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INTEGRATING THE ORGANIC AMENDMENT WITH IRON FERTILIZATION FOR IMPROVING PRODUCTIVITY AND Fe BIOFORTIFICATION IN RICE UNDER ACIDIFIED CALCAREOUS SOIL

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Iron (Fe) bioavailability is the major issue of highly calcareous soils. Iron fertilization in calcareous soils is not much effective due to their rapid conversion into unavailable form. A field experiment was conducted to evaluate the effect of Fe fertilizer along with organic amendments for improving growth, yield and Fe biofortification of rice by manipulating pH of calcareous soil. Before transplanting rice seedlings, soil pH was lowered down up to 0.5-0.6 units by using 0.25% (w/w) elemental sulfur (S). For biofortification, Fe fertilizer ($FeSO_4.7H_2O$) at the rate of 15 kg ha^{-1} was applied with biochar (BC) and poultry manure (PM) in S treated calcareous soils. Results indicated that the combined application of Fe along with BC and PM enhanced plant growth, physiology and paddy yield and improved nutritional value of grain in pH manipulated calcareous soil. Applied treatments in S treated low pH soil resulted in increased Fe mobilization and translocation from root to grain. Grain Fe concentration raised up to 2 fold and 1.8 fold in treatments where Fe was applied with BC and PM in pH manipulated soil, respectively. Ferritin concentration significantly increased up to 3 fold and 2.73 fold by using Fe with BC and PM, respectively, in pH manipulated soil. Iron applied in combination with BC decreased phytate and polyphenols contents up to 18 and 47%, respectively, in S treated soil. The results may imply that the combined use of Fe with BC increased Fe biofortification (ferritin) of rice in pH manipulated calcareous soil. This increased level of ferritin in rice grains might be helpful to eliminate Fe deficiency in humans.
Keyword: Biofortification, iron, zinc, ferritin, protein, phytate, polyphenols, rice

INTRODUCTION

Malnutrition is the deviation from the balanced nutrition in terms of deficiency or excess nutrients in human consumption which may lead to health problems. According to FAO, about 1/9th of the developing world is food insecure (FAO, 2014). This leads to micronutrient malnutrition causing hidden hunger. The main cause of this micronutrient malnutrition is consumption of monotonous food which is poor in microelements (Bouis *et al.,* 2011). Human population use variety of foods to fulfill their daily requirement of calories. A major part of daily dietary intake is provided by cereals. Among cereals, rice is second most consumed cereal worldwide after wheat. According to latest figure of FAO, rice consumption is about 509.7 million tons per year worldwide (FAO, 2015). But, rice grain has low quantities of important micronutrients like Fe and Zn (Welch and Graham, 2004).

Iron acts as a cofactor for several enzymes that takes part in the variety of metabolic processes. Susceptibility to disease also increases in Fe limiting conditions (Chatterjee *et al.,* 2006). Iron deficiency leads to loss of immunity, impaired circulatory system resulting into 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 limiting Fe availability to crops (Cifuentes and Lindemann, 1993). It is reported that Fe changes its oxidation state very quickly from soluble Fe^{+2} to relatively insoluble Fe^{+3} oxides and hydroxides leading to Fe deficiency in soil (Celik and Katkat, 2007). Besides soil factors some nutritional factors also act as potential inhibitor of Fe absorption i.e. phytate and polyphenolic compounds (Welch, 2002). Phytates make complexes with nutrient elements like Ca, Fe, Zn and Mg during digestion process and reduce their bioavailability up to a level to show 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). Various transgenic and genetic approaches have been implied to increase ferritin concentration in many crops including rice and soyabean (Vasconcelos *et al.,* 2003). Among potential approaches, maximization of ferritin concentration in wheat, cassava, rice or beans by decreasing phytate is also a possible solution of Fe malnutrition but not very practicable (Cakmak, 2010a)

Food fortification, diversification and supplementation programs required unbreakable funding and mainly targets urban population leaving behind the farmers/ poor/ majority of the population (Saltzman *et al.*, 2013). Biofortification is a cost effective approach that promises to provide sufficient mineral contents to target population and is considered one time investment (Bouis *et al.*, 2011; Saltzman *et al.*, 2013). Agronomic biofortification of food crops is considered as most sustainable approach. In highly calcareous soil, agronomic biofortification seems to be ineffective unless we lower down the soil pH. Because 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 to increase its availability, soil pH manipulation using some acidifying materials could be a useful approach. Different studies reported that microbial oxidation of elemental sulfur leads to mineral solubilization that increases nutrient availability to plants (Iqbal *et al.*, 2012; Wu *et al.*, 2014).
Organic matter is an important source of mineral nutrient but due to harsh climatic condition 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 as the best because of their long lasting effect on soil health and nutrient availability (Lorenz and Lal, 2014; de Cesare Barbosa *et al.*, 2015). Biochar application is considered a most sustainable approach to enhance soil fertility and crop productivity (Liu *et al.*, 2013). Positive effect of biochar on Fe solubilization by decreasing soil pH has also been reported (Graber *et al.*, 2014). Most recently, carbonaceous product has been used for Zn biofortification of crops (Gartler *et al.*, 2013). To the best of our knowledge, none of the study was reported to enhance Fe biofortification in rice under pH manipulated calcareous soil. The main objective of the present study was to enhance Fe concentration in grains through increased its bioavailability to rice plants by using Fe fertilizer in combination with organic amendments after acidifying natural calcareous soil.

MATERIALS AND METHODS

***Field preparation and soil properties*:** Experimental units of 4×4 m^2 sizes were prepared for rice experiment in the field area of the Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan. Prior to soil acidification and transplantation of rice seedlings, soil was homogenized to remove pieces of stones and extra particles. For soil characterization randomized soil samples (0–15 cm depth) were collected from each plot, air-dried and passed through 2 mm sieve and mixed thoroughly for measurement of various physico-chemical properties. Soil properties are mentioned in Table 1. Soil texture was determined by hydrometer method (Gee and Bauder, 1986). Saturated soil paste was measured by pH meter. Organic matter was determined by Walkley-black method (Jackson, 1962). Calcium carbonate ($CaCO_3$) estimated by acid dissolution (Allison and Moodie, 1965) method. Plant available Fe and Zn, extracted by 0.005 M DTPA (Lindsay and Norvell, 1978) and measured by atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA). Phosphorus extracted by Olsen method (Watanabe and Olsen, 1965), nitrogen by Bremner and Mulvaney (1982) and extractable potassium was measured as method described by Richards (1954).

Table 1. Some selective physico-chemical properties experimental soil and organic amendments.

Soil properties	**Value**	**Properties of organic amendments**	
Soil texture	Clay loam	Biochar	Value
Sand	39%	pH	6.84
Silt	29%	EC	1.96 dS m^{-1}
Clay	32%	Organic matter	59.1%
pH	7.71%	Total Fe	308 mg kg^{-1}
Organic matter	0.62%	Total Zn	281.8 mg kg^{-1}
Calcium carbonate	5.9%	Poultry manure	-------
DTPA-Fe	4.1 mg kg^{-1}	pH	6.91
DTPA-Zn	0.52 mg kg^{-1}	EC	5.1 dS m^{-1}
N	113 mg kg^{-1}	Organic matter	53.3%
P	7.6 mg kg^{-1}	Total Fe	291 mg kg^{-1}
K	101 mg kg^{-1}	Total Zn	501.3 mg kg^{-1}

***Soil acidification*:** Soil acidification was done under aerobic condition by using elemental sulfur (S). Elemental sulfur was manually crushed with a plastic spatula to obtain the homogeneous powder, sieved (<200 µm) and weighted. Sulfur was applied at the rate of 0.25% (w/w) as a single treatment and with combination of two organic amendments (BC and PM). Sulfur is a cost effective acidifying matter and its oxidation decreases soil pH (Iqbal *et al*., 2012). Each mole of S produces two moles of hydrogen ions (H^+) in the soil, reducing soil pH which ultimately dissolves nutrients from mineral and organic matter surfaces (Modaihsh *et al*., 1989; Kaplan and Orman, 1998).

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

After addition of S, soil was manually manipulated for complete oxidation of S. The soil samples were then suspended in deionized water at a soil:solution ratio of 1:2.5 to measure change in pH. Data was calculated on weekly basis after standardizing pH meter (JENCO pH meter, 671 P model). After and before S application, soil pH was calculated as described in Table 2.
***Nursery preparation and transplantation of rice*:** Seeds of rice (super basmati kernel) were obtained from Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan. Healthy, uniform and clean seeds were transferred into gunny

Table 2. Sulfur effect on soil pH as applied with iron (15 kg ha^{-1}), biochar (1% w/w) and poultry manure (1% w/w).

Treatments	Abbreviation	Initial soil pH	Iron solubilizing agent (Elemental Sulfur)		Soil pH before crop sowing
			Sulfur (%)	Corresponding H^+ ions (mmol kg^{-1} soil)	
Control	C	7.7±0.02	–	–	7.7±0.02
Biochar	BC	7.7±0.02	–	–	7.5±0.03
Poultry Manure	PM	7.7±0.02	–	–	7.6±0.02
Iron	Fe	7.7±0.02	–	–	7.7±0.02
Iron + Biochar	Fe + BC	7.7±0.02	–	–	7.5±0.03
Iron + Poultry Manure	Fe + PM	7.7±0.02	–	–	7.6±0.02
Sulfur	S	7.7±0.02	0.25	156.25	7.2±0.02
Sulfur + Biochar	S + BC	7.7±0.02	0.25	156.25	7.2±0.01
Sulfur + Poultry Manure	S + PM	7.7±0.02	0.25	156.25	7.2±0.02
Sulfur + Iron	S + Fe	7.7±0.02	0.25	156.25	7.2±0.01
Sulfur + Iron + Biochar	S + Fe + BC	7.7±0.02	0.25	156.25	7.1±0.02
Sulfur + Iron + Poultry Manure	S + Fe + PM	7.7±0.02	0.25	156.25	7.1±0.03

Sulfur applied with treatments in each plot having 4*4 m^2 area and its effect on soil pH after sulfur oxidation as observed in each sub-plots. Data of soil pH against each treatment is the mean value of three replications ± SE.

bags and soaked in the water for 24 hours. After soaking all seeds placed under shade and covered for 48 hours, sprouted seeds were grown in small plots. The experimental area was divided into three blocks with total of 36 plots and 25-days old uniform seedlings from nursery were transplanted into each plot maintaining row-to-row and plant-to-plant distance of 22.5 cm as treatment plan (Table 2). The 15 kg ha^{-1} Fe was applied by using $FeSO_4.7H_2O$ as Fe source. Two organic amendments i.e. BC and PM were applied at the rate of 1% (w/w). Biochar was made from Eucalyptus twigs at 400°C temperatures. Physico-chemical properties of biochar are described in table 1. Poultry manure was collected from the poultry farm of the University of Agriculture Faisalabad. Physico-chemical properties of poultry manure are described in Table 1. Recommended doses of nitrogen (urea), phosphorus (single super phosphate) and potassium (sulfate of potash) at the rate of 120, 90, 60 kg ha^{-1}, respectively, were applied. Full doses of phosphorus and potassium were applied at the time of sowing but nitrogen was applied in 3 splits (at the time of sowing, 30 and 45 days after planting). At booting stage photosynthetic parameter such as rate of photosynthesis (A), stomatal conductance (g_s), sub-stomatal conductance (C_i) and transpiration rate (E) were measured by CIRAS-3 (PP System, Amesbury, MN, USA). At maturity, when 90% of grain became golden yellow in color then rice crop was harvested. After shad drying, the samples were washed with distilled water and kept in forced-air-driven oven (Tokyo Rikakikai, Eyela WFO-600 ND, Tokyo, Japan) at 60°C until constant weight achieved. The paddy was threshed and dry biomass recorded.

***Grain analysis*:** Whole grain samples were ground in a mill (IKA Werke, MF 10 Basic, Staufen, Germany) and passed through a 0.5-mm sieve. One gram of grain sample was placed in muffle furnace at 550°C for complete oxidation of organic matter until the appearance of gray white ash (AOAC, 2003). Ground subsamples, one gram each, weight 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 atomic absorption spectrophotometer (PerkinElmer, AAnalyst 100, Waltham, USA).

Total protein concentration in grains was determined following 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_2SO_4 and then with dilute KOH solution (AOAC, 2003). Fat was determined by dry extraction method using Soxhlet apparatus (AOAC, 2003). 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, iodine test was performed by using glucose as standard and following the procedure describe by Sullivan (1935). The concentration of starch was noted through absorbance at 660 nm on spectrophotometer. For phytate determination, each 60 mg sample of finely ground grains was extracted with 10 mL of 0.2 N HCl at room temperature after 2 h continuous shaking. Phytate in the extract was determined by an indirect method (Haug and Lantzsch, 1983) and final concentration was measured on spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan). For total polyphenol determination in whole rice grain sample was prepared as method described by Gómez-Alonso *et al.* (2007). From 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 the calibration curve of gallic acid and expressed as gallic acid equivalent. For ferritin quantification, 5 g ground grain sample was prepared as seed method described by Lukac *et al.* (2009) with slight modification. Ferritin concentration was measured by developing direct ELISA (enzyme linked immunosorbent assay) using anti-body (Rabbit anti-ferritin) coated microtiter wells and mouse monoclonal anti-ferritin antibody as antibody enzyme (Horeseradish peroxidase) conjugate solution and absorbance was observed at 450 nm (Catalog Number: BC-1025, California). All devices used for chemical and biochemical analysis were soaked in diluted HNO_3 (pro analysis quality, Merck) and washed with deionized water.

***Statistical analysis*:** The field experiment was conducted in randomized complete block design. The data obtained regarding plant growth, physiology, chemical and biochemical parameters were subjective 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 ($P < 0.05$).

RESULTS

***Plant growth and yield*:** Application of Fe fertilizer with organic amendments (PM and BC) significantly improved plant growth and yield attributes as compared to control in S treated low pH soil (Table 3). In untreated. normal pH soil, separate application of Fe, BC and PM increased 1000 grain weight by 13.5, 11 and 7%, respectively, as compared to control. While in S treated soil, using Fe in combination with BC and PM improved 1000 grain weight by 34 and 30%, respectively, compared to control (Table 3). In S treated soil, application of Fe, BC and PM separately and in combination significantly improved straw yield of rice plants. It was observed that the application of Fe with BC increased up to 47 and 80% straw yield, respectively, as compared to control in untreated and S treated soil (Table 3). Use of Fe with PM showed significant increase of 70% in straw yield over control in S applied soil. Separate application of Fe improved 17% paddy yield as compared to control in S untreated soil (Table 3). Iron application combined with BC and PM increased paddy yield by 34 and 27%, respectively, compared to control in normal soil. Maximum increase in paddy yield was observed by Fe fertilization with BC (56%) followed by Fe + PM (46%) in S applied soil over control.

***Photosynthesis measurements*:** Lowering of soil pH using S together with provision of Fe ($FeSO_4.7H_2O$ @ 15 kg ha^{-1}) tended to improve all photosynthetic parameters of rice plant. Integration of organic amendments with Fe application further improved photosynthetic activity of rice plants in S treated soil (Table 3). Maximum increase in photosynthetic rate (93%) was observed by the application of Fe + BC followed by Fe + PM (81%) compared to control in S treated soil. Iron application along with PM and BC significantly improved transpiration rate (Table 3). Separate application of Fe gave 18 and 31% increase in transpiration rate in untreated and S treated soil, respectively, compared to control. Likewise, application of Fe with BC and PM increased transpiration rate by 39 and 34%, respectively, as compared to control in S treated soil. Application of Fe with PM significantly improved stomatal conductance by 45% while same treatment showed 59% increase in stomatal conductance in S treated soil over control. However, maximum increased in stomatal conductance up to 64% was obtained by BC + Fe + S as compared to control (Table 3). Data regarding substomatal conductance in Table 3 revealed a decrease up to 19%, 23% and 20% by Fe alone, Fe + BC and Fe + PM,

Table 3. Growth, yield and photosynthetic measurements of rice crop as influenced by combined application of Fe, BC and PM in normal and S treated soil.

Treatment	1000 GW (g)	SY	PY (ton ha^{-1})	A (µmol CO_2 m^{-2} s^{-1})	E (µmol H_2O $m^{-2}s^{-1}$)	gs (mmol H_2O m^{-2} s^{-1})	Ci (µmol mol^{-1})
C	20.0 e	8.4 f	4.1 e	12.1 h	3.8 f	315.0 e	245.0 a
BC	22.2 c-e	9.9 ef	4.6 c-e	14.7 e-h	4.1 d-f	351.0 e	231.7 a-c
PM	21.4 de	9.6 ef	4.5 de	14.1 f-h	3.9 ef	333.7 e	236.0 ab
Fe	22.7 c-e	10.7 de	4.8 c-e	17.3 c-e	4.5 cd	410.7 cd	198.0 b-e
Fe + BC	24.7 a-c	12.4 b-d	5.5 bc	19.1 b-d	4.6 bc	453.0 bc	188.0 de
Fe + PM	24.3 a-d	12.3 b-d	5.2 b-d	17.2 c-f	4.5 c	459.3 bc	195.0 c-e
S	21.6 de	10.3 d-f	4.5 de	13.9 gh	3.9 f	322.7 e	224.7 a-d
S + BC	23.9 b-d	11.6 c-e	4.8 c-e	16.1 d-g	4.3 c-e	369.3 de	214.0 a-e
S + PM	22.8 c-e	10.8 de	4.7 c-e	15.4 e-g	4 ef	353.7 e	212.7 a-e
S + Fe	24.9 a-c	13.4 a-c	5.9 ab	20.2 a-c	5 ab	493.7 ab	180.3 de
S + Fe + BC	26.9 a	15.2 a	6.4 a	23.4 a	5.3 a	518.3 a	186.7 e
S + Fe + PM	26.1 ab	14.3 ab	6 ab	21.9 ab	5.1 a	501.3 ab	186.3 de
$HSD_{0.05}$ `	2.9	2.2	0.9	3.2	0.4	55.2	39.4

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 experimental plots (4*4 m^2); BC: Biochar (1%), PM: Poultry manure (1%), Fe: Iron (15 kg ha^{-1}).

Table 4. Grain minerals and biological attributes as influenced by combined application of Fe, BC and PM in normal and S treated soil.

Treatment	Fe	Zn	Ash	Fat	Fiber	Starch
	(mg kg^{-1} DW)		(%)			
C	33.0 gh	19.3 de	0.43 h	0.76 f	0.34 f	48.5 e
BC	30.9 h	18.7 e	0.52 gh	0.87 d-f	0.39 d-f	52.9 de
PM	37.5 f-g	21.6 b-e	0.56 hg	0.9 c-f	0.42 d-f	53.6 de
Fe	53 d	18.5 e	0.66 c-f	0.96 b-e	0.47 c-e	55.6 c-e
Fe + BC	69.1 c	19.6 c-e	0.71 b-d	1 a-d	0.5 a-d	63.7 a-c
Fe + PM	64 c	22.9 a-c	0.69 c-e	1.1 ab	0.49 b-e	64.8 ab
S	41.6 e-g	20.7 b-e	0.48 gh	0.81 ef	0.37 ef	51.3 de
S + BC	45.6 d-f	19.3 de	0.6 d-g	0.94 b-e	0.44 d-f	53.1 de
S + PM	51.5 de	23.4 ab	0.58 e-g	0.91 c-f	0.43 d-f	54.9 c-e
S + Fe	86.8 b	20.9 b-e	0.73 bc	1.08 a-c	0.58 a-c	59 b-c
S + Fe + BC	99.6 a	22.6 a-d	0.87 a	1.2 a	0.62 a	68.4 a
S + Fe + PM	92.8 ab	25.6 a	0.83 ab	1.1 ab	0.6 ab	66.5 ab
$HSD_{0.05}$	10.6	3.4	0.13	0.17	0.13	9.1

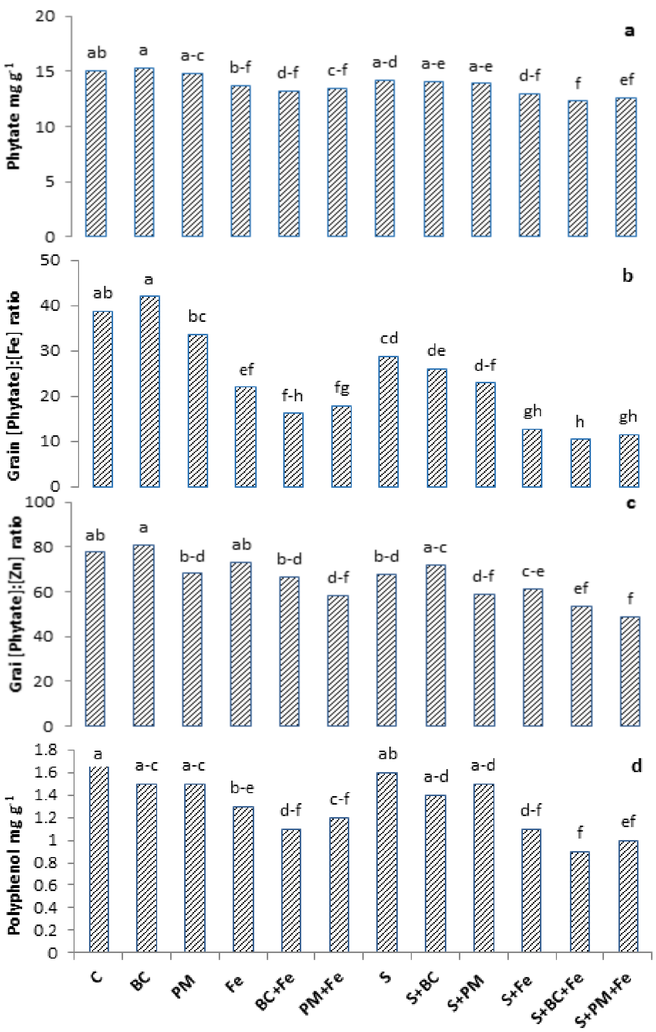
Quantities sharing similar letters are statistically similar to each other at $p \leq 0.05$; BC: Biochar (1%), PM: Poultry manure (1%), Fe: Iron (15 kg ha^{-1}).

respectively, over control in S applied soil. Highest decrease in sub-stomatal conductance up to 31% was recorded in treatment where Fe was applied with BC in S treated soil.

***Grain Fe and Zn concentration*:** Grain mineral concentration (Zn, Fe) of rice plant significantly improved by the combined application of Fe with BC and PM in S applied soil (Table 4). Separate application of Fe and PM increased grain Fe by 60 and 13%, respectively, over control in non S treated soil (Table 4). Grain Fe content was increased up to 1.8 fold compared to control when Fe fertilizer was applied with PM in S treated soil. Maximum increase in grain Fe concentration was observed by the combined use of Fe and BC in S applied soil and that was 2 fold higher over control. Regarding grain Zn concentration (Table 4), except treatment Fe + PM, S + PM, S + Fe + BC and S + Fe + PM, all other treatments showed statistically non-significant response compared to control. Maximum increase in grain Zn concentration up to 32% was obtained by using Fe + PM followed by Fe + BC in S treated soil as compared to control.

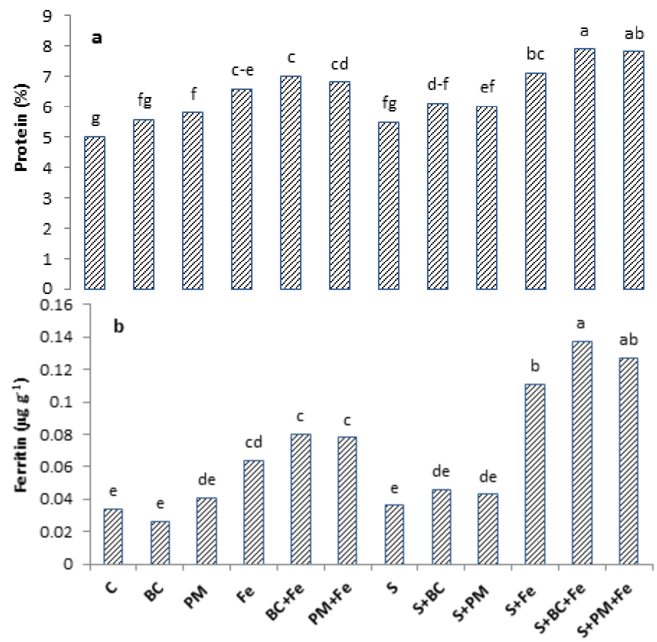
***Grain quality parameters (ash, fat, fiber and starch)*:** Quality of rice grain was markedly influenced by the application of Fe, BC and PM in S treated soil. Ash contents of rice grain was improved up to 53 and 60% when Fe was applied alone and with PM, respectively, in non S treated soil (Table 4) over control. Biochar improved ash contents by 65% when used with Fe, as compared to control, in untreated soil. By lowering soil pH with S, PM and Fe increased ash contents up to 93% over control. Highest increase in ash contents about 102% was observed over control when Fe was applied with BC in S treated soil. Iron applied with BC and poultry manure increased fat content up to 32 and 45%, respectively, as compared to control (Table 4). By lowering of soil pH with S, fat contents of rice gain were significantly varied by Fe + BC up to 58% followed by Fe + PM compared to control. Application of Fe with BC and PM significantly influenced fiber contents of rice grain in untreated and S treated soil (Table 4). In non S treated soil, use of Fe with BC and PM increased fiber contents by 47 and 44%, respectively, over control. However, in S treated low pH soil, fiber contents were improved by 71 and 82% by using Fe with PM and BC, respectively, as compared to control. In case of starch contents, separate application of BC, PM and Fe fertilization increased fat content up to 9, 10 and 14%, respectively, as compared to control (Table 4) in non S treated soil. Maximum increase in starch contents about 41% was observed in treatment where Fe was applied with BC in S treated soil over control.

***Phytate, phytate/Fe and phytate/Zn molar ratio, polyphenol*:** Application of Fe with organic amendments (BC and PM) in S treated soil significantly influenced anti-nutrient and bioavailable contents of Fe in rice grain [Fig. 1, 2]. Separate application of Fe reduced phytate contents by 9 and 14%, respectively, in untreated and S treated soil over control (Fig. 1a). Application of Fe with BC and PM reduced phytate contents by 18 and 16%, respectively, over control in S applied soil. Likewise, [phytate]:[Fe] ratio significantly influenced by the application of Fe with PM and BC in S treated soil (Fig. 1b). The [phytate]:[Fe] ratio was reduced by 67 and 43% when Fe was applied separately in normal and S treated low pH soil as compared to control. Application of Fe with BC and PM reduced [phytate]:[Fe] ratio by 58 and 54%, respectively, as compared to control in non S treated soil while in S treated soil, this reduction was up to 73 and 70%, respectively, over control. As far as [phytate]:[Zn] ratio is concerned, decrease by combined application of Fe with BC and PM by 14 and 25%, respectively, over control in non S treated soil was observed (Fig. 1c). Similarly, combined application of Fe with BC and PM reduced [phytate]:[Zn] ratio by 31 and 37%, respectively, compared to control in S applied soil. Concentration of total polyphenol in rice grain was also significantly influenced by soil pH, BC and PM (Fig. 1d). Data suggested that application of Fe with BC and PM significantly reduced polyphenol of rice grain by 35 and 29%, respectively, over control in non S treated soil. However, S treated soil significantly reduced polyphenol by 47 and 41%, respectively, when Fe was applied with BC and PM as compared to control.



[$HSD_{0.05}$= phytate (a) 1.5, Grain [phytate]:[Fe] ratio (b) 6.7, Grain [phytate]:[Zn] ratio (c) 11.4, polyphenol (c) 0.35

Figure 1. Phytate, polyphenol conc. (mg/g seed). [phyate]:[Fe] ratio and [phytate]:[Zn] ratio as determined in rice grain with various iron (Fe), Biochar (BC), poultry manure (PM) and sulfur (S) treatments under alkaline calcareous soil. Sulfur applied 4 weeks, before transplanting of seedlings, for complete oxidation of elemental sulfur.



($HSD_{0.05}$ Protein (a) 0.75, Ferritin (b) 0.025

Figure 2. Protein (%) and ferritin (µg/g) contents as determined in rice grain with various iron (Fe), Biochar (BC), poultry manure (PM) and sulfur (S) treatments under alkaline calcareous soil. Sulfur applied 4 weeks, before transplanting of seedlings, for complete oxidation of elemental sulfur.

***Ferritin and protein contents*:** Protein contents of rice grain were significantly influenced by Fe + BC and Fe + PM in normal and S applied soil (Fig. 2a). Application of Fe alone and with BC increased protein contents by 40 and 32%, respectively, in non S treated soil. Maximum increase up to 58 and 56%, respectively, in grain protein contents were obtained in treatment where Fe was used with BC and PM over control in S treated soil. Fe fertilization with BC and PM in S treated soil significantly increased ferritin contents (bioavailable Fe contents) (Fig. 2b). Application of Fe with BC increased ferritin contents by 1.35 folds over control in non S treated soil. Iron applied alone and with S increased ferritin content up to 88% and 2.3 fold, respectively, as compared to control. Highest increase of about 3 fold in ferritin content over control was observed when Fe was applied with BC in S treated soil.

DISCUSSION

The most recent challenge that we are facing is “hidden hunger” and it needs special attention. The root cause of hidden hunger is the provision of food 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 that "Biofortification is a win-win scenario".

Major issue regarding Fe is not its deficiency but its availability. As Fe is not deficient in mineral soil but high pH (Tazeh *et al.,* 2012), high carbonate and hydrogen carbonates (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 it bioavailability in human digestive tract (Welch, 2002) and cause Fe malnutrition. Thus, a sustainable approach is needed to increase its availability in soil for plant uptake and resultantly improved Fe nutrition in human body as well (Chojnacka *et al.,* 2011). Phytic acid is a potent barrier of Fe, Zn and Ca as it makes complexes with these mineral nutrients (Reinhold *et al.,* 1973). In the current study, we evaluated the effect of carbonaceous product (BC, PM) and Fe fertilizer effect on growth, yield, Fe biofortification and reduced anti-nutrients of rice in non-acidified and acidified calcareous soils.

At high pH Fe^{+2} ions (plants available form) converted into less soluble Fe^{+3} oxides and hydroxides and disappears from the soil solution (Cakmak, 2010a). Thus to cut this process soil pH manipulation might be a good approach, and it was our purposed hypothesis. Slow and steady soil acidification and nutrient mobilization by elemental sulfur is considered more practical and economical approach (Iqbal *et al.,* 2012) compared to breeding and genetic engineering. Results indicated that the application of S at the rate of 0.25% (w/w), brought the soil pH towards neutrality after four week incubation. Microbial oxidation of elemental sulfur produced enough sulfuric acid and lowered the soil pH; this might be one of the reason for this pH manipulation.

In the present study, all physiological parameters like photosynthetic rate, transpiration rate, stomatal and sub-stomatal conductance were significantly influenced by Fe applied with BC and PM in S treated soil. The reason might be Fe solubilization and mobilization by manipulation of soil pH. Lowering the soil pH, H^+ are produced to displace Fe adsorbed on BC surface (Iqbal *et al.,* 2012). Fe is a key component of porphyrin ring of chlorophyll. As soon as Fe solubilization increases, the amount of available Fe for plant uptake increases which might have ultimately improved photosynthetic activity of plant. Another reason may be the proliferation of *Thiobacillus* in the presence of biochar. As it is a well-known fact that biochar supports microbial community by providing nutrients, carbon source and shelter (Wardle *et al.,* 2008). *Thiobacillus* produce sulfuric acid that lowers down the soil pH (Ullah *et al.,* 2013).

Poultry manure is known to have plant nutrients who can play an important role in crop productivity (Slaton *et al.,* 2013). Watts *et al.* (2010) studied that poultry manure can be considered as soil conditioner that improves soil organic matter contents. Thus, release of nutrients upon mineralization of PM, supply Fe for plant uptake which improves physiology of rice plant.

In the same way, transpiration rate and stomatal conductance of rice plant was significantly improved due to the lowering of soil pH and application of Fe fertilizer with BC and PM. Stomatal conductance and internal CO_2 concentration also greatly influenced by positive plant microbe interaction (Yandigeri *et al.,* 2012) with BC application in acidified soil. Application of Fe with BC and PM significantly improved paddy yield by 56 and 46%, respectively, in acidified soil. It has been reported that biochar application may improve fertilizer use efficiency (van Zwieten *et al.,* 2010). Lowering of soil pH, releases most of the essential nutrients from BC surface along with Fe that are needed for plant growth. Biochar is known to improve soil physical properties that ultimately improve plant health and yield. Our results are in line with Rajkovich *et al.* (2012), Zhang *et al.* (2012) and Akhtar *et al.* (2015) where application of biochar increased yield of corn, maize and wheat, respectively. Similarly, PM is a source of nutrients and its application significantly improved plant growth and yield (Kaiser *et al.,* 2010). Many essential nutrients like N, and P are provided by PM that could increase plant growth and yield. Also it could help in improving water contents needed for plants (Watts *et al.,* 2010). Our results are in accordance to the Woli *et al.* (2015) and Ruiz Diaz *et al.* (2011) where grain yield was improved by the application of PM.

Metal analysis (Fe, Zn) of effects of BC on agronomic crops (i.e. wheat, rice, maize etc.) have been performed and positive impacts are concluded (Hammond *et al.,* 2013; Graber *et al.,* 2014). It was observed that grain Fe contents were significantly improved in treatment having BC and Fe both in S treated soil. The H^+ ion release lowered the soil pH due to S application and solubilized the metal ion i.e. Fe (Wang *et al.,* 2006). Also, effectiveness of inorganic fertilizer may have been improved when used with BC (Schulz and Glaser, 2012; Alburquerque *et al.,* 2013). This might have increase the mineral content of grains as observed in present study. Very recently, Hoefer *et al.* (2015) reported increased nutrient solubilization by the application of sulfur.

On the other hand, application of Fe fertilizer along with BC lowered the Zn contents of rice grain. Biochar might have increased the mineral contents of rhizosphere and in turn enhanced the phyto-availability of Fe and Zn. However, due to the competition of similar ion for plant uptake Zn contents are lower than Fe but greater than control. Similar results were reported by Beesley and Marmiroli (2011) where application of BC reduced the Zn contents due to its high sorption capacity. Application of Fe with PM increased the Fe contents of rice grain. Poultry manure is known to have variety of nutrients because many mineral elements are present in the feed of animals thus application of manure can enrich soil with certain elements i.e. Ca, Mg, Zn etc. (Kingery *et al.,* 1994; Uprety *et al.,* 2009). This could be the reason for high

Fe concentration of rice grain. Our results are inconsistent with previous reports of Demir *et al.* (2010) where fruit Zn contents were significantly improved by the application of PM.

The ultimate objective of Fe biofortification is whether to increase the bioavailable portion of Fe or decrease the anti-nutrients i.e. phytate or polyphenolics. In current study, we used BC and PM to enrich rice grain with Fe in pH manipulated soil. Several mechanisms might be operating i.e. solubilization of Fe by lowering of rhizosphere soil pH to promote translocation of Fe into plants, also desorption of Fe from the BC surface by organic acids produce by plants roots. Biochar provides microhabitat to microbes that produce Fe chelating compounds that further regulate its mobility to grain. Thus total available portion of Fe increased and phytate contents decreased. Some genotypic characters may be involved or soil native condition i.e. organic matter contents may increase Fe translocation into seed (Chandel *et al.,* 2010). Findings of Reddy *et al.* (2000) support our results where phytate contents decreased as ferritin concentration increased in grains. Previously, biochar has been used for the Zn biofortification of crops by Gartler *et al.* (2013). After phytate, polyphenolic compounds are also potential inhibitors of Fe absorption in human body (Scholz-Ahrens and Schrezenmeir, 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 pH manipulated soil. Studies also supported our findings as investigated by Tako and Glahn (2011), who reported more bioavailable Fe in white bean as compared to red been because of low polyphenol 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.

As we observed that application of Fe along with BC and PM increased bioavailable Fe and decreased phytate and polyphenol compounds. Mineral bioavailability is quite complex and least explored area although a simplified molar ratio of [Phytate]:[Fe] or [Phytate]:[Zn] can be used to access bioavailability of Fe or Zn in food (Hussain *et al.,* 2012). In current study, [Phytate]:[Fe] ratio was decreased significantly by applying Fe fertilizer along with BC and PM. Reason could be the supply of Fe in the form of fertilizer, Fe released from BC surface by lowering of soil pH and also from the mineralization of PM. Oxidation of elemental S produces enough sulfuric acid that solubilizes and mobilizes Fe. [Phytate]:[Zn] ratio may be dropped as we increased Fe supply. The [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.* (2012) where Zn supply dropped the [Phytate]:[Zn] ratio significantly.

Modern agriculture emphasizes not only on the quantity of produce but also the quality of produce at top priorities. As known, protein and ash contents are usually introduced to evaluate the nutritional value of cereal food, such as wheat, rice and legume (Graham, 1999). Although rice is poor in protein contents i.e. 8% in brown and 7% in milled rice as compared to other cereals like wheat, corn etc. (Anjum, 2007). Application of Fe with BC and PM in pH manipulated soil significantly improved all quality parameters like ash, fat, protein, starch and ferritin up to 102, 58, 58, 41% and 3 fold, respectively. Our results are supported by the findings of Fang *et al.* (2008) and Yadav *et al.* (2013), they found that optimum quantity of Fe improved nutritional quality of grains.

Conclusions**:** The research findings suggest that Fe bioavailability and nutritional quality of rice can be enhanced by combined use of Fe with organic amendments by acidification of calcareous soil. For slow and steady soil acidification with elemental S is considered a best and cheap source. As clear from the findings that 15 kg Fe ($FeSO_4.7H_2O$) ha^{-1} applied with 1% (w/w) BC increased grain Fe up to 2 fold, protein up to 58%, ferritin up to 3 fold in S treated calcareous soil as compared to control. Maximum decrease in anti-nutrients in rice grain was observed in treatments where Fe was applied with BC in S treated soil. For Fe biofortification, application of Fe could be more effective when applied with BC in S treated soil as compared to PM or where Fe was applied alone.

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