications. Ferritin concentration was measured by direct ELISA (enzyme linked immunosorbent assay) using
antibody (Rabbit anti-ferritin) coated microtiter wells and mouse monoclonal anti-ferritin antibody as antibody enzyme (Horseradish peroxidase) conjugate solution and absorbance was measured at 450 nm (Catalog Number: BC-1025). All devices used for chemical and biochemical analysis were soaked in diluted HNO₃ (pro analysis quality, Merck) and were washed with deionized water.

4.2.4 Statistical analysis

A completely randomized design was used to conduct pot experiment. The data obtained for plant growth, physiological, chemical, and biochemical parameters were subjected to one-way analysis of variance (ANOVA) using Statistix 8.1 software. Significance difference of treatment means were separated by post hoc Tukey’s test (P < 0.05).

4.3 Results

4.3.1 Soil acidification

Four different rates (1.5, 2, 2.5, 3 g kg⁻¹ soil) of S were applied to soil and incubated in laboratory conditions. Soil pH decreased gradually in the pots receiving 2.5 g S kg⁻¹ soil and remained stable after 4 weeks of soil incubation in normal and organic amended soil. Application of S in soil with BC and PM lowered soil pH consistently as shown in Table 4.1. Addition of S decreased soil pH about 1.3-1.5 units in biochar and poultry manure amended soil with 2.5 g S kg⁻¹ soil, but without organic amendments (BC and PM 1% w/w) S lowered soil pH up to 1.3-1.5 units in non-planted pots. Data of soil pH and treatments under experiment are presented in Table 4.1.

4.3.2 Root-shoot dry mass and grain weight

Root and shoot dry mass was significantly increased by combined application of Fe and BC in S-treated low pH soil by up to 69 and 86%, respectively compared to the control (Table 4.2). Iron applied with S and with PM + S increased root dry weight up to 43 and 60%, respectively and shoot dry weight 60 and 73%, respectively compared to the control. The minimum 17% increase in shoot dry weight and 9% increase in root dry weight were
observed when only S was applied. Iron applied with BC and PM significantly increased grain yield in both unamended and S treated soil compared to the control, as depicted in Table 4.2. Among these treatments, Fe + BC combined with S remained best compared to other treatments, which increased grain weight 28% compared to the control.

### 4.2.3 Gaseous exchange measurements

Gaseous exchange measurements of maize plants showed substantial increase with soil applied Fe and organic amendments (BC and PM) with and without S (Table 4.2). Significant increase in the photosynthetic rate (A) of maize was observed by using Fe with BC in S treated low pH soil, which was 74% higher than the control. Separate application of Fe and Fe + PM increased by 49 and 64% the photosynthetic rate, respectively compared to the control in S treated low pH soil. Iron applied with PM in S treated low pH soil increased transpiration rate up to 49% compared to the control. Among all treatments Fe + BC in S treated low pH soil increased maximum transpiration rate 57% over the control. Iron applied as a single treatment and applied with BC increased stomatal conductance up to 18 and 26%, respectively in unamended soil. However, in S treated low pH soil the same treatments showed 27 and 33% increase, respectively, compared to the control. Fe applied with PM in S treated low pH soil increased by 31% the stomatal conductance compared to the control. Data in Table 4.2 showed that Fe applied as a single treatment, and with S, decreased sub-stomatal conductance up to 14 and 23%, respectively compared to the control. The highest decrease of 35% relative to the control for sub-stomatal conductance was observed in the treatment where Fe was applied with BC in S treated low pH soil.
Table 4.1 Effect of sulfur applied with iron, biochar and poultry manure on soil pH

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Abbreviation</th>
<th>Iron solubilizing agent (Elemental S)</th>
<th>Soil pH before crop sowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfur (g kg(^{-1}) soil)</td>
<td>Corresponding H(^+) ions (mmol kg(^{-1}) soil)</td>
</tr>
<tr>
<td>Control</td>
<td>C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Biochar</td>
<td>BC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Poultry Manure</td>
<td>PM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Biochar</td>
<td>Fe + BC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Poultry Manure</td>
<td>Fe + PM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sulfur</td>
<td>S</td>
<td>2.5</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Biochar</td>
<td>S + BC</td>
<td>2.5</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Poultry Manure</td>
<td>S + PM</td>
<td>2.5</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron</td>
<td>S + Fe</td>
<td>2.5</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron + Biochar</td>
<td>S + Fe + BC</td>
<td>2.5</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron + Poultry Manure</td>
<td>S + Fe + PM</td>
<td>2.5</td>
<td>156.25</td>
</tr>
</tbody>
</table>

Sulfur applied with treatments in each pot having 12 kg soil and its effect on soil pH after 4 week of sulfur oxidation as observed in each pot before sowing crop.

BC: biochar (1% w/w), PM: poultry manure (1% w/w), Fe: iron (15 kg ha\(^{-1}\))
Table 4.2 Growth and physiological attributes of maize as affected by different organic and inorganic amendments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot DW</th>
<th>Root DW</th>
<th>Grain Weight</th>
<th>A (μmol CO$_2$ m$^{-2}$s$^{-1}$)</th>
<th>E (μmol H$_2$O m$^{-2}$s$^{-1}$)</th>
<th>gs (μmol m$^{-2}$s$^{-1}$)</th>
<th>Ci (μmol mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>9.5 f</td>
<td>3.5 d</td>
<td>100.3 e</td>
<td>14.8 g</td>
<td>7.9 e</td>
<td>216.7 e</td>
<td>165 a</td>
</tr>
<tr>
<td>BC</td>
<td>11.7 d-f</td>
<td>4 cd</td>
<td>105.8 de</td>
<td>16.2 e-g</td>
<td>8.9 c-e</td>
<td>231 de</td>
<td>153 ab</td>
</tr>
<tr>
<td>PM</td>
<td>11.3 ef</td>
<td>3.9 cd</td>
<td>105 de</td>
<td>15.6 fg</td>
<td>8.8 de</td>
<td>225.3 de</td>
<td>155.7 ab</td>
</tr>
<tr>
<td>Fe</td>
<td>13.2 b-e</td>
<td>4.3 b-d</td>
<td>112.6 c-e</td>
<td>17.8 d-g</td>
<td>9.9 b-e</td>
<td>255.3 b-d</td>
<td>142 b-e</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>14.4 a-d</td>
<td>4.9 a-c</td>
<td>119.1 a-c</td>
<td>20.8 b-d</td>
<td>10.5 a-d</td>
<td>273 ab</td>
<td>133 c-e</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>13.6 b-e</td>
<td>4.6 b-d</td>
<td>116.3 b-d</td>
<td>19.4 c-f</td>
<td>10 b-e</td>
<td>267.3 a-c</td>
<td>137.3 b-e</td>
</tr>
<tr>
<td>S</td>
<td>11.1 ef</td>
<td>3.8 cd</td>
<td>106.3 de</td>
<td>17.5 d-g</td>
<td>8.9 c-e</td>
<td>223 e</td>
<td>158 ab</td>
</tr>
<tr>
<td>S + BC</td>
<td>12.5 c-e</td>
<td>4.4 b-d</td>
<td>111.6 c-e</td>
<td>19.9 c-e</td>
<td>9.6 b-e</td>
<td>237 c-e</td>
<td>143.3 a-d</td>
</tr>
<tr>
<td>S + PM</td>
<td>12.0 d-f</td>
<td>4.2 cd</td>
<td>110.3 c-e</td>
<td>18.7 c-f</td>
<td>9.3 c-e</td>
<td>231.3 de</td>
<td>147 a-d</td>
</tr>
<tr>
<td>S + Fe</td>
<td>15.2 a-c</td>
<td>5 a-c</td>
<td>121.8 a-c</td>
<td>22.1 a-c</td>
<td>11.1 a-c</td>
<td>275.3 ab</td>
<td>127 d-f</td>
</tr>
<tr>
<td>S + Fe + BC</td>
<td>16.3 a</td>
<td>5.9 a</td>
<td>128.8 a</td>
<td>25.8 a</td>
<td>12.4 a</td>
<td>288.67 a</td>
<td>107.7 f</td>
</tr>
<tr>
<td>S + Fe + PM</td>
<td>15.7 ab</td>
<td>5.6 ab</td>
<td>125.6 ab</td>
<td>24.3 ab</td>
<td>11.8 ab</td>
<td>285 ab</td>
<td>119.7 ef</td>
</tr>
<tr>
<td>HSD$_{0.05}$</td>
<td>2.8</td>
<td>1.4</td>
<td>12.4</td>
<td>4.1</td>
<td>2.2</td>
<td>32</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.05$

BC: biochar (1%), PM: poultry manure (1%), Fe: iron (15 kg ha$^{-1}$), S: sulfur (2.5 g kg$^{-1}$ soil).
4.2.4 Mineral concentration of grains

Iron, Zn, and Mn contents in maize grains were significantly improved by using soil applied Fe and organic amendments (BC and PM) with and without S compared to the control (Table 4.3). Iron applied as a single treatments increased Fe contents in maize grain up to 47% but Fe applied in S amended soil, grain Fe contents increased up to 86%, over control. Biochar and PM applied with Fe, increased grain Fe contents up to 59 and 66%, respectively, while BC and PM applied with Fe in S amended soil increase grain Fe contents up to 116% and 102%, respectively, over control. Among all treatments, Fe + BC combined with S remained the best for total Fe in grain, compared to other treatments, compared to the control.

Sulfur applied in soil as a single treatment and with BC or PM increased Zn concentration 21, 14, and 40%, respectively compared to the control. Grain Zn concentration was increased up to 8, 14, and 19%, when Fe was applied alone, with BC, or with PM in S treated low pH soil, respectively compared to the control. Iron applied with BC and PM in S treated low pH soil increased Mn concentration up to 40 and 46%, respectively compared to the control. The highest increase in Mn concentration was observed in treatment where organic amendments (BC and PM) were applied in S treated low pH soil, which was 49 and 60% higher, than the control.

4.3.5 Biochemical contents (starch, fat, and protein) in grain

Application of Fe with organic amendments (BC and PM) in the presence and absence of S significantly improved starch, fat, protein, and ferritin contents in grain compared to the control (Figure 4.2). Among all treatments, combined allocation of Fe + BC with S remained best compared to other treatments, and increased starch 34%, protein 64% and fat 110% in grain compared to the control. Iron and Fe + PM added in S treated low pH soil increased starch 21 and 27%, fat 69 and 90%, and protein 42 and 55%, respectively relative to the control.
Table 4.3 Grain minerals and biological attributes as affected by different organic and inorganic amendments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fe</th>
<th>Zn (mg kg(^{-1}) DW)</th>
<th>Mn</th>
<th>Starch</th>
<th>Protein</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>41.3 g</td>
<td>28.2 c-e</td>
<td>14.9 ef</td>
<td>41.9 e</td>
<td>5.3 e</td>
<td>2.16 e</td>
</tr>
<tr>
<td>BC</td>
<td>40.9 g</td>
<td>30.3 b-d</td>
<td>16.7 d-f</td>
<td>42.3 de</td>
<td>5.9 de</td>
<td>2.53 c-e</td>
</tr>
<tr>
<td>PM</td>
<td>48.1 fg</td>
<td>33.3 a-c</td>
<td>19.4 b-d</td>
<td>43.6 c-e</td>
<td>6.1 c-e</td>
<td>2.63 c-e</td>
</tr>
<tr>
<td>Fe</td>
<td>60.9 de</td>
<td>25.1 de</td>
<td>15.1 ef</td>
<td>45.6 b-e</td>
<td>6.5 c-e</td>
<td>2.93 cd</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>65.7 d</td>
<td>22.8 e</td>
<td>14.1 f</td>
<td>48.9 a-d</td>
<td>6.9 b-d</td>
<td>3.43 b-d</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>68.7 cd</td>
<td>24.5 de</td>
<td>14.9 f</td>
<td>49.7 a-d</td>
<td>7.1 b-d</td>
<td>2.43 c-e</td>
</tr>
<tr>
<td>S</td>
<td>48.1 fg</td>
<td>34.2 a-c</td>
<td>18.8 b-e</td>
<td>42.2 de</td>
<td>5.7 de</td>
<td>2.96 cd</td>
</tr>
<tr>
<td>S + BC</td>
<td>54.6 ef</td>
<td>32.1 bc</td>
<td>22.2 ab</td>
<td>44.8 c-e</td>
<td>6.5 c-e</td>
<td>2.8 cd</td>
</tr>
<tr>
<td>S + PM</td>
<td>51.4 ef</td>
<td>39.7 a</td>
<td>23.9 a</td>
<td>45.4 c-e</td>
<td>6.4 c-e</td>
<td>2.66 bc</td>
</tr>
<tr>
<td>S + Fe</td>
<td>77.2 bc</td>
<td>30.4 b-d</td>
<td>17.9 c-f</td>
<td>50.7 a-c</td>
<td>7.5 a-c</td>
<td>3.66 bc</td>
</tr>
<tr>
<td>S + Fe + BC</td>
<td>89.3 a</td>
<td>32.5 bc</td>
<td>20.8 a-c</td>
<td>56.2 a</td>
<td>8.7 a</td>
<td>4.36 a</td>
</tr>
<tr>
<td>S + Fe + PM</td>
<td>83.7 ab</td>
<td>33.5 a-c</td>
<td>21.8 a-c</td>
<td>53.1 ab</td>
<td>8.2 ab</td>
<td>4.1ab</td>
</tr>
<tr>
<td>HSD(_{0.05})</td>
<td>9.9</td>
<td>6.9</td>
<td>3.8</td>
<td>7.7</td>
<td>1.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Quantities sharing similar letters are statistically similar to each other at \( p \leq 0.05 \)

BC: biochar (1% w/w), PM: poultry manure (1% w/w), Fe: iron (15 kg ha\(^{-1}\)), S: sulfur (2.5 g kg\(^{-1}\) soil)
4.3.6 Polyphenol, phytate, \([\text{phytate}] : [\text{Fe}]\), and \([\text{phytate}] : [\text{Zn}]\) ratio

A significant decrease of polyphenol contents in maize grain was observed when Fe and organic amendments with S were applied [Fig. 4.1 (d)]. Treatments where Fe and Fe + BC and Fe + PM were applied in S treated low pH soil decreased polyphenol contents up to 30, 40, and 36%, respectively compared to the control. Iron applied alone and with S decreased phytate content up to 12 and 23%, respectively. The maximum decrease in phytate content of 25 and 29%, respectively relative to the control occurred in treatments where Fe was applied with BC and with PM in S treated low pH soil. In all treatments where Fe was applied with organic and inorganic amendments (BC, PM, and S) \([\text{phytate}] : [\text{Fe}]\) ratio significantly decreased [Fig. 4.1 (b)]. Iron applied as a single treatment and with S decreased \([\text{phytate}] : [\text{Fe}]\) ratio 40 and 58%, respectively compared to the control. Maximum decrease of 67% in \([\text{phytate}] : [\text{Fe}]\) ratio was observed in the treatment where Fe was applied with BC followed by treatment where Fe was applied with PM (63%) in low pH soil compared to the control. In S treated low pH soil, \([\text{phytate}] : [\text{Zn}]\) ratio decreased significantly in treatments where organic amendments were applied alone or with Fe [Fig. 4.1 (c)]. Maximum decrease in \([\text{phytate}] : [\text{Zn}]\) ratio of 37% was observed when Fe + BC was applied followed by treatments where Fe was applied with PM in S treated low pH soil compared to the control (a 37% decrease).

4.3.7 Ferritin in grain

Soil applied Fe and organic amendments (BC and PM) with and without S significantly improved ferritin contents in maize grain (Fig. 4.2). Fe applied as a single treatment and combined with S increased ferritin contents 105 and 131%, respectively compared to the control. Fe application along with BC and PM in S amended low pH soil significantly increased ferritin contents up to 170 and 160 %, respectively more than the control.
(HSD value: phytate 1.9, polyphenol 1.7, [phytate]:[Fe] ratio 7.3, [phytate]:[Zn] ratio 9.7)

Fig. 4.1 a- d. Phytate, Grain [phytate]:[Fe] and [phytate]:[Zn] ratio, and polyphenol concentration in maize grain measured in various Fe treatments under unamended and pH manipulated calcareous soil. Values are the means and different letters indicate significant differences at p<0.05 (n=3).
(HSD value: Grain ferritin concentration 0.1)

Figure 4.2 Ferritin concentration in maize grain measured in various Fe treatments in unamended and acidified calcareous soil. Values are the means and different letters indicate significant treatment differences at p<0.05 (n=3).

4.4 Discussion

Today, different approaches are used to increase Fe content and bioavailability in cereals and other crops. On a long term basis, Fe content can be improved by increasing Fe solubilization in soil for plant uptake and storage in edible parts of plants (Grusak and Della Penna, 1999). Increased levels of reducing agents, chelating agents, siderophores and enzymes in the rhizosphere and transporter proteins in roots could increase Fe uptake from soil to plant body. Nutrient interaction can change their absorption rate and compete during translocation into the plant body. Thus, attempts have been directed to enhance absorption of essential mineral elements (Fe, Zn, Cu, Mn) in maize and reduce the concentration of anti-nutrients that interfere with mineral nutrient absorption (White and Broadley, 2009).
In under developed countries increasing food insecurity is a common problem causing malnutrition so, a more productive, more nutritional and sustainable agriculture system is an urgent need.

In the current study, the potential of inorganic and organic amendments was evaluated for improving growth and Fe biofortification of maize in S treated low pH soil. Organic amendments significantly enhanced plant growth and development by increasing soil organic matter, availability of plant nutrients, and improved soil physical properties such as structure, porosity and water holding capacity (Azeez et al., 2010; Demir et al., 2010). Biochar application positively affected growth and yield of maize (Uzoma et al., 2011) and rice (Asai et al., 2009). Roots are known to excrete hydroxyl ions and protons in response to excess uptake of anion and cations, respectively, and to solubilize mineral nutrient ions in response to mineral nutrient deficiency (Hinsinger et al., 2009).

Novak et al. (2009) stated that soil amended with biochar increased bioavailability of Zn, K and Mn. The increased concentrations of Fe, Zn and Mn in the present study were presumably caused by nutrient solubilization and enhanced plant growth with PM and BC. Laird et al. (2010) reported that physical and chemical properties of BC, such as high surface area, high internal porosity, and presence of polar and non-polar surface sites and high cation exchange capacity can lead to nutrient absorption and availability. In my study Fe added with organic amendments (BC and PM) in acidified calcareous soil significantly increased photosynthetic parameters except sub-stomatal conductance and the results are more prominent with BC compared to PM. Increased photosynthetic parameters by Fe + BC + S treatment might be due to increased growth and elevated level of nutrients in plants. Park et al. (2011) reported improved plant growth and nutrient uptake in mustard by using chicken manure derived biochar in problem soil.

In various parts of the world, human bioavailable micronutrient deficiency is a common problem due to low micronutrient levels in soils, interaction of macro- and micronutrients, use of high analysis fertilizers with low amounts of micronutrients, use of high yielding cultivars and less attention to organic amendments (Fageria et al., 2002; Fageria et al., 2008). To improve micronutrient uptake and to enhance their bioavailability in cereal grains, strategies can be divided into two different groups. In the first group, micronutrient
bioavailability can be improved by practices such as minimizing climatic factors that affect mineral availability and managing fertilizer application practices optimized based on soil properties (pH, EC etc.) (Fageria et al., 2002). In the present study, added micronutrient fertilizers were efficiently utilized and recovered (in grains) with integrated use of organic amendments (BC and PM) and S in calcareous soil. In the second group, crop genotypes within species can be used that work efficiently in micronutrient uptake and translocation of largely into grain (Fageria et al., 2008).

In the present study, ferritin concentration increased up to 170% as Fe concentration increased in grain. Ferritin-Fe is bioavailable to humans as FeSO₄ and does not denature in the human elementary tract (Theil et al., 2004). The main function of ferritin in seeds is protection against free Fe damage through Fenton oxidation (Briat et al., 2010b). Thus, it can be seen that a number of different barriers affect Fe translocation and accumulation into grain. Iron accumulation in the grain was not only dependent on Fe uptake from soil but also depended on its translocation from xylem to phloem. Sulfur enhances transportation of Fe in the xylem of plants because it decreases pH in the apoplast and because of its effect on nicotinamide formation which enhance Fe transport in phloem (Hu and Xu, 2002; Na and Salt, 2011). Sulfur affects the regulation of apoplast pH and Eh and is reported to increase the transport of Fe in phloem of plants, as well as enhance the deposited form of Fe in the apoplast (Toulon et al., 1992). Research suggests that while maize tends to maintain nutrient concentration in the grain within predetermined limits, application of fertilizers could alter the balance (Rengel et al., 1999), so understanding the factors that influence the balance might be important when selecting for accumulation of a specific nutrient, especially for Fe or Zn. However, in the present study, we observed significantly higher Fe and Zn bioavailability with integrated use of chemical fertilizer and organic amendments in S treated low pH calcareous soil. As seed developed in the parent plant, concentration of nutrients depended on soil type, nutrient availability, crop species and to the lesser extent, season and cultivar (Ascher et al., 1994). Contrary to our results, Gregorio et al. (2000) illustrated that nutrient concentration in grains is highly dependent on crop cultivars/genotypes rather than nutrient fertilizer management.
Mineral bioavailability from grains is quite complex but [phytate]:[Fe] and [phytate]:[Zn] ratio can be used to access bioavailability of Fe or Zn in food (Hallberg et al., 1989; Hussain et al., 2012a). In the current study, as the Fe and Zn concentration increased in the grain of maize, that ultimately reduced the phytate, [phytate]:[Fe] and [phytate]:[Zn] ratio. This might be due to the higher uptake of these nutrients in maize. Polyphenols and phytate in the plant body act as strong chelators and bind positively charged minerals (Zn$^{2+}$, Mn$^{2+}$, Fe$^{2+}$), proteins and amino acids in insoluble form by forming insoluble complexes in the plant body as well as in vitro digestion (Cheryan, 1980). In the present study, phytate and polyphenol decreased while protein, fat, and starch increased significantly with Fe application with BC in acidified calcareous soil. Our results are supported by the findings of Fang et al. (2008) and Yadav et al. (2013) where all quality parameter were significantly improved by the supply of micronutrients and organic amendments but ferritin enrichment in grains is novelty of present experiment.

4.5 Conclusions

Combined application of Fe with organic amendments (BC and PM) has high potential to confer beneficial effects on maize growth and it can be assumed that plant growth promotion and Fe bioavailability are due to mineral solubilization and uptake in S amended low pH soil. Among the organic amendments, BC remains as best for plant growth and yield. The Fe biofortified maize grains by using Fe with BC in acidified calcareous soil could serve as a valuable strategy to fight malnutrition problem in the world. However, multi-sites field trials with this approach need to be performed to warrant successful performance in the field.
Chapter 5

Evaluating growth, yield, and iron bioavailability of rice in acidified calcareous soil

Abstract

Iron (Fe) bioavailability is a major issue of high pH calcareous soils. Iron fertilization in calcareous soils is ineffective due to its rapid conversion into unavailable form. This study evaluated the effect of Fe fertilizer with organic amendments by manipulating calcareous soil pH. Before transplanting rice seedlings, soil pH was lowered 0.5-0.6 units by using 0.25% elemental sulfur (S). For biofortification, Fe fertilizer (FeSO$_4$·7H$_2$O) 0.0075 g kg$^{-1}$ was applied with biochar (BC) and poultry manure (PM) as treatment plan. The combined application of Fe along with BC and PM enhanced plant growth, physiology, and yield as well as improved nutritional value of grain in acidified calcareous soil. Data also suggested that in S treated low pH soil treatments increased Fe mobilization and translocation from root to grain. Grain Fe concentration raised up to 200% and 180% in acidified soils where Fe was applied with BC and with PM. Ferritin concentration significantly increased 300% and 273% by using Fe with BC with PM, respectively in acidified soil. Iron applied with BC decreased phytate and polyphenol up to 18% and 47%, respectively in S treated low pH soil. As reported earlier, a small fraction (2-15%) of the Fe from plant sources is bioavailable to humans. It can be concluded that increasing levels of ferritin in rice will be helpful at eliminating Fe deficiency in humans.

Keyword: biofortification, Zn, phytate, polyphenol, protein, ferritin
5.1 Introduction

Food is a basic necessity of life. Most recent statistics from the FAO indicate that 1/9th of the developing world is food insecure (FAO, 2014). This leads to micronutrient malnutrition and causes hidden hunger. The main cause of this micronutrient malnutrition is consumption of monotonous food that is poor in microelements (Bouis et al., 2011). Human population use various foods to fulfill their daily requirement of calories. A major part of daily dietary intake is provided by cereals. Among cereals, rice is the second most consumed cereal worldwide after wheat. According to the latest figures of the FAO, rice consumption is about 509.7 million tons worldwide (FAO, 2015). But, rice is low in important micronutrients like Fe and Zn (Welch and Graham, 2004).

Iron acts as a cofactor for several enzymes that take part in various metabolic processes. Susceptibility to disease also increases in Fe-limiting conditions (Chatterjee et al., 2006). Iron deficiency leads to loss of immunity, impaired circulatory systems resulting in anemia, and poor work performance. In short, Fe malnutrition leads to devastating impacts on human health (Stein, 2010).

High pH and calcareousness are two major soil factors that limit Fe availability to crops (Cifuentes et al., 1993). Iron changes its oxidation state very quickly from soluble Fe$^{+2}$ to relatively insoluble Fe$^{+3}$ oxides and hydroxides, thus cause Fe deficiency in soil (Celik and Katkat, 2007). Along with soil factors, some nutritional factors are also potential inhibitors of Fe absorption such as phytate and polyphenolic compounds (Welch, 2002). Phytate makes complexes with nutrient elements like Ca, Fe, Zn and Mg during digestion of food and reduces their bioavailability, thus playing a role in malnutrition (Jin et al., 2009). On the other hand ferritin is a bioavailable form of Fe as FeSO$_4$ in humans (Briat et al., 2010b). Transgenic and genetic approaches have been used to increase ferritin concentration in many crops including rice and soyabean (Vasconcelos et al., 2003). Among potential approaches maximizing ferritin concentration in wheat, cassava, rice, or beans is decreasing phytate, which is also a possible solution of Fe malnutrition, but not very practical due to less farmer intrust for growing such varities (Cakmak et al., 2010a).
Food fortification, diversification, and supplementation programs require continual funding and mainly target urban populations, which excludes poor populations (Saltzman et al., 2013). Biofortification is a cost effective approach that promises to provide sufficient mineral contents to a target population (Bouis et al., 2011; Saltzman et al., 2013).

Agronomic biofortification of food crops is considered the most sustainable approach. In high pH calcareous soil agronomic biofortification will be ineffective unless one can reduce soil pH. The major issue in calcareous soil is quick transformation of soluble Fe compounds to less soluble oxides and hydroxides (Celik and Katkat, 2007). Rapid transformation of Fe and increasing its availability by soil pH manipulation using acidifying material could be a useful approach. Different studies reported that microbial oxidation of elemental sulfur leads to mineral solubilization that increases nutrient availability (Iqbal et al., 2012; Wu et al., 2014).

Organic matter is an important source of mineral nutrients but due to harsh climatic conditions of Pakistan it mineralizes soon after its application (Azam et al., 2001). Among the different forms of organic amendments, biochar and poultry manure are considered best because of their long lasting effect on soil health and nutrient availability (Lorenz and Lal, 2014; de Cesare Barbosa et al., 2015). Biochar is considered the most sustainable approach to enhancing soil fertility and crop productivity (Liu et al., 2013). Positive effects of biochar, having acidic nature, on Fe solubilization by decreasing soil pH have also been reported (Graber et al., 2014). Most recently, carbonaceous products have been used for Zn biofortification of crops (Gartler et al., 2013). To be the best of our knowledge no study has been conducted for Fe biofortification in rice in acidified soil. The aim of the present study was to evaluate the effect of Fe fertilizer in organic amended (BC and PM) in unamended and S amended calcareous soil to enhance Fe bioavailability.

5.2 Materials and Methods

5.2.1 Plot preparation and soil properties

Plots of 4×4 m² sizes were prepared for the rice experiment in a field area of the Institute of Soil and Environmental Sciences University of Agriculture Faisalabad, Pakistan. Prior to
using the soil for acidification and transplanting of rice seedlings, it was homogenized to remove stones and extra particles of crop residue. For soil characterization randomized soil samples (0–15 cm depth) were collected from each plot, air-dried, passed through a 2 mm sieve, and mixed thoroughly for measurement of various physicochemical properties. Soil texture was clay loam (sand 40%, silt 27%, clay 33%) as determined by hydrometer method (Gee and Bauder, 1986). Saturated soil paste had pH value 7.71. Organic matter was 0.62% as determined by Walkley-Black method (Jackson, 1962). Calcium carbonate (CaCO₃) as estimated by acid dissolution (Allison and Moodie, 1965) was 5.9%. Plant available Fe and Zn as extracted by 0.005 M DTPA (Lindsay and Norvell, 1978) was 4.1 and 0.52 ppm, respectively as measured on an atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA). Phosphorus extracted by the Olsen method (Watanabe and Olsen, 1965) was 7.6 mg kg⁻¹, nitrogen 113 mg kg⁻¹ (Bremner and Mulvaney, 1982), and extractable potassium 101 mg kg⁻¹ soil (Richards, 1954).

5.2.2 Soil acidification

Experimental plot was acidified with elemental sulfur (S) as previously described in chapter 3.

5.2.3 Crop experiment

Rice seed (super basmati kernel) were obtained from Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Healthy, uniform, clean seeds were transferred into cloth bags and soaked in water for 24 hours. After soaking, all seeds were placed under shade and covered for 48 hours, sprouted seeds were grown in small plots. The experiment area was divided into three blocks with a total of 36 plots and 25-days old uniform seedlings were transplanted into each plot maintaining row-to-row and plant-to-plant distance of 22.5 cm. 0.0075 g kg⁻¹ Fe was applied by using FeSO₄·7H₂O . Two organic amendments (i.e. BC and PM) were applied at a 1% (w/w) rate. Biochar was made by using Eucalyptus as a feed stock at 400 C temperature. Biochar used in the experiment had the following properties: pH 6.84, EC 1.96, organic matter 59.1%, Fe 308 mg kg⁻¹, Zn 281.8 mg kg⁻¹.
Poultry manure was collected from the poultry farm of the University of Agriculture Faisalabad. Poultry manure properties were: pH 6.91, EC 5.1, organic matter 53.3 %, Fe 291.9 mg kg$^{-1}$, Zn 501.3 mg kg$^{-1}$. Recommended doses of nitrogen (urea), phosphorus (single super phosphate), and potassium (sulfate of potash) (i.e, 0.06, 0.045, 0.03 g kg$^{-1}$) respectively were applied. Full doses of phosphorus and potassium were applied at the time of sowing but nitrogen was applied in three different doses. At booting stage, photosynthetic parameters such as photosynthesis (A), stomata conductance (gs), sub-stomatal conduction (Ci), and transpiration (E) rate were measured by a CIRAS-3 (PP System. Amesbury, MN, USA). When 90% grain became golden yellow the rice crop was harvested. After shade drying and washing with distilled water samples were placed in a forced-air-driven oven (Tokyo Rikakikai, Eyela WFO-600 ND, Tokyo, Japan) at 60°C until constant weight was obtained, then dry weight of shoot and grain were recorded.

5.2.4 Grain analysis

Whole grain samples were ground in a mill (IKA Werke, MF 10 Basic, Staufen, Germany) to pass through a 0.5-mm sieve. Known sample was placed in muffle furnace at 550 Cº for complete oxidation of organic matter until the appearances of gray white ash (AOAC, 2003). Ground subsamples of known weights were digested in a di-acid mixture (HNO$_3$:HClO$_4$ ratio of 2:1) for metal analysis (Jones and Case, 1990). Iron, Zn, and Mn concentrations in the digest were measured by an atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA). Total protein concentration in grains was determined with the Bradford colorimetric method (Bradford, 1976). For fiber determination a moisture free and ether extracted sample of fiber made of cellulose was first digested with dilute H$_2$SO$_4$ and then with dilute KOH solution. Fat was determined by dry extraction method using a Soxhlet apparatus (AOAC, 2003).
Table 5.1 Sulfur effect on soil pH amended with Fe (0.0075 g kg\(^{-1}\)), biochar (1% w/w), and poultry manure (1% w/w).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Abbreviation</th>
<th>Iron solubilizing agent (Elemental Sulfur)</th>
<th>Soil pH before crop sowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulfur (% w/w)</td>
<td>Corresponding H(^+) ions (mmol kg(^{-1}) soil)</td>
</tr>
<tr>
<td>Control</td>
<td>C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Biochar</td>
<td>BC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Poultry Manure</td>
<td>PM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Biochar</td>
<td>Fe + BC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Iron + Poultry Manure</td>
<td>Fe + PM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sulfur</td>
<td>S</td>
<td>0.25</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Biochar</td>
<td>S + BC</td>
<td>0.25</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Poultry Manure</td>
<td>S + PM</td>
<td>0.25</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron</td>
<td>S + Fe</td>
<td>0.25</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron + Biochar</td>
<td>S + Fe + BC</td>
<td>0.25</td>
<td>156.25</td>
</tr>
<tr>
<td>Sulfur + Iron + Poultry Manure</td>
<td>S + Fe + PM</td>
<td>0.25</td>
<td>156.25</td>
</tr>
</tbody>
</table>

Sulfur applied with treatments in each plot having a 4*4 m\(^2\) area and its effect on soil pH after 4-5 weeks of sulfur oxidation as observed in each sub-plots.
The undigested residue collected after digestion was ignited and loss in weight after ignition was registered as crude fiber (AOAC, 2003). For starch estimation in whole grain the iodine test was used using glucose as a standard (Sullivan, 1935). The starch concentration was measured at 660 nm on a spectrophotometer. For phytate determination, each 60 mg sample of finely ground grain was extracted with 10 mL of 0.2 N HCl at room temperature for 2 h with continuous shaking. Phytate in the extract was determined by an indirect method (Haug and Lantzsch, 1983) and the final concentration was measured on a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan). Total polyphenol determination in rice grain sample was prepared as described by Gómez-Alonso et al. (2007). From the prepared grain sample polyphenol was measured by using the Folin–Ciocalteau method (Aguilar-Garcia et al., 2007). Absorbance was measured by spectrophotometer at 760 nm via a calibration curve of gallic acid and expressed as gallic acid equivalent. For ferritin quantification 5 g whole grain sample was prepared as seed method as described by Lukac et al. (2009) with slight modification. Ferritin concentration was measured by developing direct ELISA (enzyme linked immunosorbet assay) using anti-body (rabbit anti-ferritin) coated microtiter wells and mouse monoclonal anti-ferritin antibody as antibody enzyme (horseradish peroxidase) conjugate solution and absorbance was measured at 450 nm (Catalog Number: BC-1025, California). All devices used for chemical and biochemical analysis were soaked in diluted HNO₃ (pro analysis quality, Merck, Germany) and washed with deionized water.

5.2.5 Statistical analysis

The field experiment was conducted in a completely randomized block design. The data obtained with respect to growth, physiological, chemical, and biochemical parameters were subjective to one-way analysis of variance (ANOVA) using statitix 8.1® software. Significant differences of treatment means were separated by post hoc Tukey’s test (P < 0.05).

5.3 Results

5.3.1 Plant growth and yield
Application of Fe fertilizer with organic amendments (PM and BC) significantly improved plant growth and yield attributes. In nonacidified soil, separate application of Fe, BC, and PM increased 1000 grain weight by 13.5, 11 and 7%, respectively compared to the control. Meanwhile in S treated low pH soil, compared to the control, using Fe in combination with BC and PM improved 1000 grain weight by 34 and 30%, respectively (Table 5.2). Using Fe with BC increased straw yield 47% in unacidified soil (Table 5.2) compared to the control. On the other hand in S amended acidified soil, an 80% improvement in straw yield was obtained by using Fe with BC compared to its respective control. Compared to the control, Fe application combined with BC increased paddy yield 34% in unamended soil. Maximum increase in paddy yield was observed by using Fe fertilization with BC (56%) in S amended acidified soil compared to the control.

5.3.2 Photosynthetic measurements

Lowering of soil pH together with provision of Fe tended to improve all photosynthetic parameters (Table 5.2). Application of Fe with BC improved photosynthetic rate by 93% in S amended soil compared to the control. The use of poultry manure along with Fe accelerated the light reaction of rice by 81% in S treated acidified soil compared to the control. Compared to the control, application of Fe alone gave only a 18% increase in transpiration rate in unacidified soil. Likewise, compared to the control, application of Fe with BC and PM increased transpiration rate by 39 and 34 %, respectively in acidified soil. In unacidified soil, application of Fe with PM significantly improved stomatal conductance by 45% while the same treatment showed a 59% increase in stomatal conductance in acidified soil compared to the control. In unacidified soil reduction in sub-stomatal conductance was 19.2%, 23.3%, and 20.4% by using Fe alone, Fe and BC, and Fe and PM, respectively compared to the control in S-acidified soil. Compared to the control, the greatest decrease in sub-stomatal conductance (31.1%) was recorded in treatments where Fe was applied with BC in S-acidified soil.
5.3.3 Grain Zn and Fe concentration

Mineral concentration (Zn, Fe) of rice was significantly improved by using Fe along with organic amendments (BC and PM) in S–acidified soil (Table 5.3). Iron fertilization alone was inefficient in unacidified soil as there was not a marked change in grain Fe (i.e. 60%). Results depicted in Table 3 show that in unacidified soil, treatment receiving PM alone increase grain Fe concentration by 13% over the control. The effect of PM was improved in S-acidified soil. Compared to the control, grain Fe increased up to 180% when Fe was applied with PM in S-acidified soil. On the other hand, the highest increase in grain Fe concentration was observed by combined use of Fe and BC in S-acidified soil and that was 200% significantly high over control. Separate application of BC and Fe reduced grain Zn concentration 3 and 4%, respectively in unamended soil compared to the control. Among the organic amendments, PM had a positive effect on grain Zn contents (i.e.11% greater compared to the control). Maximum increase in grain Zn (i.e. 32% greater concentration) was obtained by using Fe with PM in acidified soil as compared to the control.

5.3.4 Grain quality parameters (Ash, Fat, Fiber and Starch)

Quality of rice grain was markedly influenced by application of Fe, BC, and PM in S-acidified soil (table 5.3). Ash contents of rice grain were improved when Fe was used with (60%) and without (30%) PM in unacidified soil compared to the control. The greatest increase in ash contents (i.e.102%) was observed when Fe was applied with BC in S – acidified soil over its respective control. In unacidified soil, use of Fe with BC and PM increased fiber contents by 47.1 and 44.1%, respectively compared to the control. In S acidified soil, fiber contents were improved 70.8 and 82.4%, respectively by using Fe with PM and with BC compared to the control. In the case of starch contents, in unacidified soil starch contents were 9, 10, and 14% greater by separate application of BC, PM, and Fe fertilization, respectively compared to the control. Maximum increase in starch contents (i.e. 41%) was observed in treatment where Fe was applied with BC in S-acidified soil compared to the control.
Table 5.2 Growth, yield and photosynthetic measurements of rice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1000 GW Yield (g)</th>
<th>Straw Yield (ton ha$^{-1}$)</th>
<th>Grain Yield (g)</th>
<th>A ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</th>
<th>E ($\mu$mol H$_2$O m$^{-2}$ s$^{-1}$)</th>
<th>Gs (mmol H$_2$O m$^{-2}$ s$^{-1}$)</th>
<th>Ci (µmol mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>20 e</td>
<td>8.4 f</td>
<td>4.1 e</td>
<td>12.1 h</td>
<td>3.8 f</td>
<td>315 e</td>
<td>245 a</td>
</tr>
<tr>
<td>BC</td>
<td>22.2 c-e</td>
<td>9.9 ef</td>
<td>4.6 c-e</td>
<td>14.7 e-h</td>
<td>4.1 d-f</td>
<td>351 e</td>
<td>231.7 a-c</td>
</tr>
<tr>
<td>PM</td>
<td>21.4 de</td>
<td>9.6 ef</td>
<td>4.5 de</td>
<td>14.1 f-h</td>
<td>3.9 ef</td>
<td>333.7 e</td>
<td>236 ab</td>
</tr>
<tr>
<td>Fe</td>
<td>22.7 c-e</td>
<td>10.7 de</td>
<td>4.8 c-e</td>
<td>17.3 c-e</td>
<td>4.5 cd</td>
<td>410.7 cd</td>
<td>198 b-e</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>24.7 a-c</td>
<td>12.4 b-d</td>
<td>5.5 bc</td>
<td>19.1 b-d</td>
<td>4.6 bc</td>
<td>453 bc</td>
<td>188 de</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>24.3 a-d</td>
<td>12.3 b-d</td>
<td>5.2 b-d</td>
<td>17.2 c-f</td>
<td>4.5 c</td>
<td>459.3 bc</td>
<td>195 c-e</td>
</tr>
<tr>
<td>S</td>
<td>21.6 de</td>
<td>10.3 d-f</td>
<td>4.5 de</td>
<td>13.9 gh</td>
<td>3.9 f</td>
<td>322.7 e</td>
<td>224.7 a-d</td>
</tr>
<tr>
<td>S + BC</td>
<td>23.9 b-d</td>
<td>11.6 c-e</td>
<td>4.8 c-e</td>
<td>16.1 d-g</td>
<td>4.3 c-e</td>
<td>369.3 de</td>
<td>214 a-e</td>
</tr>
<tr>
<td>S + PM</td>
<td>22.8 c-e</td>
<td>10.8 de</td>
<td>4.7 c-e</td>
<td>15.4 e-g</td>
<td>4 ef</td>
<td>353.7 e</td>
<td>212.7 a-e</td>
</tr>
<tr>
<td>S + Fe</td>
<td>24.9 a-c</td>
<td>13.4 a-c</td>
<td>5.9 ab</td>
<td>20.2 a-c</td>
<td>5 ab</td>
<td>493.7 ab</td>
<td>180.3 de</td>
</tr>
<tr>
<td>S + Fe + BC</td>
<td>26.9 a</td>
<td>15.2 a</td>
<td>6.4 a</td>
<td>23.4 a</td>
<td>5.3 a</td>
<td>518.3 a</td>
<td>186.7 e</td>
</tr>
<tr>
<td>S + Fe + PM</td>
<td>26.1 ab</td>
<td>14.3 ab</td>
<td>6 ab</td>
<td>21.9 ab</td>
<td>5.1 a</td>
<td>501.3 ab</td>
<td>186.3 de</td>
</tr>
<tr>
<td>HSD$_{0.05}$</td>
<td>2.9</td>
<td>2.2</td>
<td>0.9</td>
<td>3.2</td>
<td>0.4</td>
<td>55.2</td>
<td>39.4</td>
</tr>
</tbody>
</table>

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.05$

1000 grain weight (GW), Straw yield (SY), paddy yield (PY), photosynthesis rate (A), transpiration rate (E), stomatal conductance (gs) and sub-stomatal conductance (Ci) measured in each experiment plots (4*4 m$^2$) as influenced by Fe fertilizer and organic amendments (BC and PM) in unamended and acidified soil.
Table 5.3 Grain minerals and biological attributes as affected by different organic and inorganic amendments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fe</th>
<th>Zn</th>
<th>Ash</th>
<th>Fat</th>
<th>Fiber</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>33  gh</td>
<td>19.3 de</td>
<td>0.43 h</td>
<td>0.76 f</td>
<td>0.34 f</td>
<td>48.5 e</td>
</tr>
<tr>
<td>BC</td>
<td>30.9 h</td>
<td>18.7 e</td>
<td>0.52 gh</td>
<td>0.87 d-f</td>
<td>0.39 d-f</td>
<td>52.9 de</td>
</tr>
<tr>
<td>PM</td>
<td>37.5 f-g</td>
<td>21.6 b-e</td>
<td>0.56 hg</td>
<td>0.9 c-f</td>
<td>0.42 d-f</td>
<td>53.6 de</td>
</tr>
<tr>
<td>Fe</td>
<td>53  d</td>
<td>18.5 e</td>
<td>0.66 c-f</td>
<td>0.96 b-e</td>
<td>0.47 c-e</td>
<td>55.6 c-e</td>
</tr>
<tr>
<td>Fe + BC</td>
<td>69.1 c</td>
<td>19.6 c-e</td>
<td>0.71 b-d</td>
<td>1 a-d</td>
<td>0.5 a-d</td>
<td>63.7 a-c</td>
</tr>
<tr>
<td>Fe + PM</td>
<td>64  c</td>
<td>22.9 a-c</td>
<td>0.69 c-e</td>
<td>1.1 ab</td>
<td>0.49 b-e</td>
<td>64.8 ab</td>
</tr>
<tr>
<td>S</td>
<td>41.6 e-g</td>
<td>20.7 b-e</td>
<td>0.48 gh</td>
<td>0.81 ef</td>
<td>0.37 ef</td>
<td>51.3 de</td>
</tr>
<tr>
<td>S + BC</td>
<td>45.6 d-f</td>
<td>19.3 de</td>
<td>0.6 d-g</td>
<td>0.94 b-e</td>
<td>0.44 d-f</td>
<td>53.1 de</td>
</tr>
<tr>
<td>S + PM</td>
<td>51.5 de</td>
<td>23.4 ab</td>
<td>0.58 e-g</td>
<td>0.91 c-f</td>
<td>0.43 d-f</td>
<td>54.9 c-e</td>
</tr>
<tr>
<td>S + Fe</td>
<td>86.8 b</td>
<td>20.9 b-e</td>
<td>0.73 bc</td>
<td>1.08 a-c</td>
<td>0.58 a-c</td>
<td>59 b-c</td>
</tr>
<tr>
<td>S + Fe + BC</td>
<td>99.6 a</td>
<td>22.6 a-d</td>
<td>0.87 a</td>
<td>1.2 a</td>
<td>0.62 a</td>
<td>68.4 a</td>
</tr>
<tr>
<td>S + Fe + PM</td>
<td>92.8 ab</td>
<td>25.6 a</td>
<td>0.83 ab</td>
<td>1.1 ab</td>
<td>0.6 ab</td>
<td>66.5 ab</td>
</tr>
<tr>
<td>HSD&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td>10.6</td>
<td>3.4</td>
<td>0.13</td>
<td>0.17</td>
<td>0.13</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Quantities sharing similar letters are statistically similar to each other at $p \leq 0.05$

Iron applied with BC and with poultry manure increased fat content up to 31.6 and 44.7%, respectively compare to the control. Maximum fat contents were obtained when Fe was used with biochar (i.e. 57.9% greater than the control).

5.3.5 Phytate, Phytate/Fe and Phytate/Zn molar ratio, Polyphenol
Application of Fe with organic amendments (BC and PM) in S-acidified soil significantly influenced anti-nutrient and bioavailable contents of Fe in rice grain [Figure 5.1, 5.2]. In unacidified soil application of Fe without any organic amendment reduced phytate contents by 9% compared to the control [Fig. 5.1 (a)]. Iron added as a single treatment increased phytate content in rice grain grown under S-amended soil by 13.9%. On lowering of soil pH with S, application of Fe with BC and with PM reduced phytate contents by 18 and 16%, respectively compared to the control in S-acidified soil.

Likewise, the phytate:Fe ratio was significantly influenced by application of Fe with PM and BC in S-acidified soil [Fig. 5.1 (b)]. In unacidified soil, when Fe was used with BC and PM, reduction in the phytate:Fe ratio was 58 and 54%, respectively compared to the control. But in acidified soil this reduction reached up to 73 and 70%, respectively compared to the control. As far as the phytate:Zn ratio was concerned, it was significantly reduced by application of Fe with organic amendments (BC and PM) in S-acidified soil [Figure 5.1 (c)]. In unacidified soil, combined application of Fe with BC and with PM reduced the phytate:Zn ratio by 13.9 and 25%, respectively compared to the control. Compared to the control, combined used of Fe with BC and PM reduced the phytate:Zn ratio by 30.8 and 37.1%, respectively in S-acidified soil. Concentrations of total polyphenol in rice grain were also influenced by soil pH, BC and PM [Figure 5.1 (d)]. Data suggested that, compared to the control, application of Fe with PM significantly reduced polyphenol of rice grain by 29% in unacidified soil. Lowering soil pH with S remarkably reduced polyphenol by 47.1 and 41.2% when Fe was applied with BC and with PM, respectively, compared to the control.

5.3.6 Ferritin and protein contents

Protein contents of rice grain were significantly influenced by application of Fe with BC and PM in unacidified and S-acidified soil as depicted in [Figure 5.2 (a)]. Lowering of soil pH with S improved protein contents in all treatments as compared to control. Maximum increased in protein contents were obtained in treatment where we Fe were used with BC i.e. 58% and with PM 56 % over control in S treated low pH soil. Results indicated that in unacidified soil, as compared to control application of Fe with BC increased ferritin contents by 135% over control [Figure 5.2 (b)].
Figure 5.1 Phytate, polyphenol conc. (mg g⁻¹ seed), phytate:Fe ratio, and phytate:Zn ratio as determined in rice grain with various iron (Fe), Biochar (BC), poultry manure (PM), and sulfur (S) treatments in alkaline calcareous soil. Sulfur was applied 4 weeks, before transplanting of seedlings for complete oxidation of elemental sulfur.
The highest increase in ferritin content was observed when Fe was applied with BC in S-acidified soil and that was 300% more than the control.

\[(HSD_{0.05} \text{ Protein (a) } 0.75, \text{ Ferritin (b) } 0.025)\]

Figure 5.2 a, b. Protein (%) and ferritin (µg/g) contents determined in rice grain with various iron (Fe), Biochar (BC), poultry manure (PM), and sulfur (S) treatments in alkaline calcareous soil. Sulfur was applied 4 weeks, before transplanting of seedlings for complete oxidation of elemental sulfur.
5.4 Discussion

The most recent challenge that we are facing is “hidden hunger” and it needs special attention. The root cause of hidden hunger is provision of food that is lacking in essential microelements (i.e. Fe, Zn). Rice biofortification is needed because it is the principal source of food for more than 75% of the world population (Anjum et al., 2007). Biofortified foods may cut the need for supplements; as Shekhar (2013) said “Biofortification is a win-win scenario”.

The major issue regarding Fe is not its deficiency but its availability. Fe is not deficient in mineral soil but high pH (Tazeh et al., 2012) and high carbonate (del Campillo and Torrent, 1992) limit its availability. In addition to these soil factors, some nutritional factors (i.e. phytic acid and phenolic compounds) also reduce Fe bioavailability in the human digestive tract (Welch, 2002) and cause Fe malnutrition. Thus, a sustainable approach is needed to increase Fe availability in soil for plant uptake and resultantly improve Fe nutrition in the human body (Chojnacka et al., 2011).

The current study was planned to investigate the effect of organic amendments (PM, BC) and Fe fertilizer on Fe biofortification of rice in unacidified and acidified calcareous soil.

At high pH Fe$^{+2}$ ions (the plant available form) convert into less soluble Fe$^{+3}$ oxides and hydroxides and disappear from the soil solution (Cakmak et al., 2010a). To reduce this process soil pH manipulation might be a good approach, which was my proposed hypothesis. For slow and steady soil acidification and nutrient availability, elemental S is considered best (Iqbal et al., 2012). The application of elemental S reduced soil pH to approximately a neutral level (pH 7) after 4 weeks. Microbial oxidation of elemental S presumably produces enough sulfuric acid to lower the soil pH.

The effect of Fe fertilization along with BC and PM, in acidified soil was observed for Fe biofortification in rice. All physiological parameters like photosynthetic, transpiration rate, stomatal and sub-stomatal conductance were significantly (p≤0.05) influenced by Fe applied with BC and PM in S-acidified soil. Photosynthetic rate was markedly influenced by BC and PM in acidified soil. By lowering of soil pH, H$^{+}$ are produce that displace metal cations
adsorbed on the BC surface. Iron is a key component of prophyrin ring of chlorophyll. As soon as Fe solubilization increases the amount of available Fe for plant uptake, it also increases the photosynthetic activity of the plant. It is a well-known fact that biochar supports the microbial community by providing nutrients, carbon source and shelter (Wardle et al., 2008). \textit{Thiobacillus} produces sulfuric acid that lowers the soil pH (Ullah et al., 2013).

Poultry manure has plant nutrients that can play an important role in crop productivity (Slaton et al., 2013). Watts et al. (2010) showed that poultry manure can be considered a soil conditioner that improves organic matter contents. Thus, release of nutrients from mineralization of PM supplies Fe for plant uptake that improves the physiology of the rice plant but more positive results were obtained when Fe was applied in PM amended soil. In the same way, transpiration rate and stomatal conductance of rice was significantly improved by lowering soil pH and application of Fe fertilizer with BC and PM.

Application of Fe with BC and PM significantly improved plant growth and yield. It is suggested that application of biochar may improve fertilizer use efficiency (van Zwieten et al., 2010). On lowering of soil pH, indirect release of all essential nutrients that are needed for plant growth from BC surface along with Fe could have improved plant growth. Biochar is known to improve soil physical properties that ultimately improve plant health and yield. My results are in line with Rajkovich et al. (2012), Zhang et al. (2012), and Akhtar et al. (2015) in which application of biochar increased yield of maize and wheat. Improvement in straw yield and grain weight might also have the same mechanisms. Application of PM also significantly improved plant growth and yield as PM is considered a source of nutrients (Kaiser et al., 2010). Many essential nutrients like N, P are provided by PM that could increase plant growth and yield. Also PM could help to improve water content needed for plants (Watts et al., 2010). Our results are in accordance to the reporting of Woli et al. (2015) and Ruiz Diaz et al. (2011) where grain yield was improved by the application of PM.

Metal analysis for the effects of BC on agronomic crops (i.e. wheat, rice, maize etc.) has been done and positive impacts have been observed (Hammond et al., 2013; Graber et al., 2014). In the current study we used BC and Fe alone and in combination to investigate the grain mineral (Fe, Zn) contents of rice. Grain Fe contents were significantly improved in treatments having both BC and Fe in acidified soil. The effectiveness of inorganic fertilizer
may have been improved when used with BC (Schulz and Glaser, 2012; Alburquerque et al., 2013). This could have been increased the mineral content of grain in our study. This confirmed the investigation of Hoefer et al. (2015) in which nutrient solubilization increased by the application of S.

Application of Fe fertilizer along with BC reduced the Zn contents of rice grain. Biochar might increase the mineral contents of the rhizosphere that in turn enhance the phytoavailability of Fe and Zn. But due to the competition of similar ions for plant such as Zn contents may be less than Fe, but greater than the control. Similar results were reported by Beesley and Marmiroli (2011) in which application of BC reduced the Zn contents due to its high sorption capacity and low Zn content in soil. Application of Fe with PM increased the Fe contents of rice grain. Our results are inconsistent with previous reports of Demir et al. (2010) in which fruit Zn contents were significantly improved by the application of PM.

The ultimate objective of Fe biofortification is either to increase the bioavailable portion of Fe or decrease anti-nutrients (i.e. phytate or polyphenolics). In the current study I used BC and PM to enrich rice grain with Fe in acidified soil. Biochar provides microhabitats to microbes that produce Fe chelating compounds that further regulate Fe mobility to grain. Thus, the total available portion of Fe increased and phytate contents decreased. Some genotypic characteristics (YSI, YS2) may be involved or soil native conditions (i.e. organic matter contents may increase Fe translocation into seed) (Chandel et al., 2010). Findings of Reddy et al. (2000) support our results in which phytate contents decreased as ferritin concentration increased in grain.

Previously, biochar has been used for Zn biofortification of crops by Gartler et al. (2013). After phytate, polyphenolic compounds are also potential inhibitors of Fe absorption in the human body (Scholz-Ahrens and Schrenzenmeir, 2007; Gautam et al., 2010). In the present study we found that as bioavailable Fe content increased, polyphenol concentration decreased significantly by using Fe with BC in acidified soil. Studies also support our findings as investigated by Tako and Glahn (2010), in which white bean had more bioavailable Fe compared to red been because of low polyphenol content in white bean. Increased Fe concentration can limit the polyphenol inhibitory effect on Fe absorption provided that polyphenol concentration remains constant as Fe content increases.
The Phytate:Zn ratio may decline as one increases the Fe supply. A phytate:Zn ratio <20 is generally desirable for improving human nutrition (Turnlund et al., 1984; Weaver and Kannan, 2002). Our results are similar to the results of Hussain et al. (2012a) in which Zn supply reduced the Phytate:Zn ratio significantly.

Modern agriculture emphasizes not only the quantity of produce but the quality of produce. Protein and ash contents are usually examined to evaluate the nutritional value of cereal food, such as wheat, rice, and legumes (Graham, 1999) Rice is poor in protein (i.e. 8% in brown and 7% in milled rice) compared to other cereals like wheat, corn, etc. (Anjum, 2007). Our results are supported by the findings of Fang et al. (2008) and Yadav et al. (2013) who found that the optimum quantity of Fe improved nutritional quality of grains but reduction of phytate in grain and ferritin enrichment is a novel approach in our study.

5.5 Conclusions

The research finding suggests that Fe bioavailability and nutritional quality of rice can be enhanced by combined use of Fe with organic amendments in acidified calcareous soil. Among the organic amendments (BC and PM), Fe applied with BC (1% w/w) show more pronounced effect on plant growth and yield in S-amended calcareous soil. Iron applied in unamended soil is not much effective as affective in S-amended calcareous soil. However, for Fe biofortification, application of Fe is more effective when applied with BC in acidified soil compared to PM or where Fe was applied alone.
Chapter 6

Iron bioavailability from rice: Rat study for determination of iron absorption

Abstract
The main purpose of this paper was to investigate the fate of iron (Fe) biofortified grain in alleviating Fe deficiency anemia (IDA). For this purpose, an experiment was conducted in which Fe biofortified rice grain containing different concentration of Fe (40, 60, 85, 100 mg kg\(^{-1}\) DW) were used as a feed source. As part of this experiment, two rat studies were selected. Further, each study was comprised of five rat groups as anemic and non-anemic. Rice grain purchased from the open market and containing 26 mg Fe kg\(^{-1}\) DW was used as a control. Rats were slaughtered after eight weeks of feeding to examine organ weight, iron content in organs, hematological parameters, serum chemistry, and ferritin in liver and serum. The experiment showed that 60, 85 and 100 mg Fe kg\(^{-1}\) produced effective restorative action returning the hemoglobin (Hb), mean corpuscular volume (MCV), red blood cells (RBC), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), serum and liver ferritin in anemic rats to normal values or in some cases better than normal value. Iron biofortified rice grain had a significant positive effective on Fe concentration in organs and serum biochemical parameters compared to the controls. It was concluded that the Fe biofortified rice grain containing a high concentration of Fe had significantly alleviated iron deficiency anemia. The results of this research demonstrate that Fe biofortified rice grain should be further exploited as a safe, efficient, and a new means to eradicate Fe deficiency anemia in human.

Key Words: biofortification, anemia, hematology, serum biochemistry, ferritin
6.1 Introduction

Worldwide, rice is the most widely used staple food as it comprises 50% of the world’s consumption of staple food (Fitzgerald et al., 2009). In 2014, the Food and Agriculture Organization of the United Nations reported worldwide rice consumption of about 509.7 million tons (FAO, 2014). However, rice based food lacks richness of essential micronutrients, such as Fe (Bouis et al., 2011). Consequently, this leads to a common hematological disorder and it has been estimated that 46% of children between the age of 5 to 14 and 56% of pregnant women suffer from anemia (Low et al., 2013). Compared to developed nations, most of the population in developing countries does not have adequate access to the recommended intake of Fe, resulting in Fe malnutrition and this has impacted two billion people (54%) of the planet (WHO, 2011; Sperotto et al., 2012).

Gârban et al. (2013) reported that Fe is a vital trace element in living matter, which plays an important role in the biosynthesis of hemoglobin and myoglobin. They further mentioned that Fe acts as a cofactor for several enzymes that takes part in variety of metabolic processes. Some fatal consequences of Fe deficiency reported in the literature are; loss of immunity, damages to the human immune system, impaired circulatory systems, problems with pregnancy, and decelerated intellectual development of babies, anemia, and poor work performance (Black et al., 2008; Stein, 2010). The previous research also supported the evidence that low hemoglobin can lead to symptoms such as low energy, faintness/dizziness, painful perineal sutures, and tingling of fingers (Albacar et al., 2001; Etebary et al., 2010).

Iron deficiency could be due to two reasons: first, inadequate iron intake; second, its insufficient absorption by the body (Naigamwalla et al., 2012). They further reported that either of these will result in Fe deficiency anemia. On the other hand, Duque et al. (2014) described various factors that influence the bioavailability and Fe absorption from the daily diet. For example, the quantity and type of Fe in food, presence of inhibitors, and promoters of Fe absorption affect an individual’s Fe status. Similarly, Wo´jciak et al. (2012) referred to previous research on this topic and reported that due to low Fe intake, low tissue Fe was also observed.
Rice contains strong anti-nutrients such as phytic acid that lead to anemia in developed and developing countries (Mayer et al., 2008). Therefore, Gautam et al. (2010) reported that phytate is the major inhibitor of Fe bio-availability. On the other hand, phytoferritin, an Fe storage protein, which can preserve thousands of Fe atoms in non-toxic form, and ferritin Fe are bio-available to humans as ferrous sulphate (Arosio et al., 2009).

A large stream of researchers (e.g., Bhutta and Haider, 2009; Hess and King, 2009) have argued that Fe supplements have always been available in the world. However, various efforts for supplementation programs, food fortification, and diversification require substantial funding and resources (Saltzman et al., 2013). Therefore, very often such programs end serving urban population whereas rural or poor population are left unserved deprived. One solution to this is the use of ferrous sulfate (FeSO$_4$) teblet, because it is of low cost and, therefore, the most commonly used iron supplement. However, the use of ferrous sulfate can cause various gastrointestinal side effects such as, nausea, abdominal pain and diarrhea (Mimura et al., 2008).

The research community also argues that Fe deficiency cannot always be fulfilled by supplements. Because it can develop undesirable consequences, for example, high doses of Fe can have toxic effects on the human body (Bread et al., 2005; Golub et al., 2009). Similarly, Powell et al. (2013) reported the negative impact of dietary fortified Fe intake on patients’ lives and its low bioavailability and an antagonistic mechanism with other metals, making it undesirable as a supplement.

Pro-oxidative properties of Fe are developed by catalyzing the conversion of hydrogen peroxide and superoxide into hydroxyl radicals in the Fenton reaction (Jomova and Valko, 2011). However, King et al. (2008) cautioned that high doses of Fe can cause lipid peroxidation and oxidative damage of body cells, particularly liver cells due to accumulation of this element. The previous argument is further reinforced by Hori et al. (2010) who also warned that this element can lead to protein and DNA damage.

Among different strategies to combat Fe deficiency in humans, Saltzman et al. (2013) argued that biofortification is a cost effective approach that enables the provision of sufficient amounts of mineral contents to the target population with a one time investment. Similarly,
Bouis et al. (2011) noted that biofortified staple foods might not deliver sufficient amounts of minerals and vitamins per day, however, they can enhance micronutrient intake for resource deprived people who consume them on a daily basis and, therefore, complement existing approaches.

Rats are widely used as proxies for testing purposes particularly for agents that have high toxic effect on human body. For example, the toxicity of metals and most likely preventive and therapeutic effects (Brzóska et al., 2012; Al-Rejaie et al., 2013).

To the best of our knowledge, no comprehensive study has investigated whether different concentrations of Fe biofortified grains can eradicate anemia and what concentration of Fe is best for such purpose. Thus, the aim of this research was to investigate the potential effects of Fe biofortified rice grain on anemic and non-anemic rats to with respect to iron deficiency anemia elimination, hematological Fe status, biochemical parameters, and variation in body organ mass. Furthermore, ferritin deposits in liver and serum of rat were also mapped.

6.2 Methodology

6.2.1 Iron biofortified grain production

Iron biofortified rice grain was produced in a field area of the Institute of Soil and Environmental Sciences (ISES), University of Agriculture of Faisalabad, Pakistan. For production of Fe biofortified rice grain, soil pH was decreased with elemental S and rice seedlings were grown with Fe fertilizer (FeSO₄·7H₂O) along with two organic amendments i.e. biochar and poultry manure. After harvesting the rice, it was analyzed and grouped into four different categories of grain having different levels of Fe (i.e. 40, 60, 85 and 100 mg kg⁻¹ DW). Rice were also purchased from the market that was not Fe biofortified and these grains were considered the control having an Fe concentration of only 26 mg kg⁻¹ DW. Chemical analysis of Fe in rice was performed in the nutritional laboratory ISES, University of Agriculture Faisalabad, Pakistan.

6.2.2 Animal
Eighty male Wistar strain albino rats having initial body weights of 100 ± 10 g were purchased from the Institute of Pharmacy, Physiology, and Pharmacology in the University of Agriculture Faisalabad, Pakistan. The rats were acclimated for 5 days prior to experiments with an evaluation of their health status.

6.2.3 Anemia development

Forty male Albino rats were subjected to the procedure of anaemisation (by the method of exclusion Fe from their diet). The Fe limited diet was prepared according to AIN (American Institute of Nutrition) standards (Borel et al., 1991) eliminating iron citrate (III) as a source of iron. For this purpose AIN-76A semipurified diet was mixed in rice grains diet to develop anemia. Hemoglobin level in rat blood was monitored by collecting blood every seven days. After 4 weeks of anaemisation, hemoglobin concentration in rat blood decreased to an average value of ≤9.57 g dL⁻¹, while normal rats have Hb concentration ≥11 g dL⁻¹.

6.2.4 Experiment setup

The research was conducted in the Institute of Pharmacy, Physiology, and Pharmacology, University of Agriculture Faisalabad, Pakistan. Rats were divided into two major groups (non-anemic and anemic) and each major group was subdivided into five subgroups on the basis of Fe concentration in grain, and treatments are:

Control: Rats fed a diet containing 26 mg Fe kg⁻¹ DW of rice
G1: Rats fed a diet containing 40 mg Fe kg⁻¹ DW of rice
G2: Rats fed a diet containing 60 mg Fe kg⁻¹ DW of rice
G3: Rats fed a diet containing 85 mg Fe kg⁻¹ DW of rice
G4: Rats fed a diet containing 100 mg Fe kg⁻¹ DW of rice

The animals were kept in metabolic cages and maintained in an environmentally controlled room at 24±2 C, with relative air humidity of 55±5%, and optimum lighting in a daily cycle, (i.e., 12 h light/12 h darkness). The experiments were performed according to the rules and protocols accepted by Local Ethical Commission for Investigations on Animals. Rats were maintained in collective cages (4 rats per cage) as treatment plan. Rat feed was made by following AIN standards and during the whole period of the experiment all rats were supplied with deionized water to eliminate additional Fe sources. The animals with induced
Fe deficiency anemia before the beginning of experiment were divided into experimental groups in such a way that the standard deviations in hemoglobin concentration inside the groups were minimized.

All animals were observed twice a day for general appearance, behavior, signs of morbidity and mortality. After feeding 8 weeks of Fe biofortified rice grains and a control diet, which was not Fe biofortified, the animals were subjected to dislocation and decapitation. Rats organs (liver, kidney, hurt and spleen) were collected and weighed.

6.2.5 Hematological Parameters

Blood was collected in EDTA-K2 solution tubes for the determination of red blood cell (RBC) hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and haemoglobin (Hb) concentration using an automated hematology analyzer ( Sysmex, Tokyo, Japan).

6.2.6 Serum analysis

A few mL of blood was allowed to stand for 2 hours and thereafter centrifuged at 1500g for 10 min to separate serum. The serum was stored at −80 °C until further analysis. Serum urea, protein, cholesterol, albumin, creatinine, and glucose were measured by kit method (Merck Specialties Pvt. Ltd. Germany) according to the instructions from the manufacturer.

6.2.7 Determination of iron in rat organs and serum

Approximately 1 g of sample was wet mineralized in spectrally pure concentrated nitric acid (65% HNO₃ GR ISO Merck) in a microwave system MARS-5 (CEM). The resultant clear mineralisate was quantitatively moved to 25-ml polypropylene flasks (PP) and made to volume with deionized water. Iron content in mineralisate was determined using flame atomic absorption spectrometry, with a wavelength of 248.3 nm and gap width of 0.15 nm, (PerkinElmer, A Analyst 100, Waltham, USA) (Zielińska-Dawidziak et al., 2014).

6.2.8 Ferritin quantification

The content of ferritin was determined in centrifuged blood serum and liver sample. Analyses were performed by using a kit (Catalog Number: BC-1025, California) for rat ferritin
determination by the sandwich ELISA method. Ferritin isolation from the liver was conducted according to the procedure described by Drysdale and Ramsay (1964).

6.2.9 Statistical analysis

The study analyses of results were conducted by employing descriptive statistics and one-factor analysis of variance in SPSS Version 22 statistical software. Values are given as means ± SD for eight rats in each group. Significant means were separated by Duncan’s Multiple Range Test (DMRT) with significant probability (p < 0.05).

6.3 Results

6.3.1 Rat body organ weight

Among different body organs, liver weight was significantly increased in the non-anemic group compared to the anemic group; however reduction in remaining body organs weight was observed in the non-anemic group relative to the anemic group (Table 6.1). Maximum increase (21%) in liver weight was observed in the group feed with diet containing 100 mg Fe relative to the control in the non-anemic group. The minimum increase (5%) in liver weight was observed in group feed with diet containing 40 mg Fe relative to the control in the anemic group. Similarly, kidney weight was significantly increased (31%) within the group feed with diet containing 85 mg Fe and group feed with diet containing 100 mg Fe compared to the control in the non-anemic group; however in the anemic group, kidney weight decreased with the Fe containing diet and the maximum decrease (7%) was observed in group feed with diet containing 100 mg Fe compared to the control. Similar results were found in heart and spleen weight; the maximum increase in weight was observed (as a % of body mass) in the control of the anemic rats compared to the rest of the rats groups (Table 6.1).
Table 6.1 Organ weight with respect to percent body weight. Group of animals fed with diet containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4)

<table>
<thead>
<tr>
<th>Rats category</th>
<th>Treatments</th>
<th>Liver weight</th>
<th>Kidney weight</th>
<th>Heart weight</th>
<th>Spleen weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-anemic</td>
<td>26 mg Fe</td>
<td>2.94±0.12 c</td>
<td>0.82±0.04 e</td>
<td>0.38±0.03 d</td>
<td>0.13±0.04 f</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>3.2±0.14 b</td>
<td>0.99±0.03 d</td>
<td>0.43±0.02 c</td>
<td>0.15±0.02 e</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>3.32±0.12b</td>
<td>1.11±0.03c</td>
<td>0.45±0.04bc</td>
<td>0.17±0.02 de</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>3.33±0.14b</td>
<td>1.18±0.05bc</td>
<td>0.46±0.06bc</td>
<td>0.18±0.02 cd</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>3.56±0.32a</td>
<td>1.18±0.05bc</td>
<td>0.47±0.03b</td>
<td>0.19±0.01 b-d</td>
</tr>
</tbody>
</table>

| Anemic        | 26 mg Fe   | 2.53±0.06 e  | 1.19±0.04 a   | 0.51±0.03 a  | 0.22±0.01 a   |
|               | 40 mg Fe   | 2.65±0.1de   | 1.2±0.06 a    | 0.48±0.04ab  | 0.22±0.02 a   |
|               | 60 mg Fe   | 2.69±0.06d   | 1.14±0.02bc   | .48±0.02 ab  | 0.2±0.02 a-c  |
|               | 85 mg Fe   | 2.71±0.09d   | 1.16±0.04bc   | 0.48±0.01ab  | 0.2±0.01 a-c  |
|               | 100 mg Fe  | 2.73±0.06d   | 1.11±0.02c    | 0.45±0.03bc  | 0.19±0.02 b-d |

Different letters in a column show statistically significant differences at P < 0.05.

6.3.2 Hematology of rat blood

The general status of blood organelles was poor in the anemic group relative to the non-anemic group (Table 6.2). Hemoglobin (Hb) concentration was significantly lowered in the anemic group and the minimum concentration was found in the control (23%) compared to group feed with diet containing 100 mg Fe.
However, a significant increase (12%) in Hb concentration was observed in group feed with diet containing 100 mg Fe relative to its control in the non-anemic group (Figure 6.1). Nevertheless, red blood cells (RBC) were significantly increased (28%) in rat blood with the group feed with diet containing 100 mg Fe relative to the control in the anemic group. The maximum increase (25%) in RBC was observed in the non-anemic group feed with diet containing 100 mg Fe compared to the control (Table 6.2). Among other morphological parameters, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) showed higher concentration in rat blood in the non-anemic groups compared to the anemic groups (Table 6.2). But in rats fed with Fe biofortified rice grain, the percent increase in all these parameters was much higher in the anemic group compared to the non-anemic group. There was a 44% increase in MCV observed in rat blood with group feed with diet containing 100 mg Fe relative to its control in the anemic group while only a 17% increase in MCV was observed in rat blood with the group feed with diet containing 100 mg Fe relative to its control in non-anemic conditions. All these parameters are considered indicators of improvement in hematology in anemic rats with Fe biofortified rice grain having a high concentration of bioavailable Fe.

6.3.3 Iron concentration in rat organs (liver, kidney, heart, and spleen) and serum

Iron concentration in different body organs remained meager in Fe deficient rats (anemic group) and Fe recovery also remain on the lower side even with Fe application at different concentrations (Table 6.3). Among different body organs like the liver, kidney, spleen, heart, and serum, Fe concentration was significantly increased with Fe application in both anemic and non-anemic groups; however, the increment was variable among both groups. Among these organs the largest deposit of Fe was found in the spleen. Maximum increase in spleen Fe (96%), liver (36%), kidney (64%), heart (39%) and serum (43%) was observed with the group feed with diet containing 100 mg Fe, of anemic rats compared to their respective control. In non-anemic rats, the highest deposited Fe in organs and serum was found in that group of rat feed with diet containing 100 mg Fe, when compared with the control.
Table 6.2 Hematological parameters of rats in different groups. RBC - red blood cell, HCT - hematocrit, MCV - mean corpuscular volume, MCH - mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration. Group of animals fed with diets containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4).

<table>
<thead>
<tr>
<th>Rats category</th>
<th>Treatments</th>
<th>RBC (%)</th>
<th>HCT (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-anemic</td>
<td>26 mg Fe</td>
<td>5.13±0.13d</td>
<td>29.69±3.06 cd</td>
<td>47.41±4.55 cd</td>
<td>15.25±1.23 bc</td>
<td>26.4±2.19 cd</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>5.5±0.19 c</td>
<td>32.03±2.65 bc</td>
<td>50.50±8.33 bc</td>
<td>15.75±1.52 bc</td>
<td>27.95±2.64 bc</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>5.64±0.15 c</td>
<td>33.63±3.01 ab</td>
<td>54.05±4.95 ab</td>
<td>16.63±2.55 ab</td>
<td>29.88±2.94 b</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>6.35±0.25 b</td>
<td>34.83±3.46 ab</td>
<td>56.74±4.69 a</td>
<td>16.97±1.96 ab</td>
<td>33.26±3.02 a</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>6.86±0.26 a</td>
<td>35.5±3.76 a</td>
<td>57.85±6.1 a</td>
<td>17.93±2.36 a</td>
<td>32.75±2.97 a</td>
</tr>
<tr>
<td>Anemic</td>
<td>26 mg Fe</td>
<td>3.53±0.09 h</td>
<td>23.05±1.98 e</td>
<td>25.18±3.07 f</td>
<td>11.23±1.37 e</td>
<td>22.43±2.23 e</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>4.11±0.26 g</td>
<td>25±2.28 e</td>
<td>27.9±3.19 f</td>
<td>12.31±1.19 e</td>
<td>22.91±2.67 e</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>4.53±0.23 f</td>
<td>28±2.58 d</td>
<td>35.49±3.64 e</td>
<td>12.78±1.21 de</td>
<td>24.91±2.43 de</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>4.67±0.2 ef</td>
<td>32±2.98 bc</td>
<td>38.39±3.51 e</td>
<td>14.3±1.21 cd</td>
<td>24.98±2.67 de</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>4.87±0.22 e</td>
<td>35±3.63 ab</td>
<td>45.25±4.06 d</td>
<td>14.8±1.78 c</td>
<td>28.01±2.79 bc</td>
</tr>
</tbody>
</table>

Different letters in a column show statistically significant differences at P < 0.05.
Figure 6.1 Hemoglobin concentration (g/dL) in rats at the end of experiment. Group of animals fed with diets containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4).

6.3.4 Ferritin contents in serum and liver

Rats fed with Fe biofortified grain significantly increased serum ferritin in all groups of non-anemic rats compared to the control (Figure 6.2a). The highest increase of serum ferritin was in the group feed with diet containing 100 mg Fe of non-anemic rats which was 55% higher than the control. A significant increase in serum ferritin was observed in the anemic rat groups when compared with their control. The mean maximum value of serum ferritin was calculated in group feed with diet containing 85 mg Fe and group feed with diet containing 100 mg Fe, anemic rats (1.93 and 2.63 µg/ml, respectively). A strong accumulation of ferritin in the liver of anemic and non-anemic groups of rats was observed after feeding Fe biofortified rice grain, and results show significant variation when compared with their respective controls (Figure 6.2b). In non-anemic rats, liver ferritin increased up to 41 and 61% in group feed with diet containing 85 mg Fe and group feed with diet containing 100 mg Fe, respectively, over the anemic control.
Table 6.3 Iron reserves in rat organs and serum. Group of animals fed with a diet containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4).

<table>
<thead>
<tr>
<th>Rats category</th>
<th>Treatments</th>
<th>Fe in spleen (mg)</th>
<th>Fe in kidney (mg)</th>
<th>Fe in liver (mg)</th>
<th>Fe in heart (mg)</th>
<th>Fe in serum (ug/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-anemic</td>
<td>26 mg Fe</td>
<td>2.11±0.14 f</td>
<td>2.11±0.14 f</td>
<td>1.2±0.04 e</td>
<td>0.44±0.03 c</td>
<td>170.1±11.95 c</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>2.9±0.32 d</td>
<td>2.9±0.32 d</td>
<td>1.4±0.07 d</td>
<td>0.46±0.3 c</td>
<td>194.3±15.88 b</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>3.21±0.14 c</td>
<td>3.21±0.14 c</td>
<td>1.79±0.09 c</td>
<td>0.5±0.06 b</td>
<td>202.5±19.57 b</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>3.68±0.16 b</td>
<td>3.68±0.16 b</td>
<td>2.19±0.11b</td>
<td>0.52±0.04 b</td>
<td>207.8±18.19 ab</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>4.09±0.18 a</td>
<td>4.09±0.18 a</td>
<td>2.35±0.15 a</td>
<td>0.62±0.08 a</td>
<td>222.1±21.7 a</td>
</tr>
<tr>
<td>Anemic</td>
<td>26 mg Fe</td>
<td>1.2±0.08 i</td>
<td>1.2±0.08 i</td>
<td>0.88±0.06 h</td>
<td>0.33±0.03 f</td>
<td>142.1±13.54 d</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>1.45±0.08 h</td>
<td>1.45±0.08 h</td>
<td>0.99±0.05 g</td>
<td>0.37±0.02 e</td>
<td>149.1±13.76 c</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>1.77±0.13 g</td>
<td>1.77±0.13 g</td>
<td>1.03±0.06 fg</td>
<td>039±0.03 de</td>
<td>169±14.83 c</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>2.03±0.1 f</td>
<td>2.03±0.1 f</td>
<td>1.08±0.08 g</td>
<td>0.42±0.04 cd</td>
<td>176.1±17.43 c</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>2.36±0.18 e</td>
<td>2.36±0.18 e</td>
<td>1.2±0.06 e</td>
<td>0.46±0.03 c</td>
<td>203.8±17.75 b</td>
</tr>
</tbody>
</table>

Different letters in a column show statistically significant differences at P < 0.05.
6.3.5 Serum biochemical analysis

In relation to the control group, rats with Fe biofortified rice grain (60, 85, and 100 mg Fe), had significantly greater levels of serum urea, cholesterol, protein, albumin, and creatinine in both anemic and non-anemic rats compared to the respective controls (Table 6.4). In non-anemic rat groups the highest increase in serum urea (49%), cholesterol (42%), protein (23%), albumin (41%) and creatinine (39%) was observed in the group feed with diet containing 100 mg Fe compared to the control. Among anemic group, the group feed with diet containing 85 mg Fe and group feed with diet containing 100 mg Fe, remained best, with maximum values of these biochemical parameters equal to or more than the non-anemic control and group feed with diet containing 40 mg Fe. Elevated levels of glucose were observed in groups of anemic rats compared to non-anemic rats. In control and group feed with diet containing 40 mg Fe, of anemic rats, the highest glucose levels were calculated compared to the group feed with diet containing 60 mg Fe, group feed with diet containing 85 mg Fe and group feed with diet containing 100 mg Fe, of anemic and all non-anemic rats groups. The maximum mean value of glucose (115.75 mg/dl) was observed in the control of anemic rats and that was 43 and 40% higher than the group feed with diet containing 100 mg Fe of anemic and non-anemic rats groups, respectively.
Figure 6.2 a, b: Ferritin contents in serum and liver of rats as affected by various treatments. Group of animals fed with diet containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4).
Table 6.4 Rat serum biochemical analysis. Group of animals fed with diets containing: 26 mg Fe (control), 40 mg Fe (G1), 60 mg Fe (G2), 85 mg Fe (G3) and 100 mg Fe (G4).

<table>
<thead>
<tr>
<th>Rats category</th>
<th>Treatments</th>
<th>Serum Urea (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-anemic</td>
<td>26 mg Fe</td>
<td>37.88±2.5 d</td>
<td>60.5±3.25 de</td>
<td>4.80±0.09 de</td>
<td>3.41±0.05 g</td>
<td>0.43±0.03 cd</td>
<td>65.75±5.47 e</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>40.38± 2.5 d</td>
<td>61.88±4.7 d</td>
<td>5.03±0.16 c</td>
<td>3.52±0.13 f</td>
<td>0.47±0.04 c</td>
<td>69.75±4.23 e</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>46.63±3.02 bc</td>
<td>68.5±4.87 c</td>
<td>5.61±0.15 b</td>
<td>3.76±0.07 c</td>
<td>0.47±0.04 c</td>
<td>76±4.2 d</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>48.88±3.4 b</td>
<td>76.63±5.68 b</td>
<td>5.94±0.16 a</td>
<td>4.42±0.06 b</td>
<td>0.56±0.05 b</td>
<td>77.63±3.02 d</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>56.63±5.45 a</td>
<td>86.25±5.73 a</td>
<td>5.94±0.21 a</td>
<td>4.84±0.12 a</td>
<td>0.6±0.05 a</td>
<td>82.38±4.66 cd</td>
</tr>
<tr>
<td>Anemic</td>
<td>26 mg Fe</td>
<td>29.75±2.87 e</td>
<td>56.5±3.78 e</td>
<td>4.19±0.13 f</td>
<td>3.32±0.11 g</td>
<td>0.39±0.03 d</td>
<td>115.75±4.8 a</td>
</tr>
<tr>
<td></td>
<td>40 mg Fe</td>
<td>32.5±2.39 e</td>
<td>61.75±5.15 d</td>
<td>4.19±0.24 f</td>
<td>3.53±0.11 ef</td>
<td>0.42± 0.05 d</td>
<td>111.5±6.61 a</td>
</tr>
<tr>
<td></td>
<td>60 mg Fe</td>
<td>37.75±3.77 d</td>
<td>64.5±4.99 cd</td>
<td>4.67±0.14 e</td>
<td>3.59±0.14 d-f</td>
<td>0.46±0.04 c</td>
<td>95±12.31 b</td>
</tr>
<tr>
<td></td>
<td>85 mg Fe</td>
<td>40.5±3.42 d</td>
<td>62.25±3.49 cd</td>
<td>4.93±0.12 cd</td>
<td>3.63±0.1d e</td>
<td>0.47±0.04 c</td>
<td>86.75±5.47 c</td>
</tr>
<tr>
<td></td>
<td>100 mg Fe</td>
<td>44±3.55 c</td>
<td>69.25±5.18 c</td>
<td>5.09±0.11 c</td>
<td>3.69±0.1 cd</td>
<td>0.53±0.04 b</td>
<td>80.75±3.58 cd</td>
</tr>
</tbody>
</table>

Different letters in a row show statistically significant differences at P < 0.05
6.4 Discussion

The main purpose of this research was to investigate the potential effects of Fe biofortified rice grain containing different concentration of Fe on anemic and non-anemic rats. Rats with induced Fe deficiency anemia showed poor general status and were apathetic, due to loss of appetite due to daily feed diet. Macroscopic results further indicated that the anemic rats also suffered from hair loss and were lethargic. This condition can be attributed to the inefficiency and ineffectiveness in the blood circulatory system which is responsible for oxygen and nutrient supply to body tissues and hair follicles (body cells). These findings corroborate with Ebuehi and Mbara (2011). Rats fed with Fe biofortified grains had significantly improved appetite and overall condition. This finding is also positively correlated with observations made by Yun et al. (2011) and Zielin’ska-Dawidziak et al. (2012).

In my experiment, significant increase in liver weight was observed in non-anemic rats while reduction in volume and liver weight was observed in anemic rats. Iron biofortified rice grain having high concentrations of Fe caused hyperplasia in the liver compared to anemic rats. Similarly, Zielin’ska-Dawidziak et al. (2012) and Yun et al. (2011) also reported their observation of liver volume reduction caused by iron deficiency and increased liver weight after Fe supplementation. Hypertrophy of the spleen was observed in rats of anemic groups because iron deficiency activated spleen cells and cell proliferation (Kuvibidila et al., 2012). In contrast, it was also observed in this research that the anemic rats also had a higher organ coefficient of heart as compare to non-anemic rats. This finding is also supported by Hedge et al (2006) who reported that heart weight increase in iron deficient animals is a common outcome - cardiomegaly. The findings of this research and those of Hedge et al. (2006) further supports Zielińska-Dawidziak et al. (2014) who reported similar results of their research.

In terms of percent body mass, anemic animals of the control observed with the highest increase in kidney mass. This increased coefficient in anemic rats was partly due to the oedema stimulated by iron deficiency anemia. This previous argument and finding further corroborates with researchers such as Zielińska-Dawidziak et al. (2014) and Wang et al. (2015).
The main aim of this experiment was to examine Fe bioavailability from Fe biofortified grain. Iron therapy is recommended for patients who suffer from severe or intravascular hemolysis. In this situation, a persistent hemoglobinuria turns into significant Fe loss (Ebuehi and Mbara, 2011). Iron is known as vital for hemoglobin synthesis and it can be reasonably assumed that any inadequacy in Fe level will result in a slower hemoglobin synthesis rat (Gârban, et al., 2013). Following this assumption, anemic group rats of control group and group feed with diet containing 40 mg Fe continued to be anemic in the whole experiment. Hence, the high concentration of hemoglobin in these anemic groups was due to the enhanced supply of iron and increased consumption. Results obtained under such assumptions also support the observations made by Zielińska-Dawidziak et al. (2012) and Tang et al. (2014). These previous researchers made similar observations from rats that were supplied with FeSO₄ and heme iron enriched peptide, respectively.

Throughout the experiment, anemic rats which were fed with a 26 and 40 mg kg⁻¹ Fe containing diet, showed the lowest average values of some parameters associated with blood counts such as MCV, RBC, HCT, MCH, and MCHC (Table 6.2). On the other hand, the highest values were found from animals fed with a 85 and 100 mg Fe containing diet. These findings also support Zielińska-Dawidziak et al. (2012) and Tang et al. (2014) who obtained similar results from animals that were treated with heme iron enriched peptide and with iron ferritin isolate.

Red blood cells (RBC) play an important role supporting tissue metabolism. Therefore, normal RBC should be present to sustain tissue oxygenation and to maintain a normal acid-base balance in the system (Carley, 2003). However, often hemoglobin production is restrained due to the absence of sufficient Fe, consequently, RBC are impacted. Further, Carley (2003) argued that the RBC decline in equal proportion to the hemoglobin concentration particularly in Fe deficient anemia. As Table 6.2 shows, the RBC counts of non-anemic rats groups were higher that the anemic rat groups. Because, the anemic rat groups showed significantly lower readings (p<0.05). However, the RBC numbers of anemic rat groups were found to be dramatically increased after feeding Fe biofortified rice grain. These results and findings also support Tang et al. (2014). They also found increased RBC after feeding Fe enriched peptide to the rats.
The proportion of the blood that is occupied by the RBC is indicated by HCT (Modepalli et al., 2013). This further depends upon the concentration of hemoglobin in the RBC. Table 6.2 also shows that the hematocrit value of anemic rats was significantly lower (p < 0.05) than non-anemic rats, but significantly increased after feeding Fe biofortified rice grain. Hematocrit increased significantly compared to the remaining groups of anemic and non-anemic rats. The high concentration of Fe in Fe biofortified rice feed was the reason of this increase. These groups also showed an increase in MCV value, which further reflects an increase in the plasma volume (Table 6.2). These findings corroborate with those of Smol 2001. Zielińska-Dawidziak et al. (2012) also found the same results from animals previously treated with Fe ferritin isolate.

Knight et al. (1983) reported the mean corpuscular hemoglobin (MCH) as a good haematological indicator of Fe deficiency. In my experiment due to the low Fe concentration in the diet of control and G1 group of anemic rats, MCH and MCHC increased non-significantly. Further, rats supplied with Fe biofortified rice grain containing enhanced Fe concentration (60, 85, or 100 mg Fe) significantly increased MCH and MCHC in anemic rats compared to anemic controls.

Generally, it was observed that feeding Fe (60, 85, or 100 mg Fe) biofortified rice grain brought improvements in anemic rats in their hemoglobin RBC, MCV, HCT, MCH and MCHC from normal to better. Similar observations were reported by Tang et al. (2014) and Bertinato et al. (2014) after feeding heme Fe enriched peptide and diet containing a 70 mg Fe kg⁻¹ diet.

Angelucci et al. (2000) suggested that to test the complete body Fe store, liver Fe concentration is a reliable indicator. Similarly, Emerit et al. (2001) mentioned that the spleen and liver are two main Fe storage sites. In this research, non-anemic rat group had higher Fe contents in their organs (liver, spleen, kidney and heart) compared to anemic rat groups (Table 6.3). The highest iron deposition was observed in the spleen, followed by the liver, kidney, and heart irrespective of groups. It is suggested by the authors that depletion of Fe storage from rat organs can be ameliorated by adding Fe in their diet (Zielin’ska-Dawidziak et al.,
This research confirms the hypothesis that the rats fed with Fe biofortified rice grain containing greater concentrations of Fe (60, 85, or 100 mg kg\(^{-1}\) DW) improved the depleted Fe storage in rat organs. Cancado et al. (2001) indicated that serum Fe concentration is a measure of the total quantity of Fe in the serum and the concentration increases with the supply of dietary Fe.

Ferritin is a protein that operates in vivo as an Fe storage complex. The level of Fe body stores can be reflected by serum ferritin; where serum ferritin level is low it indicates Fe deficiency (Wang et al., 2014). The literature suggested serum ferritin levels are a reliable indicator for Fe stores in the body that can be mobilized, and further reliable measurements in finding Fe deficiency at a very early stage and usually directly proportional to body Fe stores (Reddy et al., 2006).

Pollock et al. (1978) and Powell et al. (2013) reported that Fe deficiency treatment is quickly reflected in serum ferritin, which further reflects the storage size of the Fe compartment. This research found lower serum ferritin in the control of anemic rats as compared to all rats groups feeding with Fe biofortified grains. The observations made in our research further support studies by Xiao et al. (2015) and Zielin’ska-Dawidziak et al. (2012). They reported that enhanced levels of Fe in diet serum ferritin levels also increased and returned to normal. An increase in ferritin levels in the liver is due to its increased synthesis rate and does not depend on its degradation rate. This showed that the production of anemia did not result in a complete Fe reserve decline in the liver. Therefore, based upon changes in ferritin level, it is hypothesized that Fe biofortified rice grain has a positive impact in Fe reserve reconstruction.

This research further demonstrated that in all groups of non-anemic rats there was a maximum concentration of serum urea, cholesterol, protein, albumin and creatinine compared to anemic rats (Table 6.4). It is also believed that changes in lipid metabolism, in serum levels, and in tissue lipids are usually caused by high doses of Fe. Further, it is also reported that changes in the gene expression of hepatic enzymes mainly HMG-COA reductase also causes an Fe induced increase in cholesterol in serum and tissues.

Kojima et al. (2004) reported that heavy metals stimulate changes in the gene expression of HMG-COA reductase. On the other hand, Gielen and Tiekink, (2005) and Gârban et al.
(1989) reported serum creatinine increases in the circumstance of multiple metal compound administration can be elaborated by their interaction with phosphate groups. Katsumata et al. (2009) reported that Fe deficiency anemia decreases the proportion of serum total protein and albumin and increased with enough Fe supplied diet, similar observation was noticed in our experiment. An increased glucose level was observed in control anemic rats that were supplied with a 26 and 40 mg Fe diet, respectively. Further, these values were statistically significant compared to the remaining anemic and non-anemic rat groups. Kamei et al. (2010) reported that increases in serum glucose levels in Fe deficient rats are due to the increases in the initial stages of gluconeogenesis. They also reported that, in the case of anemia, serum glucose level increase and further that the Fe deficiency potentially stimulates gluconeogenesis instead of glycolysis. Thses results showa that Fe biofortified rice grains have a positive effect on rats health and CBC recovery.

6.5 Conclusions

Due to the toxic effects of high doses of Fe and the negative impacts of dietary fortified Fe, it is believed that Fe deficiencies are not able to be compensated by fortification and supplementation. Biofortification is reported as a cost effective process that enables sufficient provision of mineral contents with a one time investment to the target population. Based on the results of this research, it can be argued that Fe absorption from Fe biofortified rice grain at the rate of 60, 85 and 100 mg Fe, leads to positive effects by eliminating Fe deficiency anemia. However, the author believes that Fe biofortified grain should be further exploited as a safe and efficient new Fe supplement approach compared to outdated supplements and fortification processes and practices. The increased deposits of Fe in non-anemic rat organs demonstrates the need for future studies in this area to further investigate the Fe biofortified rice grain effects on humans or other animals.
Chapter 7

Summary

Food security is a serious issue in developing countries because of ever-increasing population. The FAO states that in developing countries about 13.5% of the total population lacks enough food for their daily intake of calories. However, in last 40 years, agricultural research has focused only on production but not on quality of produce, which leads farmers to maximize profits by adopting the latest production technology for fewer crops, especially in developing countries. This inclusion of cash crops and high yielding varieties in cropping schemes has resulted in micronutrient malnutrition (i.e. Fe, Zn) and less food diversity. According to one estimate, every 3rd person in the world suffers from hidden hunger due to essential nutrient and vitamin deficiency, resulting in poor health conditions.

Calcareous soils are one of the main reasons for increasing micronutrient (especially Fe) malnutrition problems. High pH and high HCO$_3$ contents are two most important factors that limit Fe availability in calcareous soil. Iron is not lacking in mineral soil, but due to high pH and calcareousness it is unavailable to plants because at high pH Fe$^{+2}$ ions are converted into less soluble Fe$^{+3}$ oxides and hydroxides and disappear from the soil solution. Iron fertilization may not be effective in calcareous soil due to its rapid conversion. Mixing inorganic salts of micronutrients with different organic materials can enhance the efficacy of micronutrients. Organic matter is an important nutrient source, but due to harsh climatic conditions of Pakistan its decomposition is very quick.

Soil acidity favors the solubilization of mineral cations in soil with high calcium carbonate contents. Thus, elemental S is a common and cost effective acidifying matter. Microbial oxidation of S leads to dissolution of nutrients by releasing H$^+$. To combat these limitations we developed a strategy to increase Fe solubility in soil solution and availability for plant uptake using some acidifying agents in calcareous soil.

Phytate and polyphenols are major inhibitors of Fe present in cereals that reduce Fe bioavailability and lead to anemia in developed and developing countries; its consequences are greater in developing countries. Along with anemia, Fe deficiency results in increased susceptibility to infection, mental and psychomotor retardation in children, and impaired
immune system. In contrast, ferritin is a stable Fe storage protein present in cereals grains and doesn’t form complexes with other cations, thus increasing Fe availability to humans. Iron stored in ferritin is completely bioavailable because it doesn’t denature in the human alimentary tract.

Conventionally, both short term (i.e. supplementation, fortification, food diversification) and long term solutions (i.e. breeding, genetic engineering, and agronomic) approaches have been proosed to combat Fe deficiency in crops and in humans. Compared to these approaches biofortified crops may not provide as high a level of nutrients as per day demand, but it is a promising approach to provide adequate micronutrients throughout the life cycle. It is a cost effective approach that sustainably provide minerals and vitamins to target populations.

To combat these described limitations, we developed a strategy in which we evaluated the effect of Fe fertilizer combined with organic amendments (BC and PM) for Fe biofortification of cereals grains in S treated low pH calcareous soil. The main conclusions drawn from this research work are summarized below:

Initially, we used elemental S to reduce the soil pH and to solubilize Fe in calcareous soil. As demonstrated in Chapter 3, soil pH decreased gradually in pots receiving different doses of S and was stable after 4 weeks of incubation. Among the four organic amendments, S application in soil amended with biochar and poultry manure lowered soil pH consistently compared to soil amended with compost and farmyard manure. Addition of S decreased soil pH by about 1-1.9 units in biochar and poultry manure amended soil compared to the control. Iron is not deficient in mineral soil but due to high pH and calcareousness it is merely unavailable to plants because at high pH Fe$^{+2}$ ions converts into less soluble Fe$^{+3}$ oxides and hydroxides and disappears from soil solution. However in Chapter 3 our result showed that DTPA-extractable Fe concentration increased with biochar and poultry manure amendment along with different rates of Fe fertilizer in low pH soil compared to the control. Among the different rates of Fe fertilizer, Fe at a rate of 0.0075 g kg$^{-1}$ combined with biochar and poultry manure in low pH soil gave DTPA-extractable Fe of 10.3 and 9.9 ppm, respectively, which was double the amount as required by cereal plants to complete their life cycle.
In Chapter 3 it was concluded that soil pH manipulation with S improved Fe solubilization and its bioavailability in wheat grain. Hence, Fe application along with organic amendments could be an effective strategy to enhance Fe biofortification of wheat in pH manipulated soil. Ferritin concentration in wheat grain grown in calcareous soil was enhanced by optimizing Fe bioavailability in low pH soil.

In Chapters 4 and 5 it was observed that Fe bioavailability and nutritional quality of maize and rice grain was enhanced by combined use of Fe with organic amendments in acidified calcareous soil. Iron (FeSO$_4$.7H$_2$O, 0.0075 g kg$^{-1}$) applied with 1% biochar and 1% poultry manure increased grain Fe concentration and ferritin in low pH calcareous soil when compared with a control (in non-acidified soil). Maximum decrease in anti-nutrients in maize and rice grain was observed in treatments where Fe was applied with biochar in low pH soil. Highest increase in growth, physiology, chemical, and biochemical parameters was observed when Fe was applied with biochar in S treated low pH soil compared to unacidified soil.

It was reported that due to the toxic effects of high doses of Fe supplementation and negative impacts of dietary fortified iron, Fe deficiencies were not possible to be compensated by fortification and with supplementations. So, a rate study was conducted in anemic and non-anemic rats to find out the absorption rate of Fe from Fe biofortified rice grains.

Bioavailable Fe (ferritin) increased up to 65-230%, Fe concentrations up to 60-200%, Zn up to 9-32% and protein up to 19-58% in Fe biofortified rice grains and it was necessary to determine its fate with regards to Fe deficiency anemia elimination. So a comprehensive rate study was conducted to evaluate the Fe biofortified grains effect on rats CBC and organs.

It was concluded from Chapter 6 that out of five rate of Fe, three rates (i.e. 60, 85 and 100 mg Fe kg$^{-1}$ DW) produced effective restorative action returning the hemoglobin (Hb) up to 23%, mean corpuscular volume (MCV) up to 17%, red blood cells (RBC) up to 28%, hematocrit (HCT) up to 44%, mean corpuscular hemoglobin (MCH) 22%, mean corpuscular hemoglobin concentration (MCHC) up to 31%, serum, and liver ferritin in anemic animals to normal values or in some cases better than normal values. Furthermore, it was observed that Fe biofortified rice grain had a significant positive effect on Fe concentration in organs and serum biochemical parameters compared to controls. Based on the results of this research, it
can be argued that Fe absorption from Fe biofortified rice grain at rates of 60, 85, or 100 mg Fe, lead to positive effects by eliminating Fe deficiency anemia.

Overall, soil pH manipulation of calcareous soil with S improved Fe solubilization and its bioavailability. Iron application along with organic amendments could be an effective strategy to enhance Fe biofortification of cereals in pH manipulated calcareous soil. Ferritin concentration in wheat, maize, and rice grain grown in calcareous soil was enhanced by optimizing Fe bioavailability in low pH soil and its translocation in the plant body could serve as a valuable approach to fight the malnutrition problem in the humans.

**Future Suggestions:** The combined use of Fe fertilizer with biochar and poultry manure for improving Fe biofortification in S treated acidified soil could be an effective approach to compensate for Fe deficiency in plants and humans. However, multi-site field experiments are required through combined use of organic and inorganic amendments in acidified calcareous soils to further confirm this experimental approach. Moreover, the rate study confirmed that Fe biofortified rice grain has a positive effect on Fe deficiency anemia elimination in rats. But further trials in humans are required to determine the fate of Fe biofortification. Therefore, increased deposits of Fe in non-anemic rat organs demonstrates the need for future studies in this area to further investigate the Fe biofortified rice grain effects on humans and other animals.
References


FAO. 2014. The State of Food Insecurity in the World 2014, FAO.


Malakouti, M.J. and N. Gheibi. 1988. Determine the critical nutrients strategic and proper fertilizer recommendations in the country. Publication of agricultural education, training and equipping the human resources department of Tat, the Ministry of Agriculture, Karaj, Iran


Matz, S.A. 1991. The chemistry and technology of cereals as food and feed. AVI Book Springer


Tako, E., O.A. Hoekenga, L.V. Kochian and R.P. Glahn. 2013. High bioavailability iron maize (Zea mays L.) developed through molecular breeding provides more absorbable iron in vitro (Caco-2 model) and in vivo (Gallus gallus). Nutr. J. 12: Doi: 10.1186/1475-2891-12-3


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WHO. 2010. Progress on the health-related Millennium Development Goals MDGs


