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VFRC Report 2016/1

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VFRC Report 2016/1

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Citation

Pandey R., Sharma S., Barman M. and Bindraban P.S. 2016. *Nutrient delivery system in crop plants to augment acquisition, translocation and utilization efficiency.* VFRC Report 2016/1. Virtual Fertilizer Research Center, Washington, D.C. 93 pp.; 13 tables; 63 figs.; 30 ref.

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Contents

Acronyms and abbreviations

2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
A	anthesis
APOX	ascorbate peroxidase
bHLH	basic helix-loop-helix
CAS	chrome azurol sulphonate
CAT	catalase
cDNA	complementary DNA
CHAPS	3-[(3-**ch**olamidopropyl) dimethyl**a**mmonio]-1-**p**ropane**s**ulfonate
CPC	clay polymer composite
Cu	copper
DAP	diammonium phosphate
DAS	days after sowing
dNTP	deoxynucleotide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDDHA	ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
EDTA	ethylene diamine trichloroacetic acid
Fe	iron
FRO	Ferric Reductase Oxidase
GF	grain filling
GR	glutathione reductase
H_2O_2	hydrogen peroxide
HA	humic acid
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAR-IARI	Indian Council of Agricultural Research – Indian Agricultural Research Institute
IDT	Integrated DNA Technologies
IEF	isoelectric focusing
IRT	Iron Regulated Transporter
K	potassium
KOH	potassium hydroxide
Mn	manganese
MPP	monopotassium phosphate
N	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NRAMP	Natural Resistance Associated Macrophage Protein
O_{2^-}	superoxide radical
OA	oxalic acid
P	phosphorus
PCR	polymerase chain reaction
PF	pod filling

PHT	phosphate transporter
POX	peroxidase
PSB	phosphorus-solubilizing bacteria
RGAP	Rice Genome Annotation Project
RNA	ribonucleic acid
ROS	reactive oxygen species
RRM	RNA recognition motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
T	tillering
TAE	tris acetate-EDTA
TBARS	thiobarbituric acid-like reactive substances
TEMED	tetramethylethylenediamine
VFRC	Virtual Fertilizer Research Center
WEHS	water-extractable humic substances
XRD	X-ray diffractometer
YSL	Yellow Stripe-Like
Zn	zinc

1 Introduction

There are two systems to deliver nutrients into plants: from the root primarily and also through foliage. Foliage serves as an efficient organ to absorb nutrient elements, which enter the cells and participate in various metabolic processes. From a literature survey, we found that if the nutrient molecule is made charge-neutral, there is a possibility that it will pass through the plasma membrane unhindered (Pandey et al., 2013). Synthetic chelates, such as Fe-EDTA or Fe-EDDHA, are cheaper and more effective but their excess use has negative environmental impacts (Adesemoye et al., 2009). For iron absorption through foliage, there are prospects to chelate Fe^{3+} with artificially synthesized siderophores that are of either plant or bacterial origin. Since the leaf cells possess a Fe^{3+}-chelate reductase enzyme system to reduce the Fe^{3+}, foliar delivery of chelated Fe^{3+} is possible. One study revealed that water-extractable humic substances (WEHS) complexed with Fe improved Fe uptake when applied to roots of Fe-deficient tomato plants. When these Fe complexes were applied to the leaf discs, higher transcript levels of *LeFRO1, LeIRT1* and *Ferritin2* genes were observed (Tomasi et al., 2009). Another study proved that siderophores from bacterial strain *Chryseobacterium* C138 can effectively supply Fe to iron-starved tomato plants via roots (Radzki et al., 2013). However, both reports discussed the effect of root-applied Fe complexed with organic siderophores. Efficacy of Fe-siderophore complex as a foliar spray needs detailed investigation. Further, the passage of charge-neutral molecules through the leaf plasma membrane, which might occur via channel proteins called “porins,” needs to be confirmed.

The concentration of Pi (inorganic phosphate) in soil solution is usually 1,000 times less than that of the root cells due to P fixation. Developing a controlled-release technology to reduce P fixation and loss through runoff will therefore be helpful in enhancing P use efficiency in crops. Controlled-release technology using nano-clay polymer composites has been used to address these problems (Guo et al., 2005; Jamnongkan and Kaewpirom, 2010). The interaction of clay with surface cross-linking polymers increases the barrier property of clay composites and leads to the slow-release property of fertilizers (Basak et al., 2012). There are several reports on the release behavior of P from nano-composites, but studies to evaluate the response of crops to application of P-loaded nano-composites are almost nonexistent. Very few reports are available on positive yield response of crops toward application of polymer hydrogels (Abd El-Rehim, 2006; Talaat et al., 2008). However, the actual mechanism of nutrient mobilization to crops from these polymers is not known. This indicates that nano-clay composites can release nutrients in a controlled way, but response of crops toward application of these composites should be evaluated. The nutrient-loaded clay polymer composite has been used as fertilizer. Developing seed coating material with these composites and loading them with P would supply nutrient to the plant at the initial stages of growth. Since P is required for root growth and better seedling establishment, coating seeds with P-enriched polymers will be beneficial.

The experiments were carried out to study the nutrient delivery systems through foliage and roots (seed coating). The objectives were to *(i) study the efficacy of Fe complexed with various organic and inorganic substances and applied as a foliar spray, (ii) identify novel proteins expressed in the leaf and involved in*

absorption of Fe and its translocation, (iii) develop a seed coating material loaded with phosphorus, organic acid and phosphorus-solubilizing bacteria and study the phosphorus release behavior of the seed coating material, (iv) evaluate the efficacy of seed coating material in improving P uptake efficiency of crops, and (v) combine the best seed coating material and foliar formulation in enhancing nutrient uptake efficiency of crops under field conditions.

2 Foliar application of Fe and P with various inorganic substances on rice and soybean (Objective 1)

2.1 Foliar experiments on rice and soybean conducted in a glasshouse

Preliminary experiments were conducted on rice and soybean crops with different combinations of Fe and P. From the available literature (Ali et al., 2009; Kaya et al., 2005; Maibodi et al., 2015; Mosali et al., 2006; Mahmood et al., 2011; Pandey et al., 2013; Victoria et al., 2004; Yanyan et al., 2012), the various foliar treatments were determined at two concentrations each. Table 1 represents the details of each foliar treatment, including water spray (with surfactant) as the "control," used on the crops. The spray formulations were prepared by dissolving the required amount of chemical and adding 100 µL of surfactant (Triton X100) in 1 L of solution. The pH of each spray formulation was maintained at 6.0 using hydrochloric acid (HCl) or potassium hydroxide (KOH).

Table 1. *Details of foliar treatments, including water (with surfactant) as "control," used on rice and soybean crops grown in a glasshouse.*

S. No.	Treatments				
	Compound	Conc. (mM)	% of Compound in Solvent	% of Fe/P/K in Compound	g 500 mL^{-1}
1	Deionized water (Control)	-	-	-	-
2	Fe-Citrate	2	0.049	22.85 (Fe)	0.245
3	Fe-Citrate	4	0.098	-	0.490
4	Fe-EDTA	2	0.073	15.25 (Fe)	0.367
5	Fe-EDTA	4	0.147	-	0.734
6	$FePO_4.2H_2O$	2	0.037	29.94 (Fe) & 16.57 (P)	0.187
7	$FePO_4.2H_2O$	4	0.075	-	0.374
8	Monopotassium phosphate	2	0.027	22.79 (P) & 28.65 (K)	0.135
9	Monopotassium phosphate	4	0.054	-	0.270
10	Humic acid + $FeCl_3$	0.025 g L^{-1} + 2	0.0025 + 0.032	34.49 (Fe)	0.0125 + 0.162
11	Humic acid + $FeCl_3$	0.050 g L^{-1} + 2	0.005 + 0.032	-	0.025 + 0.162
12	Humic acid	0.025 g L^{-1}	0.0025	-	0.0125 g
13	Humic acid	0.050 g L^{-1}	0.005	-	0.025 g

2.1.1 Materials and methods

2.1.1.1 Plant growth conditions

Seeds of rice (*Oryza sativa* var. MAS-946-1) and soybean (*Glycine max* var. DS-2614) were sown in pots containing field soil. The soil properties are presented in Table 2. For growing rice plants, the larger pots

with 30 cm diameter and a capacity of 15 kg soil were used, and for soybean, smaller pots with 15 cm diameter and a capacity of 5 kg soil were used. Two plants per pot were maintained for rice and one for soybean. The plants were raised in a glasshouse with temperature maintained at 30°C during the day and 26°C during the night with 80-90% relative humidity and natural light duration.

Initially, four seeds of rice were sown per pot, and later, only two healthy plants were maintained. Similarly, two seeds of soybean were sown, and later, only one plant was maintained in each pot. Soybean seeds were soaked overnight in deionized water and treated with *Bradyrhizobium japonicum* culture before sowing. The recommended dose of fertilizer for the rice and soybean crops was added to the soil as per package of practice. For rice, the recommended dose was 120 kg N, 60 kg P_2O_5 and 40 kg K_2O per hectare, whereas for soybean, it was 60 kg P_2O_5 and 40 kg K_2O per hectare. Nitrogen was not applied to soybean since it is a legume crop. The amounts of urea, single superphosphate and muriate of potash were calculated according to the soil volume for each pot size. Urea was applied in three splits in rice (50% as a basal dose, 25% at tillering and 25% at anthesis). Plants were irrigated daily with normal tap water.

Table 2. *Properties of the soil used to grow rice and soybean plants in a glasshouse. Soil sampling was done before sowing of crops.*

S. No.	Soil Properties	Values	Remarks
1	pH	8.44	
2	EC (dS m^{-1})	0.29	
3	*Mechanical Composition*		
	i. Clay (%) ii. Silt (%) iii. Sand (%)	12.3 22.5 63.2	
4	Texture	-	Sandy Loam
5	Organic Carbon (%)	0.57	Medium
6	Available N (kg ha^{-1})	227	Low
7	Available P (kg ha^{-1})	26.8	High
8	Available K (kg ha^{-1})	371	High
9	Available Zn (mg kg^{-1} soil)	1.52	Medium
10	Available Cu (mg kg^{-1} soil)	2.29	High
11	Available Fe (mg kg^{-1} soil)	4.8	Medium
12	Available Mn (mg kg^{-1} soil)	9.60	High

For rice, two sprays were carried out, the first at maximum tillering and the second at anthesis stage, while in soybean, the first spray was done at 50% anthesis and the second at pod filling, i.e., 15 days after anthesis. The surfaces of the leaves were sprayed on both sides by completely drenching with spray fluid. All foliar treatments were completed between 8:00 a.m. and 10:00 a.m. The various parameters of the plants were recorded on the sixth day after the foliar treatment. The stage of sampling after foliar treatment was derived from the available literature, since by this time the applied nutrients were completely absorbed and metabolized by the plant and reflected in terms of growth or biomass

accumulation. Observations were recorded on biomass accumulation, total leaf area, rate of photosynthesis, total chlorophyll and tissue nutrient analysis. At maturity, the yield traits were also recorded, including number of panicles/pods per plant, grains per panicle/pod, test (500-seed) weight and grain yield.

Chlorophyll was measured by the non-maceration method using dimethyl sulfoxide and reading the absorbance with a spectrophotometer. Leaf area was estimated by leaf area meter (model LICOR-3000), while photosynthesis was measured by portable photosynthesis system (infrared gas analyzer, LICOR-6400). Tissue phosphorus concentration was estimated by the method of Murphy and Riley (1962) after wet digestion with a diacid (HNO_3:$HClO_4$) mixture, and Fe was estimated in the same extract using an atomic absorption spectrophotometer (ECIL, India). Biomass was recorded for each organ (root, stem and leaf) by drying the samples in a hot-air oven at 65°C until a constant weight was obtained and expressed as g plant^{-1}.

2.1.1.2 Statistical analysis

The experiment was laid out in a completely randomized design with three replicates for each treatment. From the replicate values, standard error of mean was calculated. The data were analyzed by one-way analysis of variance, and the significant differences between treatment effects were calculated by critical difference at $P < 0.05$.

2.1.2 Results of foliar application of Fe and P on rice in a glasshouse experiment

The effects of various foliar treatments on root, shoot and total plant biomass accumulation in rice plants at two growth stages are depicted in Fig. 1A-D. Significant difference was observed between the foliar treatments for biomass accumulation. The shoot dry weight was highest in Fe-citrate (2 mM) followed by Fe-EDTA (4 mM) during tillering stage, while the opposite trend occurred with treatment at anthesis stage (Fig. 1A). Root dry weight was also highest with Fe-citrate (2 mM) at both stages as compared to other treatments (Fig. 1B). However, the effects of other treatments, such as Fe-citrate (4 mM), Fe-EDTA (2 mM) and monopotassium phosphate (MPP; 4 mM), on root dry weight were almost similar. The total biomass accumulation per plant was also higher with the Fe-citrate (2 mM) treatment followed by Fe-EDTA (4 mM) at both stages (Fig. 1C). At harvest, the effects of foliar treatments varied significantly in terms of total plant biomass accumulation, including grain weight (Fig. 1D). The percentage increase in total plant biomass was 135% and 30% with Fe-citrate (2 mM) and 82% and 16% with Fe-EDTA (4 mM) at tillering and anthesis stages, respectively, as compared to control (Table 3). Fe-citrate (2 mM) resulted in significantly higher biomass, while the lowest biomass was recorded with the humic acid (HA)-25 mg + Fe-2 mM treatment, as compared to control (water spray). The percentage increase in biomass accumulation with foliar treatments as compared to control was 29.9% and 6.8% with Fe-citrate (2 mM) and Fe-EDTA (4 mM), respectively (Table 3).

The increases in leaf number per plant and leaf area were significantly influenced by treatment. Foliar application during tillering stage resulted in maximum leaf number with Fe-citrate (>60%) treatments followed by Fe-EDTA (4 mM; 53%) and MPP (2 mM; 26%) as compared to control (Fig. 2A). However, all other treatments resulted in a reduction of leaf number. At anthesis stage, Fe-EDTA (4 mM) and HA-25 mg + Fe-2 mM resulted in higher leaf number as compared to control. This increase may have been because new leaf emerged after tillering stage, which could be due to treatment effect. Increase in total leaf area was found only in Fe-citrate (2 mM) at tillering stage, while foliar treatment at anthesis stage resulted in a 15% and 17% increase with Fe-EDTA (4 mM) and Fe-citrate (2 mM), respectively, as compared to control (Fig. 2B). Other foliar treatments showed significant reduction in leaf area as compared to control (Table 4).

Leaf chlorophyll concentration was significantly influenced by foliar treatment. At tillering stage, all foliar treatments except Fe-EDTA (4 mM) resulted in a reduction of chlorophyll concentration, whereas treatment during anthesis led to significantly higher chlorophyll as compared to control (Fig. 2C). Although maximum chlorophyll concentration was recorded in MPP (4 mM), Fe-citrate (2 mM) also resulted in a 66% higher concentration as compared to control (Table 4). However, Fe-EDTA (4 mM) showed a 40% reduction in chlorophyll as compared to the control treatment. The increase in chlorophyll concentration at anthesis stage could be the cumulative effect of the sprays, but two treatments, HA-25 + Fe-2 mM and HA-50 + Fe-2 mM, consistently resulted in its reduction. This indicates that humic acid complexed with Fe is not favorable. Similarly, the rate of photosynthesis was also reduced significantly after foliar application during tillering stage, but it increased from 26% in HA (50 mg) to 78% with the Fe-P (2 mM) treatment at anthesis stage (Fig. 2D; Table 4). The increase in the photosynthetic rate at anthesis was due to higher chlorophyll accumulation at this stage due to foliar treatment.

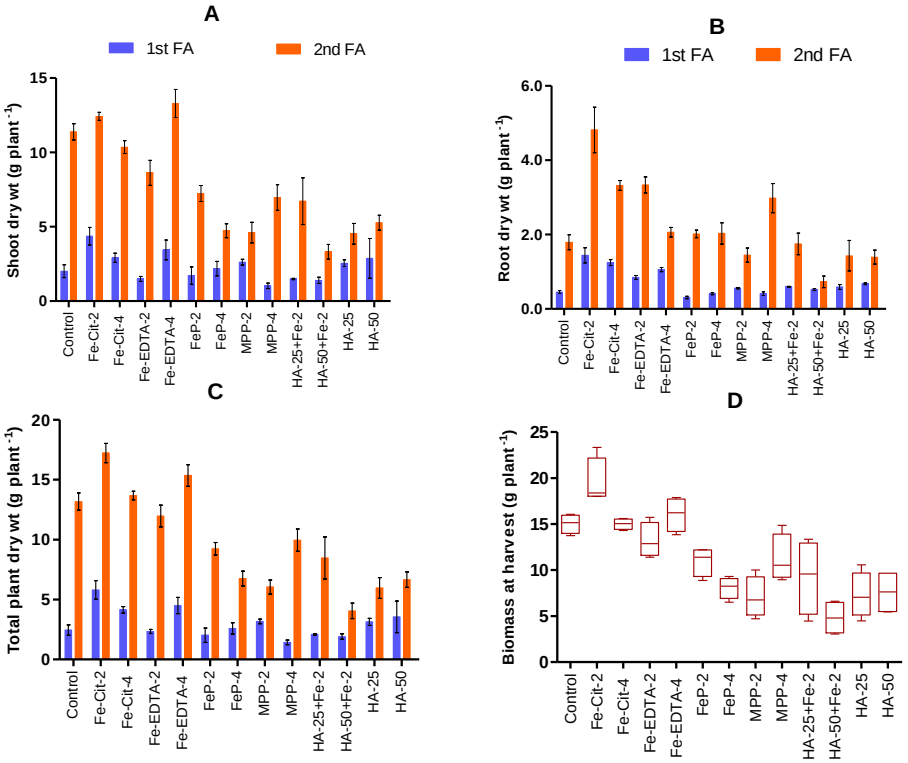

Figure 1. *Effect of different foliar treatments on (A) shoot, (B) root and (C) total biomass and (D) total biomass accumulation at harvest (including panicle weight) in rice plants grown in a glasshouse. Foliar application was performed at two stages: maximum tillering and anthesis.*

The P content in different organs of rice plants was influenced by foliar treatment (Fig. 3A). In roots, the maximum P content was recorded with Fe-citrate (both concentrations) and Fe-EDTA (2 mM), which was almost 1.5- to 2-fold, while HA treatments resulted in a reduction of root P content, compared to control. Similarly, the shoot P content was significantly higher with Fe-EDTA (4 mM) as compared to control, but the grain P content was similar, indicating that P was not sufficiently mobilized from shoot and root toward grain during the grain-filling period. All other treatments except for Fe-citrate 4 mM and HA-50 resulted in a higher grain P content as compared to control. However, high grain P is not a desirable trait as P is stored as phytate in grain, which is considered an anti-nutritional factor. Phytate chelates all other divalent cations (Fe^{2+}, Ca^{2+}, Zn^{2+}) and makes them unavailable for absorption by the human/animal body. The best treatment in terms of P uptake, therefore, would be Fe-citrate (2 mM), which resulted in balanced P distribution in all organs.

Table 3. *Percentage change in biomass accumulation in rice plants due to different foliar treatments as compared to control (water spray)*

S. No.	Treatment	Shoot Dry Weight		Root Dry Weight		Total Dry Weight		Biomass at Harvest
		1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application	
1	Control	-	-	-	-	-	-	-
2	Fe-Cit-2	117.4	9.1	215.8	168.6	135.6	30.8	29.9
3	Fe-Cit-4	44.8	-9.0	172.7	85.4	68.6	3.8	-0.1
4	Fe-EDTA-2	-25.7	-24.2	85.8	86.2	-5.0	-9.2	-12.0
5	Fe-EDTA-4	71.2	16.7	131.7	15.2	82.5	16.5	6.8
6	FeP-2	-14.7	-36.5	-32.2	12.7	-18.0	-29.9	-27.0
7	FeP-4	8.6	-58.5	-9.8	13.4	5.2	-48.7	-46.2
8	MPP-2	30.6	-59.6	22.4	-19.0	29.0	-54.1	-53.0
9	MPP-4	-49.2	-38.8	-9.8	66.2	-41.9	-24.5	-25.3
10	HA-25+Fe-2	-25.8	-41.0	30.6	-2.2	-15.2	-35.7	-38.4
11	HA-50+Fe-2	-30.6	-70.8	14.2	-59.3	-22.3	-69.2	-67.9
12	HA-25	26.7	-60.2	29.5	-19.9	27.2	-54.7	-51.5
13	HA-50	43.5	-53.8	48.6	-22.2	44.4	-49.5	-49.4

In general, the Fe content was significantly highest in roots in all treatments for rice plants (Fig. 3B). However, Fe-EDTA (4 mM) application showed a similar amount of Fe in both root and shoot as compared to other treatments. The accumulation of Fe was the highest with the Fe-citrate 4 mM treatment. There was a more than 2-fold accumulation of Fe in grain due to foliar treatment, except with the humic acid treatments (HA-25 and HA-50), as compared to control.

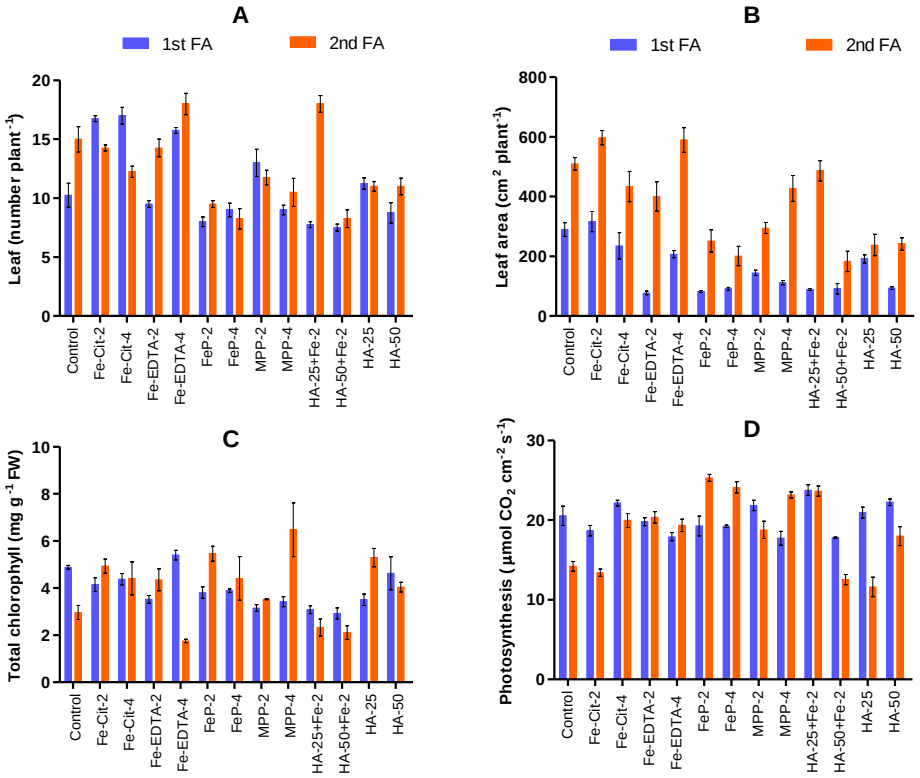

Figure 2. *Effect of different foliar treatments on (A) total number of leaves, (B) leaf area, (C) chlorophyll concentration and (D) rate of photosynthesis in rice plants grown in a glasshouse.*

Table 4. *Percentage change in leaf area, chlorophyll and rate of photosynthesis in rice plants due to different foliar treatments as compared to control (water spray).*

S. No.	Treatment	Total Leaf Area		Total Chlorophyll		Photosynthesis	
		1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application
1	Control	-	-	-	-	-	-
2	Fe-Cit-2	9.2	17.2	-15.1	66.5	-9.1	-5.7
3	Fe-Cit-4	-18.9	-14.9	-10.4	48.8	7.8	40.4
4	Fe-EDTA-2	-73.3	-21.5	-27.9	46.9	-3.6	43.2
5	Fe-EDTA-4	-28.4	15.7	10.5	-40.8	-12.8	36.1
6	FeP-2	-71.5	-50.7	-22.0	84.2	-6.2	78.0
7	FeP-4	-68.6	-60.6	-20.3	48.8	-6.4	69.7
8	MPP-2	-50.1	-42.2	-35.5	18.9	6.2	32.1
9	MPP-4	-61.4	-16.2	-30.0	118.5	-13.8	63.0
10	HA-25+Fe-2	-69.3	-4.6	-37.0	-21.3	15.6	66.4
11	HA-50+Fe-2	-68.4	-64.1	-40.1	-28.7	-13.5	-11.9
12	HA-25	-33.9	-53.3	-28.3	78.6	1.9	-18.3
13	HA-50	-67.7	-52.6	-5.4	36.3	8.2	26.6

The yield-attributing traits in rice, such as number of panicles per plant, panicle weight, percentage of filled spikelets per panicle and test weight, were significantly influenced by foliar treatment (Table 5). Only two foliar treatments resulted in higher panicle numbers as compared to control; those were Fe-citrate (2 mM) and MPP (4 mM). Weights of individual panicles from the main culm per plant were recorded and were significantly higher with most of the foliar treatments. The maximum panicle weight was found with the HA-25+Fe-2 mM treatment, followed by Fe-EDTA (2 mM). However, the percentage of filled spikelets was more than 90% with Fe-citrate (2 mM), Fe-EDTA (both concentrations) and MPP (2 mM) application as compared to control. However, the HA-25+Fe-2 mM treatment resulted in a decrease of filled spikelets per panicle compared to control. The test weight also increased due to MPP (2 mM). Although the number of panicles per plant and panicle weight were lower with this treatment, a combination of potassium and phosphorus is seen to help in proper grain filling and, thus, increase the weight of the individual spikelets, which determines grain yield.

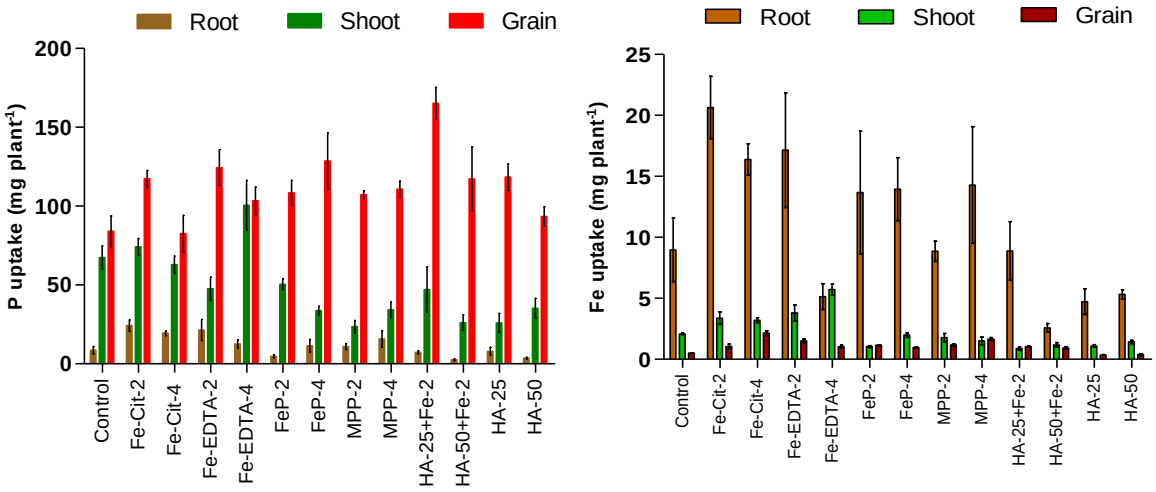

Figure 3. *Effect of different foliar treatments on (A) phosphorus uptake and (B) iron uptake in shoot, root and grain in rice plants grown in a glasshouse.*

2.1.3 Results of foliar application of Fe and P on soybean crop in a glasshouse experiment

The effect of foliar treatment on soybean showed significant variation in biomass accumulation at two stages: anthesis and pod filling (15 days after anthesis). Shoot, root and total plant biomass accumulation were higher after the second foliar treatment (Fig. 4A-C). At anthesis stage, the increase in shoot dry weight was the highest with Fe-P (2 mM; 42%), followed by MPP (2 mM; 39%) as compared to control (Table 5). However, there was a reduction in root dry weight with all foliar treatments except HA (50 mg) as compared to control (Fig. 4B). Total plant dry weight also increased significantly with Fe-P (2 mM) and MPP (2 mM) by 32% and 24.5%, respectively, as compared to control (Fig. 4C; Table 5). The root-to-shoot ratio was higher with humic acid treatments, whereas almost all other treatments resulted in a reduction as compared to control (Fig. 4D). A higher root-to-shoot ratio is desirable in plants only when

there is a stress (water, nutrient, etc.); otherwise, plants invest more fixed carbohydrates in developing roots, leading to less partitioning toward shoot or grain growth. It was interesting to note that HA (50 mg) performed consistently well in terms of shoot, root and total plant biomass and root-to-shoot ratio as compared to control (Table 6). HA-50+Fe-2 mM also resulted in consistently increased biomass except for root dry weight at grain filling in comparison to control.

Table 5. *Effect of different foliar treatments on yield traits in rice plants grown in a glasshouse. The value in parentheses is the percentage change over the control treatment.*

S. No.	Treatments	No. of Panicles Plant^{-1}	Panicle Weight (g plant^{-1})	% Filled Spikelets Panicle^{-1}	500-Seed Weight (g)
1	Control	8.2	9.68	87.4	9.5
2	Fe-Cit-2	10.3 (26.5)	12.60 (30.1)	93.1 (6.5)	9.9 (4.6)
3	Fe-Cit-4	9.2 (12.2)	10.22 (5.6)	87.0 (-0.5)	10.3 (8.7)
4	Fe-EDTA-2	9.2 (12.2)	13.64 (40.9)	94.4 (8.0)	10.7 (12.5)
5	Fe-EDTA-4	8.5 (4.1)	10.65 (10.0)	93.6 (7.0)	9.6 (1.5)
6	FeP-2	8.8 (8.2)	11.44 (18.1)	84.4 (-3.5)	10.1 (6.7)
7	FeP-4	8.8 (8.2)	11.75 (21.3)	89.0 (1.7)	10.3 (8.7)
8	MPP-2	6.3 (-22.4)	9.84 (1.6)	93.1 (6.5)	11.6 (22.1)
9	MPP-4	10.7 (30.6)	12.43 (28.3)	83.7 (-4.3)	10.8 (13.8)
10	HA-25+Fe-2	8.2 (0)	17.74 (83.2)	77.4 (-11.5)	10.6 (11.5)
11	HA-50+Fe-2	7.3 (-10.2)	11.85 (22.3)	86.2 (-1.4)	10.4 (9.7)
12	HA-25	9.3 (14.3)	11.51 (18.9)	87.4 (0.0)	9.8 (3.5)
13	HA-50	8.2 (0)	9.30 (-3.9)	85.3 (-2.4)	10.9 (15.1)
	Mean	**8.4**	**11.21**	**87.5**	**10.3**
	CD ($P < 0.05$)	1.276	1.495	8.167	1.010

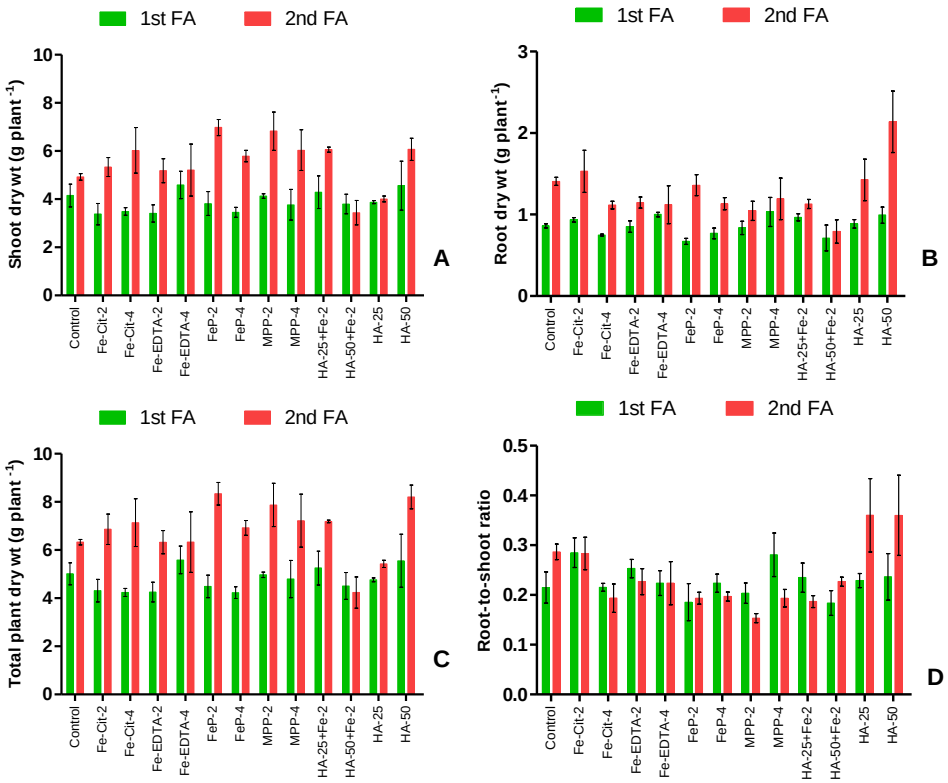

Figure 4. *Effect of different foliar treatments on (A) shoot, (B) root and (C) total biomass and (D) root-to-shoot ratio in soybean plants grown in a glasshouse.*

Total leaf area, chlorophyll and rate of photosynthesis were significantly influenced by foliar treatment. Leaf area was significantly higher at grain-filling stage in Fe-citrate (4 mM), Fe-EDTA (2 mM), Fe-P and MPP treatments as well as control (Fig. 5A). Other treatments showed a reduction or non-significant effect on leaf area. Among the treatments, Fe-P (2 mM), MPP (2 mM) and MPP (4 mM) resulted in maximum leaf expansion as compared to control with an increase of 31.6%, 22.6% and 21.3%, respectively, at grain-filling stage (Table 6). The HA treatments resulted in a marked reduction in leaf area. Total chlorophyll concentration in leaf tissue was higher at anthesis stage but decreased during grain filling with all treatments. This is a growth-related trait, as with age the plant enters the senescence stage. But interestingly, the treatment with Fe-P (2 mM), Fe-EDTA (2 mM) and MPP (2 mM) retained an almost similar concentration of chlorophyll at both stages of crop growth (Fig. 5B), indicating that their application can delay senescence or increase the leaf area duration. The percentage increase in chlorophyll at anthesis stage was 38.1, 32.6 and 22.3 in Fe-P (2 mM), Fe-EDTA (2 mM) and MPP (2 mM), respectively, as compared to control (Table 6). The rate of photosynthesis was higher with foliar treatments except for Fe-citrate and Fe-EDTA (Fig. 5C). After the first foliar treatment, there was a significant increase in the rate of photosynthesis, whereas after the second foliar application, only a few treatments showed a significant increase (Fig. 5C). The increase in rate of photosynthesis was 51% with Fe-P (2 mM) as compared to control (Table 7). Fe-P at the lower concentration was seen to increase leaf

area and retain a higher chlorophyll concentration at pod-filling stage, which could be the reason for maintaining higher photosynthesis at this stage.

Table 6. *Percentage change in biomass accumulation in soybean with different foliar treatments as compared to water spray (control).*

S. No.	Treatments	Shoot Dry Weight		Root Dry Weight		Total Plant Dry Weight	
		1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application
1	Control	-	-	-	-	-	-
2	Fe-Cit-2	-18.7	8.4	8.6	8.8	-14.0	8.5
3	Fe-Cit-4	-15.9	22.4	-13.1	-20.6	-15.4	12.8
4	Fe-EDTA-2	-18.0	5.3	-1.2	-18.5	-15.2	-0.1
5	Fe-EDTA-4	10.5	5.8	16.1	-20.4	11.5	0.1
6	FeP-2	-8.1	41.8	-22.2	-3.3	-10.5	31.8
7	FeP-4	-16.7	17.7	-10.7	-19.5	-15.7	9.4
8	MPP-2	-0.5	38.8	-2.8	-25.7	-0.9	24.5
9	MPP-4	-9.3	22.6	19.6	-15.2	-4.3	14.1
10	HA-25+Fe-2	3.3	23.2	12.0	-19.7	4.8	13.7
11	HA-50+Fe-2	-8.6	-30.1	-17.4	-43.7	-10.1	-33.1
12	HA-25	-6.7	-18.6	2.9	1.3	-5.1	-14.2
13	HA-50	9.9	23.4	15.2	51.9	10.8	29.7

Table 7. *Percentage change in leaf area, chlorophyll and photosynthesis rate in soybean with different foliar treatments compared to water spray (control)*

S. No.	Treatments	Leaf Area		Total Chlorophyll		Photosynthesis	
		1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application	1st Foliar Application	2nd Foliar Application
1	Control	-	-	-	-	-	-
2	Fe-Cit-2	-3.3	-14.1	4.0	-27.7	19.3	-7.9
3	Fe-Cit-4	4.3	3.5	20.4	1.0	-12.6	-3.7
4	Fe-EDTA-2	-17.3	-10.7	15.5	32.6	29.1	19.3
5	Fe-EDTA-4	19.1	-8.2	8.5	8.2	0.7	5.7
6	FeP-2	19.3	31.6	-3.5	38.1	3.9	51.0
7	FeP-4	-0.4	3.8	27.9	-6.0	20.1	4.4
8	MPP-2	24.5	22.6	4.5	22.3	42.6	-15.3
9	MPP-4	26.6	21.3	4.7	7.1	65.3	11.0
10	HA-25+Fe-2	19.3	-2.0	2.4	-9.0	69.4	0.8
11	HA-50+Fe-2	-4.4	-51.8	8.1	4.3	70.5	31.6
12	HA-25	0.4	-43.3	19.1	-13.6	10.7	21.0
13	HA-50	30.7	3.2	14.8	-11.9	77.4	-4.5

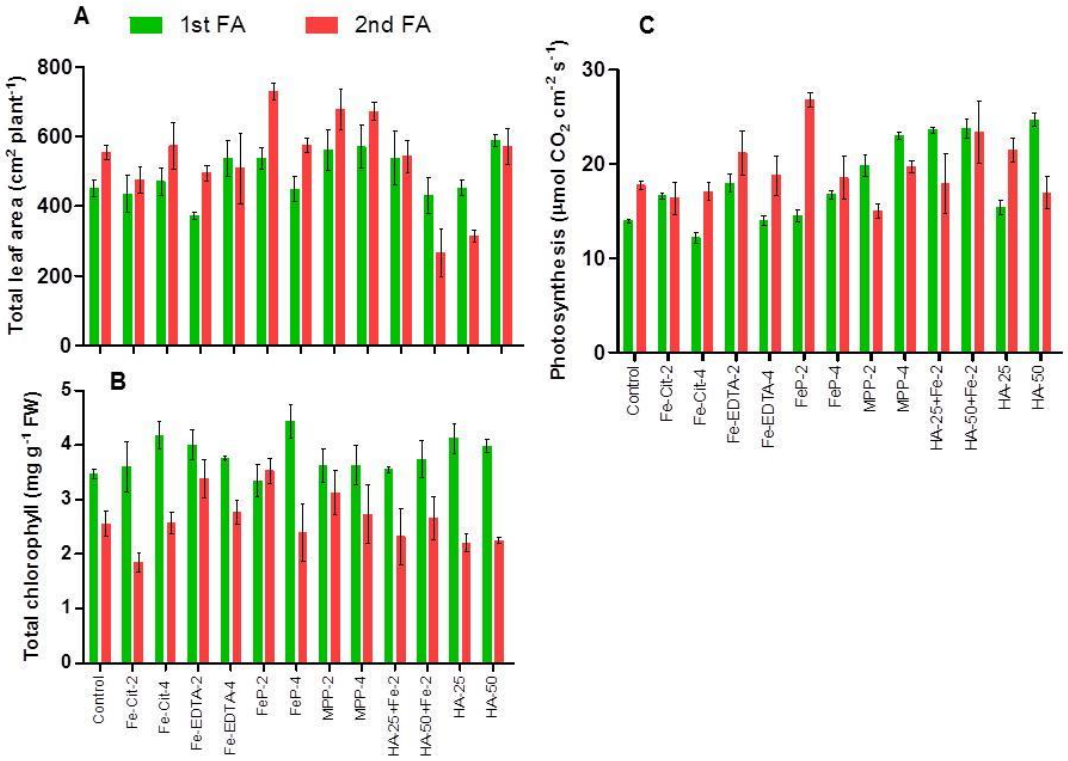

Figure 5. *Effect of different foliar treatments on (A) leaf area (B) chlorophyll concentration and (C) rate of photosynthesis in soybean plants grown in a glasshouse.*

The P content in different plant organs, such as root, shoot (stem + leaves) and seed, in soybean plants was significantly influenced by foliar treatment (Fig. 6A). Among different treatments, Fe-P (2 mM) resulted in the highest P content in all organs as compared to control. The P content in seed was also found to be higher with HA-25+Fe-2 mM, but the P content in root and shoot was less than with the Fe-P (2 mM) treatment. The Fe content in the different organs of soybean was significantly influenced by foliar treatment (Fig. 6B). The root retained the highest amount of Fe, while there was a differential response for leaf, stem and grain Fe content.

The yield traits of soybean, such as number of pods, total seed weight and test weight, were recorded (Table 8). The number of pods and total seed weight per plant were statistically the same among the foliar treatments, including control. With Fe-citrate (4 mM) and Fe-P (2 mM), the percentage change was more than 10.0 as compared to control; however, the critical difference between the treatments was not significant. The test weight, or 100-seed weight, was significantly higher with almost all foliar treatments, except for Fe-P (4 mM) and MPP (2 mM), than control. The higher test weight was obtained with HA-50+Fe-2, Fe-citrate (2 mM and 4 mM) and Fe-EDTA (2 mM).

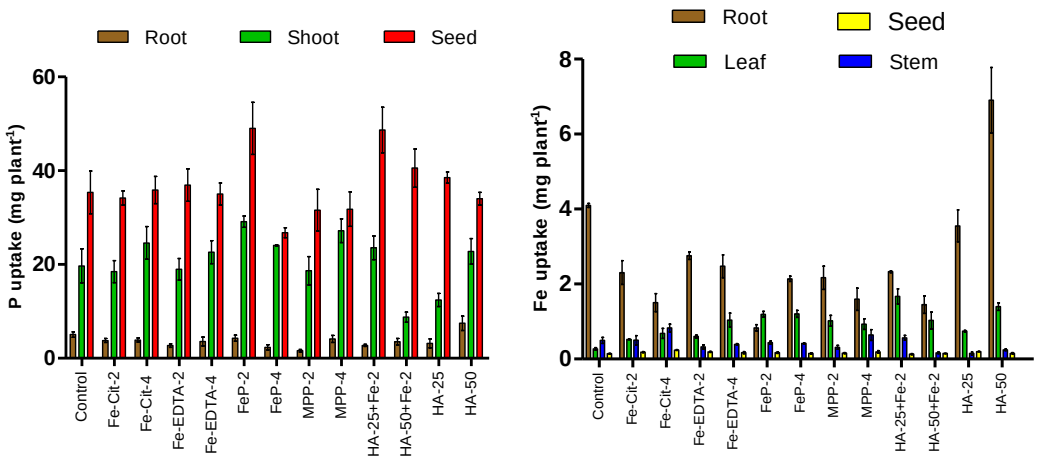

Figure 6. *Effect of different foliar treatments on (A) phosphorus uptake and (B) iron uptake in different organs of soybean plants grown in a glasshouse.*

Table 8. *Effect of different foliar treatments on yield traits in soybean plants grown in glasshouse. The value in parentheses is the percentage change over the control treatment.*

S. No.	Treatments	No. of Pods Plant^{-1}	Seed Weight (g plant^{-1})	100-Seed Weight (g)
1	Control	16.83	2.08	6.07
2	Fe-Cit-2	16.67 (-0.95)	2.21 (6.3)	8.25 (35.9)
3	Fe-Cit-4	19.75 (17.3)	2.45 (17.8)	8.21 (35.3)
4	Fe-EDTA-2	18.92 (12.4)	2.23 (7.2)	7.73 (27.4)
5	Fe-EDTA-4	15.42 (-8.4)	2.03 (-2.4)	8.24 (35.8)
6	FeP-2	18.58 (10.4)	2.35 (13.0)	7.58 (24.9)
7	FeP-4	14.58 (-13.4)	1.52 (-26.9)	6.14 (1.2)
8	MPP-2	18.75 (11.4)	1.90 (-8.6)	6.34 (4.5)
9	MPP-4	19.00 (12.9)	2.07 (-0.5)	7.03 (15.8)
10	HA-25+Fe-2	17.92 (6.5)	1.99 (-4.3)	7.33 (20.8)
11	HA-50+Fe-2	14.17 (-15.8)	1.73 (-16.8)	8.40 (38.4)
12	HA-25	17.75 (5.5)	2.05 (-1.4)	7.44 (22.6)
13	HA-50	18.33 (8.9)	2.03 (-2.4)	6.82 (12.4)
	Mean	**17.59**	**2.06**	**7.35**
	CD (5%)	2.992	0.488	0.476

2.1.4 Summary

Rice crop

- The second spray, i.e., during anthesis, was more effective in improving physiological traits.
- Biomass increased significantly with treatments Fe-Cit-2 > Fe-EDTA-4 > Fe-Cit-4 > Fe-EDTA-2.

- Leaf area and chlorophyll were higher with treatments Fe-Cit-2 > Fe-EDTA-4 > Fe-Cit-4 > Fe-EDTA-2 > HA-25+Fe-2.
- Photosynthesis was higher with treatments FeP-2 > HA-25+Fe-2 > MPP-4 > FeCit-4 > FeEDTA-2.
- P content in grain influenced by treatments HA-25+Fe-2 > FeEDTA-2.
- Partitioning of P toward grain was higher in treatments HA-50+Fe-2 > HA-25 > HA-25+Fe-2 > HA-50.
- Percentage filled grains influenced by Fe-EDTA-2 > MPP-2 > Fe-Cit-2.
- Test weight (500-seed weight) was higher with MPP-2.

Soybean crop

- The second spray, i.e., during pod filling, was more effective in improving physiological traits.
- Biomass increased significantly with treatments FeP-2 > HA-50 > MPP-2 > MPP-4.
- Leaf area, chlorophyll and photosynthesis were higher with treatments FeP-2 > HA-50+Fe-2 > Fe-EDTA-2.
- P content in different organs was influenced by treatments FeP-2 > HA-25+Fe-2.
- Partitioning of P toward grain was higher in treatments HA-50+Fe-2 > HA-25 > HA-25+Fe-2 > HA-50.
- Yield traits were influenced by Fe-Cit-4 > FeP-2.

2.2 Foliar application of inorganic Fe on rice and soybean conducted in the field

Results from the preliminary experiments conducted in a glasshouse on rice and soybean were used to determine treatments for the next set of experiments to be conducted in the field. As per discussion with the Executive Director, VFRC, we included a treatment of nano-Fe (Sigma SKU 544884-5G) at two concentrations (derived from literature). The final list of compounds used in these experiments is presented in Table 9. As pointed out, the results obtained in the glasshouse experiments may be due to the cumulative effects of foliar application at two growth stages. Therefore, to identify the right crop stage that responds to foliar application with maximum growth, the design of experiment was modified accordingly. A total of three sprays in rice (at tillering, anthesis and grain filling) and two in soybean (at anthesis and pod filling) were planned (Fig. 7A, B). In rice, the plants in sets 1, 2 and 3 received a single spray at tillering, anthesis and grain-filling stages, respectively, while plants in set 4 were sprayed twice (at anthesis and grain-filling stages) and those in set 5 were sprayed at all three growth stages. Similarly, for soybean, plants in sets 1 and 2 were sprayed at anthesis and pod-filling stages, respectively, and set 3 was sprayed at both stages.

Another important observation from our glasshouse experiments pertaining to growth (leaf area and biomass) in both crops was that there was a negative effect in some of the treatments as compared to the control. This indicates that the plant might have undergone stress due to the foliar treatment. This could have been due to the effect of chemicals on the cellular process, which may have affected cell division or cell enlargement, or at the physiological level, such as production of more oxy-radicals and exposure of the plants to oxidative stress. To analyze this, we planned to study the complete antioxidant scavenging system, including enzymes and metabolites, in both crops at all sampling stages. This

information provided an important clue regarding the chemical compound that exposes the plants to oxidative stress and that may not be suitable for foliar application. Such information was not available in the literature.

Table 9. *Foliar treatments for the second set of experiments to be conducted in the field on rice and soybean.*

S. No.	Treatment		
	Compound	Concentration (mM)	g 500 mL^{-1}
Rice			
FT.1	Deionized water (Control)	00	00
FT.2	Fe-Citrate	2	0.245
FT.3	Fe-Citrate	4	0.490
FT.4	Fe-EDTA	2	0.367
FT.5	$FePO_4.2H_2O$	4	0.374
FT.6	Monopotassium phosphate	4	0.270
FT.7	Humic acid + $FeCl_3$	25 mg L^{-1} + 2	0.012 + 0.162
FT.8	Nano-Fe	2	0.159
FT.9	Nano-Fe	4	0.319
Soybean			
FT.1	Deionized water (Control)	00	00
FT.2	Fe-Citrate	4	0.490
FT.3	$FePO_4.2H_2O$	2	0.187
FT.4	Monopotassium phosphate	2	0.135
FT.5	Humic acid + $FeCl_3$	25 mg L^{-1} + 2	0.012 + 0.162
FT.6	Humic acid	50 mg L^{-1}	0.025
FT.7	Nano-Fe	4	0.319

2.2.1 Materials and methods

The second round of experiments was conducted with rice (*Oryza sativa* var. MAS 946-1) and soybean (*Glycine max* var. DS-2614) under the natural environment in pots/field to validate the results of preliminary experiments conducted at the National Phytotron Facility (NPF), New Delhi. For rice, the nursery was prepared in the second week of June, and 25-day-old plants were transferred to the pots in July 2015. The pots were filled with soil collected from 0-30 cm depth and sieved to remove debris before filling. Each pot (30 cm diameter) contained 15 kg of soil. The properties of the soil are mentioned in Table 2. The recommended dose of fertilizer, i.e., 120 kg N, 60 kg P_2O_5 and 40 kg K_2O per hectare, was added to each pot. Urea was applied in three splits (50% as a basal dose, 25% at tillering and 25% at anthesis). Two healthy plants were maintained in each pot. Plants were irrigated on alternate days with normal tap water to maintain standing water. The complete setup was divided into five sets with an equal number of pots, and each set differed in terms of number of sprays applied at different growth stages.

Figure 7A represents the layout of treatment for rice crop. The plants in sets 1, 2 and 3 received single spray at tillering, anthesis and grain-filling stages, respectively, while plants in set 4 were sprayed twice (anthesis and grain-filling stages) and those in set 5 were sprayed at all three stages of growth. The experiment was carried out in a completely randomized block design with five replications for each treatment.

Soybean seed was sown directly in the field under natural conditions in mid-July 2015. Planting was done on ridges with a row-to-row spacing of 30 cm and plant-to-plant distance of 15 cm. Seeds were sown by dibbling method. Similar to rice, soybean plants were divided into three sets with an equal number of plants in each set (Fig. 7B). Set 1 was sprayed at anthesis stage, set 2 received foliar application of nutrient at pod-filling stage, and set 3 received foliar application at both stages.

(A) Rice

SET 1	SET 2	SET 3	SET4	SET 5
Tillering				Tillering
	Anthesis		Anthesis	Anthesis
		Grain filling	Grain filling	Grain filling

(B) Soybean

SET 1	SET 2	SET 3
Anthesis		Anthesis
	Pod filling	Pod filling

Figure 7. *Foliar spraying schedule at different growth stages of (A) rice and (B) soybean*

2.2.1.1 Foliar treatments and sampling schedule

From the 13 treatments in the preliminary experiments conducted in the glasshouse at two concentrations (2 and 4 mM) of nutrients (Fe and P), we selected nine spray formulations for rice and seven for soybean. The spray formulations were prepared by dissolving the required amount of chemical compound and adding 100 µl of surfactant (Triton X100) in 1.0 L of solution. A water spray as the “control” was also included. The pH of each spray formulation was maintained at 6.0 using HCl or KOH. Plants were sprayed before noon between 8:00 to 10:00 a.m. The sampling was done for growth and physiological traits in rice and soybean on the sixth day after foliar treatment. For rice, the data for all five sets were collected at grain-filling stage, while for soybean, the data for all three sets were collected at pod-filling stage. A comparison with respect to crop performance was made between different sets receiving single, double or multiple sprays in both crops. Observations on growth parameters and yield traits were recorded as mentioned for preliminary experiments conducted in a glasshouse.

Rice seedlings 15 days after transplanting

Soybean crop sown in field

Rice plants at maximum tillering stage

Soybean plants at flowering stage

Rice plants at anthesis stage

Physiological maturity stage in soybean

Figure 8. *Rice and soybean experiments under natural environmental conditions in soil conducted during July 2015.*

2.2.1.2 Antioxidant enzymes

To understand the physiological response of plants to the applied chemical compounds, the antioxidant enzyme system was studied in both rice and soybean. The fully expanded young leaf was collected after

the sixth day of foliar application. The reactive oxygen species (ROS), such as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), are formed when the plants experience any kind of stress. These ROS, if not scavenged properly, damage the lipid membrane of the cells and produce thiobarbituric acid-like reactive substances (TBARS). As a result, the cell membrane loses specificity and becomes less functional. The antioxidant enzyme system involves superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and glutathione reductase (GR).

The superoxide radical was estimated according to the method of Chaitanya and Naithani (1994) by taking 0.5 g of leaf tissue and homogenizing it in 5 mL of pre-cooled 0.5 M phosphate buffer (pH 7.2) containing 1 mM diethyldithiocarbamate. The homogenate solution was centrifuged at 5,000 rpm for 5 minutes. In the supernatant, superoxide radical was measured by its capacity to reduce nitroblue tetrazolium and form blue color formazones. Hydrogen peroxide was estimated by measuring the intensity of light yellow-colored titanium-hydro-peroxide complex with titanium reagent at 415 nm (Rao et al., 1996). Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid-reactive substances, according to Heath and Packer (1968). The lipid peroxides were expressed as nmol TBARS g^{-1} FW using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The buffer used for extraction of enzymes SOD, CAT, POX and GR from leaf tissue was 0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA. Leaf tissue was ground in liquid nitrogen and then homogenized in 10 mL of pre-cooled extraction buffer. After centrifugation for 20 minutes at 15,000 g, the supernatant was taken in a fresh tube and used for enzyme assay. Superoxide dismutase activity was assayed by the method described by Dhindsa et al. (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13.33 mM methionine, 75 µM nitroblue tetrazolium chloride, 0.1 mM EDTA and 50 mM sodium carbonate in a total reaction volume of 3.0 mL. Enzyme activity was estimated by recording the decrease in optical density of formazone at 560 nm produced by superoxide radical and nitroblue tetrazolium dye. For measuring CAT activity, the reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 12.5 mM of hydrogen peroxide in a total volume of 3.0 mL (Aebi, 1984). The reduction of hydrogen peroxide to water and molecular oxygen catalyzed by CAT was measured as a decrease in absorbance at 240 nm over time. The POX activity was assayed in a total 3.0 mL reaction mixture containing 50 mM phosphate buffer (pH 6.1), 16 mM guaiacol and 2 mM hydrogen peroxide (Castillo et al., 1984). POX activity was measured as an increase in optical density due to the oxidation of guaiacol to tetra-guaiacol. For glutathione reductase activity, in a total volume of 3.0 mL reaction mixture, 66.67 mM phosphate buffer (pH 7.5), 0.33 mM EDTA, 0.5 mM DTNB, 66.67 µM NADPH and 666.67 µM oxidized glutathione were added (Smith et al., 1988). The GR activity was measured by recording the increase in absorbance at 412 nm.

2.2.2 Results of foliar application of Fe and P on rice and soybean in a field experiment

2.2.2.1 Effect of foliar treatments on tissue Fe and P concentration and uptake in rice

The main aim of this study was to test the ability of various foliar-sprayed Fe formulations at different growth stages to increase grain yield and grain Fe content. The foliar sprays under study succeeded in elevating grain Fe concentration at all spraying stages (tillering [T], anthesis [A], grain filling [GF], A+GF, T+A+GF) as compared to control (Fig. 9A). A more than 5-fold increase in grain Fe concentration was recorded for HA+$FeCl_3$ and Fe-EDTA, while all others were shown to have more than a 4-fold increase except $FePO_4$ (3.7-fold). Interestingly, this maximum hike was observed when spraying was performed at all three stages (T+A+GF), while single spray at tillering (T) showed the least rise in grain Fe concentration and total grain Fe content as compared to control. Total grain Fe content increased by more than 7-fold for Fe-citrate (2 mM), Fe-EDTA and HA+$FeCl_3$ (Fig. 9B). For other treatments, it increased by more than 5-fold. A different trend was observed for Fe-citrate (4 mM), for which the maximum increase for both these parameters occurred at anthesis stage.

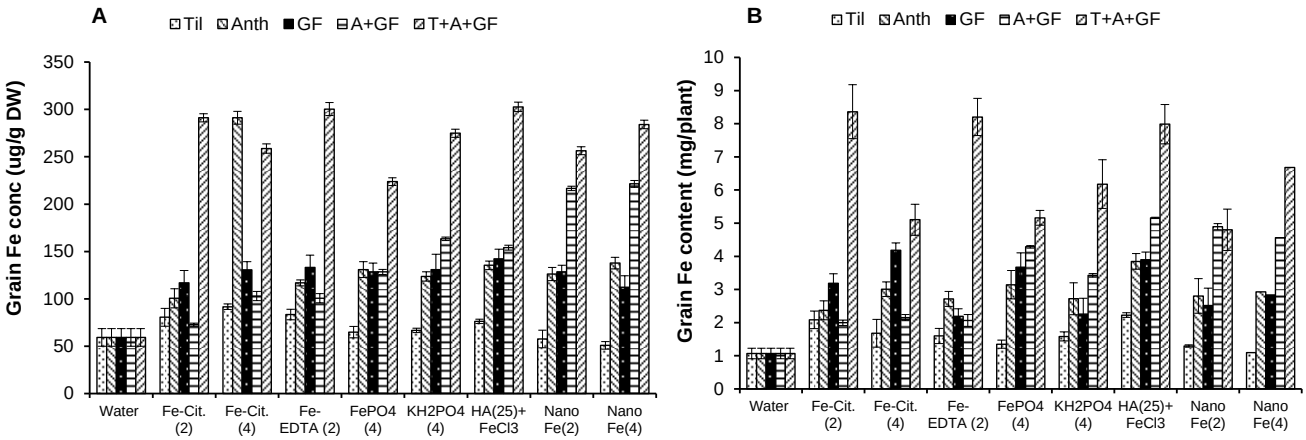

Figure 9. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) grain Fe concentration and (B) total Fe content in grain. Til – tillering, Anth – anthesis, GF – grain filling. Bar represents mean ± SEm.*

For most of the treatments, total Fe uptake per plant was maximum with spraying at tillering, followed by spraying at all three stages, with a few exceptions. Maximum increase in total Fe uptake by plants was recorded for Fe-citrate (4 mM) and $FePO_4$ when sprayed at GF and A+GF stages, respectively (Fig. 10A). Comparing all the treatments, nano-Fe (4 mM) and KH_2PO_4 had the maximum hike in total Fe uptake by 81% and 79.6%, respectively, while the least was recorded for $FePO_4$ (4.5%). These increases ranged from 7% to 81% with spraying at tillering and 9% to 42% with spraying at T+A+GF. Similar to total Fe uptake, root Fe uptake was also highest at tillering with the exception of Fe-citrate (4 mM) and $FePO_4$, the values of which decreased by 27% and 19%, respectively, at this stage as compared to control (Fig. 10B). Moreover, plants sprayed with $FePO_4$ failed to increase in root Fe uptake at other stages. Root Fe

uptake increase at other stages was not on par with tillering spray. The least root Fe uptake occurred with Fe-EDTA (5.5%), while the maximum was shown by KH_2PO_4 (83.6%), followed by Fe-citrate (4 mM; 56%) and nano-Fe (4 mM; 42.8%). All of the treatments had the largest increase in shoot Fe uptake at either T or T+A+GF, except HA+$FeCl_3$, which showed its maximum hike (99%) at GF due to the highest shoot and root biomass obtained at this stage (Fig. 10C). Shoot Fe uptake increase was maximum for both nano-Fe treatments, while it was the least for Fe-EDTA and KH_2PO_4.

The Fe concentration in root and shoot tissue also varied significantly due to various foliar treatments applied at different growth stages (Fig. 10B). It is noteworthy to mention that the percentage increase in root (10-40%) and shoot (31-157%) Fe tissue concentration was the same at T and T+A+GF stages, though the increase was higher for shoot Fe concentration, as compared to control. For most of the treatments, Fe concentration was maximum at these stages, except for Fe-citrate (4 mM), HA+$FeCl_3$ and nano-Fe (2 mM), which had the highest root Fe concentration at GF or A stage. Root Fe concentration was highest with KH_2PO_4 application, followed by Fe-citrate (2 mM), while the lowest was recorded with $FePO_4$, nano-Fe (2 mM) and Fe-citrate (4 mM); the reverse was seen for shoot Fe concentration.

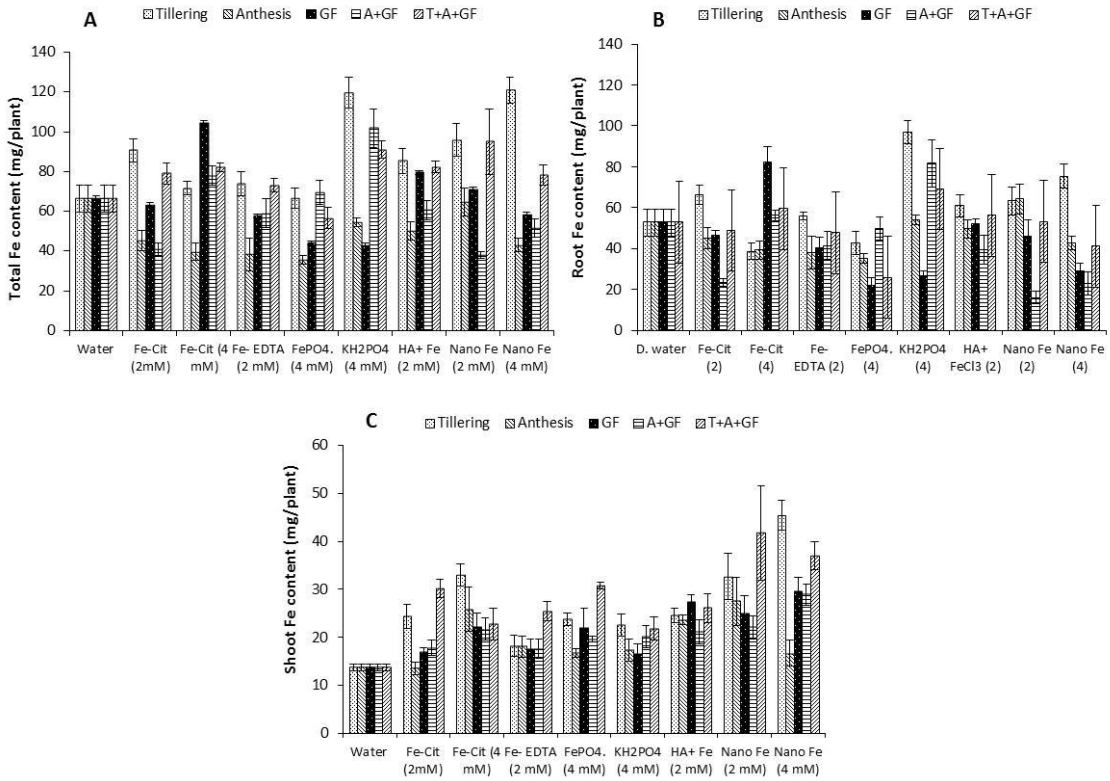

Figure 10. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) total Fe content per plant, (B) Fe content in shoot and (C) Fe content in root.*

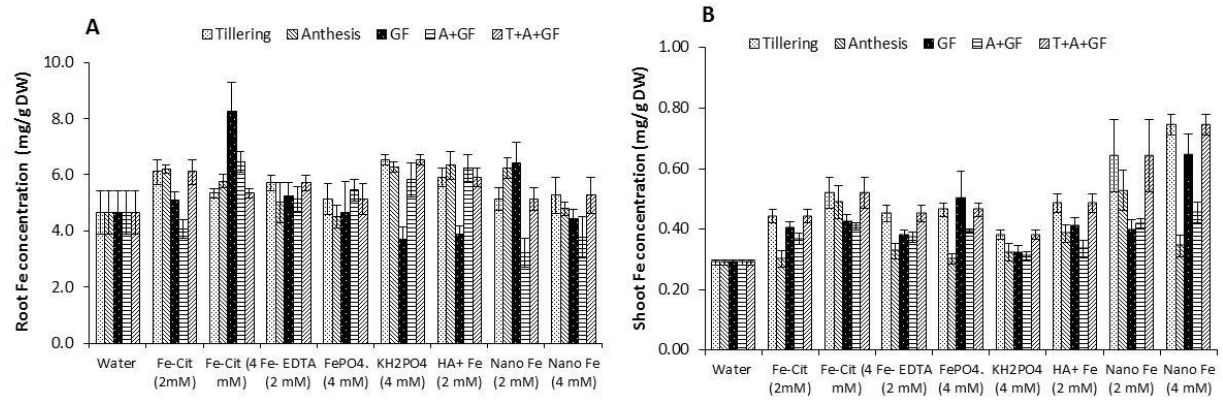

Figure 11. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on tissue Fe concentration in (A) root and (B) shoot.*

The maximum P concentration in rice grain was obtained when plants were sprayed once at tillering (1.7- to 2.4-fold), anthesis (1.3- to 2.7-fold) or grain filling (1.6- to 2.8-fold) stages for all treatments as compared to control, though the increase was not as high as grain Fe content (5-fold; Fig. 12A). Increase in grain P concentration over control was less when spraying was done at two or three stages; however, the increase among treatments did not vary much. A similar trend was seen for total grain P uptake and content, which were highest compared to control when plants were sprayed at different stages individually (Fig. 12B). Maximum increase in total grain P content was recorded in foliar treatment with $FePO_4$ (2.8-fold), followed by Fe-citrate (2, 4 mM; 2.8-fold) and HA+$FeCl_3$ (2.6-fold); however, the increase was less when plants received foliar application at two or three stages.

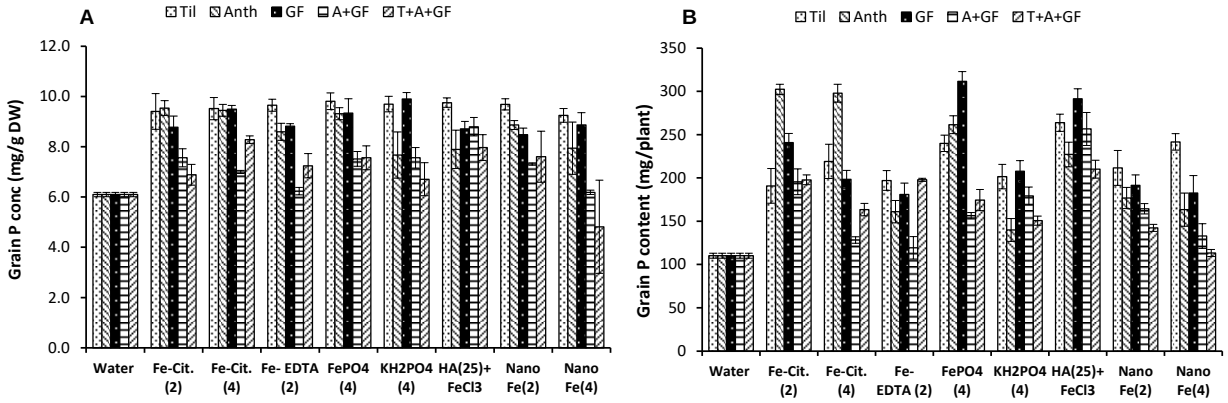

Figure 12. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) grain P concentration and (B) total P content in grain.*

Shoot P content was higher at T and A+GF, while root P uptake was maximum when spraying was done at two or three stages (Fig. 13). KH_2PO_4 and nano-Fe (2 mM) treatments showed maximum increase in shoot P uptake when sprayed at A+GF stage (Fig. 13B). When sprayed at T+A+GF stage, Fe-citrate (4 mM), KH_2PO_4 and HA+$FeCl_3$ showed a reduction in shoot P uptake by 52%, 19% and 54%,

respectively, as compared to control. Root P uptake was highest for KH_2PO_4 (6.4-fold) and lowest (1.3-fold) for Fe-citrate (4 mM) and HA+$FeCl_3$ (Fig. 13A). Total P uptake was maximum with a single spray for Fe-citrate (2, 4 mM), HA+$FeCl_3$ and nano-Fe (2 mM) (Fig. 13C). For other treatments, total P uptake per plant was maximum with spraying at either A+GF or T+A+GF stage.

2.2.2.2 Effect of foliar treatment on physiological parameters in rice

Compared to control, increase in total biomass with HA+$FeCl_3$, KH_2PO_4 and Fe-citrate (2 mM) was 30%; while it was the lowest for Fe-EDTA at 10% (Fig. 14A). Significant variability was observed in the performance of these treatments at different growth stages. Higher (4 mM) concentrations of Fe-citrate and nano-Fe failed to show any increase in total biomass with spraying at T+A+GF and the maximum was with spraying at tillering stage, while for Fe-EDTA, $FePO_4$, Fe-citrate (2 mM) and nano-Fe (2 mM), the maximum was with spraying at T+A+GF. These treatments also produced the highest shoot biomass when spraying was done at two or three stages. For KH_2PO_4 (32%, 36%) and HA+$FeCl_3$ (36%, 40%), total and shoot biomass were highest at A+GF and GF stages, respectively (Fig. 14B). Increase in shoot biomass was lowest (19%) with Fe-EDTA, while the increase was greater than 40% with Fe-citrate (2 mM), $FePO_4$ and HA+$FeCl_3$ as compared to control.

Root biomass was not positively influenced by foliar spraying since root biomass was decreased at A and T+A+GF stages with all treatments (Fig. 14C). Moreover, for Fe-citrate, Fe-EDTA and $FePO_4$, the decrease was also recorded at the other three stages (T, GF and A+GF). However, KH_2PO_4 had a 26% and 19% increase in root biomass at T and A+GF stages, respectively. The plants sprayed with nano-Fe also had increased root biomass at tillering stage, though it was higher for 4 mM (22%) than for 2 mM (4%). At GF stage, only HA+$FeCl_3$ had a higher root biomass (16%) than control.

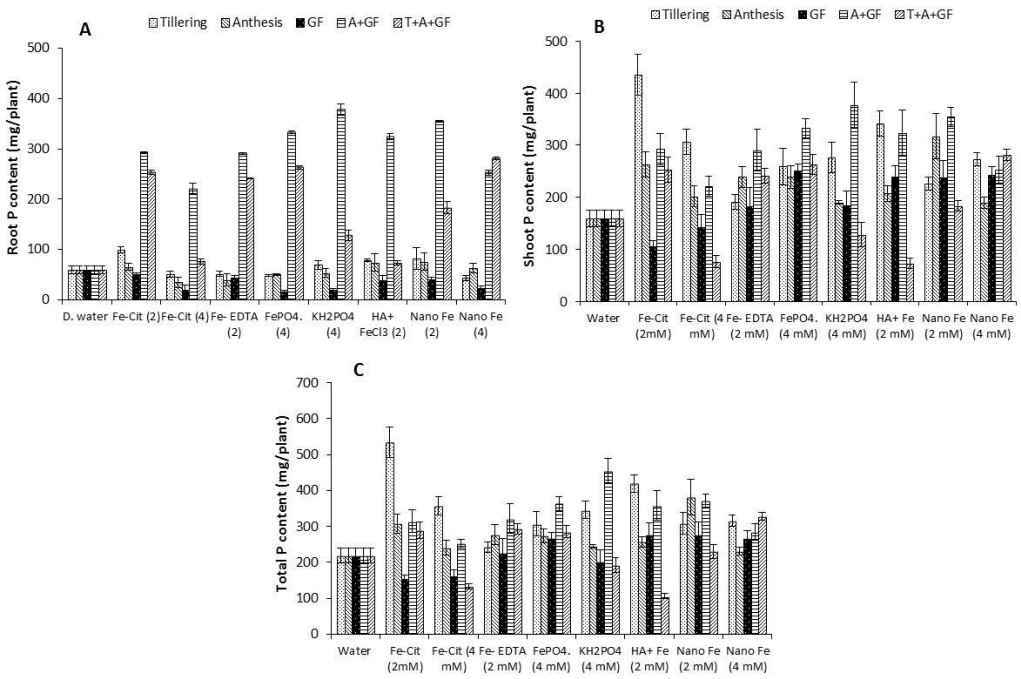

Figure 13. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on phosphorus uptake or content in (A) root (B) shoot and (C) total plant P.*

The increase in leaf area (Fig. 15A) and chlorophyll (Fig. 15B) of plants sprayed with the lower concentration (2 mM) was higher when spraying was performed at two or three stages (A+GF or T+A+GF) rather than an individual stage, but the reverse was true for the higher concentration. $FePO_4$ resulted in the highest increase (2.4-fold) in leaf area, followed by similar increases (1.5-fold) for Fe-citrate (4 mM), HA+$FeCl_3$ and Fe-citrate (2 mM); the increase was lowest for nano-Fe (2 mM). Total chlorophyll was highest for nano-Fe (4 mM), followed by $FePO_4$, while the lowest increase was recorded with Fe-citrate (4 mM). Increase in leaf area was not accompanied by an increase in chlorophyll at respective growth stages: the leaf area of plants treated with $FePO_4$ and nano-Fe (2 mM) was reduced at tillering stage as compared to control, while the chlorophyll content was maximum at this particular stage.

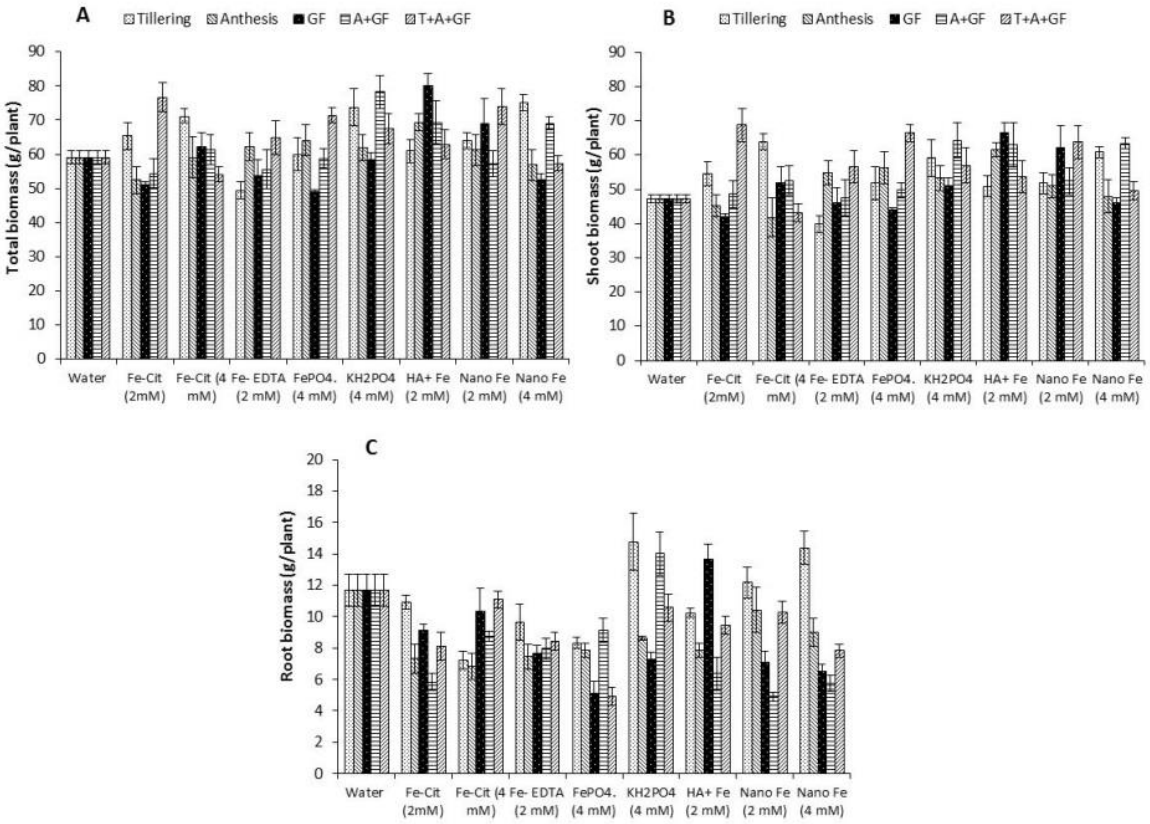

Figure 14. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) total plant biomass (B) shoot biomass and (C) root biomass.*

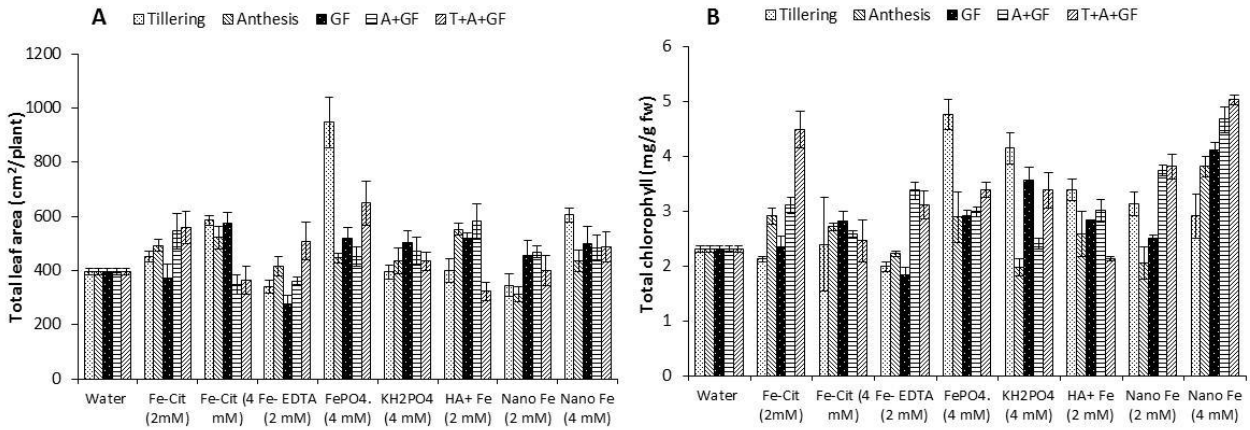

Figure 15. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) total leaf area and (B) total leaf chlorophyll concentration.*

2.2.2.3 Effect of foliar treatment on yield parameters in rice

The yield parameters were significantly influenced by the different foliar treatments at the different growth stages. The maximum increase in total grain weight was recorded with application of HA+$FeCl_3$ (86%) followed by $FePO_4$ (85%), Fe-citrate (2 mM; 75%) and Fe-citrate (4 mM; 74%), while the least was

recorded for KH_2PO_4 (31%) and nano-Fe (2 mM; 25%), as compared to control (Fig. 16A). For all treatments, the highest increases in grain weight per panicle (Fig. 16B) and total grain number per panicle (Fig. 16C) were in the range of 32-45% and 11-20%, respectively. The number of panicles per plant was highest for nano-Fe (4 mM; 36%) as compared to control (Fig. 16D). Total grain number per panicle was maximum for all treatments at T+A+GF. For Fe-EDTA, the number of panicles per plant and total grain weight were also highest when sprayed at all three stages. Just like dry weight, total grain weight was also highest for Fe-citrate at anthesis stage, while the number of panicles per plant was maximum at A+GF. For nano-Fe (4 mM), the number of panicles per plant was maximum (36%) at anthesis stage, while total number of grains per panicle and grain weight per panicle were highest at T+A+GF.

At harvesting, the increases in plant dry weight, number of panicles per plant and total grain number per panicle were lowest compared to control when spraying was done at tillering stage (Fig. 17A). The maximum increase in dry weight was recorded for $HA+FeCl_3$ (41%) followed by KH_2PO_4 (34%) and $FePO_4$ (25%) at GF stage, while for Fe-citrate (2 and 4 mM), it was highest at anthesis stage. At T+A+GF stage, Fe-EDTA and nano-Fe (4 mM) had a more than 20% increase, while Fe-citrate, $FePO_4$ and $HA+FeCl_3$ had the lowest increase.

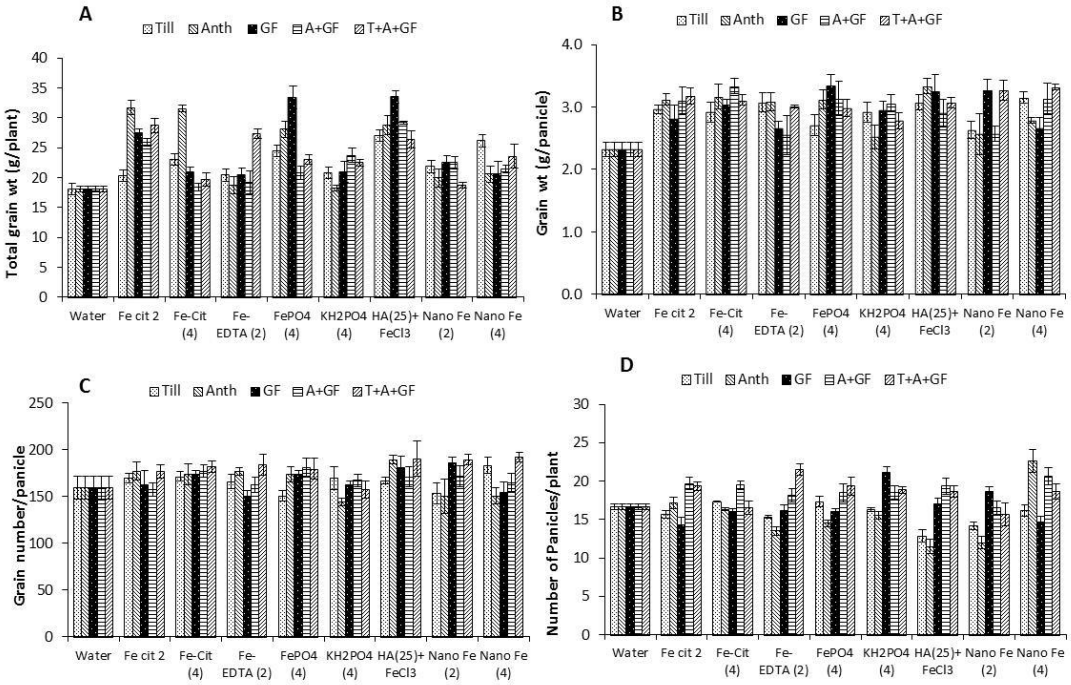

Figure 16. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) total grain weight per plant, (B) grain weight per panicle, (C) number of grains per panicle and (D) number of panicles per plant.*

Increases in plant dry weight, grain number per panicle and total grain weight were maximum for $HA+FeCl_3$ (41%, 19%, and 85%, respectively), while not much variation among the treatments was observed in percentage of grain filling (Fig. 17B) and grain weight per panicle (Fig. 16B). Also, the

increase observed for all the foliar treatments with respect to these parameters was not consistent with the growth stages. Both nano-Fe concentrations showed an increase in total grain number per panicle similar to HA+$FeCl_3$. The number of panicles per plant had the highest increase with nano-Fe (4 mM), followed by Fe-EDTA and KH_2PO_4, while the lowest increase was with nano-Fe (2 mM). Three (nano-Fe [2 and 4 mM] and KH_2PO_4) out of eight treatments showed a maximum increase in number of panicles per plant when foliar application was done at an individual stage (A or GF). The test weight (500-seed weight) increased significantly by 21% with all foliar treatments as compared to control, but no difference was observed when spraying was done at different growth stages (Fig. 17C).

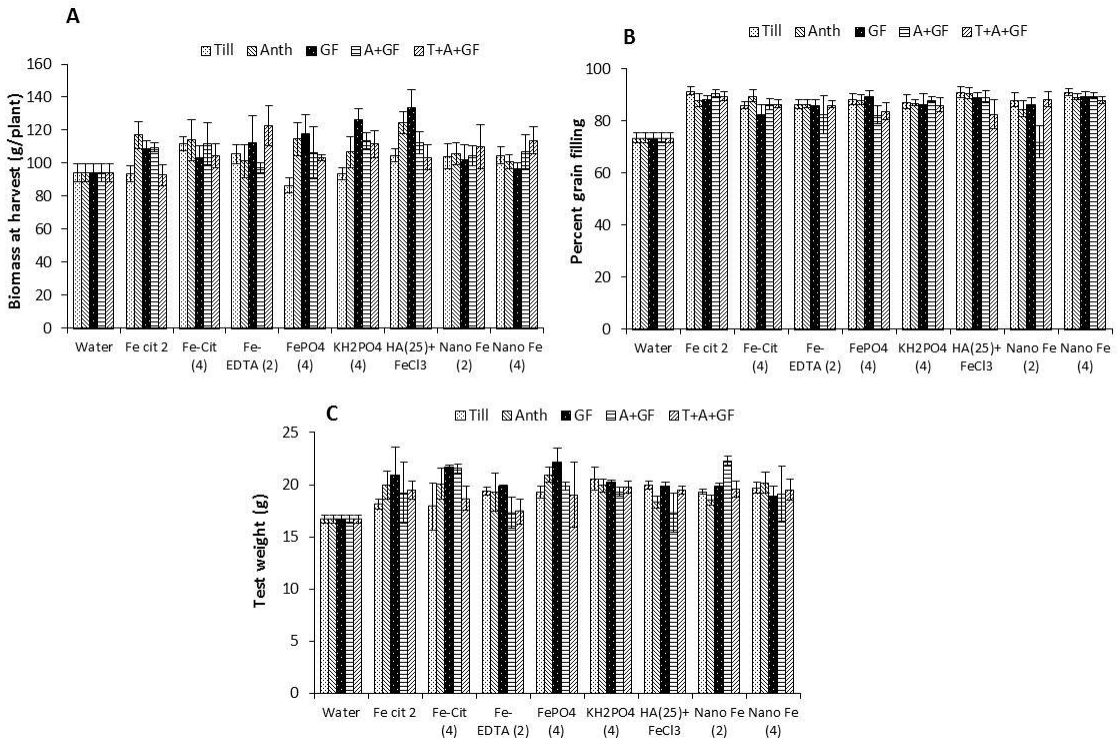

Figure 17. *Effect of foliar application of various Fe and P compounds on rice at different growth stages on (A) aboveground biomass at harvest stage, (B) grain filling percentage and (C) test weight*

2.2.2.4 Summary of rice experiment

- Maximum total grain Fe content was obtained at T+A+GF and minimum at A+GF stage.
- Total grain Fe content increased more than 7-fold with HA+$FeCl_3$, Fe-citrate (2 mM) and Fe-EDTA foliar treatments. With other treatments, a more than 4.5-fold increase was observed.
- Total biomass was highest with application of HA+$FeCl_3$ (at GF), KH_2PO_4 (at A+GF) and Fe-citrate (2 mM) at T+A+GF, but the least biomass was recorded with Fe-EDTA.
- Total Fe uptake was maximum when plants were sprayed only once at tillering stage. $FePO_4$ application resulted in the lowest total Fe uptake.

- Total P uptake was maximum with Fe-citrate (2 and 4 mM), HA+$FeCl_3$ and nano-Fe (2 mM) with a single spray.

2.2.2.5 Effect of foliar treatment on tissue Fe and P concentration and uptake in soybean

Significant variation was observed for Fe and P tissue concentration and uptake due to foliar application in soybean. Maximum Fe concentration in leaf tissue was obtained when plants were sprayed twice, at anthesis and pod-filling (A+PF), and the lowest at anthesis stage for all foliar treatments (Fig. 18A). However, HA (25 mg) + $FeCl_3$ (2 mM) showed increased leaf Fe concentration at anthesis stage. Leaf Fe concentration decreased by 12% for KH_2PO_4 (2 mM) at PF stage and increased by 16% and 38% for HA (25 mg) + $FeCl_3$ (2 Mm) and nano-Fe (4 mM), respectively. None of the treatments except HA (25 mg) + $FeCl_3$ (2 mM) showed more than a 37% increase in leaf area at anthesis stage. Similarly, Fe concentration in stem decreased by 23%, 12% and 5% for KH_2PO_4 (2 mM) at A+PF, anthesis and PF stage, respectively (Fig. 18B). A more than 50% increase in stem Fe concentration was recorded for treatment with Fe-citrate (4 mM) and KH_2PO_4 (2 mM) at anthesis and PF stages, while HA (25 mg) + $FeCl_3$ (2 mM) and nano-Fe (4 mM) showed increased stem Fe concentration at all stages. The increase was higher with spraying once at anthesis or PF stage as compared to spraying twice at A+PF. The increase in stem Fe concentration was lowest with $FePO_4.2H_2O$ (2 mM) treatment at all stages (24-38%).

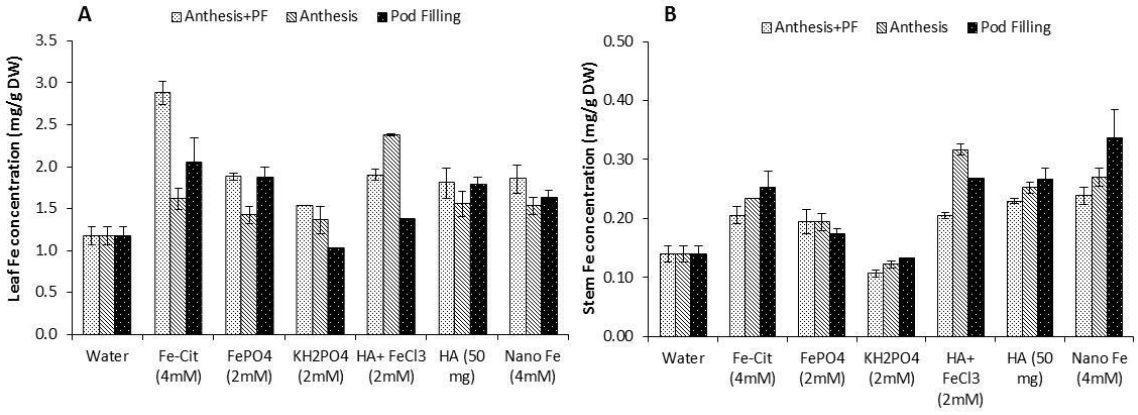

Figure 18. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on tissue Fe concentration of (A) leaf and (B) stem. PF – pod filling. Bar represents mean ± SEm.*

Fe content in leaf was increased at all stages with all foliar treatments except KH_2PO_4 (2 mM), which decreased leaf Fe content by 22% at PF stage (Fig. 19A). Moreover, the leaf Fe content with KH_2PO_4 (2 mM) application at A+PF and anthesis was lowest as compared to other treatments. The leaf Fe content increased by more than 50% with Fe-citrate (4 mM at A+PF and PF stages), $FePO_4.2H_2O$ (2 mM at all stages), HA (25 mg) + $FeCl_3$ (2 Mm) (at anthesis), HA (50 mg at all stages) and nano-Fe (4 mM at A+PF). Stem Fe content also decreased with KH_2PO_4 (2 mM) application at all stages (Fig. 19B). A more than 100% increase in stem Fe was recorded with HA (25 mg) +$FeCl_3$ (2 mM, at anthesis and PF), followed by HA (50 mg at anthesis, PF), nano-Fe (4 mM at PF) and $FePO_4.2H_2O$ (2 mM at anthesis) as compared to control.

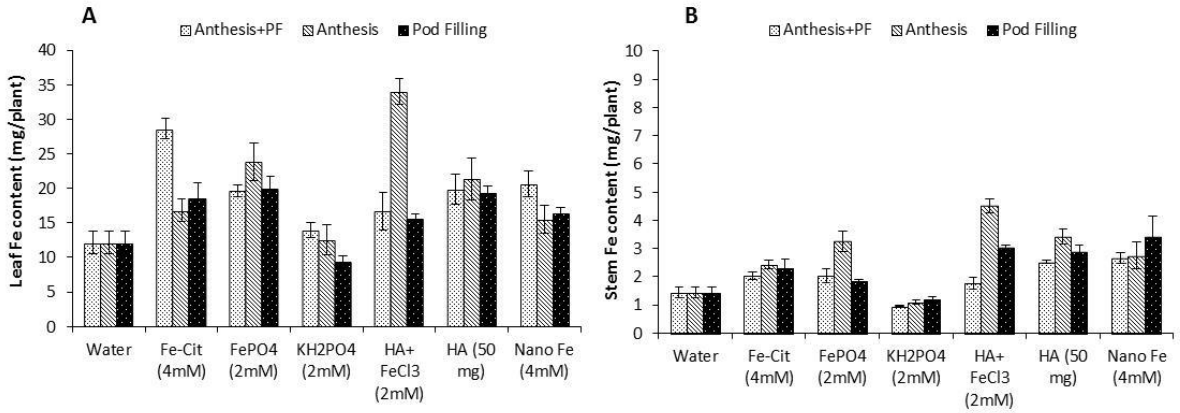

Figure 19. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on Fe content or uptake in (A) leaf and (B) stem. PF – pod filling.*

The largest increase in leaf tissue P concentration was recorded with application of nano-Fe (2 mM; 28%) and HA (50 mg; 15%) at A+PF and anthesis stages, respectively, over control (Fig. 20A). Spraying only at PF stage led to a P leaf concentration decreased by 36% and 12% for Fe-citrate (4 mM) and nano-Fe (4 mM), respectively, as compared to control. HA (25 mg) + $FeCl_3$ (2 mM; 11.5% and 11.5%), along with HA (50 mg; 6.16% and 7.31%), had similar increases in leaf P concentration at A+PF and PF stages. Leaf P concentration decreased in plants sprayed with Fe-citrate (4 mM) by 36% at PF stage, with KH_2PO_4 (2 Mm) by 18% at A+PF and with nano-Fe (4 mM) by 20% at PF stage. In stem tissue, P concentration was lowest with application of nano-Fe (4 mM) at all stages (Fig. 20B). The highest increase in stem P concentration was with Fe-citrate (4 mM; 49%) and $FePO_4.2H_2O$ (2 mM; 60%) at anthesis stage, with KH_2PO_4 (2 mM) at anthesis (65%) and PF (56%) stages, and with HA (25 mg) + $FeCl_3$ (2 mM; 50%) at A+PF. Stem P concentration decreased only with application of HA (50 mg) by 16% at PF stage and nano-Fe (4 mM) by 6.3% at anthesis and 8.8% at PF stages. Stem P averaged over stages was maximum with the KH_2PO_4 (2 mM) treatment.

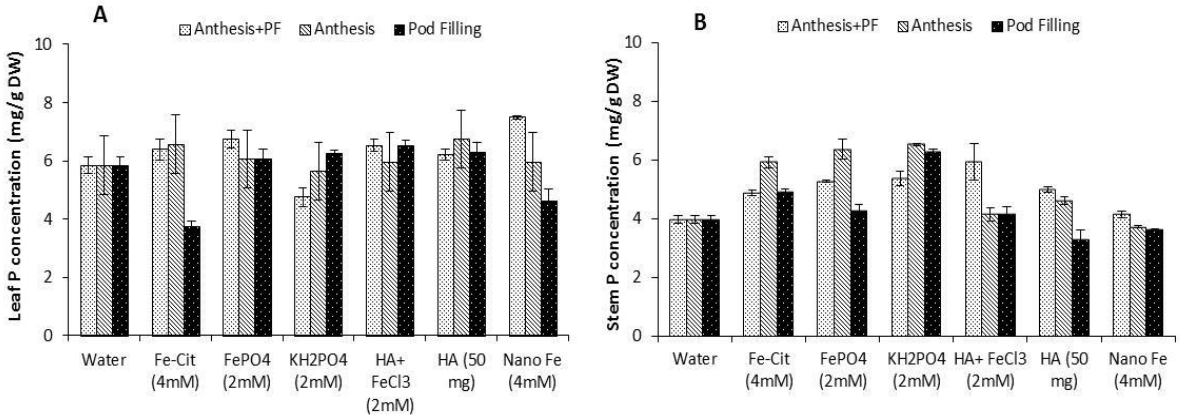

Figure 20. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on tissue phosphorus concentration of (A) leaf and (B) stem.*

Similar to tissue P concentration, the leaf P content also decreased with KH_2PO_4 (2 mM) at all stages (Fig. 21A). Foliar application of Fe-citrate (4 mM) and nano-Fe (4 mM) at PF stage resulted in a decrease in leaf P content of 41% and 22%, respectively. Among treatments, the highest increase in leaf P uptake was recorded with HA (50 mg) and HA (25 mg) + $FeCl_3$ (2 mM) at 52% and 43%, respectively, at anthesis stage as compared to control. However, it was lowest with KH_2PO_4 (2 mM) in all the stages. P content in stem decreased by 11% at PF and 7% at anthesis with HA (50 mg) and nano-Fe (4 mM), respectively (Fig. 21B). The highest increase was recorded for all the treatments at anthesis stage, and except for KH_2PO_4 (2 mM), all treatments resulted in a higher stem P content at A+PF as compared to PF alone. Among treatments, the highest increase in stem P uptake was recorded with $FePO_4.2H_2O$ (2 mM) at 160%, followed by HA (50 mg) at 54%, Fe-citrate (4 mM) at 51%, and KH_2PO_4 (2 mM) at 46% and HA (25 mg) + $FeCl_3$ (2 mM) at 46%.

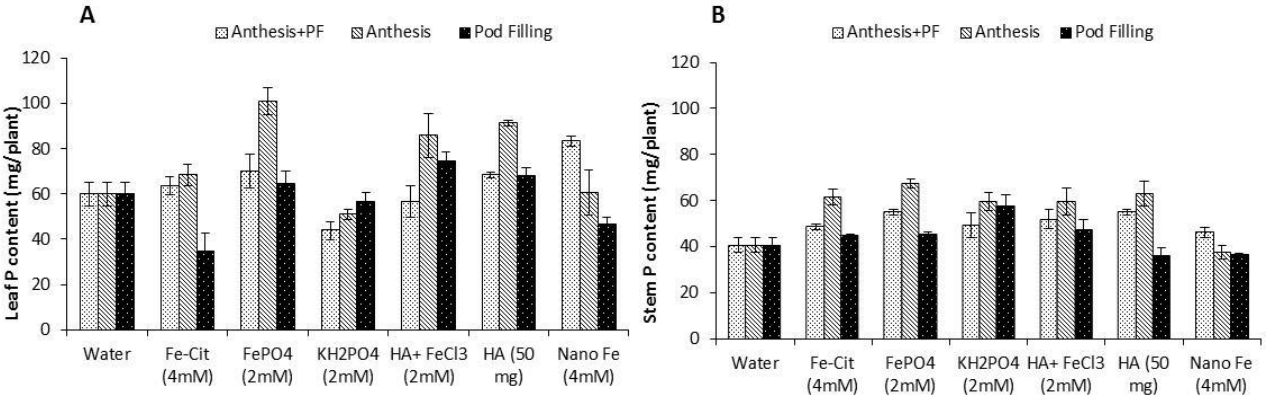

Figure 21. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on phosphorus content or uptake in (A) leaf and (B) stem.*

Total shoot P uptake (stem and leaf P content) decreased by 20%, 7%, 2% and 17% with application of Fe-citrate (4 mM) at PF stage, KH_2PO_4 (2 Mm) at A+PF and nano-Fe (4 mM) at anthesis and PF stages, respectively (Fig. 22B). Similar to leaf Fe uptake, total shoot Fe uptake also increased by more than 50% with Fe-citrate (4 mM) at A+PF and PF stages, with $FePO_4.2H_2O$ (2 mM) at all stages, with HA (25 mg) + $FeCl_3$ (2 mM) at anthesis, with HA (50 mg) at all stages and with nano-Fe (4 mM) at A+PF (Fig. 22A). The increase was highest with HA (25 mg) + $FeCl_3$. The lowest total shoot Fe content was recorded with KH_2PO_4 (2 mM) application as compared to all other treatments.

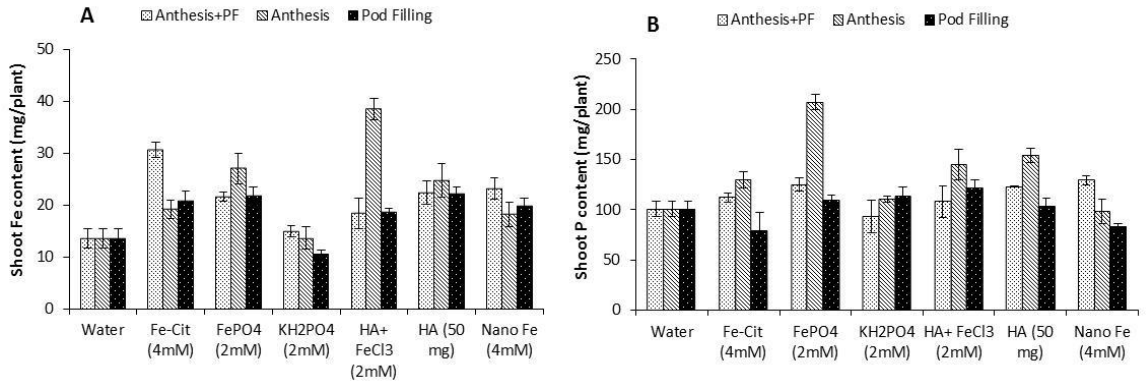

Figure 22. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on (A) Fe content in shoot and (B) P content in shoot*

The seed Fe concentration and total content in seed was significantly influenced by foliar treatment (Fig. 23). The maximum increase in seed Fe concentration (39%) was recorded with Fe-citrate (4 mM) application at A+PF as compared to control (Fig. 23A). With $FePO_4.2H_2O$ (2 mM), the increase in seed Fe concentration was 11-13% at all growth stages. Application of nano-Fe (4 mM) resulted in a more than 20% increase in seed Fe concentration at all stages, while HA (50 mg) had the highest increase at A+PF (13%). The total seed Fe content or uptake increased at A+PF by more than 2.3-fold with HA (25 mg) + $FeCl_3$ (2 mM), Fe-citrate (4 mM) and nano-Fe (4 mM; Fig. 23B). There was a 9% reduction in total seed Fe content with HA (50 mg) application at A+PF stage, though it had the highest increase among the treatments at anthesis stage. A decrease in total seed Fe content was recorded with Fe-citrate (4 mM), KH_2PO_4 (2 mM), HA (50 mg) and nano-Fe (4 mM) at PF stage. Maximum seed Fe content was recorded at A+PF stage with all foliar treatments except HA (50 mg), which had its maximum increase at anthesis, as compared to control.

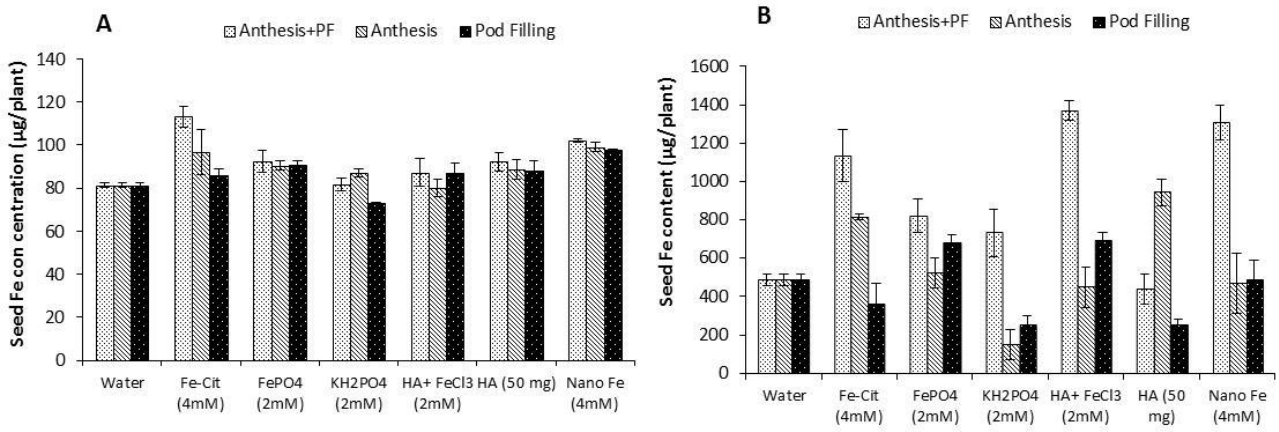

Figure 23. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on (A) Fe concentration in seed and (B) total Fe content in seed*

The P concentration (Fig. 24A) in seed was not significantly influenced by foliar treatment, while total seed P content was significantly affected by treatment and growth stage. Increased seed P content was recorded in plants sprayed at A+PF with all treatments except HA (50 mg), which showed higher seed P content at anthesis stage, as compared to control (Fig. 24B). Seed P content increased by more than 90% with nano-Fe (4 mM) and HA (25 mg) + $FeCl_3$ (2 mM), both at A+PF, while a 40-65% increase was recorded with Fe-citrate (4 mM) at A+PF and anthesis, $FePO_4.2H_2O$ (2 mM) at A+PF and HA (50 mg) at anthesis stage.

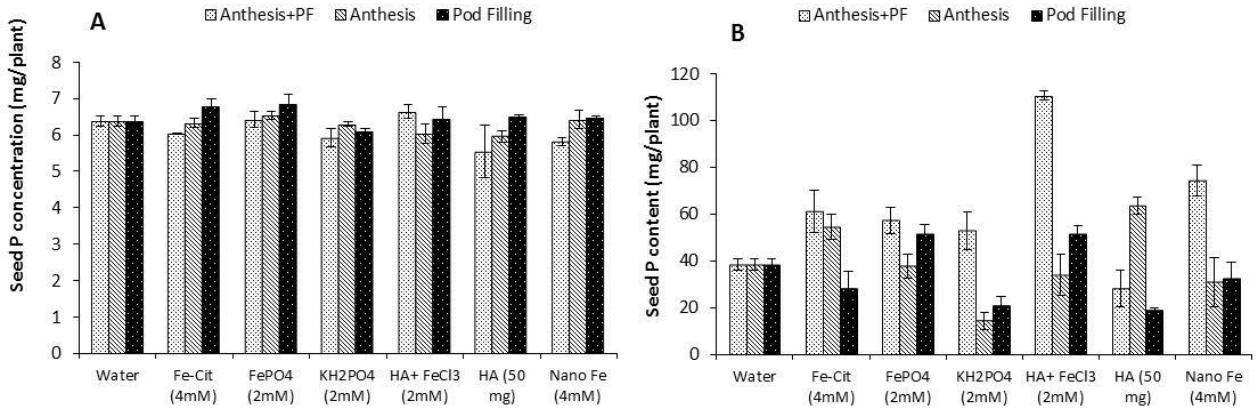

Figure 24. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on (A) P concentration in seed and (B) total P content in seed*

2.2.2.6 Effect of foliar treatment on physiological parameters in soybean

Total shoot biomass (leaf and stem) increased at PF stage with $FePO_4.2H_2O$ (2 mM) application by 63% compared to control, followed by HA (25 mg) + $FeCl_3$ (2 mM) at 40% and HA (50 mg) at 33% (Fig. 25A). At other stages, shoot biomass either decreased or increased very little as compared to control. The lowest shoot biomass was recorded with KH_2PO_4 (2 mM) at all stages.

All foliar treatments resulted in a significant increase in total leaf area as compared to control (Fig. 25B). Maximum leaf area was recorded at anthesis stage with all treatments except nano-Fe (4 mM). Foliar application of Fe-citrate (4 mM) and $FePO_4.2H_2O$ (2 mM) resulted in a more than 80% increase in leaf area at all stages (A, PF and A+PF), while with HA (25 mg) + $FeCl_3$ (2 mM) application, increase in leaf area was recorded only at anthesis stage. However, HA (25 mg) + $FeCl_3$ (2 mM) also resulted in a more than 50% increase in leaf area at other stages. Treatment with nano-Fe (4 mM) showed the lowest increase.

Total chlorophyll concentration was not significantly influenced by any treatment except KH_2PO_4 (2 mM). There was a reduction in chlorophyll by 12% and 15% at anthesis and PF, respectively, with HA (50 mg) treatment (Fig. 25C). Chlorophyll increased in plants sprayed with KH_2PO_4 (2 mM) at PF stage.

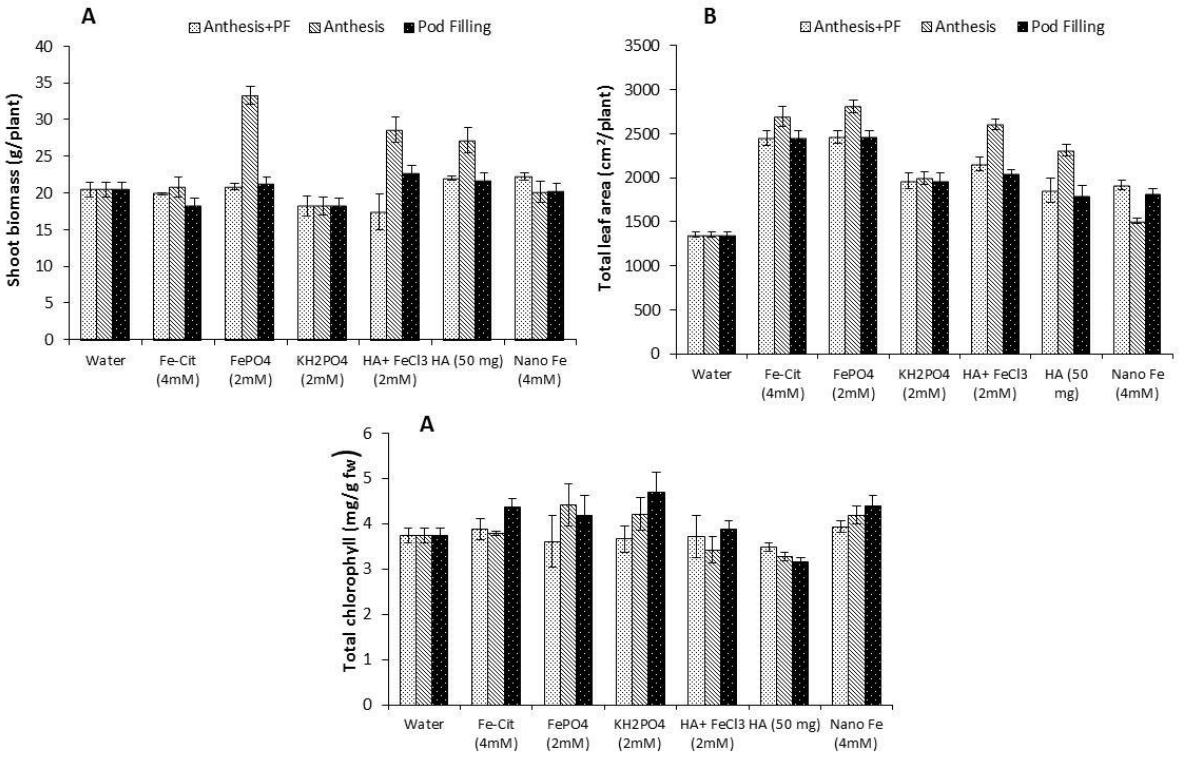

Figure 25. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on (A) total shoot biomass, (B) total leaf area and (C) leaf chlorophyll concentration*

2.2.2.7 Effect of foliar treatment on yield parameters in soybean

Significant influence of foliar treatment and growth stage was observed on yield traits of soybean (Fig. 26). The number of pods decreased by 34%, 21% and 17% with KH_2PO_4 (2 mM) at anthesis, HA (50 mg) at PF and nano-Fe (4 mM) at anthesis, respectively (Fig. 26A). The maximum increase in pod number was obtained at A+PF stage with all treatments except HA (50 mg). The highest pod number per plant was achieved with HA (25 mg) + $FeCl_3$ (2 mM), followed by nano-Fe (4 mM) and $FePO_4.2H_2O$ (2 mM).

Total pod weight (Fig. 26B), which includes pod cover and seeds, and total seed weight per plant (Fig. 26C) increased significantly at A+PF stage with all treatments except HA (50 mg). Both of these traits showed a similar trend with the maximum increase with HA (25 mg) + $FeCl_3$ (2 mM) by 154%, followed by nano-Fe (4 mM) by 95% over control. However, spraying with KH_2PO_4 (2 mM) resulted in a reduction in seed weight. Test weight, or 100-seed weight, significantly increased for all treatments at all stages as compared to control (Fig. 26D). Test weight increased by more than 30% with HA (50 mg) applied at A+PF and anthesis stages, nano-Fe (4 mM) applied at A+PF stage and $FePO_4.2H_2O$ (2 mM) applied at PF stage.

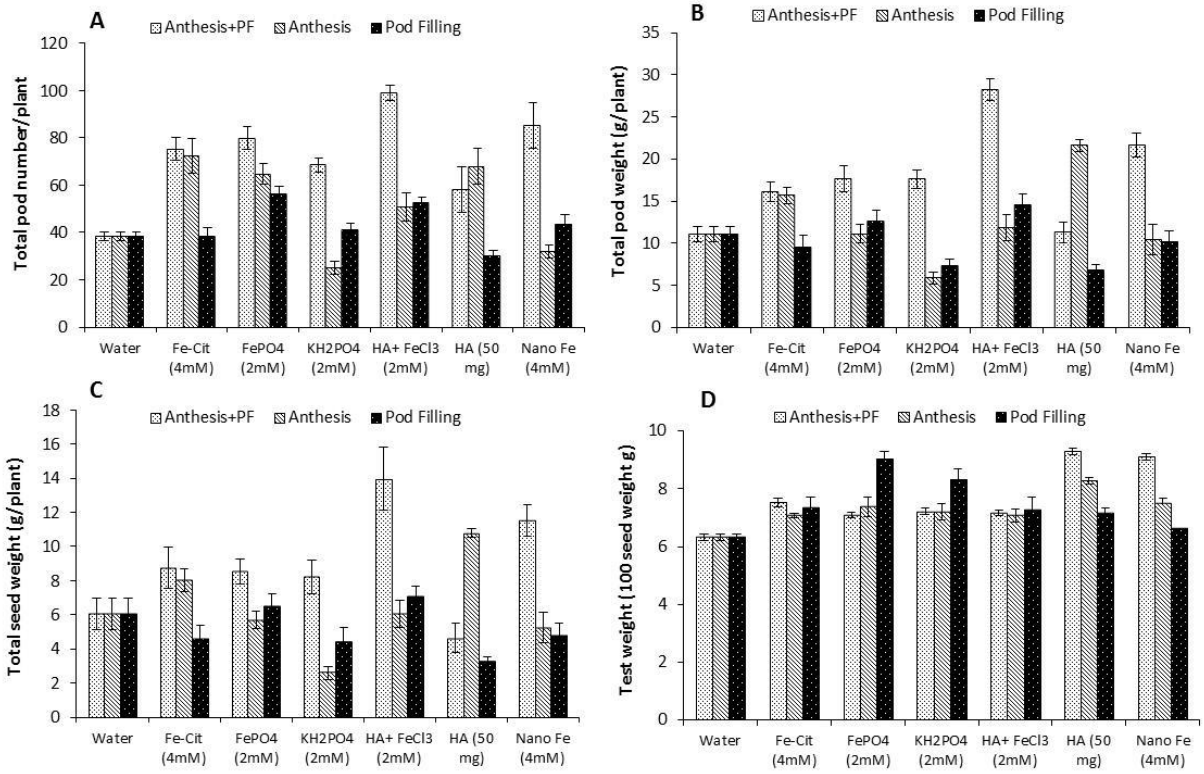

Figure 26. *Effect of foliar application of various Fe and P compounds on soybean at different growth stages on yield attributes: (A) total pod number per plant, (B) total pod weight including seed, (C) total seed yield per plant and (D) test weight.*

2.2.2.8 Summary of soybean experiment

- Maximum grain Fe and P content were observed when spraying was done at both anthesis and pod-filling stages, so this is the best way in which to increase grain Fe content.
- HA at the higher concentration (50 mg) sprayed at a single stage (anthesis) is the best treatment to increase grain Fe concentration.
- Foliar application of HA+$FeCl_3$ resulted in increased total seed weight per plant, pod number and pod weight, which might be due to increased translocation of Fe and P toward grain. This treatment resulted in reduced shoot biomass but increased leaf area.
- Fe-citrate (4 mM) resulted in increased grain Fe and P content – less than with HA+$FeCl_3$ but more than with $FePO_4$. Shoot biomass decreased, and leaf area increased.
- Fe uptake in stem was the same with $FePO_4$ and Fe-citrate (4 mM). Fe-citrate had higher Fe uptake in shoot but lower P uptake in shoot than $FePO_4$.
- Finally, application at A+PF is the best way to obtain maximum Fe partitioning toward grain and plant growth; among treatments, the chemical compounds found to be better for soybean were in the order HA+$FeCl_3$ > Fe-citrate > $FePO_4$.

2.3 Foliar application of organic Fe (Fe enriched bacteriosiderophore) on soybean conducted in the field

2.3.1 Materials and methods

2.3.1.1 Evaluation of bacterial strain producing siderophore and enriching with Fe

Siderophores are low molecular weight (~400-1000 Da) iron-binding protein molecules that bind with ferric iron and transport it into the plant cell. Twenty siderophore-producing bacterial cultures (Table 10) were collected from the Division of Microbiology, ICAR-IARI, New Delhi, and screened for bacteriosiderophore production.

2.3.1.2 Quantification of siderophore

Bacterial cultures were grown in Luria agar plates at 37°C for 24 hours and sub-cultured in Luria broth by picking a single colony from plates. The medium was composed of 6.0 g L^{-1} K_2HPO_4, 3.0 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4$, 1.0 g L^{-1} NH_4SO_4 and 4.0 g L^{-1} succinic acid with a pH of 7.0. For siderophore production, 0.1 mL of culture from each strain was inoculated into 50 mL of iron-deficient SM medium and incubated for 48 hours at 37°C with constant shaking at 120 rpm (Sayyed et al., 2004). Following incubation, bacterial growth was measured by reading absorbance at $OD_{600\ nm}$, and an OD of 1.0 was used for siderophore detection.

Table 10. *Bacterial strains used for evaluation of siderophore production in minimal media*

S. No.	Strain No.	Bacteria	Accession No.
1	IARI-R-6	*Aeromonas hydrophila*	JX429038
2	IARI-R-12	*Pseudomonas xanthomarina*	JX429041
3	IARI-R-45	*Arthrobacter sp.*	JX429024
4	IARI-R-46	*Arthrobacter psychrolactophilus*	JX429025
5	IARI-R-48	*Pseudomonas extremaustralis*	JX429042
6	IARI-R-50	*Janthinobacterium lividum*	JX429043
7	IARI-R-53	*Pseudomonas cedrina*	JX429044
8	IARI-R-57	*Pseudomonas fragi*	JX429045
9	IARI-R-59	*Pseudomonas tolaasii*	JX429046
10	IARI-R-64	*Pseudomonas trivialis*	JX429047
11	IARI-ABL-35	*Brachybacterium sp.*	KC581678
12	IARI-ABL-29	*Klebsiella sp.*	KC581672
13	IARI-L-2	*Lysinibacillus fusiformis*	JF343177
14	IARI-L-16	*Arthrobacter sulfonivorans*	JN411436
15	IARI-L-21	*Bacillus firmus*	JF343183
16	IARI-L-46	*Desemzia incerta*	JF343203
17	IARI-L-54	*Bacillus pumilus*	JF343193
18	IARI-L-109	*Pseudomonas putida*	JN411453
19	IARI-B-6	*Bacillus sp.*	JN411378
20	IARI-S-24	*Chryseobacterium haifense*	JX460826

Detection of siderophore was carried out quantitatively by liquid chrome azurol sulphonate (CAS) assay (Schwyn and Neilands, 1986). The bacterial cells were pelleted by centrifugation at 3,000 rpm for 15 minutes, and 0.5 mL of supernatant was mixed with 0.5 mL of CAS solution and 10 µL of sulfosalicylic acid. Absorbance was recorded at 630 nm after incubation for 20 minutes in the dark. The change in color of the blue CAS assay solution to purple-orange indicated the presence of siderophore. A blank (minimal medium) and reference solution (minimal medium, CAS dye and sulfosalicylic acid) were used. The amount of the siderophore released was determined using the following formula:

$$Percentage\ siderophore = \frac{Absorbance\ reference - Absorbance\ sample}{Absorbance\ reference}\ x\ 100$$

Based on the quantity of siderophore production and growth of bacteria, we selected two contrasting bacterial strains, one with high siderophore production, *Lysinibacillus fusiformis* (Acc. No. JF343177, BS13) and another with low siderophore production, *Arthrobacter sp.* (Acc. No. JX429024, BS3) (Fig. 27). For foliar application, 1.0 mL of culture of these strains in Luria broth were separately inoculated into each 1.0 L SM medium and incubated for 48 hours at 37°C with constant shaking. Siderophore content was checked by CAS assay. To enrich the bacterial culture with Fe, 500 mL of each culture was incubated with 2 mM of $FeCl_3$ for 90 minutes at room temperature, and the remaining 500 mL of each culture was used as such. Therefore, four siderophore treatments (*L. fusiformis* and *Arthrobacter* sp. with and without Fe) were used with water spray as the control. The pH of the spray solution was made 6.0 using HCl or KOH and adding 50 µl of surfactant (Triton X100) before application on plants.

The experiment on the soybean crop was carried out in the experimental field during the summer season of 2016. Soybean (var. DS-2614) seeds were sown in the field. At flowering stage, soybean plants were sprayed with four bacteriosiderophore (two Fe-enriched, two without Fe) solutions along with a water control before noon. Growth and yield parameters were recorded in the same manner as mentioned earlier.

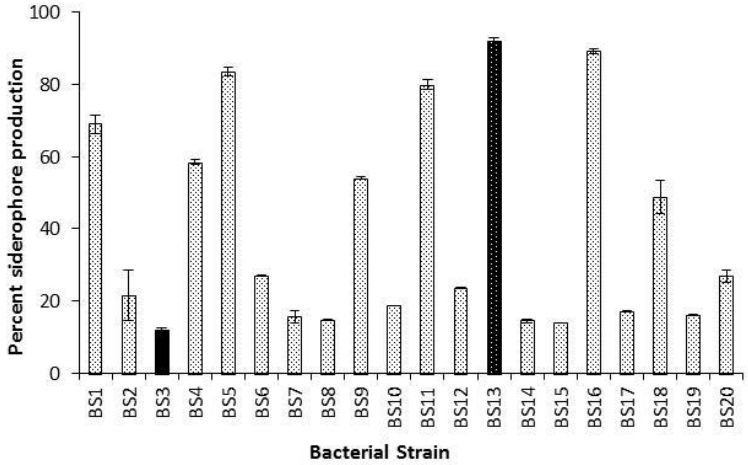

Figure 27. *Quantification of siderophore production by different bacterial strains in Fe-deficient media. Dark color bars indicate for iron enrichment and foliar application.*

2.3.2 Results of organic Fe application

Application of organic Fe (bacteriosiderophore-Fe) as a foliar treatment shows significant variation in terms of physiological and yield traits. Figure 28 presents the phenotype of soybean plants sprayed with bacteriosiderophore with and those without Fe enrichment. An increase in leaf area by 23% (Fig. 29A) and total aboveground biomass by 35% (Fig. 29B) were recorded in plants sprayed with *L. fusiformis* + $FeCl_3$ as compared to control. The lowest values for both of these parameters were observed in plants sprayed with *L. fusiformis* without Fe as compared to control.

Figure 28. *Soybean plants sprayed using bacteriosiderophores enriched with iron or without iron. High BS – high siderophore-producing bacterial strain (L. fusiformis), Low BS - low siderophore-producing bacterial strain (Arthrobacter sp.).*

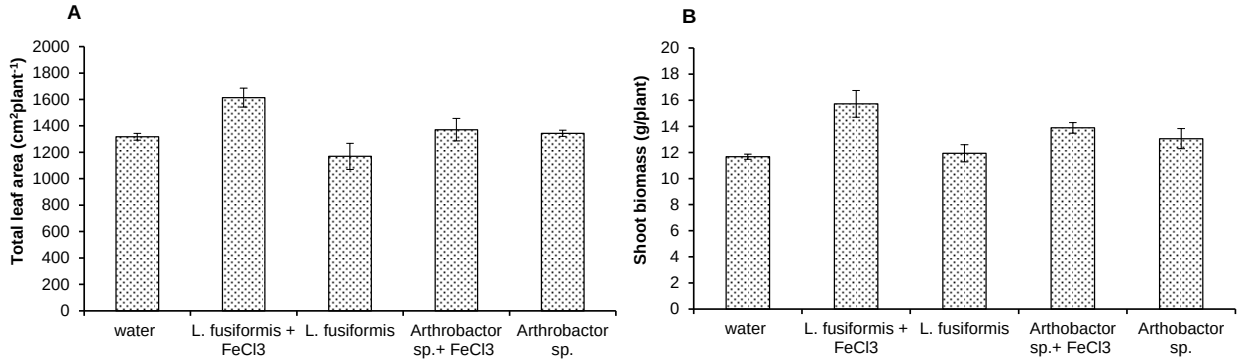

Figure 29. *Effect of bacteriosiderophore + Fe enrichment on soybean plant (A) total leaf area and (B) total aboveground biomass. L. fusiformis – high siderophore-producing bacterial strain; Arthrobacter sp. – low siderophore-producing bacterial strain.*

The leaf and stem tissue concentrations of P and Fe were significantly influenced by bacteriosiderophore spray (Fig. 30A, B). As compared to control, there was a slight increase in leaf P concentration when plants were sprayed with *L. fusiformis* + $FeCl_3$ (8%) and *Arthrobacter sp.*+ $FeCl_3$ (5%). However, stem P concentration was highest using *Arthrobacter sp.* with or without Fe enrichment. Interestingly, increased Fe concentration in leaf (19%) and stem (138%) was observed with *L. fusiformis* + $FeCl_3$ as compared to control.

Bacteriosiderophore foliar application significantly influenced the content of P and Fe in soybean plants (Fig. 31A, B). A similar trend was observed for total content of P and Fe in leaf, stem and total shoot in plants sprayed with either of the bacterial strains without Fe enrichment. However, addition of $FeCl_3$ to bacteriosiderophore showed increased content of P and Fe as compared to control. The plants sprayed with *L. fusiformis* + $FeCl_3$ had a 24%, 45% and 33% increase in leaf, stem and shoot P concentration, respectively, and Fe content increased by more than 1.6-fold.

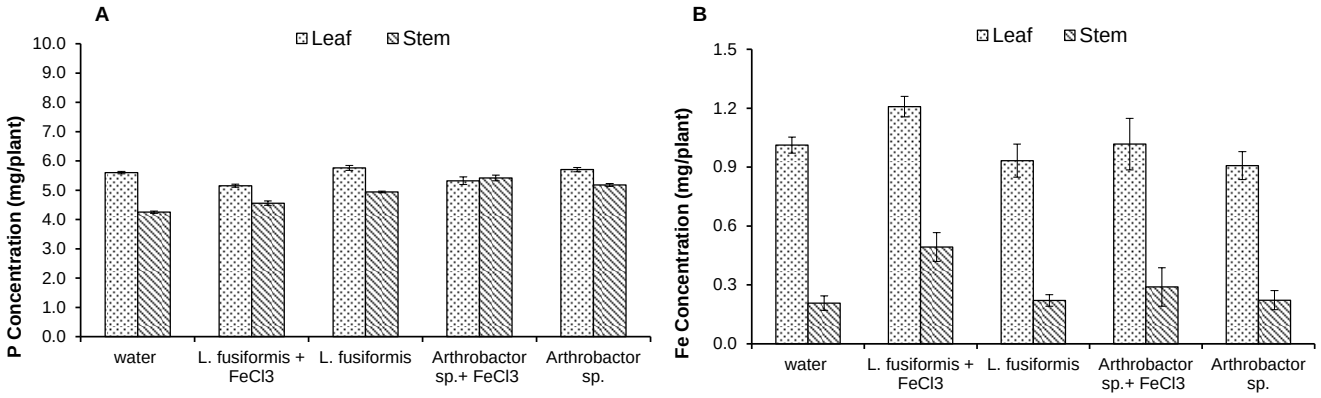

Figure 30. *Effect of bacteriosiderophore + Fe enrichment on soybean plants (A) tissue P concentration and (B) tissue Fe concentration. L. fusiformis – high siderophore-producing bacterial strain; Arthrobacter sp. – low siderophore-producing bacterial strain*

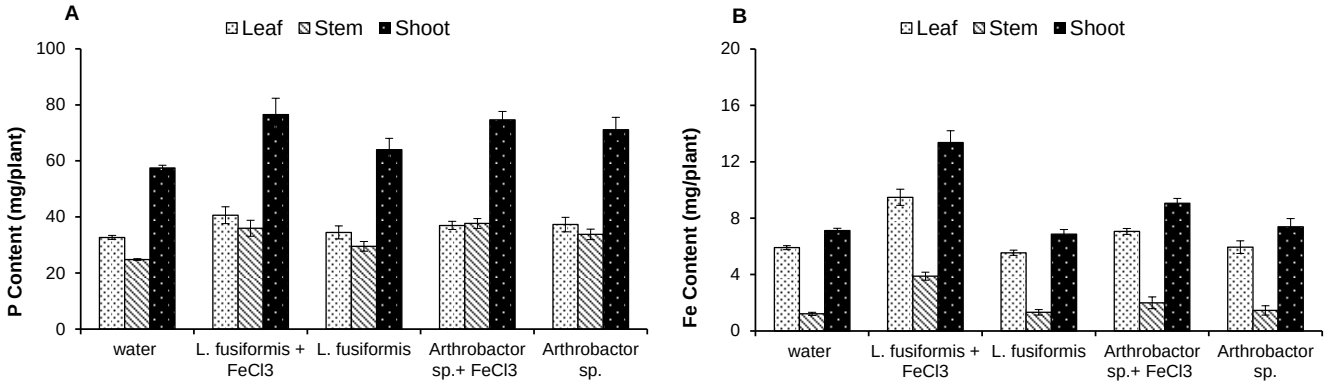

Figure 31. *Effect of bacteriosiderophore + Fe enrichment on soybean plant (A) P uptake or content and (B) Fe uptake or content. L. fusiformis – high siderophore-producing bacterial strain; Arthrobacter sp. – low siderophore-producing bacterial strain*

Along with the increase in leaf P and Fe content, a substantial increase in pod number, total seed weight per plant and 100-seed weight was recorded with *L. fusiformis* + $FeCl_3$ at 34%, 21% and 45%, respectively, as compared to control (Fig. 32A, B, C).

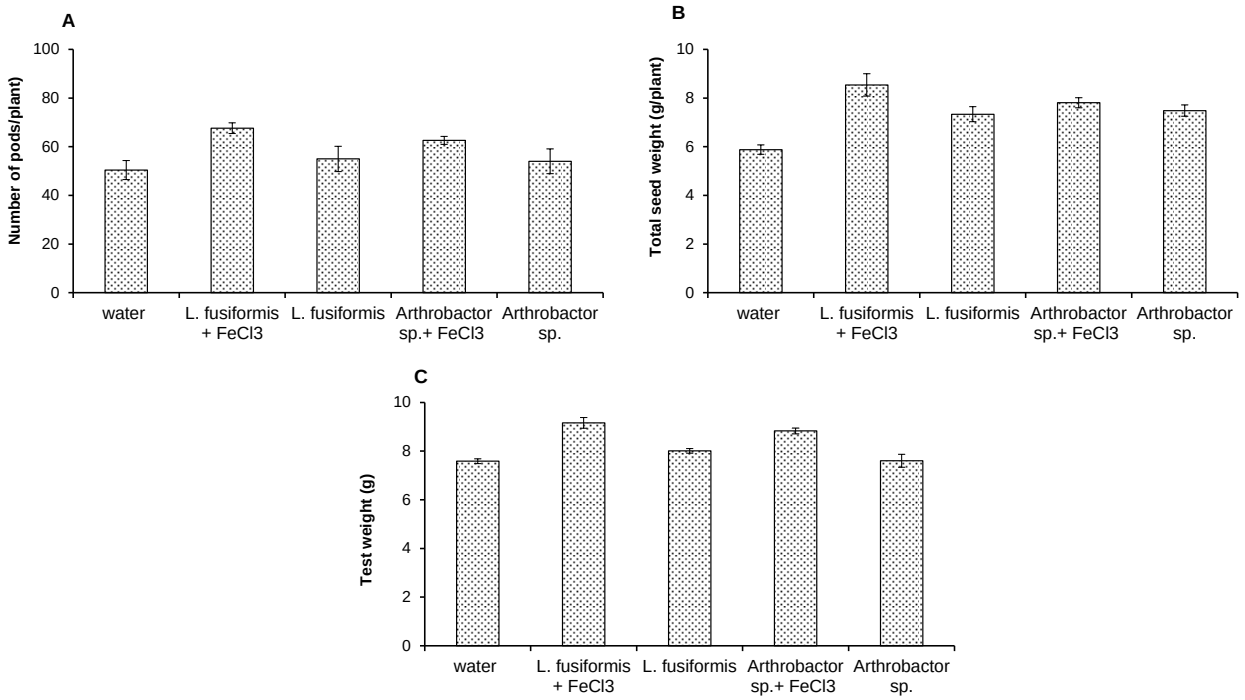

Figure 32. *Effect of bacteriosiderophore + Fe enrichment on yield traits of soybean plant (A) number of pods per plant, (B) total seed weight per plant and (C) 100-seed weight. L. fusiformis – high siderophore-producing bacterial strain; Arthrobacter sp. – low siderophore-producing bacterial strain*

Thus, these data suggest that adding Fe to *L. fusiformis,* the high bacteriosiderophore-producing strain, improves the penetration of Fe after its foliar spray. There are reports of bacteriosiderophore application improving plant growth and nutrient uptake (P and Fe) by its application in soil, but no report is available on its application as a foliar spray after enrichment with Fe. Bacteriosiderophores also contain some plant growth-regulating hormone, which helps improve the growth of plants. Therefore, for both the bacterial strains, we took cultures enriched with Fe and those without Fe and observed that addition of Fe significantly improves growth, nutrient uptake and yield in soybean. This is also evident from the increased Fe content in plants. Also, in terms of yield, the performance of *L. fusiformis* + $FeCl_3$ was the best among the four treatments.

The highest Fe concentration was recorded in the leaf tissue of plants sprayed with *Arthrobacter sp.* + $FeCl_3$, while the lowest Fe was recorded in the leaf of the plants sprayed with the high siderophore-producing strain *L. fusiformis* + $FeCl_3$. The percentage increase of Fe concentration was highest in the stem (113%), pod cover (39%) and seeds (50%) of plants sprayed with *L. fusiformis* + $FeCl_3$ as compared

to control. The concentration of Fe in the stem, pod cover and seeds of plants sprayed with *Arthrobacter sp.* +$FeCl_3$ increased by only 94%, 12% and 17%, respectively. This indicates that the *L. fusiformis* strain helps in translocation of Fe toward the seed, while in the case of *Arthrobacter sp.*, most of the Fe did not move toward the seed but remained in the leaf.

The maximum increase in P concentration in leaf tissue was recorded with application of *L. fusiformis* (176%), followed by *Arthrobacter sp.* (139%) and *Arthrobacter sp*. + $FeCl_3$ (91%), while the lowest was recorded for *L. fusiformis* + $FeCl_3$ (73%), as compared to control. Treatment with *Arthrobacter sp.* and *L. fusiformis* showed maximum increase in stem P concentration by 117% and 94% as compared with control. The concentration of P in seed also increased with all treatments as compared to control; however, not much difference was observed among them. Foliar application of *L. fusiformis* + $FeCl_3$, *L. fusiformis, Arthrobacter sp*. + $FeCl_3$ and *Arthrobacter sp.* increased P concentration in seed by 61%, 69%, 67% and 76%, respectively, as compared to control.

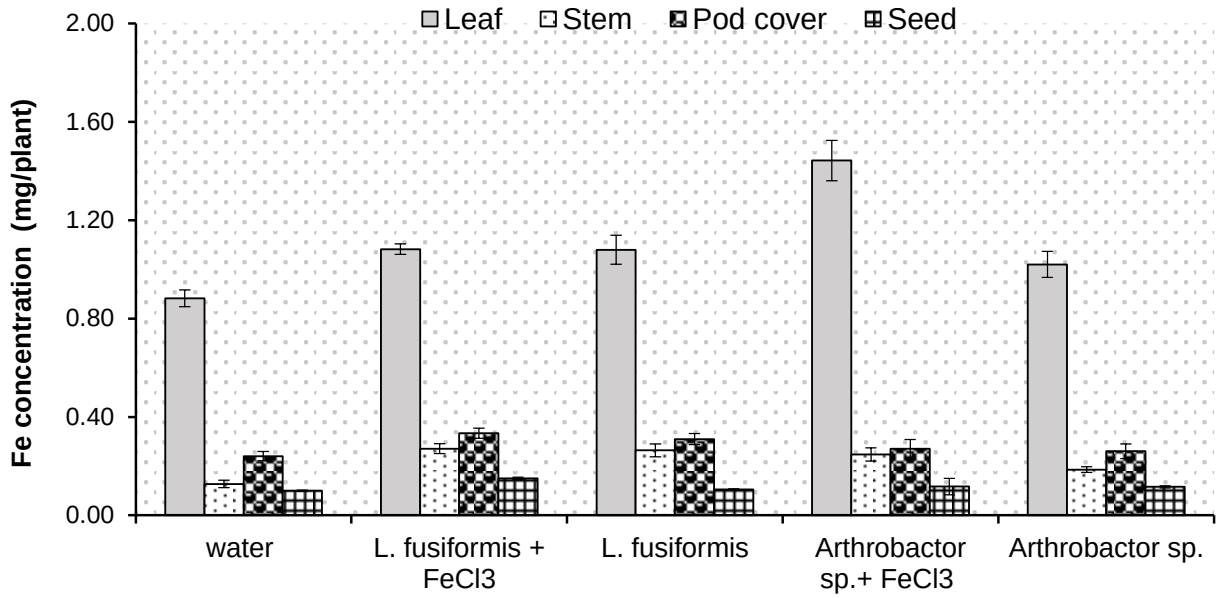

Figure 33. *Influence of foliar application of bacteriosiderophore with and without Fe enrichment on Fe concentration and Fe content in seed per plant of soybean. Data correspond to mean ± SEm (n=5).*

2.4 Physiological basis of selection of chemical compounds suitable for use as foliar spray on crop plants

Spraying foliage with chemical compounds might subject the plants to stress, which could be oxidative stress. We studied the antioxidant scavenging system by analyzing the generation of reactive oxygen species (ROS; superoxide radical, hydrogen peroxide, hydroxyl radical) and the various enzymes (SOD, CAT, ascorbate peroxidase [APOX], GR) involved in detoxification. The excessive production of ROS due to stress causes oxidative damage to the cell membranes and, thus, cell death. The superoxide

radical is scavenged by SOD to H_2O_2, which is converted to water by action of CAT, APOX and GR enzymes. Ascorbate scavenges H_2O_2 non-enzymatically via the ascorbate-glutathione pathway. The experiment was conducted on soybean with the objective to understand the response of plants to the chemical compounds applied as foliar spray and the way plants respond to the concentration of these compounds. This study will help to select the chemical compounds and their concentration suitable for foliar application. The methodology was described previously.

Results show that spraying with all Fe formulations decreased the quantity of superoxide radical production as compared to control. The maximum reduction was recorded with Fe-citrate (4 mM), followed by $FePO_4$ (2 mM) and HA (50 mg; Fig. 34A). However, foliar application of KH_2PO_4 (2 mM) and nano-Fe (4 mM) produced the same level of superoxide radical production as control. The greatest increase in H_2O_2 level was recorded with HA (50 mg), followed by Fe-citrate (4 mM; Fig. 34B). High levels of H_2O_2 in cells indicate that it was not converted to water. A reduction in TBARS was observed for all treatments, except KH_2PO_4 (2 mM) and nano-Fe (4 mM), which showed increased TBARS content by 35% and 32%, respectively (Fig. 34C). Foliar application of $FePO_4$ (2 mM), Fe-citrate (4 mM), HA (50 mg) and HA (25 mg) + $FeCl_3$ (2 mM) resulted in either a decrease or non-significant effect in ascorbate levels as compared to control (Fig. 34D). But foliar application of nano-Fe and KH_2PO_4 resulted in higher ascorbate levels, indicating they are not efficiently utilized in neutralizing the H_2O_2, which is also justified as the GR activity in these treatments was very low. Production of excess ROS indicates that the plant cells undergo oxidative stress. Higher levels of TBARS indicate higher lipid peroxidation, leading to cell membrane damage, thereby a loss of membrane selectivity.

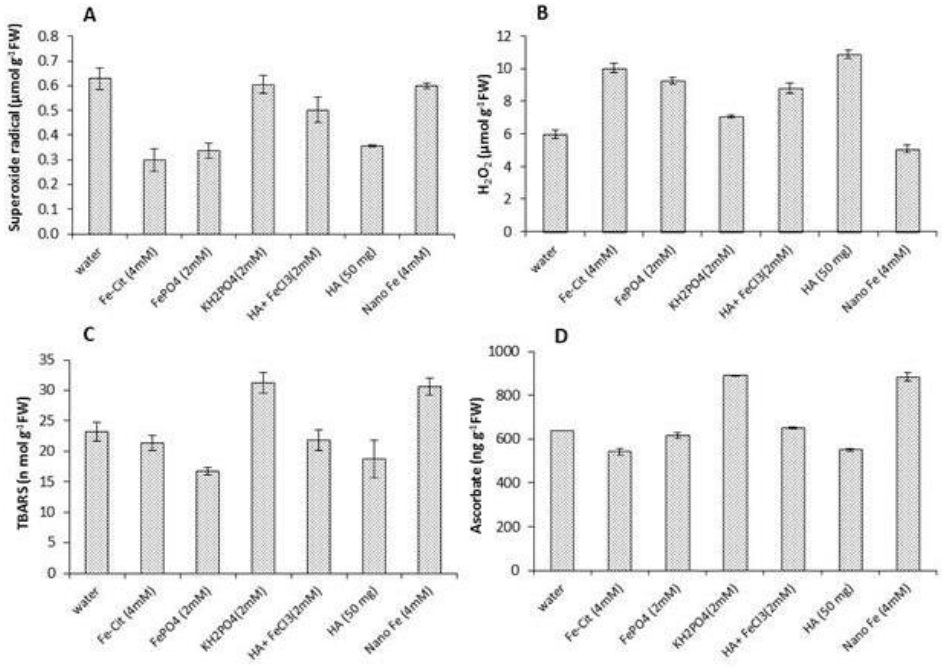

Figure 34. *Effect of various chemical compounds applied as foliar spray on soybean leaves in terms of (A) superoxide radical production, (B) hydrogen peroxide generation, (C) thiobarbituric acid reactive substances and (D) ascorbate*

All foliar Fe treatments in this study showed a significant effect on the activities of antioxidant enzymes, viz., SOD, CAT, APOX and GR (Fig. 35A-D). Nano-Fe (4 mM) did not show any increase in the levels of SOD, GR and APX activities, but an increase in CAT activity by 19% as compared to control was observed (Fig. 35A). The highest increase in CAT and SOD activities was recorded with Fe-citrate (4 mM) at 49% and 41%, followed by HA (50 mg) at 47% and 51%, respectively. APOX activity decreased by 30% and 50% in leaves of plants sprayed with KH_2PO_4 (2 mM) and nano-Fe (4 mM), respectively, and the highest APOX activity was recorded with $FePO_4$ (100%; Fig. 35C), indicating that plants efficiently scavenged the ROS. Similarly, the maximum increase in GR activity was recorded with HA (50 mg) by 3.2-fold, followed by HA (25 mg) + $FeCl_3$ (2 mM) by 2.7-fold, while reduced activity was observed with KH_2PO_4 and nano-Fe (4 mM) application (Fig. 35D), indicating that these two chemicals are not favorable as foliar spray in these concentrations.

In conclusion, SOD activity was maximum with Fe-citrate, but APOX activity was lower than with $FePO_4$ and HA+$FeCl_3$, so the H_2O_2 concentration was higher. GR activity was also maximum with HA + $FeCl_3$. Plants sprayed with these three treatments were able to combat oxidative stress due to the activation of one or more attributes of the antioxidant defense system, while maximum membrane damage due to lipid peroxidation in plants sprayed with nano-Fe (4 mM) and KH_2PO_4 was seen. Further, analysis of the antioxidant-scavenging enzyme system indicates that a higher concentration of Fe or P applied to foliage subjected the plants to oxidative stress, resulting in less biomass accumulation and/or yield. Thus, the results suggest the difference in antioxidant enzyme activities as one of the physiological bases of plant response to foliar application of nutrients.

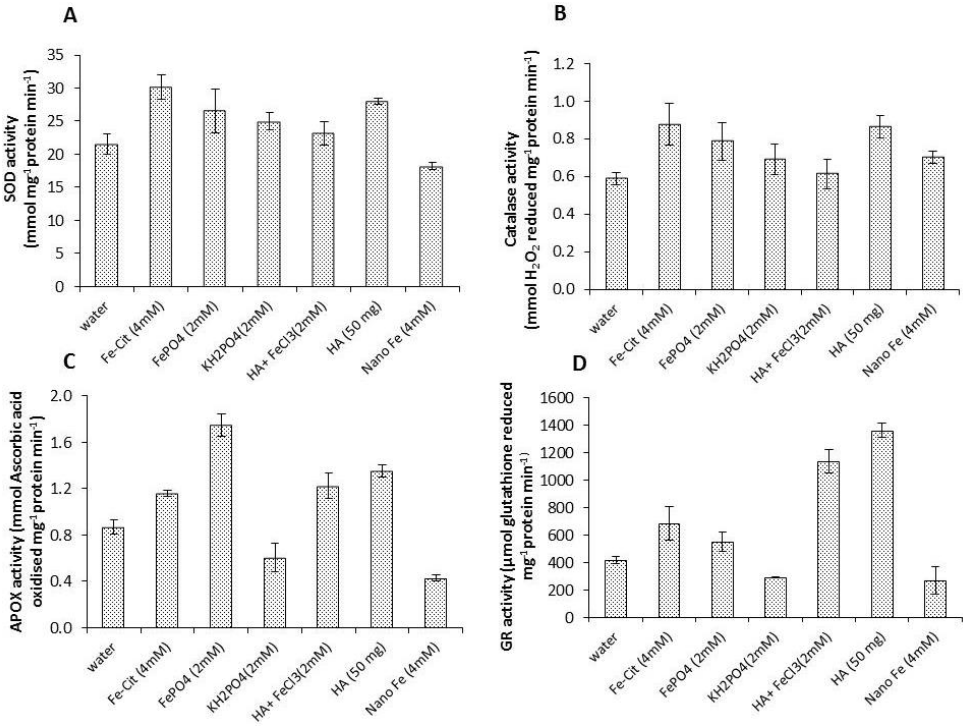

Figure 35. *Effect of various chemical compounds applied as foliar spray on soybean leaves in terms of enzyme activity: (A) superoxide dismutase, (B) catalase, (C) ascorbate peroxidase and (D) glutathione reductase*

3 Identification of novel proteins expressed in leaf and involved in Fe absorption and translocation (Objective 2)

3.1 Materials and methods

3.1.1 Plant material and growth conditions

Seeds of rice and soybean varieties were washed with double distilled water and surface sterilized using 0.1% $HgCl_2$ for 2 minutes. The surface-sterilized seeds were rolled in germination paper moistened with 0.1M $CaCl_2$ and kept inside an incubator at 28°C for germination. After emergence of coleoptiles, plants were transferred to complete nutrition solution, which was changed every third day. The solution was aerated continuously. The experiment was conducted in a glasshouse at National Phytotron Facility (NPF), New Delhi. Fifteen days after transplanting, the nutrient solution was changed based on the Fe or P depletion experiments. The treatments include Fe deficient (-Fe), Fe and P deficient (-Fe/-P) and a control (with complete nutrients). For the recovery experiment, the Fe/FeP-deficient plants were supplied with a 2 mM solution of Fe-citrate as an iron source and $FePO_4$ as a phosphorus and iron source. Samples were collected at different time intervals (in hours), i.e., 0 (before foliar spray), 0.5, 2, 24 and 48, after foliar application and stored in a deep freezer until further use.

3.1.2 Isolation of RNA and cDNA synthesis

Total RNA isolation from leaf tissue was done using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. The purified RNA after DNase treatment was re-suspended in 30 µL of RNAse-free water. The purity of extracted RNA was checked on 1% agarose gel (Fig. 36), and quantification was done using a NanoDrop2000 spectrophotometer. cDNA were synthesized from each sample using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and stored at -20°C for further study.

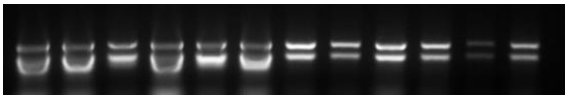

Figure 36. *Extraction of RNA from soybean leaves with Fe- and P-deficient treatments and those under restoration conditions.*

3.1.3 Primer designing

The gene-specific primers were designed for candidate genes involved in Fe uptake (bHLH, IRT, FRO_2, NRAMP and YSL) and P uptake (PHT1.2, PHT1.4, PHT1.6, PHT1.8 and PT1). Sequences of candidate genes were obtained from the Rice Genome Annotation Project (RGAP), National Center for Biotechnology Information (NCBI) and "Soybase." Gene-specific primers were designed using the Real-Time qPCR tool (Primer3 version2.2.3) from Integrated DNA Technologies (IDT; http://eu.idtdna.com/scitools/Applications/RealTimePCR).

3.1.4 Expression analysis of candidate genes

Expression of candidate genes from samples collected at different time intervals was performed by polymerase chain reaction (PCR) using gene-specific primers. The PCR reaction mixture in a total volume of 20 µL contained 2 µL buffer, 2 µL $MgCl_2$, 1 µL dNTP, 1 µL of each forward and reverse primer, 0.5 µL of Taq polymerase, 2 µL of cDNA and 10.5 µL water. The cycling parameters were as follows: 2 minutes of pre-denaturation at 95°C, 35 cycles of 15 seconds at 95°C for denaturation, 15 seconds at 58°C for annealing and 30 seconds at 72°C for extension, followed by followed by 1 minute at 72°C for final extension. PCR products were electrophoresed on 2% (w/v) agarose gel in 0.5X TAE Buffer and stained with ethidium bromide (0.5 µg mL^{-1}). Gels with amplification fragments were visualized and photographed under UV light. After observing the intensity and clarity of bands, we selected a 24-hour sample for proteome profiling.

3.1.5 Protein isolation, quantification and enrichment of low abundance protein

The steps taken during proteome profiling of leaf tissues are shown in Figure 37. For protein extraction, 2 g of frozen leaf tissue was ground in liquid nitrogen using a mortar and pestle. Ground samples were thoroughly homogenized in 10 mL of extraction buffer containing 50 mM HEPES at pH 7.5, 40% (w/v) sucrose and 0.1% (v/v) β-mercaptoethanol. After vortexing for 5-10 minutes, the tube was placed in a rocker for 30 minutes and then centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant-containing protein was carefully transferred to a fresh 50 mL Oak Ridge tube, to which 40 mL of precipitation buffer (0.1 M ammonium acetate in 100% (v/v) methanol) was added, and incubated overnight at -20°C. The protein pellet, obtained by centrifugation at 15,000 rpm for 30 minutes at 4°C, was washed thrice with 40 mL of 80% (v/v) acetone. The protein pellet was dissolved in solubilizing buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 400 mM DTT and 1% (v/v) ampholyte (Bio-Lyte pH 4-7). After 1 hour of gentle stirring at room temperature, samples were centrifuged at 15,000 rpm for 30 minutes at 4°C. Supernatant was used for protein quantification using Bradford reagent (Bradford, 1976). To enrich the samples with low-abundance protein, the samples were passed through a Seppro Rubisco Spin Column (Sigma-Aldrich, Cat: SEP070) to remove Rubisco protein, which constitutes ~40% of leaf protein before performing 2D-PAGE.

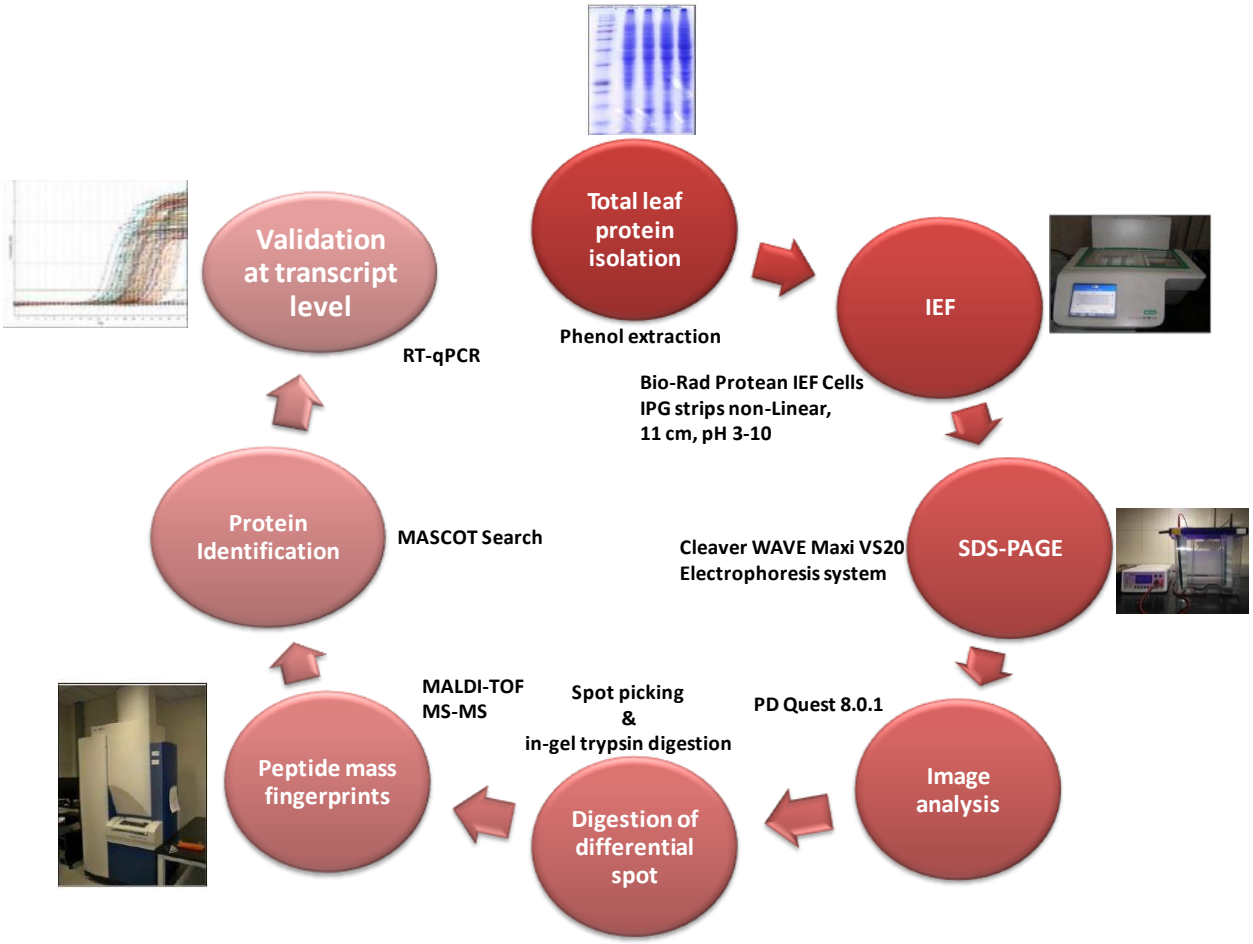

Figure 37. *Steps followed during proteome profiling of rice and soybean leaf tissue.*

3.1.6 Isoelectric focusing gel electrophoresis (separation of proteins according isoelectric point)

Isoelectric-focusing (IEF) electrophoresis of leaf protein samples was performed using Protean IEF cells (Bio-Rad, USA) with 400 µg of protein loaded onto the IPG strips (pH 3-10, non-linear, 11 cm). The IPG strips were passively rehydrated for 12-16 hours with 185 µL of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT and 0.2 % Bio-Lyte ampholytes) containing 300 µg of protein. The voltage program for IEF run was 200 V for 3 hours, 500 V for 30 minutes, 1,000 V for 30 minutes, 6,000 V for 1 hour and 6,000 V for rest of the time to reach a total of 28 kVh. During the running of program, the current was initially kept at 50 µA, which changed automatically with decreasing resistance. During the IEF run, evaporation of the protein sample was prevented by pouring 5-8 mL of mineral oil on the IEF tray. After completion of the IEF run, excess mineral oil was removed and strips were immediately stored in a deep freezer (-80°C) or processed for the next step. Following the run, IEF strips containing focused protein were treated with 5 mL of freshly made equilibration buffer I (or reduction buffer) containing 6 M urea, 0.375 M Tris HCl pH 8.8, 2% SDS, 20% glycerol and 2% DTT. After completion of the reduction step,

strips were removed from the reduction buffer and treated with 5 mL of equilibration buffer II (or alkylation buffer) for 15 minutes. The composition of alkylation buffer was same as the reduction buffer except 2% DTT was replaced with 2.5% iodoacetamide.

3.1.7 SDS-PAGE to gel electrophoresis

The SDS-PAGE separating gel was composed of 12.5% (w/v) acrylamide, 1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.04% TEMED. After all components were mixed by gentle pipetting, it was poured into the gel slabs. When gel became polymerized, the equilibrated IPG strips were loaded on top of the separating gel and overlaid with a mixture of 1% (w/v) agarose and 0.1% (w/v) bromophenol blue tracking dye. Separation was performed in a cleaver maxi electrophoresis unit (WAVESYS-CU VS20 WAVE Maxi) at a constant 50 V. When bromophenol blue dye reached in the base of the gel, the current supply was terminated. Gels were washed with double distilled water and incubated for 1 hour in fixing solution containing 50% (v/v) methanol and 10% (v/v) glacial acetic acid. The gels were kept in staining solution (0.1% Coomassie Brilliant Blue [CBB G-250], 40% (v/v) methanol and 10% (v/v) glacial acetic acid) overnight and then placed in destaining solution containing 50% (v/v) methanol and 10% (v/v) glacial acetic acid until the background was destained and protein spots were visible.

3.1.8 Scanning and image analysis of gels

For further analysis, gels were scanned in an Epson scanner system (Epson Expression 11000XL). The gels were stored at 4°C in storage solution containing 5% (v/v) glacial acetic acid. The pattern of proteins spot distribution on 2-D gels was compared with PDQuest software version 8.0.1 (Bio-Rad, USA).

3.1.9 Spot picking and in-gel trypsin digestion

After PDQuest analysis of the gels of both rice and soybean, the soybean crop gels were used for further work. The proteins of interest were carefully picked from the 2-D gel and transferred in separate wells in 96 well micro-plates. The excised gel pieces containing protein were washed twice with 100 µl of Milli-Q water and centrifuged at 1,100 rpm for 20 minutes. Each spot was washed with 100 µL of 50% (v/v) acetonitrile solution and then centrifuged at 1,100 rpm for 20 minutes. After centrifugation, the supernatant was discarded and pellets were incubated at 45°C for 60 minutes after adding 100 µL reducing buffer (10 mM DTT in 25 mM ammonium bicarbonate). Reducing buffer was discarded after incubation, and protein spots were incubated in the dark for 15 minutes at 30°C after adding 100 µL of alkylation buffer (10 mM iodoacetamide in 25 mM ammonium bicarbonate). After reduction and alkylation, protein samples were washed two times with 100 µL and 50 µL of 50% (v/v) acetonitrile solution and then centrifuged at 1,100 rpm for 20 minutes. After centrifugation, pellets were allowed to dry thoroughly. 50 µl of 10 µg/mL trypsin solution, dissolved into 25 mM ammonium bicarbonate, was added to each well, which was incubated overnight at 35°C. After completion of digestion, samples were centrifuged at 1,100 rpm for 20 minutes. Supernatant was transferred into new tubes and stored in a deep freezer (-80°C) for 4 hours and then lyophilized. Lyophilized peptides were homogenized in 1% (w/v) tetrafluoroacetic acid in 50% (v/v) acetonitrile before mass spectrometry was performed.

3.1.10 Protein identification MALDI-LC MS/MS

An ABI Sciex 5800 TOF/TOF System with MALDI-LC was used for collection of the peptide mass fingerprints of differentially expressed proteins. The parameters were set as Mass Spectrometry (precursor-ion) peak filtering: mass tolerance 250 ppm, m/z interval 800-4,000, minimum signal-to-noise ratio (S/N) 10, monositonic; digestion enzyme: trypsin with one missed cleavage; fixed modification: Carbamidomethyl (C); database: Viridiplantae taxonomic sub-database of NCBInr. Peptide mass fingerprints and MS/MS ion peaks were searched against the database using the Mascot algorithm (http://www.matrixscience.com). Differential proteins with a Mascot score greater than 65 were considered significant ($P < 0.05$) hits and chosen to validate their gene expression levels under Fe/P stress and recovery conditions.

3.1.11 Protein annotation: In-silico analysis

The bioinformatics platform Blast2GO (Conesa and Götz, 2008) was used for functional annotation of the Mascot search-identified protein sequences. Based on the protein homology from other or the same species, determined by BLAST, enzyme codes and generic gene ontology (GO) were assigned for the identified protein. The ANNEX program was used for further elaboration of annotation (Myhre et al., 2006). BLAST searches (TBLASTX, nr database) were used for each protein sequence followed by mapping and annotation. The GO terms were arranged on the corresponding Plant GO Slim terms and KEGG pathway was also mapped from annotation enzymes.

3.1.12 Primer design and Real-Time PCR for validation of proteomics results of soybean

The Real-Time qPCR tool (Primer3 version 2.2.3) from IDT was used for designing the gene-specific primer (http://eu.idtdna.com/scitools/Applications/RealTimePCR). RT-qPCR was carried out using the KAPA SYBR® FAST qPCR Kit (KAPA Biosystem) on a PikoReal Real-Time PCR System (ThermoFisher Scientific). RT-qPCR was performed in triplicates of 10 µL reactions containing 5 µL of KAPA SYBR FAST master mix, 1 µL of primer mixture comprised of 0.5 µL forward primer (0.5 pmol μL^{-1}) and 0.5 µL reverse primer (0.5 pmol μL^{-1}), 1 µL of cDNA template and 3.0 µL of nuclease-free water. The cycling parameters were as follows: 3 minutes of pre-denaturation at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C, followed by melting curve analysis (at 60°C and then ramped up to 95°C). All cDNA template samples were normalized to contain an equal concentration (25 ng μL^{-1}) of cDNA confirmed by the amplification of the reference gene "*GmEF1α*" (elongation factor 1α), and amplification fragments were visualized on 3% (w/v) agarose gel. The comparative cycle threshold (ct) was calculated according Schmittgen and Livak (2008).

3.2 Expression analysis of leaf-proteome in response to foliar application of Fe and P

3.2.1 Expression analysis of soybean leaf proteome

The leaf protein pattern obtained by 2-D gel electrophoresis depicted 126, 134, 146 and 154 protein spots in control, Fe stress, P stress and dual stress (FeP) conditions, respectively (Fig. 38). The intensity of 35, 32 and 36 spots showed more than 2-fold changes in Fe stress, P stress and dual stress conditions, respectively, in comparison to control (Fig. 39A). Thirty-eight spots showed more than 2-fold variation in plants sprayed with Fe-citrate (Fe recovery) compared to the Fe stress plant. From these 38 proteins, 25 proteins were up-regulated, and the remaining were down-regulated in Fe-recovery conditions as compared to Fe stress. In the case of P treatments, a total of 42 differently expressed proteins were observed, from which 32 proteins were down-regulated in P recovery ($FePO_4$-sprayed plants) as compared to P-stress conditions. Twenty-eight proteins showed up-regulation and seven proteins showed down-regulation in dual stress conditions as compared to control. However, 16 and 14 spots showed more than 2-fold up-regulation, and 11 and 19 spots showed down-regulation when dual stress (FeP stress) plants were sprayed separately with Fe-citrate and $FePO_4$, respectively (Fig. 39B).

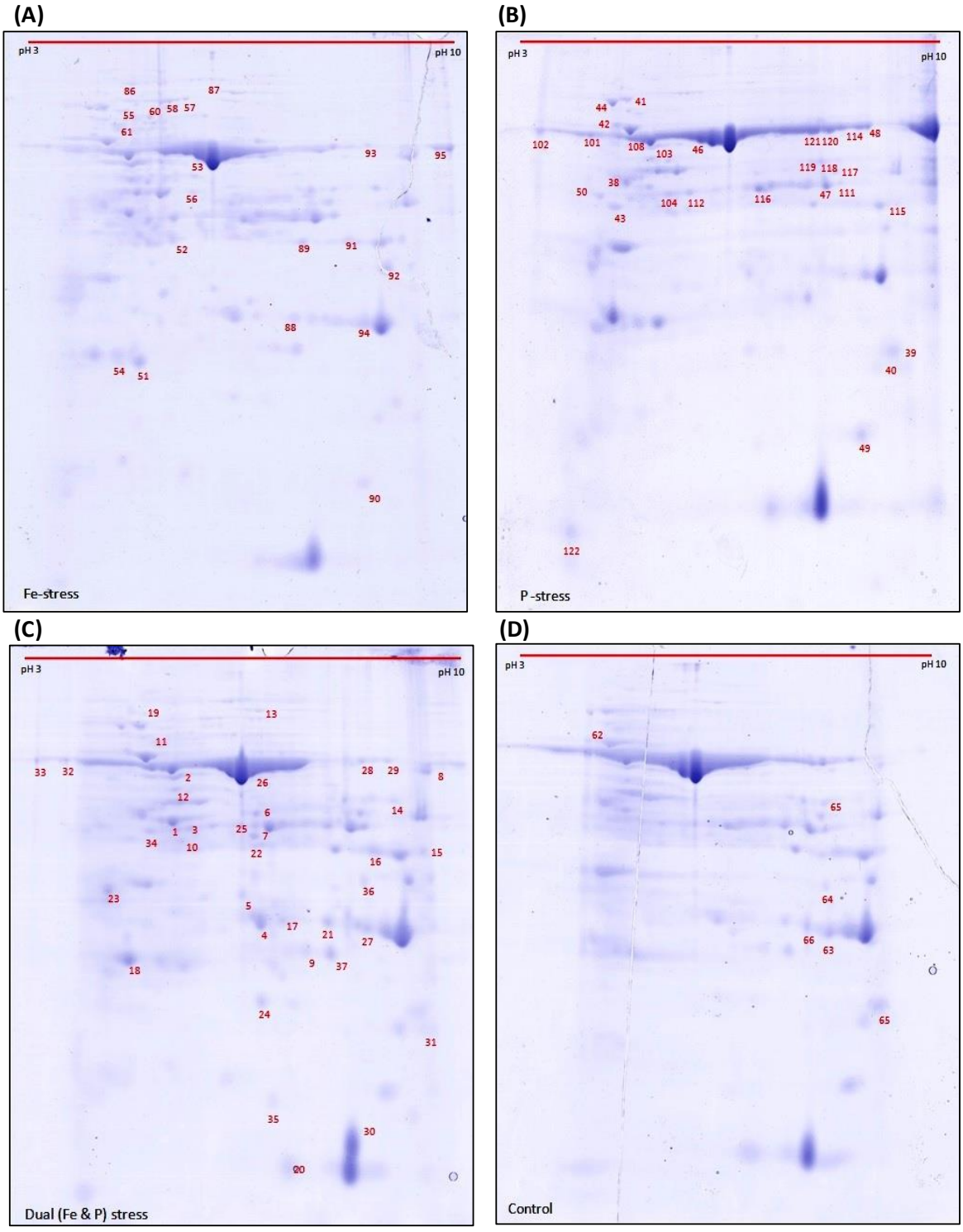

Figure 38. *Two-dimension gels of leaf protein of soybean crops grown under (A) Fe stress, (B) P stress, (C) dual (Fe and P) stress and (D) control nutrients solution under hydroponic conditions. Numbers in the gels denote differently expressed proteins analyzed using PDQuest software.*

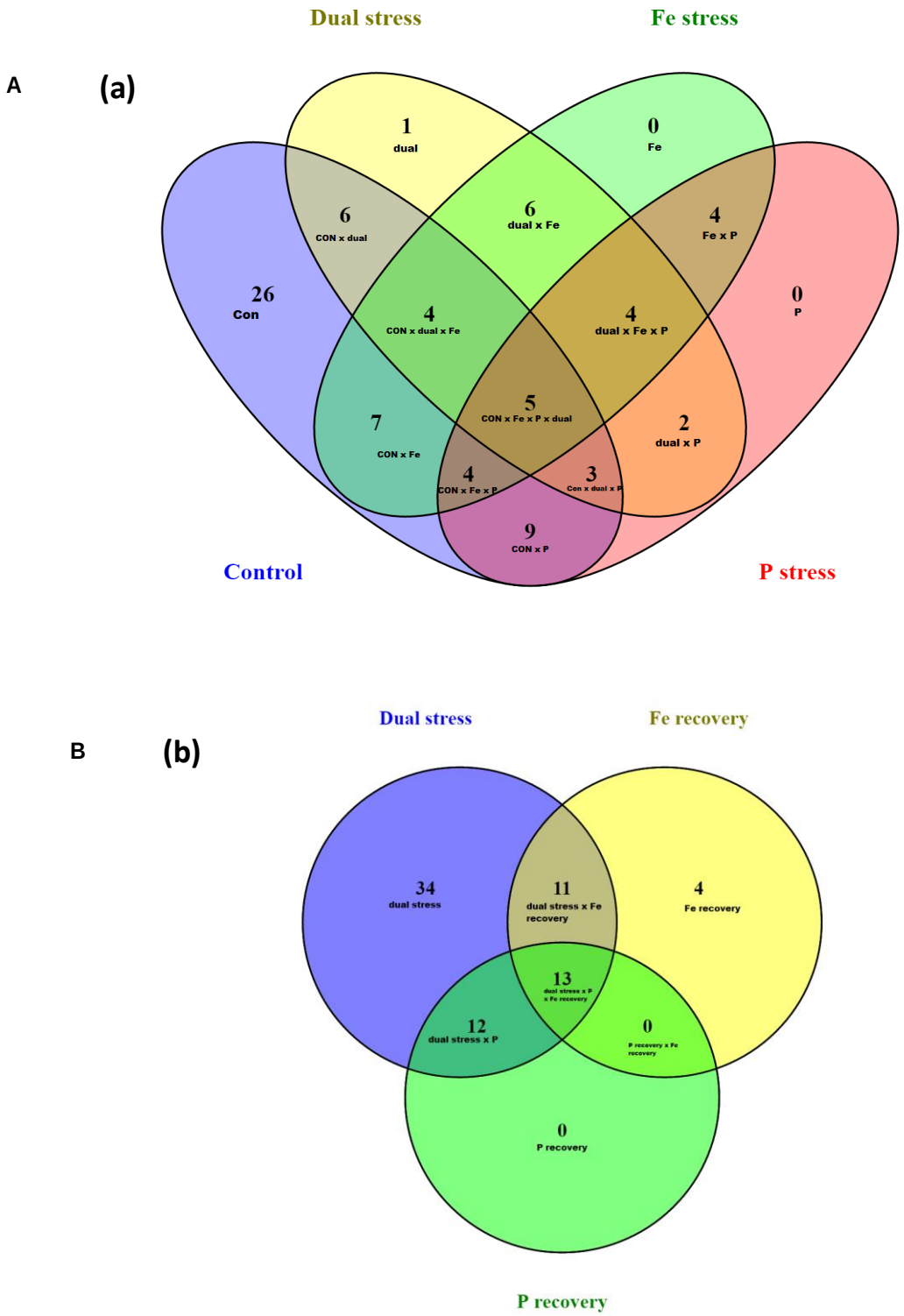

Figure 39. *Venn diagram showing the number of differentially expressed proteins on 2-D gels at dual stress comparing with (a) control vs. stress and (B) stress vs. recovery.*

3.2.2 Expression analysis of rice leaf proteome

The Coomassie Brilliant Blue dye-stained 2-D gels of rice proteomes showed 105, 99, 111 and 121 protein spots in control, Fe-stress, P-stress and dual stress conditions, respectively (Fig. 40). As compared to control, the expression of 48, 30 and 27 proteins spots displayed more than 2-fold changes under Fe-stress, P-stress and dual stress conditions. Of 48 differently expressed protein spots, 42 proteins were up-regulated under Fe stress conditions as compared to control, while only six proteins spots showed down-regulation under Fe stress. The intensity of 21 spots was more than 2-fold up-regulated under P stress as compared to control. Twenty-seven proteins showed more than 2-fold differential expression under dual stress conditions as compared to control. Of these 27 proteins, 20 proteins were up-regulated, while only seven were down-regulated in dual stress plants. A total of 97 proteins spots were visualized in Coomassie Brilliant Blue-stained 2-D gel of plants sprayed with Fe-citrate (Fe recovery). From these 97 proteins, only 17 spots showed more than 2-fold differential expressions as compared to Fe stress. A total of 52 spots showed more than 2-fold variations in plants sprayed with Fe-phosphate (P recovery) in comparison with P stress plants. Of these 52 differential expressed proteins, 43 proteins spots showed more than 2-fold down-regulation under P recovery (plants sprayed with Fe-P) as compared to P stress, while only nine proteins were up-regulated in P-recovery conditions. A total of 118 and 113 proteins spots were detected in dual stress Fe-recovery and dual stress P-recovery gels. Dual stress plants were recovered by Fe and P separately; 33 and 34 proteins showed more than 2-fold down-regulation under Fe recovery and P recovery conditions, respectively, as compared to dual stress (Fig. 41).

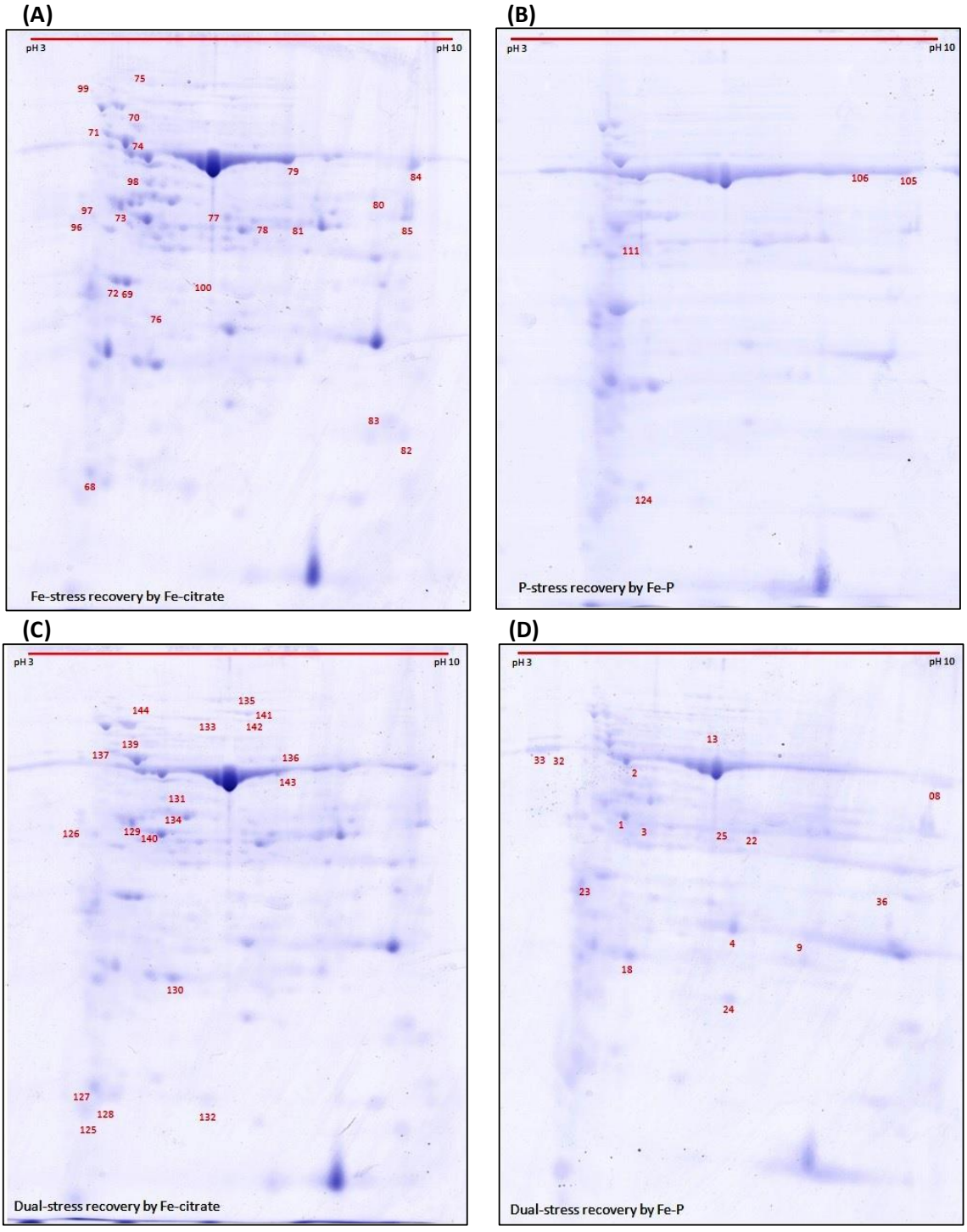

Figure 40. *Two-dimension gels of leaf protein of soybean crops grown under (A) Fe stress recovered by foliar spray of Fe-citrate, (B) P stress recovered by foliar spray of Fe-P, (C) dual stress recovered by foliar spray of Fe-citrate and (D) dual stress recovered by foliar spray of Fe-P under hydroponic conditions. Numbers in the gels denote differently expressed proteins analyzed using PDQuest software*

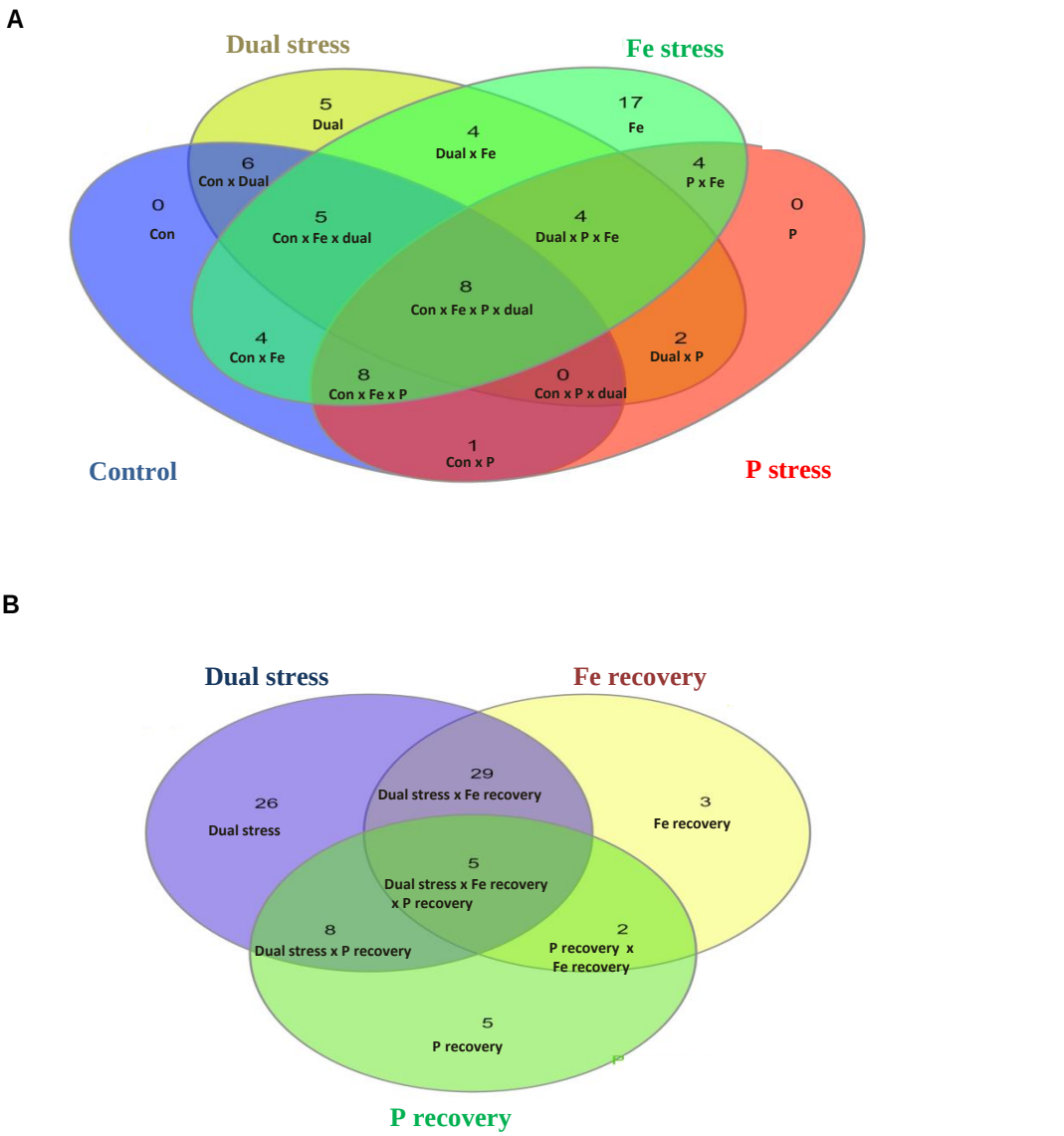

Figure 41. *Venn diagram showing the number of differentially expressed rice leaf proteins on 2-D gels at dual stress comparing (A) control vs. stress and (B) stress vs. recovery*

3.3 Proteome profiling and identification

After PDQuest analysis of the gels of both rice and soybean crops, the soybean crop gels were used for further work. Soybean leaf protein gels of all treatments were analyzed using PDQuest software followed by spot picking, in-gel trypsin digestion and peptide mass fingerprinting by MALDI/TOF MS/MS. After identification of proteins by MALDI MS/MS and Mascot search, their involvement in the different metabolic processes, such as biosynthetic pathways, signal transduction or generation of precursor molecules, was annotated based on gene ontology. Details of differently expressed proteins in different treatments are documented as follows.

3.3.1 Fe stress vs. control

The staining of the 2-D gel using Coomassie Brilliant Blue dye visualized 126 and 134 protein spots in control and Fe stress gels, respectively. Among these spots, 36 proteins showed more than a 2-fold change in expression at Fe-stress conditions. All 36 proteins were predicted from soybean genome sequence; however, some were hypothetical or uncharacterized proteins. Proteins up-regulated at Fe-stress conditions were nitrophenylphosphatase, adenosine triphosphatase, transcription initiation factor IIB, 13-hydroxylupanine O-tigloyltransferase, ADP-ribose diphosphatase, fructose-bisphosphate aldolase, cellulose synthase, 3-beta-hydroxysteroid-4-alpha-carboxylate 3-dehydrogenase, phospholipase D, peptidylprolyl isomerase, cytochrome-b5 reductase, ribulose-bisphosphate carboxylase, and phosphatidylinositol-4-phosphate 5-kinase. There were seven uncharacterized proteins also showing more than 2-fold up-regulation under Fe-stress conditions. However, nine proteins, such as Rec-P21, glycerol-3-phosphate dehydrogenase, ribulose-bisphosphate carboxylase, photosystem I subunit PsaD, and 14-3-3 protein SGF14f, were down-regulated under Fe-stress conditions in comparison to control conditions (Fig. 42).

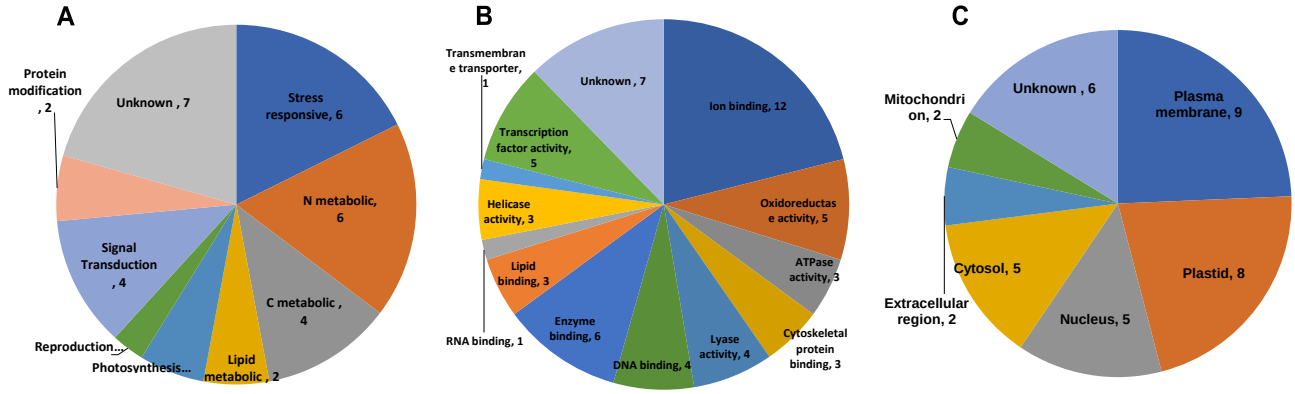

Figure 42. *Gene ontology distribution based on the role in (A) biological process, (B) molecular function and (C) cellular localization under control vs. Fe stress*

3.3.2 P stress vs. control

A total of 146 and 126 proteins spots were detected in P-stress and control gels, respectively. Thirty-two proteins showed more than 2-fold changes, and all proteins were predicted from soybean genome. Of these 32 proteins, 24 proteins showed up-regulation while eight proteins showed down-regulation at P-stress conditions. The up-regulated proteins were acid phosphatase, adenosine triphosphatase, heat shock cognate protein, NADP-retinol dehydrogenase, adenosine triphosphatase, transcription initiation factor IIB, abscisic acid receptor PYL2-like, while down-regulated proteins were microtubule-severing ATPase, transferring phosphorus-containing groups and others that were uncharacterized or predicted proteins (Fig. 43).

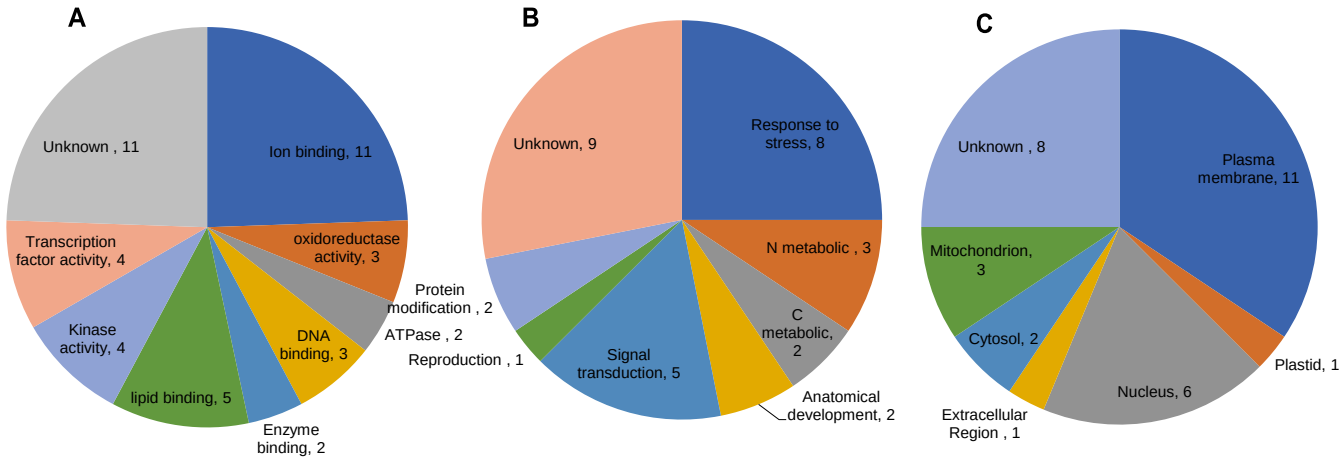

Figure 43. *Gene ontology distribution based on the role in (A) biological process, (B) molecular function and (C) cellular localization under control vs. P stress*

3.3.3 Dual stress vs. control

After staining with Coomassie Brilliant Blue, 154 spots were visualized in the dual stress gel. Thirty proteins showed more than 2-fold up-regulation, while only six proteins showed 2-fold down-regulation under dual stress conditions. The up-regulated proteins were involved in different cellular pathways including nitrogen metabolism (glutathione transferase, protein REVEILLE 1, ADP-ribose diphosphatase), carbohydrate metabolism (glycerol-3-phosphate dehydrogenase [NAD^+], fructose-bisphosphate aldolase, cellulose synthase [UDP-forming], ribulose-bisphosphate carboxylase), anatomical structure development (3-beta-hydroxysteroid-4-alpha-carboxylate 3-dehydrogenase, glycosyltransferases, adenosine triphosphatase), signal transduction (phospholipase D, nitrate reductase), and protein metabolism (peptidylprolyl isomerase, glycosyltransferases, transferring) (Fig. 44).

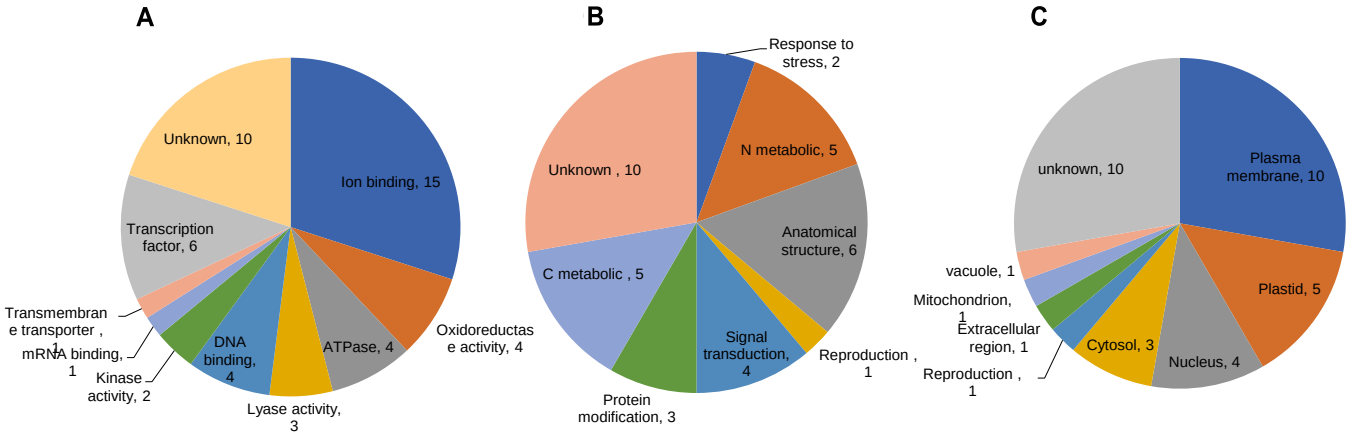

Figure 44. *Gene ontology distribution based on the role in (A) biological process, (B) molecular function and (C) cellular localization under control vs. dual stress*

3.3.4 Fe stress vs. Fe recovery

Under Fe-recovery (foliar spray of Fe-citrate solution in Fe-stress plants) conditions, a total of 130 protein spots were detected after Coomassie Brilliant Blue staining. Among these spots, 37 proteins spot showed more than 2-fold changes in expression under Fe recovery as the comparison of Fe-stress gels. Twenty-five proteins showed 2-fold up-regulation, while 12 spots intensities were 2-fold less under Fe-recovery conditions. Some of the up-regulated proteins were Stem 31 kDa glycoprotein precursor, DEAD-box ATP-dependent RNA helicase 47A-like, peptidyl-prolyl cis-trans isomerase 1-like, kinesin-like protein NACK1 isoform X1, oxygen-evolving enhancer protein 1, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1A-like isoform X2 and t-RNA dimethylallyltransferase (Table 13). Of 12 down-regulated proteins, eight proteins were uncharacterized; however, all peptide sequences have significant Mascot scores and all were predicted from soybean genomes (Fig. 45).

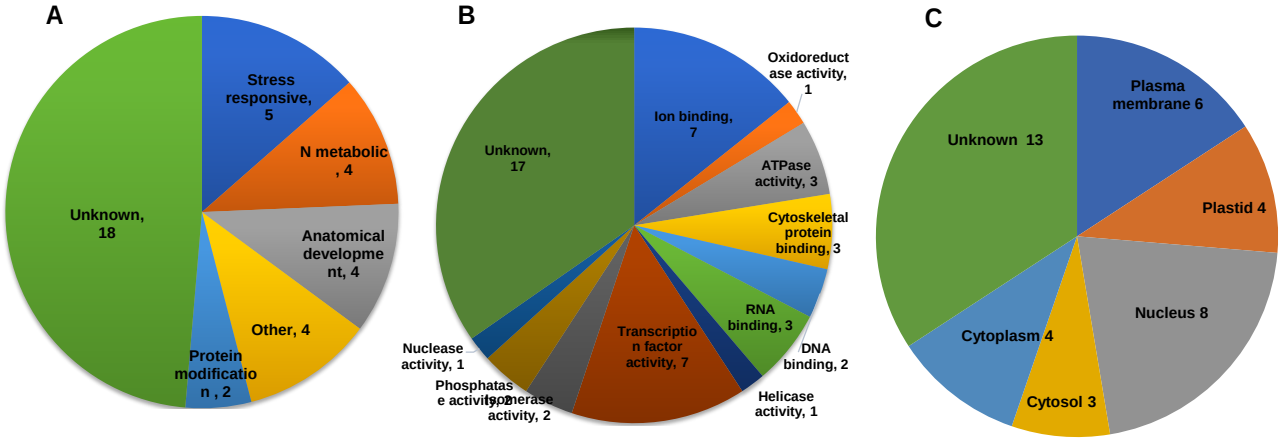

Figure 45. *Gene ontology distribution based on role the in (A) biological process, (B) molecular function and (C) cellular localization under Fe stress vs. Fe recovery*

3.3.5 P stress vs. P recovery

P-deficient plants were recovered by foliar spray of Fe-P solution, and total leaf proteins were run in the 2-D gel. A total of 135 spots were detected in P recovery gels. Among these proteins, 21 proteins were 2-fold down-regulated under P-recovery conditions; some of the down-regulated proteins included fructose-bisphosphate aldolase, carbonate dehydratase, carboxylesterase, formin-like protein 4, abscisic acid receptor PYL2-like, U-box domain-containing protein 3-like isoform X2, galactoside 2-alpha-L-fucosyltransferase, and RNA recognition motif (RRM) superfamily protein. Some of the up-regulated proteins included glycine hydroxymethyltransferase, O-glucosyltransferase Rumi-like isoform X1, E3 ubiquitin-protein ligase, and syntaxin-31-like(Fig. 46).

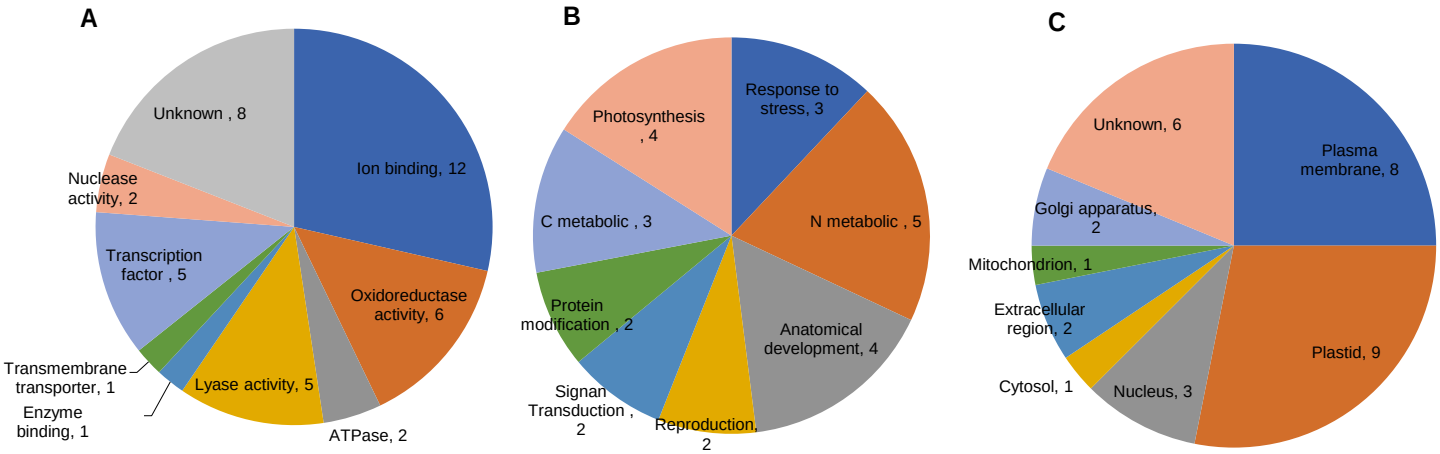

Figure 46. *Gene ontology distribution based on the role in (A) biological process (B) molecular function and (C) cellular localization under P stress vs. P recovery*

3.3.6 Dual stress vs. Fe and P recovery

The Coomassie Brilliant Blue dye-stained 2-D gels showed 118 and 98 protein spots in Fe recovery and P recovery gels, respectively. When dual stress plants were recovered by Fe and P separately, 24 and 27 proteins showed more than a 2-fold change in expression under Fe-recovery and P-recovery conditions, respectively (Fig. 47). Among these proteins, four similar proteins were up-regulated (>2-fold) and three similar proteins were down-regulated (>2-fold) in both conditions, while two proteins showed the opposite expression level in both conditions. In both conditions, nine uncharacterized or predicted proteins were found; however, all peptide sequences have significant Mascot scores and all were predicted from soybean genomes.

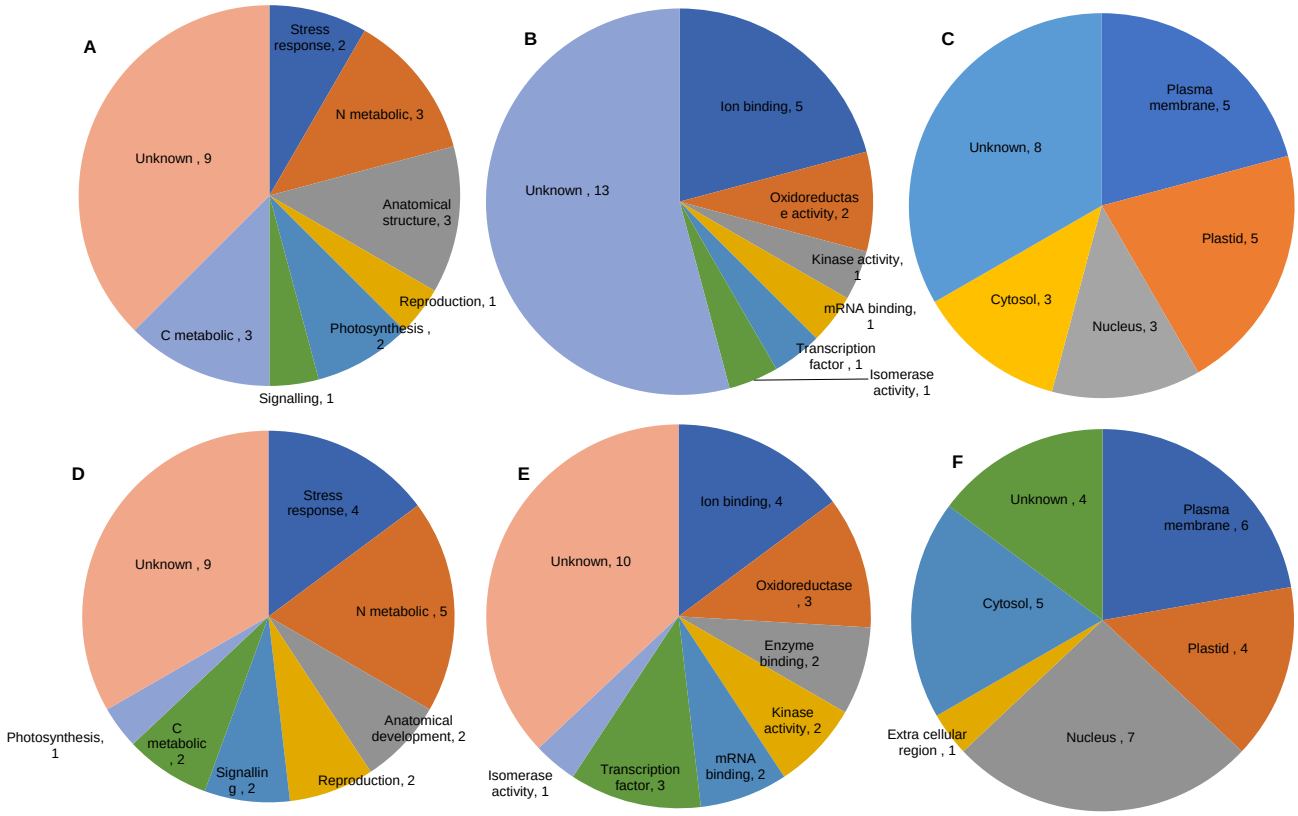

Figure 47. *Gene ontology distribution based on the role in (A) biological process, (B) molecular function and (C) cellular localization under dual stress vs. Fe recovery and (D) biological process, (E) molecular function and (F) cellular localization under dual stress vs. P recovery*

3.4 Validation of protein expression in transcription level

RT-qPCR analysis was performed to validate the expression at transcript levels of 75 differently expressed proteins that displayed differential expression under different nutrients stress (Fe-, P- and dual stress) and recovery conditions. Of the 75 selected genes, 53 (71%) genes showed trends at transcription level that were similar with proteins at expression level, while 22 (29%) genes showed transcription trends opposite to the trends recorded in proteome profile (Figs. 47 and 48).

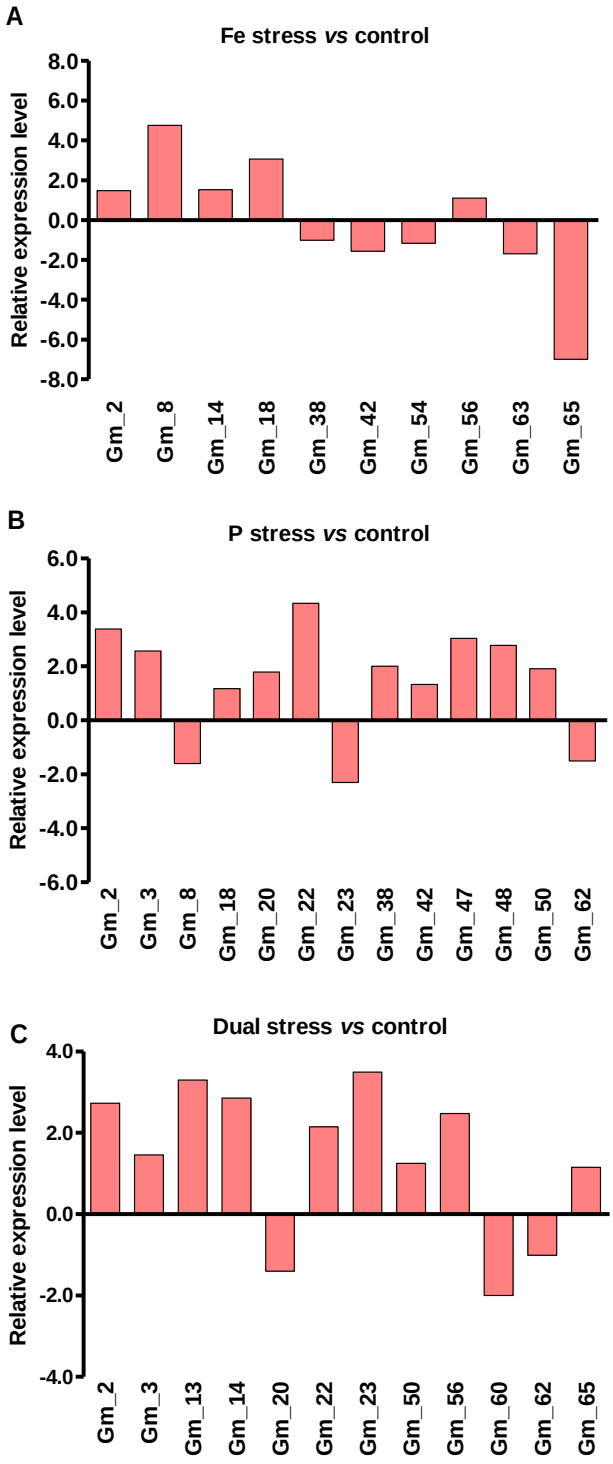

Figure 48. *Relative transcript levels of differently expressed proteins by more than 2-fold at (A) Fe stress vs. control, (B) P stress vs. control and (C) dual stress vs. control in the leaf of soybean.*

3.4.1 Fe stress vs. control

A total of 35 proteins showed more than a 2-fold change in expression under Fe-stress condition as compared to control. Of these 35 protein spots, 10 were validated at gene level using RT-qPCR analysis (Fig. 48A). Among these 10 validated spots, nine genes and their products, *viz.*, *Gm_02* (hypothetical protein), *Gm_08* (predicted protein REVEILLE 1), *Gm_14* (uncharacterized protein LOC100818855), Gm_18 (hypothetical protein), *Gm_38* (Glyma_09G253700), *Gm_54* (1-phosphatidylinositol-4-phosphate 5-kinase), *Gm_56* (hypothetical protein), *Gm_63* (an unknown protein) and *Gm_65* (predicted protein LOC100776545), displayed similar trends at both gene and protein levels; however, *Gm_42* (oxysterol-binding protein-related protein 1C-like isoform X2) gene expression was down-regulated, while protein expression was up-regulated under Fe-stress conditions.

3.4.2 P stress vs. control

For validation, 14 proteins were used out of a total of 32 differently expressed proteins for RT-qPCR analysis under P-stress conditions. Among these validated proteins, eight (57%) proteins showed similar expression trends at the transcriptional and translational levels (Fig. 48B). The genes encoding 3-beta-hydroxysteroid-4-alpha-carboxylate 3-dehydrogenase (*Gm_03*), a hypothetical protein (*Gm_18*), oxysterol-binding protein-related protein 1C-like isoform X2 (*Gm_42*), abscisic acid receptor PYL2 (*Gm_47*), transferring phosphorus-containing groups (*Gm_48*) and two uncharacterized proteins (*Gm_50* and *Gm_44*) were up-regulated in gene as well as protein level under P-stress conditions as compared to control. The expressions of predicted protein REVEILLE 1 (*Gm_02*), hypothetical protein (*Gm_22*) and Glyma_09G253700 (*Gm_38*) were down-regulated under P-stress conditions, but transcript showed up-regulation under the same conditions (Fig. 48B).

3.4.3 Dual stress vs. control

A total of 36 proteins showed more than 2-fold differential expressions under dual stress conditions as compared to control. Among these proteins,12 were used for the validation of protein expression at the transcription level. Ten out of 12 (83%) proteins displayed similar expression trends at both protein and gene levels (Fig. 48C). Two hypothetical proteins, Glyma_06G101500 (*Gm_23*) and LOC100776545 (*Gm_65*), displayed transcription trends opposite to the trends recorded in proteome expression.

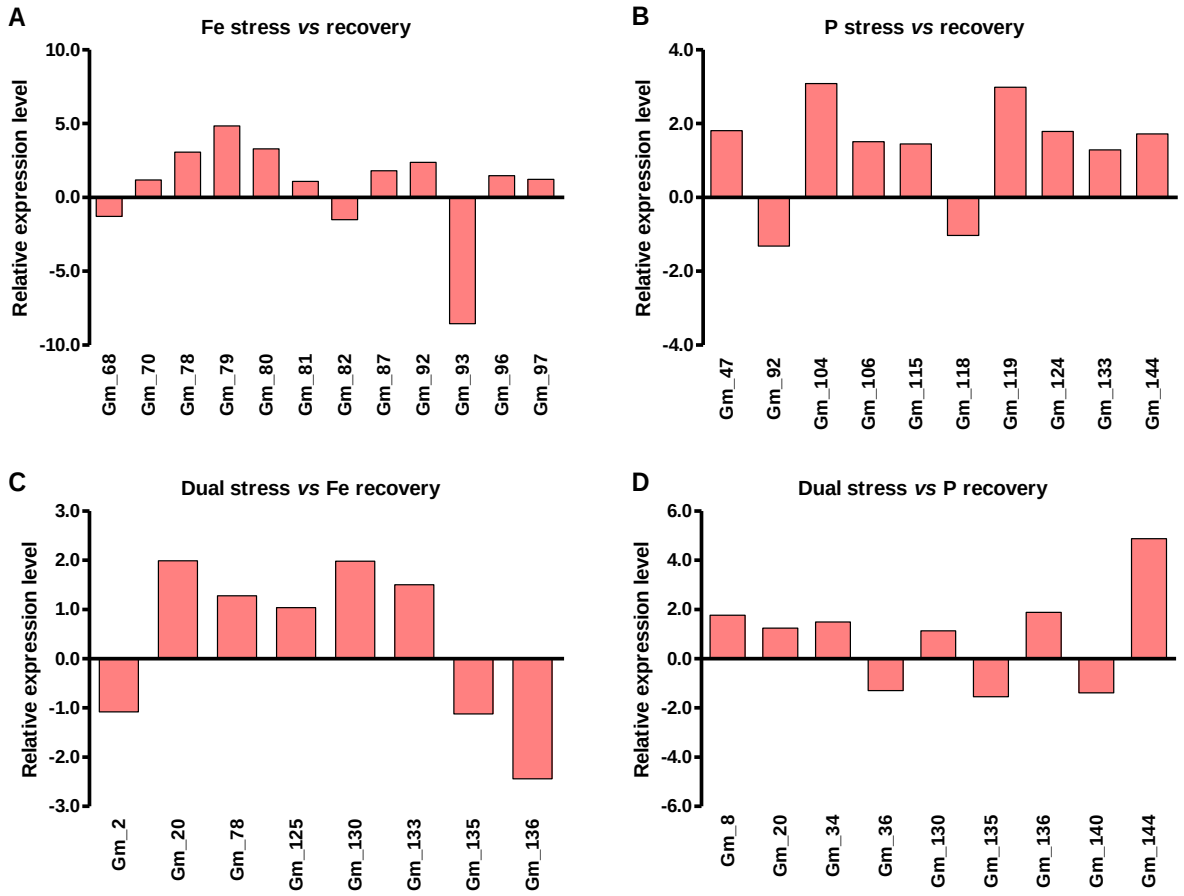

Figure 49. *Relative transcript levels of differently expressed proteins by more than 2-fold at (A) Fe stress vs. recovery, (B) P stress vs. P recovery, (C) dual stress vs. Fe recovery and (D) dual stress vs. P recovery in the leaf of soybean.*

3.4.4 Fe-stress vs. Fe recovery

In accordance with gene expression pattern, eight out of 12 genes used for RT-qPCR analysis were up-regulated under Fe-recovery conditions (Fig. 49A). The eight genes, *Gm_70*, *Gm_78*, *Gm_79, Gm_80*, *Gm_81, Gm_93, Gm_96* and *Gm_97*, showed similar trends in both gene and protein levels under Fe-recovery conditions; however, all genes encoded hypothetical or predicted proteins. Transcripts of peptidyl-prolyl cis-trans isomerase 1-like (*Gm_82*) and three uncharacterized proteins (*Gm_68, Gm_87* and *Gm_92*) displayed reverse expression trends compared to their protein expression pattern (Fig. 49A).

3.4.5 P stress vs. P recovery

A total of 32 proteins showed more than 2-fold expressions under P-recovery conditions as compared to P-stress conditions. Ten genes, *Gm_47, Gm_92, Gm_104, Gm_106, Gm_115, Gm_118, Gm_119, Gm_124, Gm_133* and *Gm_144,* were used for confirmation of protein expression with gene expression (Fig. 49B). Of these 10 genes, five genes showed similar expression trends in both proteins and gene

level while five displayed the opposite. The transcripts of abscisic acid receptor PYL2-like (*Gm_47*) showed 1.8-fold up-regulation in the leaf of Fe-P foliar-sprayed plants; however, the accumulation of abscisic acid receptor PYL2-like protein was recorded at more than 2-fold less in P-recovery conditions.

3.4.6 Dual stress vs. Fe and P recovery

When Fe and P were supplied to dual stress plants by foliar spray, 24 and 27 proteins displayed more than 2-fold differential expression in Fe-recovery and P-recovery conditions, respectively (Fig. 49C-D). Under Fe-recovery conditions, eight genes (*Gm_2*, *Gm_20*, *Gm_78, Gm_125*, *Gm_130*, *Gm_133*, *Gm_135* and *Gm_136*) were used for RT-qPCR analysis, and all genes showed similar trends in both gene and protein expression levels. However, for validation of protein pattern at transcript levels, nine differentially expressed proteins were used for P-recovery conditions. The gene *Gm_135* encoding adenosine-triphosphatase was down-regulated by more than 2-fold when dual stress plants were sprayed by either with Fe-citrate (Fe recovery) or Fe-phosphate (P recovery). The transcript level of gene *Gm_20* was up-regulated in both recovery conditions; however, their product "*transferring phosphorus-containing groups*" was up-regulated more than 2-fold in Fe-recovery conditions but down-regulated under P-recovery conditions.

4 Developing seed coating material loaded with P, organic acid and phosphorus-solubilizing bacteria (PSB) and study of P-release behavior from the coating material (Objective 3)

4.1 Preparation of clay polymer composites using polyacrylate and polyacrylamide (polyacrylate-CPC)

An effort was made to prepare seed-coating material with clay and different polymers that will act as a slow-release carrier of P in soil. A series of polyacrylate clay polymer composites (CPCs), with varying concentrations of cross-linker and neutralization percentages, was prepared using polyacrylate and polyacrylamide following the method of Liang and Liu (2007). Commercially available bentonite clay was used for the preparation of CPCs. Bentonite powder contains 60-70% nano-size particles. Typically, 5.5 mL of acrylic acid was neutralized by slow addition of varying amounts of concentrated ammonia solution in a four-neck flask equipped with a stirrer and a nitrogen line. In this solution, 1.15 g of acrylamide was added. Then, 0.55 g of bentonite was added and dispersed in the above partially neutralized monomer solution. Under a nitrogen atmosphere, the cross-linker N,N'-methylene-bis-acrylamide (varying amounts) was added to the acrylic acid/acrylamide/clay mixture solution. The mixed solution was stirred with a magnetic stirrer for 30 minutes at room temperature. The radical initiator ammonium persulfate (80 mg) was then added to the mixed solution, and the mixture was heated from 60° to 80°C. Finally, the polymer composite was formed by a vigorous endothermic process. After 2 hours, the CPCs were collected and dried in an oven at 100°C (Fig. 50).

Figure 50. ***(****A, B, and C) Different polyacrylate-CPCs and (D) a polyacrylate-CPC after grinding*

4.1.1 Loading of phosphorus, oxalic acid and PSB in polyacrylate-CPCs

Modes of P, oxalic acid and PSB loading in CPCs were evaluated and standardized. Loading of P in CPCs was done by two methods. In the first method, P was loaded in CPCs through excess amount of solution containing diammonium phosphate (DAP). But in this method, the percentage of P loading could not be maintained properly, as some P that was not absorbed by the CPCs remained in the bathing solution. In the second method, dry CPC was allowed to absorb less distilled water containing the required amount of dissolved P or ammonium oxalate, so that the whole solution would be absorbed by the CPCs. After complete absorption of the solution, the CPCs were dried and processed for further use. Loading of PSB in CPCs was done using a pure culture of PSB in dried CPCs.

4.1.2 Characterization of products developed using an X-ray diffractometer

Characterization of various formulations of CPCs was done with the help of an X-ray diffractometer (XRD). A strong peak at 6.0 was obtained for pure bentonite, as shown in Figure 50A. But when bentonite was used to make different CPCs with 10% clay, no significant peak was obtained around these domains (Fig. 51B-D). This implies that, during CPC formation, the clay particles were exfoliated, which should act as a barrier in CPCs. The exfoliation of clay is an important property as it imparts the slow-release properties in the polymer.

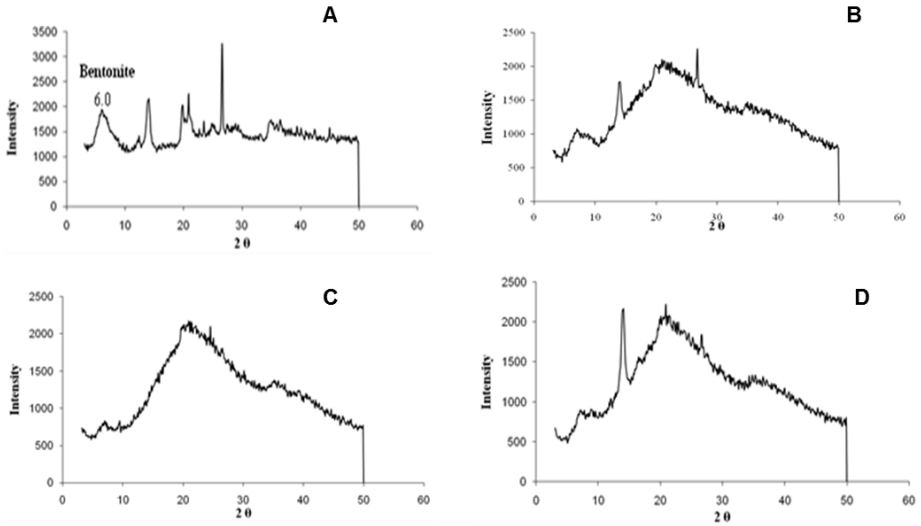

Figure 51. *Graph showing (A) XRD patterns of pure bentonite clay and (B-D) XRD patterns of different CPCs with 10% clay prepared using bentonite*

4.1.3 Incubation experiment with loaded acrylate-CPC in the laboratory

To study the effect of application of oxalic acid- and PSB-loaded CPCs in soil on P availability, an incubation experiment was conducted in the laboratory. In this, moist soil (50 g) was mixed with CPC loaded with oxalic acid and PSB separately and stored in plastic containers in replicates (Fig. 52). Soil samples were withdrawn at different intervals (5, 10, 20, 30 and 45 days) to estimate the available P in

the soil. A control soil sample (without addition of CPC) was also included for comparison. Both forms of CPCs, loaded with either oxalic acid or PSB, released significantly higher amounts of available P from soil up to 45 days as compared to control (Fig. 53). However, the CPC loaded with 40 ppm of oxalic acid resulted in significantly higher amounts of available P up to 30 days after incubation, while CPC loaded with PSB showed a gradual increase from day 5 to day 30 after incubation.

Figure 52. *Soil incubation experiment to study the P release from different CPCs loaded with oxalic acid and PSB*

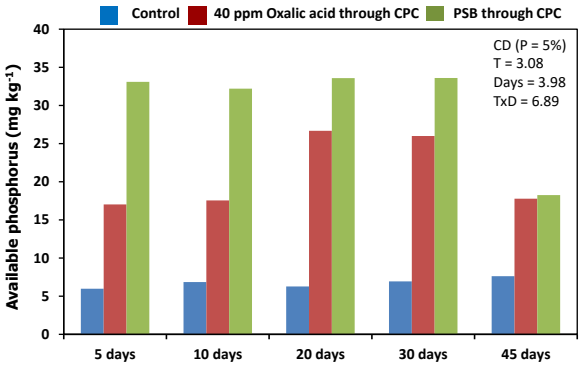

Figure 53. *Effect of oxalic acid- and PSB-loaded CPC application in soil on P availability*

4.2 Preparation of starch-grafted clay polymer composites (bio-CPC) and phosphorus release from bio-CPC in soil

In order to make CPCs economically viable, starch-based bio-CPC was prepared using varying concentrations of clay, in which 50% of the acrylic acid was replaced by a cheap source of starch. Different ratios of clay and bio-polymer were used to prepare bio-CPC (Table 11). The basic method of preparation of bio-CPC was similar to that of acrylate-CPC. The bio-CPCs were loaded with P (10%). The cumulative release of P from various P-loaded bio-CPCs in water and soil was determined. Fixed P (%) in soil was calculated under various combinations of bio-CPC. To study the P release from bio-CPC in soil, P was applied at 100 mg P kg^{-1} soil through bio-CPC. A total of 100 g soil was taken in a funnel, and 0.1 g bio-CPC (10% P-loaded) was added to it. Then the soil was subjected to leaching by adding 100 mL of 0.001 M $NaHCO_3$ solution. Leaching of soil with 0.001 M $NaHCO_3$ solution was repeated 10 times at 24-hour intervals. Phosphorus content in the leachate was analyzed using the blue color vanadomolybdate method.

Table 11. *Different ratios of clay used to prepare bio-CPC*

% Clay	% Neutralization		
	30	40	50
10	10:30 A	10:40 B	10:50 C
15	15:30 D	15:40 E	15:50 F
20	20:30 G	20:40 H	20:50 I
25	25:30 J	25:40 K	25:50 L
30	30:30 M	30:40 N	30:50 O

The cumulative values of P released from the bio-CPC in soil over time show that, among the different combinations, the ratio 10:40 (B) gave the maximum cumulative release of P (Fig. 54A). From this, the percentage of P fixation in soil was calculated (Fig. 54B). Based on the percentage of P fixation, the bio-CPC (B) was selected for seed coating and a simultaneous greenhouse experiment.

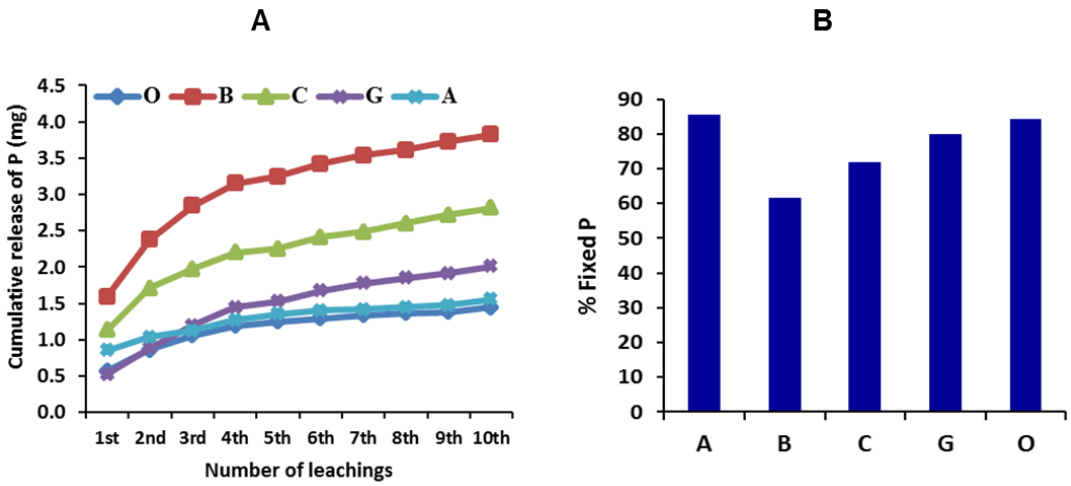

Figure 54. *(A) Release of P in soil from bio-CPC and (B) fixation of P in soil released from bio-CPC.*

4.2.1 Loading of phosphorus in bio- and polyacrylate-CPCs

To determine the maximum loading percentage of P in the CPCs, an experiment was conducted in which P was loaded on bio- and polyacrylate-CPCs externally at different loading percentages. The loading percentage of P was set up as 10%, 20%, 30%, 40% and 50% on the basis of product weight. This was carried out by immersing pre-weighed dry product (10 g) into 50 mL of solution containing 4.126, 8.252, 12.378, 16.504 and 20.63 g of DAP, respectively, at room temperature for 24 hours. CPCs were dried in an oven at 60°C. To find out the actual P loading percentage in the CPCs, a leaching experiment was conducted. One gram each of 10%, 20%, 30%, 40% and 50% P-loaded CPCs was taken in a plastic funnel and leached with 100 mL of distilled water. After 24 hours, the CPCs were dried in an oven at

60°C. Dried CPCs were digested with hydrofluoric acid and perchloric acid. Phosphorus was estimated in the digested sample and leachate. The following parameters were calculated:

(a) P loading with initial leaching (%) = P in leachate + P in digested CPC
(b) P loading without initial leaching (%) = P in direct digestion
(c) P loading (%) inside CPC = P remaining after initial leaching in digested CPC only

P loading inside the CPC particle structure is the actual P loading in the CPC without the amount of P present on the surface of the CPC. The maximum P loading inside the CPC was 5% and 4.7% in bio- and polyacrylate-CPCs, respectively (Table 12). Therefore, the P-loading percentage in the CPC was set up at 20%. Also, when loading at a higher percentage of P was tested, salt deposition on the surface of CPC was observed. This indicates that P loading in the CPC at a higher percentage is not feasible.

Table 12. *Phosphorus loading (%) in bio- and polyacrylate-CPC*

Desired P Loading in CPC (%)		P Loading with Initial Leaching (%)	P Loading without Initial Leaching (%)	P Loading Inside CPC (%)
Bio-CPC	10	9.3	4.6	3.0
	20	**13.4**	**11**	**5.0**
	30	16.5	13	3.3
	40	18.0	15	4.6
	50	18.9	13	1.1
Polyacrylate-CPC	10	12.2	6.3	4.0
	20	**14.5**	**10**	**4.7**
	30	18.3	12	3.2
	40	18.6	13	2.8
	50	0.0	0.01	0.0

5 Evaluation of efficacy of seed coating material in improving phosphorus uptake efficiency of a crop (Objective 4)

5.1 Response of soybean to oxalic acid-, phosphorus- and PSB-loaded seed coating material (greenhouse experiment)

The response of soybean crop to oxalic acid-, P- and PSB-loaded seed coating was evaluated using alluvial soil in a glasshouse experiment. Treatments consisted of only CPC, 5% oxalic acid, 10% P, 20% P, 10% P + 5% oxalic acid, 20% P + 5% oxalic acid, 10% P + 5% oxalic acid + PSB, and 20% P + 5% oxalic acid + PSB, as well as a control (soil with no P). Seed coating was prepared from both polyacrylate- and bio-CPCs. Each treatment combination was replicated five times in a completely randomized design. Plastic pots were filled with 2 kg of soil, and 10 coated seeds of soybean were sown in each pot (Fig. 55). Two weeks after germination, a uniform plant population (two plants per pot) was maintained. Pots were watered daily with deionized water. A soil sample was collected at 60 days after sowing (DAS). The plants were sampled at anthesis and maturity stages. After harvesting, plant samples were dried in a hot-air oven at 60-70°C, and after attaining constant weight, dry weight was recorded. Dried plant samples were ground using mechanical grinder made of stainless steel. Plant samples were then digested with a diacid mixture (HNO_3:$HClO_4$ at 10:4), and P content was determined by the yellow color method (Jackson, 1973). After harvesting the soybean, soil was taken out of the pots and mixed thoroughly. Soil samples were air dried, ground and sieved through a 2-mm sieve. These soil samples were used for extraction of P using 0.5 M $NaHCO_3$ solution, and P content in the extract was determined with the ascorbic acid blue color method (Watanabe and Olsen, 1965).

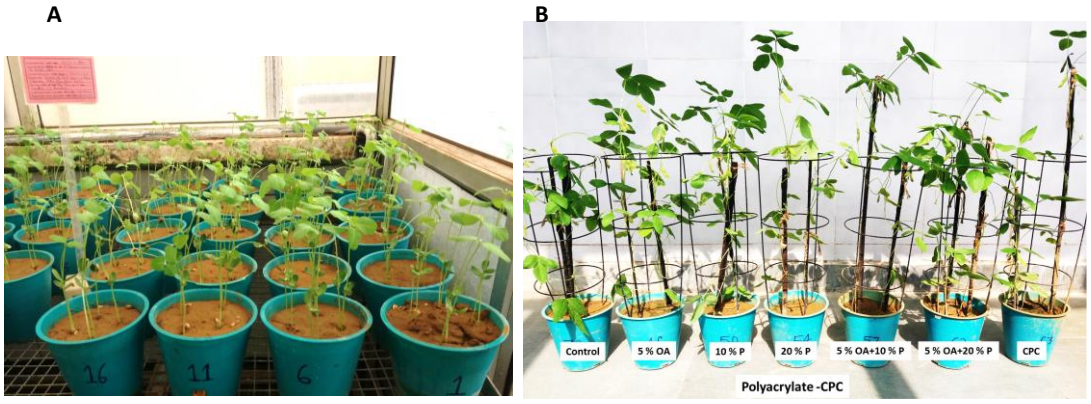

Figure 55. *Soybean seeds coated with polyacrylate- and bio-CPCs with different combinations of P, oxalic acid and PSB (for details, refer to Materials and methods section): (A) coated seeds in a glasshouse and (B) general view of plant growth of coated seeds.*

5.2 Results of seed coating with bio- and polyacrylate-CPCs

Various CPC treatments showed a non-significant effect on available soil P, while the plant growth traits were significantly influenced. Seed coating with both polyacrylate and bio-CPC significantly affected total plant biomass (Fig. 56). In seed coating with polyacrylate-CPC, the highest biomass was obtained in 20% P, followed by 10% P + 5% oxalic acid + PSB. In bio-CPC-coated seeds, the highest biomass was recorded in 10% P + 5% oxalic acid, followed by 20% P + 5% oxalic acid. Maximum P concentration in shoot tissue was observed at 10% P + 5% oxalic acid, which was statistically on par with 10% P and 10% P + 5% oxalic acid + PSB in polyacrylate-CPC (Fig. 57). In the case of bio-CPC, P concentration in shoot was highest in the 20% P + 5% oxalic acid + PSB and 10% P + 5% oxalic acid treatments. However, the common treatment 20% P + 5% oxalic acid resulted in maximum shoot P concentration in both bio- and polyacrylate-CPCs. Root tissue P concentration was also highest in the 20% P + 5% oxalic acid + PSB treatment, as compared to other treatments, in both polyacrylate- and bio-CPCs (Fig. 58).

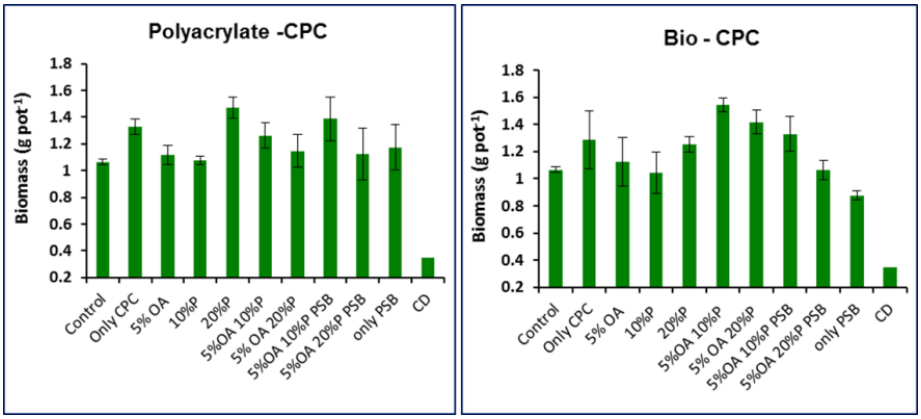

Figure 56. *Effect of P, oxalic acid and PSB application through seed coating with polyacrylate- and bio-CPCs on total plant biomass at flowering stage in soybean.*

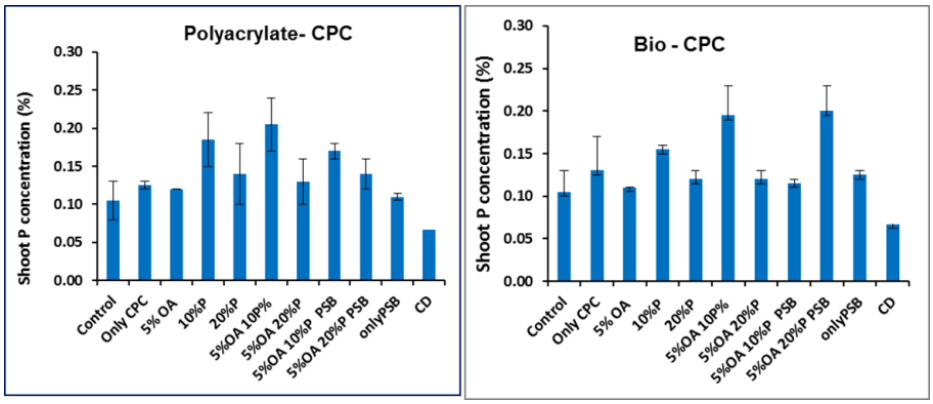

Figure 57. *Effect of P, oxalic acid and PSB application through polyacrylate- and bio-CPCs on P content in soybean shoot at flowering (%).*

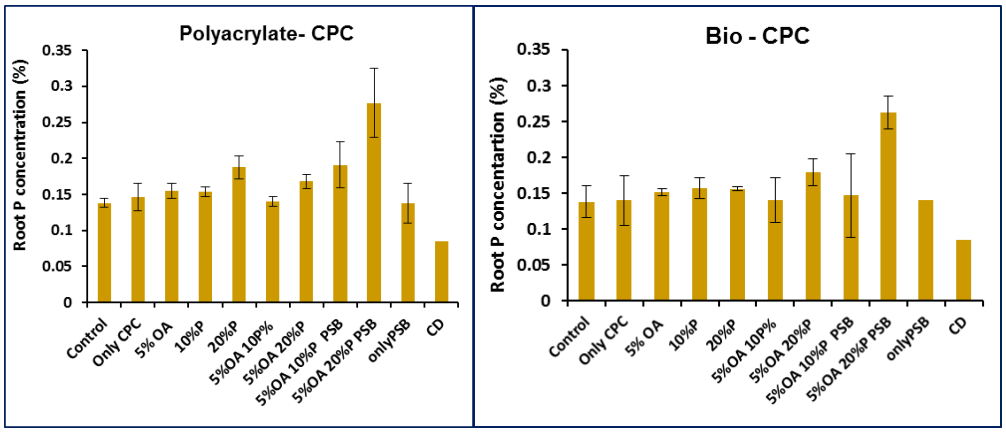

Figure 58. *Effect of P, oxalic acid and PSB application through polyacrylate- and bio-CPCs on P content in soybean root at flowering (%)*

Total seed weight of soybean was significantly and positively influenced by various treatments over control (no P) or only CPC (Fig. 59). Seed coating containing 10% P + 5% oxalic acid + PSB, either in polyacrylate-CPC or bio-CPC, resulted in maximum seed yield. However, seed coating with bio-CPC containing 10% P + 5% oxalic acid also resulted in maximum yield. High seed P concentration is not a desirable trait as P in seeds binds with divalent cations, such as zinc, manganese, and iron, in the form of phytic acid, thereby making these minerals less bioavailable for ruminants. But seed P is important for seed germination and early seedling establishment. In both polyacrylate- and bio-CPC-coated seeds, the P concentration was significantly higher in all treatments as compared to control or CPC only. Seed P concentration on par with control was recorded in 10% P + 5% oxalic acid + PSB in polyacrylate-CPC seed coating, while the same treatment resulted in the highest seed P concentration in bio-CPC seed coating (Fig. 60).

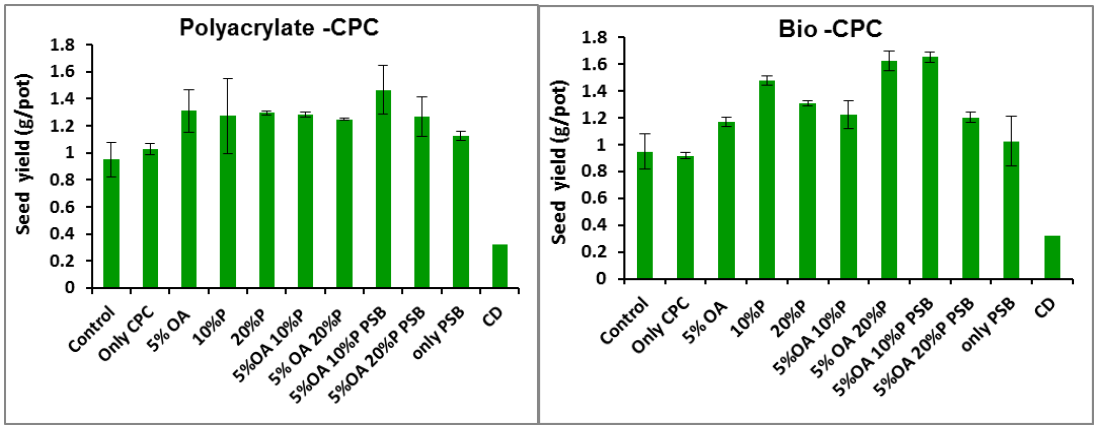

Figure 59. *Effect of P, oxalic acid and PSB application through polyacrylate- and bio-CPCs on soybean seed yield (g pot^{-1}).*

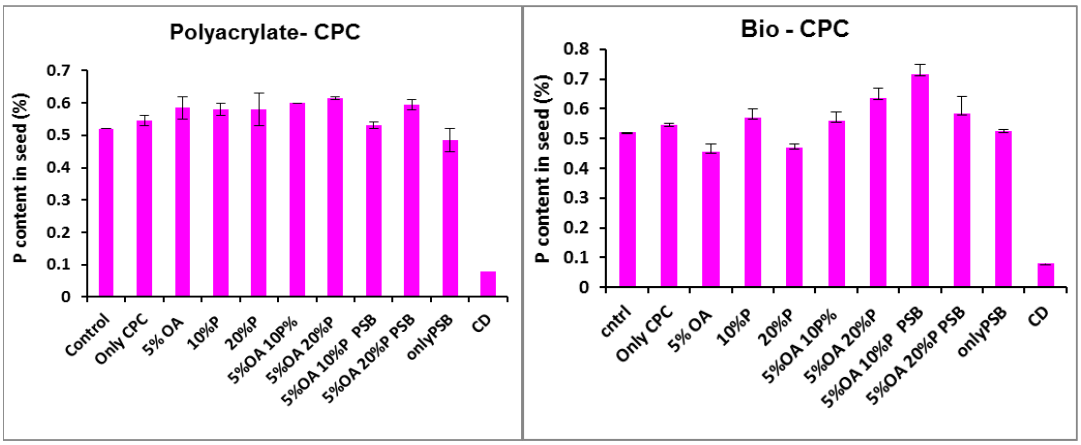

Figure 60. *Effect of P, oxalic acid and PSB application through polyacrylate- and bio-CPCs on P content in soybean seed (%)*

5.3 Conclusions of Objective 4

- Bio-CPC (at a ratio of 10:40, B) allows the lowest P fixation (%) in soil and was selected for seed coating.
- P-loading percentage in CPC was set up at 20%, as maximum P loading in CPC was achieved at this percentage.
- Application of P through seed coating with polyacrylate and bio-CPC improved yield and P uptake by soybean over control (no soil P). However, considering cost effectiveness and the environment, using starch based bio-CPC is preferred.
- Loading of 20% P in CPCs was found to be the best for seed coating and also released maximum P in the soil.

6 Combinations of the best seed coating material and foliar formulation in enhancing nutrient uptake efficiency of crops under field conditions (Objective 5)

6.1 Materials and methods

Testing was carried out in the experimental fields during summer season in 2016 using soybean variety DS-2614 in a complete randomized block design with three replicates. Each replicate consisted of two rows each with 10 plants. Coated soybean seeds containing phosphorus with a specific carrier (polyacrylate-CPC or bio-CPC) were used, as presented in Table 13 (coated seeds provided by Dr. Mandira Barman). Based on the results of the experiments of foliar application (Objective 1) and seed coating (Objectives 3 and 4), a combination of eight treatments, as well as a control (uncoated seed without foliar spray), were selected. The recommended dose of fertilizer was added to the soil before sowing. Foliar spray of HA (25 mg) + $FeCl_3$ (2 mM) was applied in selected treatments at anthesis stage only. At maturity, biomass and yield traits were recorded, including number of pods per plant, test weight, yield per plant, and Fe and P content in shoot tissue and seeds.

Table 13. *Treatments selected to study the efficacy of seed coating and foliar application in combination on soybean in the field.*

S. No.	Treatments
T0	Control (uncoated seed and without foliar)
T1	Polyacrylate-CPC + 20% Phosphorus
T2	Bio-CPC + 20% Phosphorus
T3	Polyacrylate-CPC + 20% Phosphorus + 5% Oxalic Acid
T4	Bio-CPC + 20% Phosphorus + 5% Oxalic Acid
T5	Polyacrylate-CPC + 20% Phosphorus + Foliar
T6	Bio-CPC + 20% Phosphorus + Foliar
T7	Polyacrylate-CPC + 20% Phosphorus + 5% Oxalic Acid + Foliar
T8	Bio-CPC + 20% Phosphorus + 5% Oxalic Acid + Foliar

Soybean field: combination of coated seed and best foliar spray

6.2 Results obtained with coated seed combined with foliar application

Figure 61 A-D clearly shows that the seeds coated with polyacrylate-CPC + 20% P + 5% oxalic acid (OA) in combination with foliar application of HA (25 mg) + $FeCl_3$ (2 mM), as well as its counterparts without foliar spray, at anthesis stage in soybean resulted in the maximum increase in biomass at harvest, number of pods, total pod weight and total seed weight per plant as compared to control. However, the test weight was significantly higher in plants from seed coated with bio-CPC + 20% P + 5% OA without foliar spray as compared to all other treatments (Fig. 61E). These results show that seed coating, preferably with polyacrylate-CPC + 20% P + 5% OA, and foliar spray of HA (25 mg) + $FeCl_3$ (2 mM) only once at anthesis leads to higher grain production.

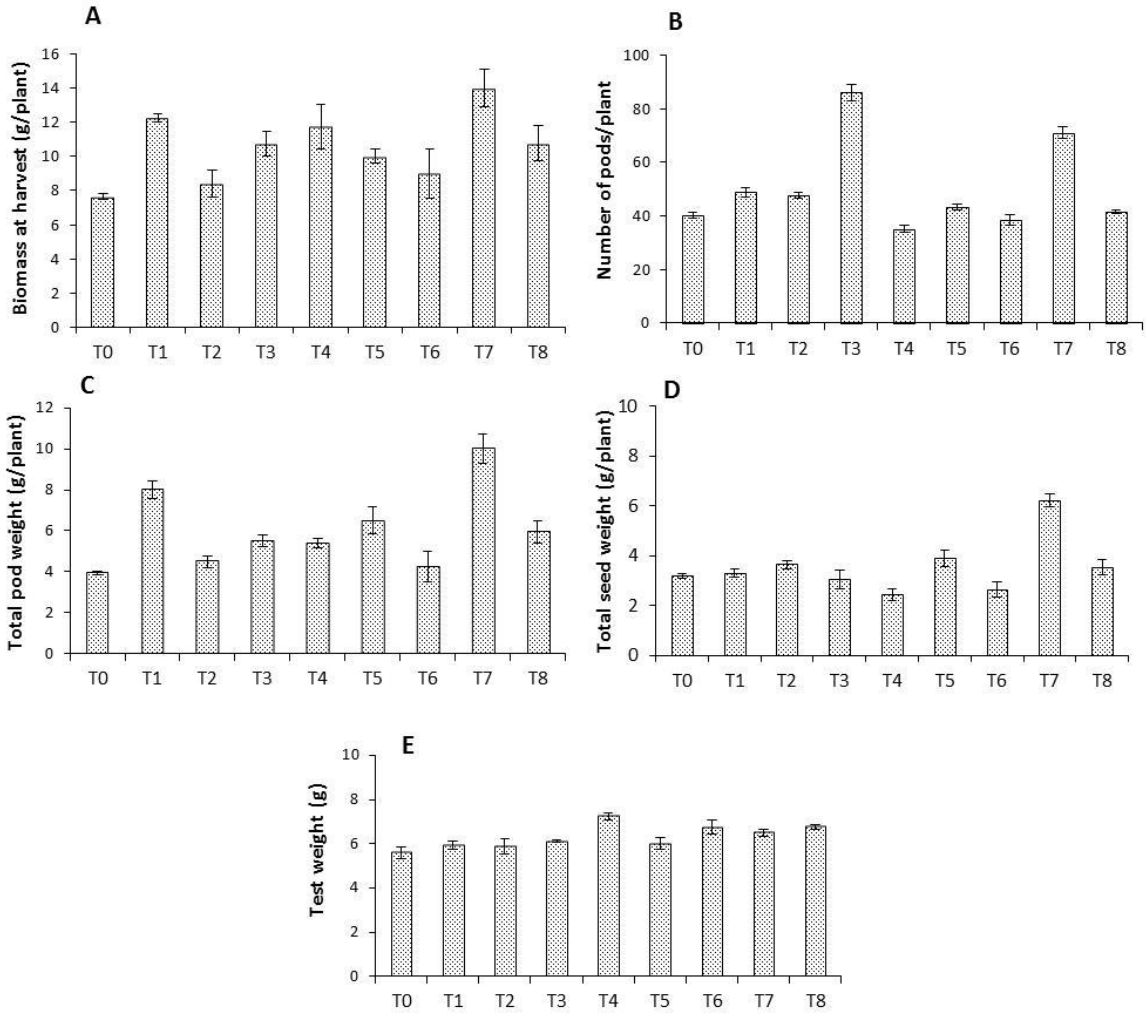

Figure 61. *Effect of coated seeds in combination with foliar spray on growth and yield of soybean grown in the field: (A) biomass at harvest, (B) number of pods per plant, (C) total pod weight including seeds, (D) seed weight per plant and (E) 100-seed weight or test weight.*

The highest P concentration in different plant tissues, such as leaf, stem, pod cover and seeds, was recorded with a combination of seed coating and humic acid foliar application (Fig. 62 A-B). Among different treatments, maximum P concentration in leaf occurred with T7, followed by T5 and T6, while T2 remained the lowest. Stem P concentration was highest with T6 and T5. Percentage increase in seed P concentration ranged from 50% to 60% as compared to control. However, there was not much difference in seed P concentration among all treatments. All of the seed coating treatments resulted in an increase in Fe concentration in different tissues as compared to control. T6, T8 and T5 increased leaf Fe concentration by 70%, 54% and 33%, respectively, over control. However, the stem Fe concentration was maximum for T3, followed by T2, T6 and T5, while least increase was recorded with T8. There was no significant difference in seed Fe concentration among all treatments, but the maximum increase was recorded with T6 (14%).

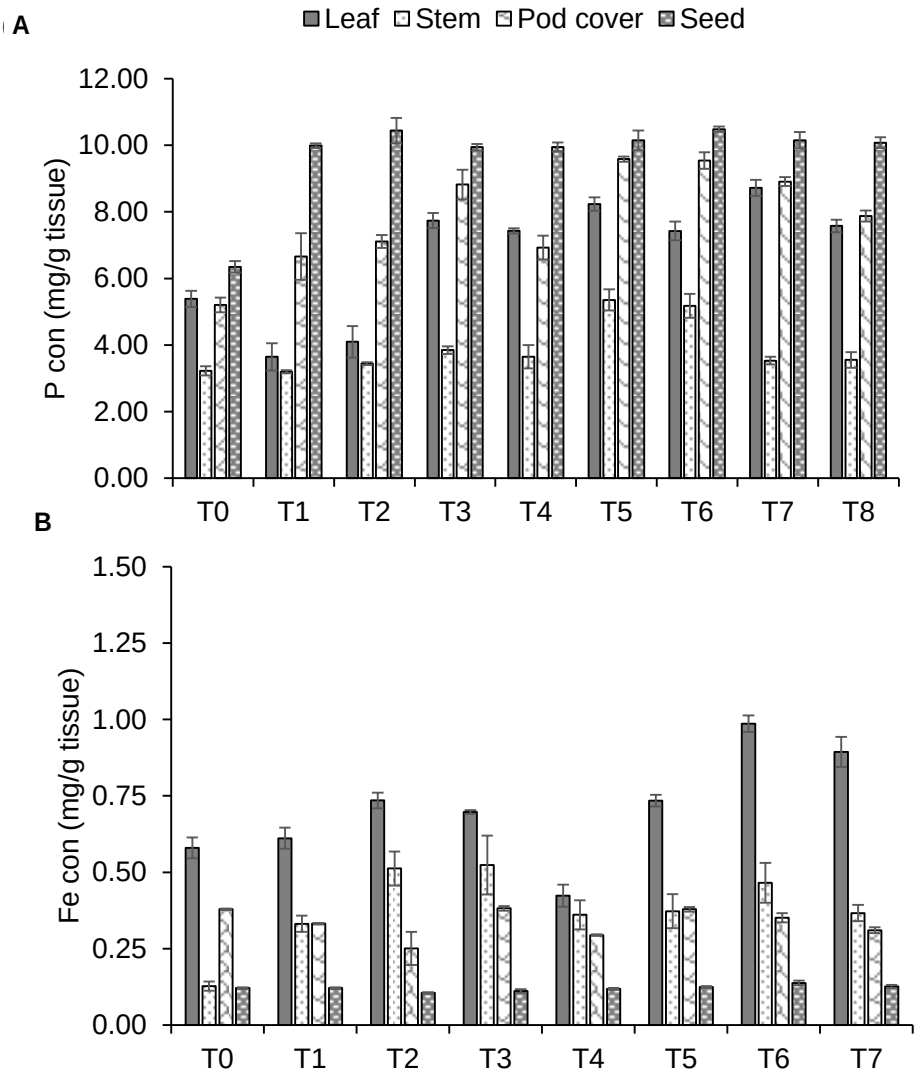

Figure 62. *Effect of coated seeds in combination with foliar spray on P and Fe concentration in different tissues of soybean.*

6.3 Activity related to lettuce crop

Lettuce seed was sown in the nursery in glasshouse on October 20, 2016, and seedlings were transplanted in the pots under natural environmental conditions.

The experiment was conducted with lettuce variety Chinese Yellow lettuce variety under natural conditions in pots. The nursery was prepared in a germination tray in a glasshouse and 25-day-old (two-leaf stage) healthy plants were transferred to the pots. Each pot (30 cm diameter) contained 15 kg of soil with the recommended dose of NPK. Plants were irrigated as per requirement with normal tap water. Three foliar treatments (HA [25 mg] + $FeCl_3$ [2 mM], $FePO_4$ [2 mM] and Fe-citrate [4 mM]) were selected from Objective 1 and used for spray. The spray formulations were prepared by dissolving the required

amount of chemical compound and adding 100 µL of surfactant (Triton X100) in 1.0 L of solution. A water spray was also included as a control. Plants were sprayed after attaining of sufficient leaf growth, i.e., in mid-December. Growth traits, such as leaf area, biomass (dry and fresh) and nutrient content (in old and new leaf), were recorded after six days of spraying. Old and new leaves correspond to the fully mature and young leaves, respectively, at the time of sampling.

Foliar application on lettuce showed significant difference in terms of physiological parameters and nutrient concentration in leaf (Fig. 63 A-E). Highest shoot fresh biomass was recorded for plants sprayed with HA + $FeCl_3$, followed by Fe-citrate and $FePO_4$. As compared to control, shoot fresh and dry weight increased by 85% and 50%, respectively, in plants sprayed with HA + $FeCl_3$. In general, all of the treatments resulted in an increase in leaf area in comparison to control, though the hike was maximum with HA + $FeCl_3$ (78%), followed by Fe-citrate (72%) and $FePO_4$ (32%). Significant difference in P and Fe concentrations between older and young leaves of lettuce plants was recorded. Fe and P concentrations were recorded to be higher in mature and young leaves, respectively. The highest Fe concentration was recorded in mature leaves of plants sprayed with HA + $FeCl_3$ (45%), followed by Fe-citrate (33%). However, leaf P concentration was the highest in $FePO_4$-sprayed plants as compared to other treatments; this was a 40% increase as compared to control.

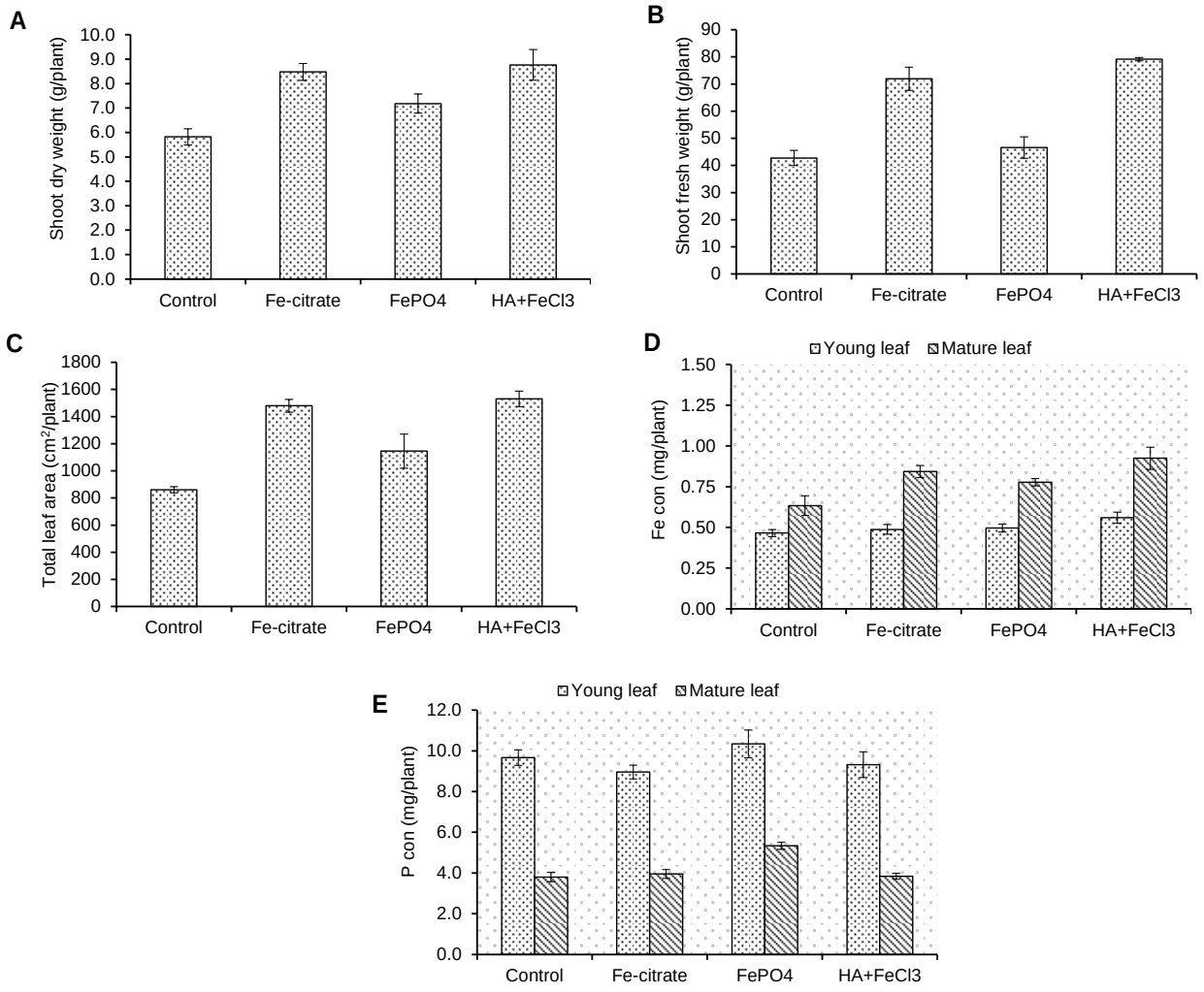

Figure 63. *Effect of foliar spray of different Fe compounds on (A) shoot dry weight, (B) shoot fresh weight, (C) total leaf area, (C) Fe concentration and (E) P concentration in young and mature leaves of lettuce.*

The response of lettuce plants to HA + $FeCl_3$ was better as compared to other treatments. The spray of HA + $FeCl_3$ resulted in higher biomass and more leaf area of lettuce plants, accompanied by a respective increase and decrease in leaf Fe and P concentrations.

7 Acknowledgements

The Principal Investigator and Co-PI gratefully acknowledge the funding received from the Virtual Fertilizer Research Center, Washington, D.C., USA. DARE, ICAR (New Delhi), and the Indian Agricultural Research Institute (New Delhi) are duly acknowledged for facilitating the research work and in-kind contributions, including infrastructure, instrumentation, and farm. The authors are also thankful to Dr. Hina Malhotra, Senior Research Fellow, NSAF Project, for help in writing the report.

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Preliminary experiments conducted on rice and soybean crops grown in a glasshouse at National Phytotron Facility

Rice seedlings after transplanting

Rice plants given foliar application

Soybean plants in glasshouse

Measuring photosynthesis on the sixth day after foliar application

Rice plants after foliar application

Rice plants in grain filling stage

Soybean after 1st foliar application

Pod filling stage

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