Plant Physiology and Biochemistry 144 (2019) 207–221

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Enhancement of nitrogen assimilation and photosynthetic efficiency by novel iron pulsing technique in *Oryza sativa* L. var Pankaj

Swarnali Dey[a], Rita Kundu[a], Geetha Gopal[b], Amitava Mukherjee[b], Anish Nag[c], Subhabrata Paul[d,*]

[a] Centre of Advance Study, Department of Botany, University of Calcutta, Kolkata, 700019, India
[b] Centre for NanoBiotechnology, VIT University, Vellore, 632014, Tamil Nadu, India
[c] Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, 560029, India
[d] Department of Life Sciences, Presidency University, Kolkata, 700073, India

ARTICLE INFO

Keywords:
Iron-pulsing
Rice
Growth
Photosynthesis
Nitrogen assimilation

ABSTRACT

Rice is a major food crop. Due to urbanization and climate change, rice production is declining, posing a threat to the increasing food demand. For this, a modified technique of priming is used to enhance plant vigor. In the present study an endogenous rice cultivar was treated with two different iron salts for 72 h and grown for 14 days in nutrient solution. This increased the iron content of the samples which further escalated the photosynthetic efficiency and carbon assimilation in the treated plants. Photosynthesis being correlated to nitrogen assimilation, nitrogen assimilation intermediates and protein content were also elevated in treated plants. Plants showed no symptoms of stress as evident from low malondialdehyde content and increased antioxidant enzymes' activity. From this study it can be inferred that, treatment with iron during germination, helps to trigger growth by facilitating photosynthesis and nitrogen assimilation.

1. Introduction

Rice (*Oryza sativa*, an annual grass of the Poaceae family), a major cereal grain, is the most widely consumed staple food for majority of the world's population. India is one of the world's largest producers of rice accounting for 20 percent of all worldwide production (158.8 million tons in 2016) (India:Country Study:C, 1995). India is the second most populated country in the world and with a population growth rate of 1.13%, it is projected to be the world's most populous country by 2030, surpassing the population of China (https://www.un.org/en/dev, 2015). India has an arable land area of 394.6 million acres which is the second highest in the world just after US. India has the largest irrigated crop area of 215.6 million acres in the world. But this area is shrinking at an alarming rate of 0.03 million hectares per year and it has been documented that between the years 2010–11 and 2015–16 average size of Indian arable land shrank by almost 6% (agricoop.nic.in/sites/def, 2016).

In 1960s, Green Revolution, an international scientific effort to diminish the threat of worldwide hunger, produced improved strains of food crops including rice. With the rapid growth of population and consequent increase in demand for food, there stands an urgent need of a 'Second Green Revolution' (Sinha, 1997). This calls for new approaches and new set of technologies as climate change is tightening up its grip and threatening food supply. With fixed rather descending amount of cultivable land, it becomes obligatory to enhance yield of the prevalent rice crops or introduce new high yielding varieties which will be user friendly and affordable to the poor farmers.

Seed priming is a technique by which seeds are subjected to controlled hydration before sowing, for certain amount of time (preferably overnight), followed by drying. This helps to 'prepare' the seeds for sowing and ensures maximum and rapid germination (Farooq et al., 2006). Rice seed priming with water (Hydropriming) is an age old practice to enhance germination of rice. Modification of this orthodox technique has given diverse forms of priming. Modern day priming techniques includes Halopriming with salts such as $Mg(NO_3)_2$, NaCl, KCl, $CaCl_3$, KNO_3, Osmopriming with osmolytes including sugar alcohols sorbitol, mannitol and PEG, Hormopriming with certain hormones like kinetin and GA3, priming with natural compounds such as wood vinegar, coconut water, coconut milk and Biopriming with *Bacillus subtilis* biofilms and Rutin, a bioflavonoid (Lutts et al., 2016; Theerakulpisut et al., 2016; Catada et al., 2016; Singh et al., 2016). Besides an emerging technique of priming is nanopriming. Several reports have been published where priming has been done with nano silica, nano iron and biocompatible nano silver particles (Adhikari et al., 2013; Mahakham et al., 2017; Guha et al., 2018). Mostly all priming techniques has shown enhanced germination rates and growth

* Corresponding author.
E-mail address: subhabrata.dbs@presiuniv.ac.in (S. Paul).

https://doi.org/10.1016/j.plaphy.2019.09.037
Received 7 July 2019; Received in revised form 21 September 2019; Accepted 22 September 2019
Available online 25 September 2019

of seedlings by several folds compared to the non primed control sets where as some techniques have also resulted in increase of biomass, photosynthetic ability, activity of α amylase enzyme, less amount of ROS and also upregulation of aquaporin gene (Adhikari et al., 2013; Guha et al., 2018). In this work we have modified priming technique and introduced a 'pulsing' treatment where we treated rice seeds with two different iron salts for 72 h and then retractedthem from the treatment and grown in hydroponics.

About 17 elements are utilized by plants for their optimal growth and development. These minerals are classified into macro and micro nutrients based on their requirements by the plants, when required in relatively high amounts, are called macronutrients and when in trace amounts, are called micronutrients. Though the micronutrients are required in relatively smaller quantities for plant growth, they are as important as macronutrient. Micronutrient deficiency can lead to growth suppression or even complete inhibition (White and Brown, 2010). Micronutrients often act as co-factors in enzyme systems and participate in redox reactions, in addition to having several other vital functions in plants. Most importantly, micronutrients are involved in the key physiological processes of photosynthesis and respiration (Marschner, 1995). Iron is one of the most essential micronutrient. Iron (Fe) deficiency is a common nutritional problem faced by many crops, rice being especially susceptible to iron deficiency. Fe is the chief nutrient responsible for chlorophyll biosynthesis (making the porphyrin backbone) and thus essential for photosynthesis. Besides it has numerous other functions in the plant as it acts as co factor of crucial enzymes required for vital metabolic process like respiration (cytochrome), nitrogen metabolism (nitrate and nitrite reductase, glutamate synthase), redox balance (catalase, peroxidase, SOD) (Rout and Sahoo, 2015).Fe is one of the most abundant mineral in soil but magnitude of its bioavailability is low owing to aerobic or calcareous soil or high pH which readily oxides iron to insoluble ferric oxides. Higher plants uptake iron via two pathways: Strategy I (mostly dicots) that involves proton extrusion and consequent conversion of ferric ions to ferrous ones, followed by uptake through Iron Regulated Transporters (IRT) present in the plasma membrane of roots and Strategy II (graminaceous plants) where a phytosiderophore named Mugineic acid (MA) is released into the rhizosphere that forms a Fe(III)-MA complex which in turn is taken up by Yellow Stripe Like (YSL) transporters. Rice can uptake iron by both the strategies which compelled us to use two different iron salts, one in the ferrous form ($FeSO_4$) and one in the ferric form ($FeCl_3$) (Kar and Kumar Panda, 2018).

We have hypothesized that, optimum Fe treatment will affect N_2 assimilation positively and that may have a cascading effect in photosynthesis, as a result overall growth and vigor will enhance, which may be translated in yield enhancement. Hence, we focused on iron pulsing treatment in order to increase growth and vigor of rice cultivar Pankaj and carry out a detailed analysis of how iron is able to do so. In this regard, we have thoroughly studied two major metabolic pathways (photosynthesis and nitrogen assimilation) and tried to draw a correlation between the two, as earlier studies have shown that iron deficiency has hindered both the processes in some way or the other. For the present study, we have selected Pankaj, (a semi-dwarf indigenous variety once rigorously grown in Bengal, but, now a days, not favored by the farmers any more, due to its poor yield), to test the efficacy of our hypothesis. We attempted to increase its growth rate, vigor by increasing photosynthesis and nitrogen assimilation with a simple, cost effective and user friendly treatment which can be easily translated from laboratory to land.

2. Materials and methods

2.1. Materials

$FeCl_3$ and $FeSO_4$ used in this study were from Merck. Rice seeds were collected from Rice Research Station, Chinsurah (Govt. of West Bengal, India). All other chemicals were procured from standard companies (Himedia, SRL, Merck, Sigma).

2.2. Iron pulse treatment

Two iron salts, $FeCl_3$ (ferric) and $FeSO_4$ (ferrous) were used in this study to treat seeds at the time of germination. Distilled water was used in place of iron salt solutions in the control sets. Three concentrations viz. 2.5 mM, 5 mM and 10 mM were used for both salts for a period of 72 h at the time of germination of seeds. Surface sterilized rice seeds were placed on moist filter paper in plastic containers with iron salt solutions and incubated in dark for 72 h at 25 °C.

The germinated seeds were washed with distilled water and kept in standard hydroponic system (Hoagland) for 14 days (Hoagland and Arnon, 1950). After that period, growth parameters along with nitrogen assimilation, and change in photosynthesis were studied by various methods.

The $FeCl_3$ and $FeSO_4$ sets were designated as C2.5, C5, C10 and S2.5, S5, S10 based on their concentration.

2.3. Growth parameters

Shoot, root lengths along with dry weights of the whole plants were taken.

2.4. Vigor index

Both vigor indexes I and II were calculated following Abdul-Baki and Anderson (1973) at 14 day time points (Abdul-Baki and Anderson, 1973).

Vigor index I = Germination % x Seedling Length (Root + Shoot)

Vigor index II = Germination % x Seedling Dry weight (Root + Shoot)

2.5. Relative water content (RWC)

RWCs were calculated by the following formula (Barrs and Weatherley, 1962).

Relative water content% = [(Fresh weight – Dry weight)/(Turgid weight –Dry weight)] X 100

2.6. Nitrogen assimilation

Nitrogen assimilation intermediates and enzymes were assayed using previously documented methods in literature (Ghosh et al., 2013; Wray and Fido, 1990).

2.6.1. Total nitrogen

Using Nessler's reagent, total and soluble nitrogen were quantified spectrophotometrically.100 mg plant tissue (shoot and root) was digested in 1 ml of conc. H_2SO_4 and cooled. Gradually 500 µl H_2O_2 was added and heated until the solutions became colorless. To 100 µl mixture of 10% NaOH and 10% sodium silicate (1:1) was added with subsequent addition of 1 ml Nessler's reagent and incubated for 30 min at room temperature. The resultant yellowish orange color was quantified in an UV-VIS spectrophotometer at 490 nm.

2.6.2. Soluble nitrogen

Sample was homogenized in 1 ml distilled water, centrifuged at 12000 rpm and the supernatants were collected. 5% TCA was added to precipitate the proteins and after centrifugation the supernatant was collected. After H_2SO_4 digestion, the nitrogen content was measured following same method, described in the earlier section.

2.6.3. Nitrate content

100 mg of tissue was boiled in 1 ml water for 30 min and centrifuged. With the collected supernatant nitrate content was measured and expressed as μg g^{-1} fresh weight shoot or root using a standard curve. 20 μl supernatant was added to 80 μl of 5% salicylic acid and incubated for 30 min in dark and then mixed with 1.9 ml of 2(N) NaOH. The resultant yellow color was measured at 430 nm in a uv–vis spectrophotometer (Shimadzu).

2.6.4. Nitrate reductase (NR, EC 1.6.6.6)

100 mg plant tissue was homogenized in 1 ml of 25 mM phosphate buffer (pH 8.8) containing 25 mM cysteine and 1 mM EDTA and centrifuged. With the collected supernatant enzyme activity was measured and expressed as μM nitrite produced g^{-1} FW min^{-1} using a standard curve of $NaNO_3$.

2.6.5. Nitrite content

100 mg of plant tissue was homogenized in 1 ml of 100 mM phosphate buffer (pH 7.7) and centrifuged. With the collected supernatant nitrate content was measured and expressed as μg g^{-1} FW shoot or root using a standard curve. With 500 μl supernatant same amounts of 1% sulphanilamide in 3(N) HCl and 0.1% NED were added and incubated at room temperature for 15 min. The resultant red color was measured at 540 nm in a uv–vis spectrophotometer (Shimadzu).

2.6.6. Nitrite reductase (NiR, EC 1.6.6.4)

100 mg of plant tissue was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.7) and centrifuged. With the collected supernatant nitrate content was measured and expressed as nitrite consumed using a standard curve. This process uses dithionate reduced viologen as artificial electron donor. To 450 μl of reaction mixture containing 2.5 mM $NaNO_2$, 3 mM methyl viologen and 200 mM Na-dithionate in 290 mM Na-bicarbonate, 50 μl of supernatant added and incubated for 30 min at room temperature and then the reaction was terminated by adding a stop solution containing 1% sulphanilamide in 3(N) HCl and 0.1% NED. Absorbance was taken at 540 nm.

2.6.7. Free amino acid content

100 mg of plant tissue was extracted in 1 ml of 80% ethanol and centrifuged. With 100 μl supernatant, free amino acid content was measured using ninhydrin reagent.The resultant blue color was measured at 570 nm in a spectrophotometer.

2.6.8. Dissolved ammonia

100 mg of plant tissue was extracted in water. With a portion of extract, equal amounts of 1% phenol containing 0.005% Na nitroprusside and 0.042% Na-hypochlorite in 0.5% NaOH was added and incubated for 20 min at room temperature. Absorbance of the resultant blue color was determined at 625 nm and expressed as μmoles NH_3 gm^{-1} FW tissue.

2.6.9. Glutamine synthetase (GS 6.3.1.2)

This enzyme activity was expressed as glutamic hydroxamate formed gm^{-1} FW min^{-1}. 100 μl of supernatant was added to 800 μl reaction mixture containing 100 mM Tris (pH 7.6), 20 mM $MgSO_4$, 30 mM glutamate, 6 mM NH_2OH, 6 mM aspartate, 4 mM EDTA and 12 mM ATP and incubated for 15 min at room temperature and then the reaction was stopped by adding 100 μl of stop solution containing 370 mM $FeCl_3$, 200 mM TCA and 670 mM HCl. The resultant γ-glutamylhydroxamate was measured at 540 nm.

2.6.10. Glutamate synthase (GOGAT 1.4.1.13)

100 mg plant tissue was extracted in 1 ml 50 mM phosphate buffer (pH 7.5) containing 2 mM EDTA, 1.5% casein, 2 mM DTT, and 1% PVP and centrifuged. 100 μl supernatant was added to 900 μl reaction mixture (40 mM phosphate buffer (pH7.5) containing 10 mM glutamine, 10 mM oxoglutamate, 140 μM NADH) and the activity was measured. The rate of NADH oxidation was measured after addition the supernatant and the enzyme activity was expressed as mM NADH oxidized gm^{-1} FW min^{-1}.

2.6.11. Glutamate dehydrogenase (GDH 1.4.1.2)

This enzyme activity was expressed as mM NADH oxidized g^{-1} FW min^{-1}. 100 μl supernatant was added to 900 μl reaction mixture containing 100 μM Tris (pH 8.2), 150 μM NH_4Cl, 1 μM $CaCl_2$, 0.3 μM NADH and 20 μM 2-oxoglutarate.

2.6.12. Protein estimation

In the enzymatic preparations, protein content was measured using BSA as standard (Lowry et al., 1951). Briefly 10 μl supernatant was added to 115 μl water and 1 ml of Lowry solution (2% sodium carbonate in 0.1 (N) NaOH: 1% sodium potassium tartarate: 1% $CuSO_4$ = 98:1:1) and incubated for 10 min. Then 125 μl of 1/3rd diluted folin ciocalteu solution was added and incubated for 30 min at dark and resultant blue color was quantified at 660 nm.

2.7. Photosynthesis

2.7.1. δ-Ala content

δ-ALA content was quantified following Masoner and Kasemir (1975) (Masoner and Kasemir, 1975).100 mg leaf tissue was homogenized in 1 ml of 0.1 (N) TCA and centrifuged. The supernatant was adjusted to pH 4.5 with 1 M sodium acetate. To 500 μl supernatant, 70 μl acetyl acetone was added and kept in boiling water bath for 10 min. After cooling 500 μl of Ehrlich reagent was added and after 20 min incubation absorbance was taken at 555 nm.

2.7.2. Chlorophyll content

50 mg finely chopped leaf was immersed in airtight containers containing 2 ml 80% acetone for overnight and absorbance was recorded in three different wavelengths (470, 646.8 and 663.2) nm in a spectrophotometer and by the following equations, amount of chlorophyll *a*, *b*, total chlorophyll (a + b) and total carotenoid (x + c) was calculated (Lichtenthaler, 1987).

$$C_a = 12.25A_{663.2} – 2.79A_{646.8}$$

$$C_b = 21.5A_{646.8} – 5.1A_{663.2}$$

$$C_{a+b} = 7.15\ A_{663.2} + 18.71A_{646.8}$$

$$C_{x+c} = (1000A_{470} – 1.82C_a – 85.02C_b)/198$$

2.7.3. Chlorophyll fluorescence

Fluorescence of the extracted chlorophyll solutions was measured. Readings were taken at excitation wavelength of 640 nm and emission wavelength 680 nm with the help of a spectrofluorimeter (Hitachi).

2.7.4. Chlorophyll activity

Hill activity was measured in terms of DCPIP reduction by illuminated chlorophylls (Bauer and Senser, 1979). 100 mg leaves were extracted in extraction buffer containing 0.4M sucrose in 0.05 M phosphate buffer (pH 6.2) and centrifuged at 1000 G for 5 min. The supernatant was taken and re-centrifuged at 5000 G for 15 min. Then pellets were taken and re-suspended in 1 ml of sucrose phosphate buffer. In the assay mixture, 125 μl chloroplast suspension was added to 0.05M phosphate buffer and 62.5 μl 0.03% DCPIP and absorbance was taken at 610 nm. The mixture was then incubated for 15 min under strong incandescence light and again absorbance was taken at 610 nm. Hill activity was finally expressed as μmole DCPIP reduced per mg chlorophyll h^{-1}.

Amount of total chlorophyll was measured. 250 μl chlorophyll solution was shaken with 1 ml of 80% alkaline acetone (acetone: sodium

carbonate = 9:1) and centrifuged. The supernatant was collected and absorbance was taken at 645 and 663 nm. Total chlorophyll was calculated by the following formula:

$Tc = [(20.2\ A_{645} + 8.02\ A_{6630})\ x\ V]/1000W$

where.

A = optical density
V = Final volume of chlorophyll extract
W = Fresh wt of leaf

2.7.5. Photosynthetic activity

Photosynthetic ability of the intact leaves was measured by infrared gas analyzer Cl-340 (CID Bio-Science). Healthy leaves were hold in the gas chamber of the handheld system and readings were taken.

2.7.6. Sugar estimation

Total sugar from the 14 day plants was estimated by phenol sulfuric acid method (Dubois et al., 1956). Reducing sugar was measured by DNSA method (Miller, 1972).

2.8. Gene and protein expression study

2.8.1. RT-PCR

Total RNA was isolated by plant RNA isolation kit (HiMedia), quantified by nano drop (Eppendorf) and quality checked by agarose gel method. First strand cDNA synthesis was done by iScript cDNA synthesis kit (BioRad) taking 1 μg total RNA as starting material. Gene specific primers for *rbcL, rbcS* and reference gene *18S* were designed by Primer3 using FASTA sequences from NCBI nucleotide and procured from IDT (Table S1). SemiQ RT-PCR and real time qPCRs were performed using GoTaq flexi (Promega) and iTaq (Biorad). PCR reaction was carried out as stated in Table S1. Semi Q RT-PCR products were run in 1.5% agarose gel and photographed in gel doc (UVP). For real time Q RT-PCR expression change of the gene expressions were quantified by $2^{-\Delta\Delta CT}$ method and relative expressions were plotted.

2.8.2. SDS-PAGE

Total protein was isolated by protein extraction buffer (PEB), quantified by Lowry method (Lowry et al., 1951), and 50 μg of total protein was separated in 10% SDS-PAGE and stained with coomassie brilliant blue G250. Expressions of large and small subunits of RuBisCO protein were calculated by densitometry using Image J software.

2.9. Stress response

2.9.1. Proline content

100 mg of plant tissue was homogenized in 1 ml of 0.1 M sulfosalicylic acid and centrifuged. 200 μl supernatant was added with equal volume of glacial acetic acid and 500 μl of 2.5% ninhydrin and followed by heating in a boiling water bath for 1 h. Then 700 μl toluene was added and then the upper toluene layer was collected. Proline content was measured by taking the absorbance at 520 nm and comparing with a standard curve (Bates et al., 1973).

2.9.2. Lipid peroxidation (TBARS assay)

100 mg of plant tissue was homogenized in 1 ml of 0.1% TCA and centrifuged. 250 μl of supernatant was mixed with 750 μl of 0.5% TBA diluted in 20% TCA and heated in boiling water bath for 30 min. The reaction was stopped by incubation in ice. Later absorbance of the resultant compound was taken at 532 nm. Absorbance at 600 nm was used to eliminate any background absorbance. Amount of malondialdehyde was calculated by the following formula and expressed as μmol g^{-1} FW tissue (Heath and Packer, 1968).

$C = \Delta A/\varepsilon L$ where, ε = 155.5 M absorption coefficient; L = 1 cm path length, ΔA = absorbance

2.9.3. Anti-oxidative enzyme assays

2.9.3.1. Crude tissue extract preparation. 1 g plant tissue was homogenized in 10 ml of 0.2 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA and centrifuged. With the supernatant all the anti oxidative enzymes were measured according to reported literatures (Elavarthi and Martin, 2010).

2.9.3.2. Superoxide dismutase (SOD, EC 1.15.1.1). 100 μl supernatant was added to 2.1 ml reaction mixture containing 80 mM Tris (pH 8.9), 0.12 mM EDTA, 10.8 mM TEMED, 0.003% BSA, 600 μM riboflavin in 5 mM KOH, 6 mM NBT. It was kept under light (150 W) for 2 min and the absorbance was recorded at 560 nm. A set without enzyme and light showed amount of NBT present in the reaction mixture. Unit SOD was calculated as 50% reduction in photo reduction of NBT g^{-1} FW min^{-1}.

2.9.3.3. Catalase (CAT, EC 1.11.1.6). 20 μl supernatant was mixed with 980 μl reaction mixture containing 50 mM phosphate buffer (pH 7) and 20 mM H_2O_2. The decomposition of H_2O_2 was recorded by measuring the absorbance at 240 nm continuously for a min. Enzyme activity was calculated using extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1}) and expressed as mM H_2O_2 g^{-1} FW min^{-1}.

2.9.3.4. Ascorbate peroxidase (APX, EC 1.11.1.11). 20 μl supernatant was added to 980 μl assay mixture containing 50 mM phosphate buffer (pH 7), 0.5 mM ascorbate, 0.5 mM H_2O_2. Decrease in absorbance was recorded at 290 nm for 1 min. Enzyme activity was calculated using extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) and expressed as mM ascorbate g^{-1} FW min^{-1}.

2.9.3.5. Glutathione reductase (GR, EC 1.6.4.2). 20 μl supernatant was added to 980 μl assay mixture containing 50 mM phosphate buffer (pH 7), 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG. Increase in absorbance at 412 nm was recorded for 1 min. Enzyme activity was calculated using extinction coefficient of product TNB (14.15 M^{-1} cm^{-1}) and expressed as mM TNB g^{-1} FW min^{-1}.

2.9.3.6. Guaiacol peroxidase (GPX, 1.11.1.9). 20 μl supernatant was added to 980 μl assay mixture containing 50 mM phosphate buffer (pH 7), 50 mM guaiacol, 10 mM H_2O_2. Increase in absorbance at 470 nm was recorded for 1 min. Enzyme activity was calculated using extinction coefficient (26.6 mM^{-1} cm^{-1}) and expressed as mM product g^{-1} FW min^{-1}.

2.10. Iron measurement by AAS

Samples were acid digested by microwave digester (50 mg in 5 ml of HNO_3). Dilution of the samples was done with equal volume of MilliQ water. Acid digested sample were further analyzed for iron estimation AAS by graphite Furnace (PerkinElmer, India) at a wavelength of 334.9 nm. Standard iron solution for AAS was used to plot calibration curve and the iron concentration of acid digested sample was calculated from the standard graph.

2.11. Statistical analysis

Experiments were performed in triplicates. Graphs were prepared by Microsoft excel. In histograms, columns represent average with bars represent standard deviation. Significance levels were checked by one way ANOVA followed by Bonferroni's multiple comparison tests. Graphs were prepared by Graphpad Prism6. Multivariate data analysis based on the Principle Component Analysis (PCA) tool is performed by Minitab 18 statistical software.

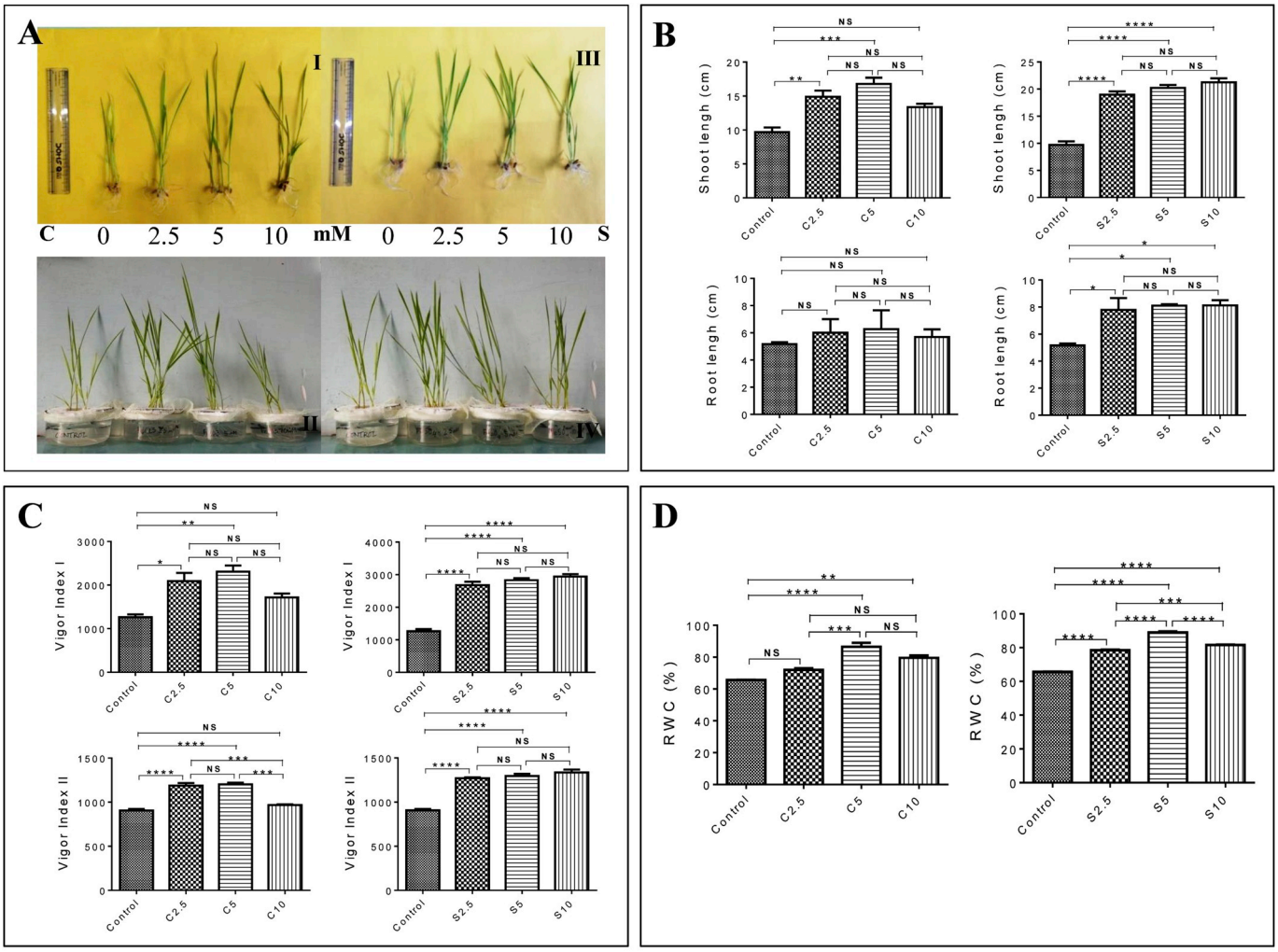

Fig. 1. Growth parameters of 14 day plants. (A) Seedlings of the 14 day plants, I & II belong to $FeCl_3$ set, III, IV belong to $FeSO_4$, (B) Shoot and root lengths (C) Vigor Index I & II and (D) Relative water content. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

3. Results

3.1. Growth enhancement

With the pulsing treatment the sets had shown promising growth enhancement (Fig. 1). Significant increase in shoot and root lengths were observed. $FeSO_4$ sets showed better results compared to the $FeCl_3$ sets. Increase in vigor indices in treated sets also confirms growth enhancement. Relative water content (RWC) is a measure to determine health and water content of the plants. Increase in RWC in treated sets also indicated better health of the treated plants.

3.2. Nitrogen assimilation

3.2.1. Total and soluble nitrogen

In treated samples total and soluble nitrogen content had increased considerably (Fig. 2) in both shoot and root. S10 set showed highest increase in total and soluble nitrogen. Comparing the overall result, the sets C5, S5 and S10, showed maximum increase in amount of total and soluble nitrogen.

3.2.2. Dissolved ammonia

Dissolved ammonia is cytotoxic. In all the treated root samples dissolved ammonia was less than that of the control set (Fig. 2). But significant increase in dissolved ammonia was found in the shoot samples of C10 set. For both the root and shoot samples, sets treated with $FeSO_4$ had shown significant decline in dissolved ammonia content.

3.2.3. Free amino acid

In both treated root and shoot samples of most of the sets increase in free amino acid content was found. In $FeCl_3$ treated sets, C5 showed significant increase in free amino acid content where as in all the $FeSO_4$ sets, the content was significantly higher than the control set with S5 being the highest (Fig. 2).

3.2.4. Nitrate content

It is a very important component of the nitrogen assimilation cycle as it is the predominant from of nitrogen absorbed by plant roots. From the experiments it's quite evident that the nitrate content increased in most of the treated sets (Fig. 3). In the $FeCl_3$ treated sets, C5 set showed highest nitrate content in shoot while roots of all the C sets (C2.5, C5 and C10) showed increased nitrate content than the control plant. In $FeSO_4$ treated sets, nitrate content has increased less in shoot, while root samples of S5 and S10 sets had showed marked increase.

3.2.5. Nitrite content

Similar to nitrate, the treated root sets showed marked increase in

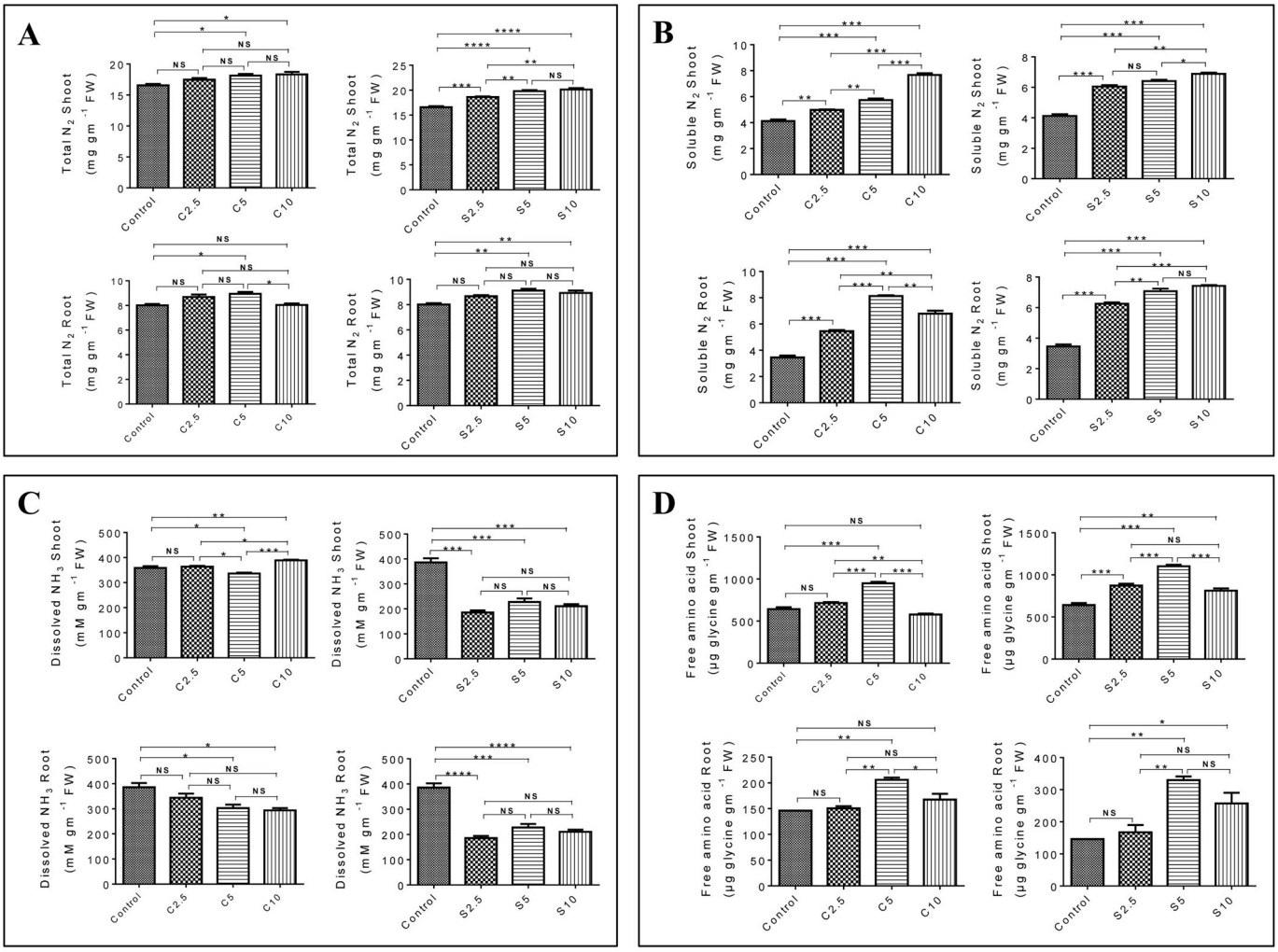

Fig. 2. Nitrogen assimilation in 14 day plants. (A) Total nitrogen, (B) soluble nitrogen, (C) dissolved ammonia and (D) free amino acid. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

nitrite content. In $FeCl_3$ treated sets, the C5 set showed highest amount of nitrite while in C10set, nitrite content in root was more than the C2.5 and control sets. The $FeSO_4$ treated sets however showed different results. In shoot samples, S2.5 set showed highest amount of nitrite while S5 set showed highest nitrite in root samples (Fig. 3).

3.2.6. Nitrate reductase

The root samples of treated sets showed higher enzyme activity than that of shoot (Fig. 3). This result was in accordance with the previous reports of activity of NADH dependent nitrate reductase in rice roots. Marked increase in enzyme activity in all the root sets was found. In shoot samples enzyme activity increased more in $FeSO_4$ treated sets.

3.2.7. Nitrite reductase

In all the treated shoot samples this enzyme activity did not increase significantly. But in root samples there was a remarkable enhancement of enzyme activity. The C2.5 and C5 sets portray highest activity among $FeCl_3$ treated samples whereas S10 showed highest activity in the $FeSO_4$ treated sets (Fig. 3).

3.2.8. GS

GS activity in roots was more than shoots. In FeCl3 treated sets, more enzyme activity was found in C2.5 and C5 sets. In $FeSO_4$ sets, dose dependent increase in enzyme activity was found (Fig. 4).

3.2.9. GOGAT

Here also dose dependent enhancement of enzyme activity was seen in $FeSO_4$ treated sets. More activity was found in treated root samples. In $FeCl_3$ treated sets, the C2.5 and C5 sets showed increased enzyme activity in both in root and shoot samples (Fig. 4).

3.2.10. GDH

GDH pathway is the alternative mechanism of ammonium incorporation. For all treated samples, enzyme activity was found to increase. However highest activity was found in S10 set (Fig. 4).

3.2.11. Total protein

The total protein content was higher in the treated sets with respect to the control with $FeSO_4$ treated sets showing maximum increase (Fig. 4).

3.3. Photosynthetic efficiency

3.3.1. δ-Amino levulinicacid (δ-ALA)

Being the starting material of chlorophyll biosynthesis, increase in δ-ALA content is directly linked with enhancement of chlorophyll content of the treated sets. Increase in δ-amino levulinic acid was found in $FeSO_4$ treated samples along with the treatment. While in $FeCl_3$ treated sets, dose dependent increase was found in C2.5 and C5 sets but

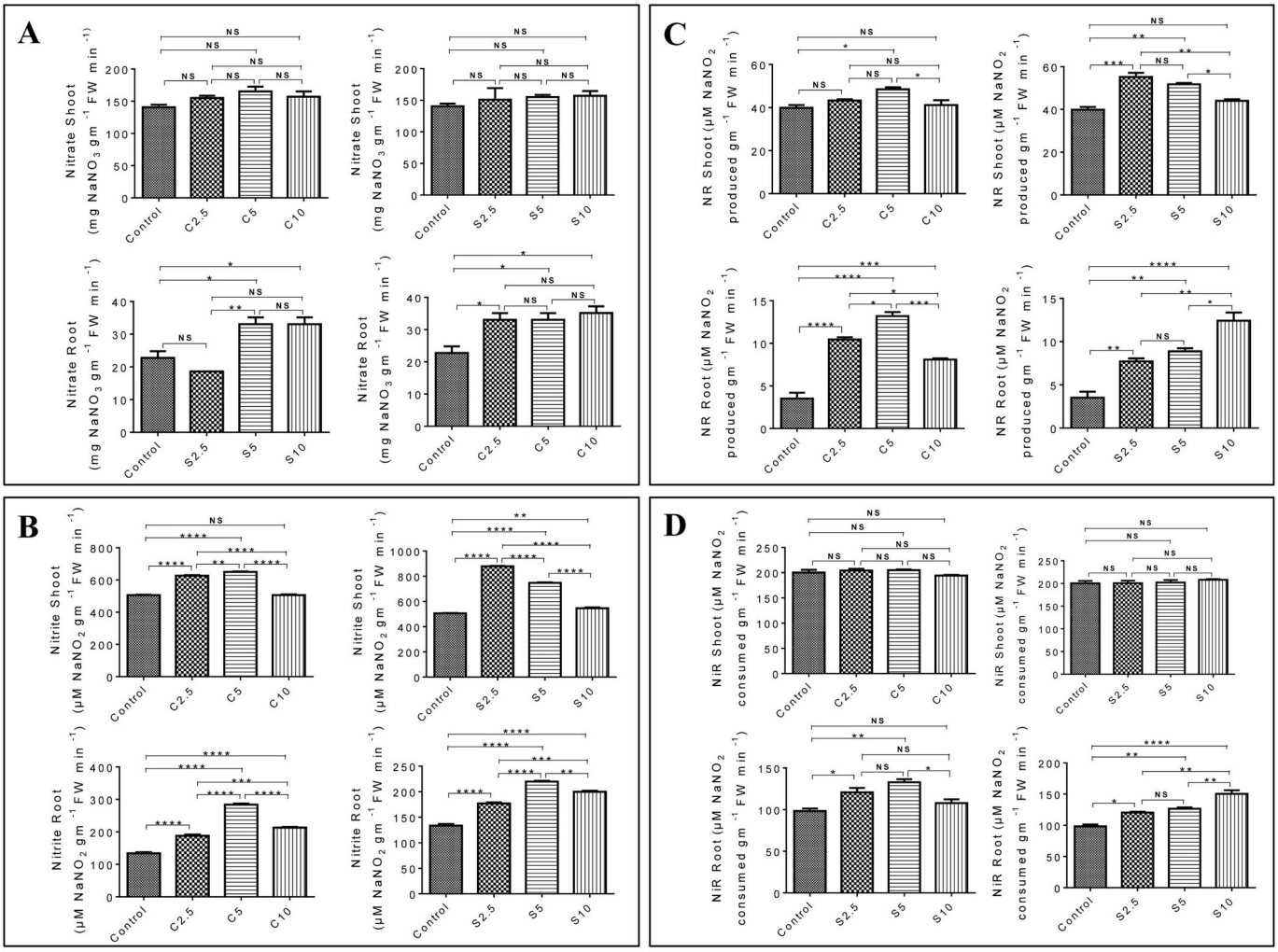

Fig. 3. Changes in nitrogen assimilation intermediates. (A) Nitrate, (B) Nitrite, (C) Nitrate reductase and (D) Nitrite reductase in 14 day plants. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

it was found to decrease in C10set. In C5 set, highest production of δ-ALA (1.56 fold) was recorded (Fig. 5).

3.3.2. Chlorophyll content

Significant increase in total chlorophyll content per gm fresh weight and per unit area leaf tissue was found in the $FeSO_4$ treated sets. While in $FeCl_3$ treated sets, huge increase in chlorophyll content (more than 3 fold) was found in C2.5 and C5 sets but less in C10 sets (Fig. 5,Table S 2,3).

3.3.3. Chlorophyll fluorescence

Higher chlorophyll fluorescence signifies high photosynthetic efficiency. Here also same type of response was found. Dose dependant increase of chlorophyll fluorescence in $FeSO_4$ treated sets was noted. In $FeCl_3$ sets, C2.5 and C5 showed higher fluorescence than that of C10 set. (Fig. 5).

3.3.4. Gene and protein expression

qRT and real time PCR shows increase in expressions of both *rbcL* and *rbcS* genes along with increase in protein expression of small and large subunits of *RuBisCO* (Fig. 5).

3.3.5. Hill activity

The C5 and S5 sets had showed highest hill activity while other sets have also shown higher activity than control set (Fig. 6).

3.3.6. Photosynthetic rate

The C5 and S10 sets showed highest photosynthetic rate among the treated sets (Fig. 6). Leaf stomatal conductance was better in C sets than in S sets. Transpiration rate was higher in all treated sets (Fig. 6).

3.3.7. Sugar content

Both soluble and reducing sugar content of the treated plants increased especially in C5 and S10 sets (Fig. 7).

3.4. Stress response

From the antioxidant enzymes and non enzymatic assays it was quite clear that the pulse treated plants have elevated anti oxidant defense mechanism. Proline was uplifted in C10 set where MDA was down regulated in all treated sets. The anti oxidative enzymes like SOD, catalase, peroxidases were increased in treated sets (Fig. 8).

3.5. Iron content

In comparison to the untreated sets, iron content has increased in all the treated sets especially in the $FeSO_4$ treated sets (Fig. 9).

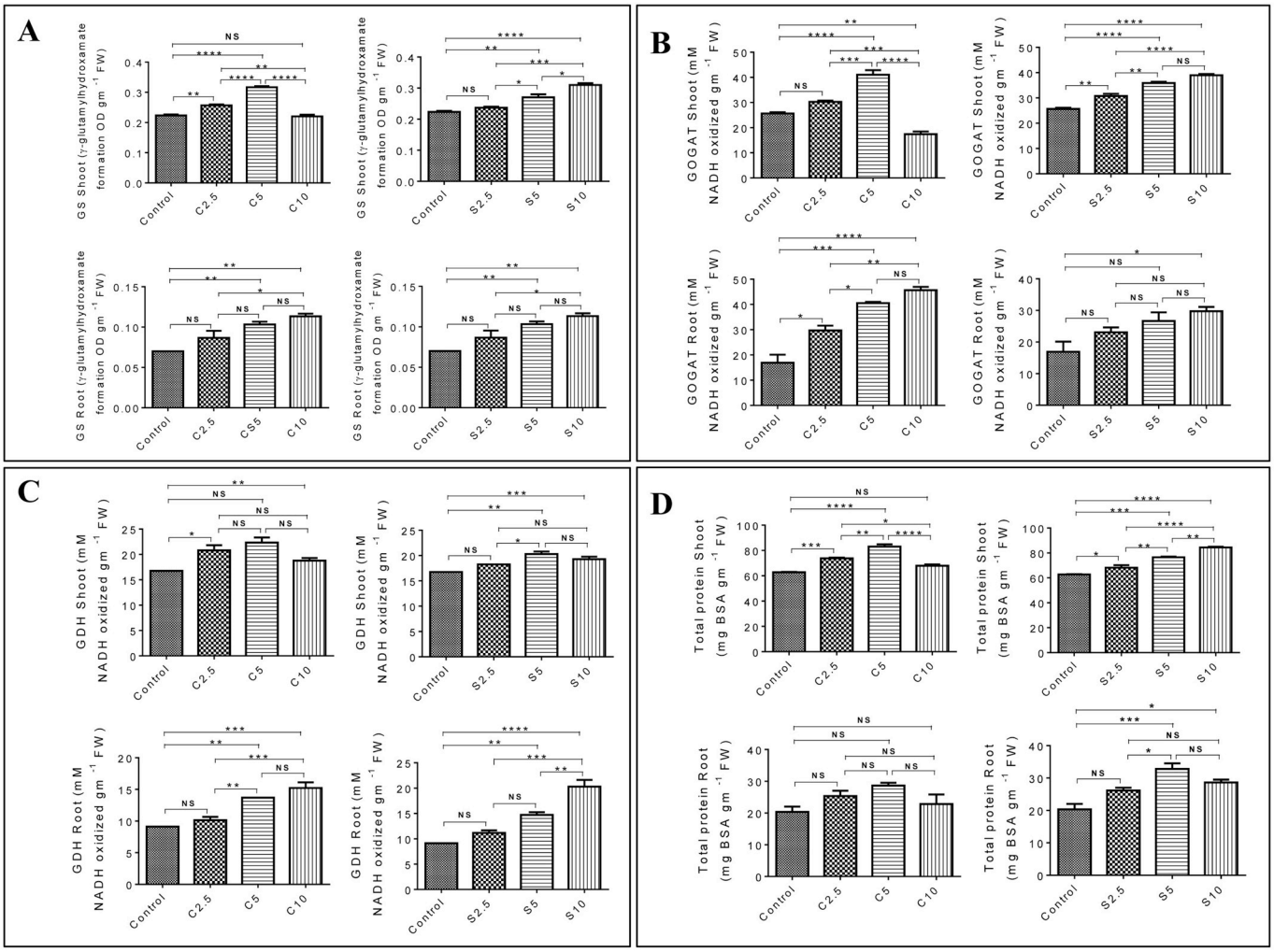

Fig. 4. Changes in amino acid biosynthesis pathway. (A) GS activity (B) GOGAT activity, (C) GDH activity and (D) Total protein in 14 day plants. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

3.6. Principle Component Analysis

To discern the quantitative relations between the studied parameters in our work, a PCA using the mean values of all the biochemical and physiological parameters were performed to determine their contributions to enhance carbon and nitrogen assimilation due to the iron pulsing (Fig. 10, Fig. S1). As shown in Fig. 10, for C5 dose, the first principal component (PC1) explained approximately 96.1% of the variance, which mainly included Chlorophyll content/gm, chlorophyll content/cm, chlorophyll fluorescence, δ-ALA, transpiration rate, hill activity, free amino acid in shoot, total nitrogen, soluble nitrogen, nitrate, nitrite, nitrate reductase, nitrite reductase, dissolved ammonia, GS, GDH, GOGAT, soluble sugar, reducing sugar and iron content. The second principal component (PC2) explained 3.2% of the variance, which included the other parameters such as net photosynthetic rate, leaf stomatal conductance and total protein in shoot. Together, the two components (PC1 and PC2) could explain 99.3% of total variance. For S5, the first principal component (PC1) explained approximately 97.6% of the variance, which mainly included all 22 variables except transpiration rate. Together, the two components (PC1 and PC2) could explain 99.6% of total variance. Further, considering the loading in both the components, three distinct clusters were observed indicating the relationship between the variables within individual cluster. This PCA analysis established that there exists quantitative relations between all 23 physiological and biochemical parameters and all the parameters for assessing augmented nitrogen and carbon fixation in rice are reliable in this study.

4. Discussion

Pulsing with two different iron salts ($FeSO_4$ & $FeCl_3$) at 3 different concentrations for each (2.5 mM, 5 mM, 10 mM) has been effective in enhancing the seedling growth at 14 days. There was a promising increment in the length of roots and shoots of the treated plantlets compared to the wild type control set. Samples treated with $FeSO_4$ showed better growth than the $FeCl_3$ samples. However, S5 and S10 for the $FeSO_4$ treated samples and C5 for the $FeCl_3$ treated samples were the best performers.

Relative water content (RWC) of plants is an important parameter to judge the water status of the plant that helps to determine the overall health of the plant (Soltys-Kalina et al., 2016). RWC of treated plants were better than the control set with C5 and S5 showing best results among the other doses. This helps to determine that the treated plants seemed healthier than the non treated ones. Seedling vigor, encompassing seedling size, health and rate of growth, is a product of several factors such as genotype of the plants and environmental influence and it can be manipulated. Higher vigor index reflects optimum growth conditions. Our treated samples has shown higher vigor index than the

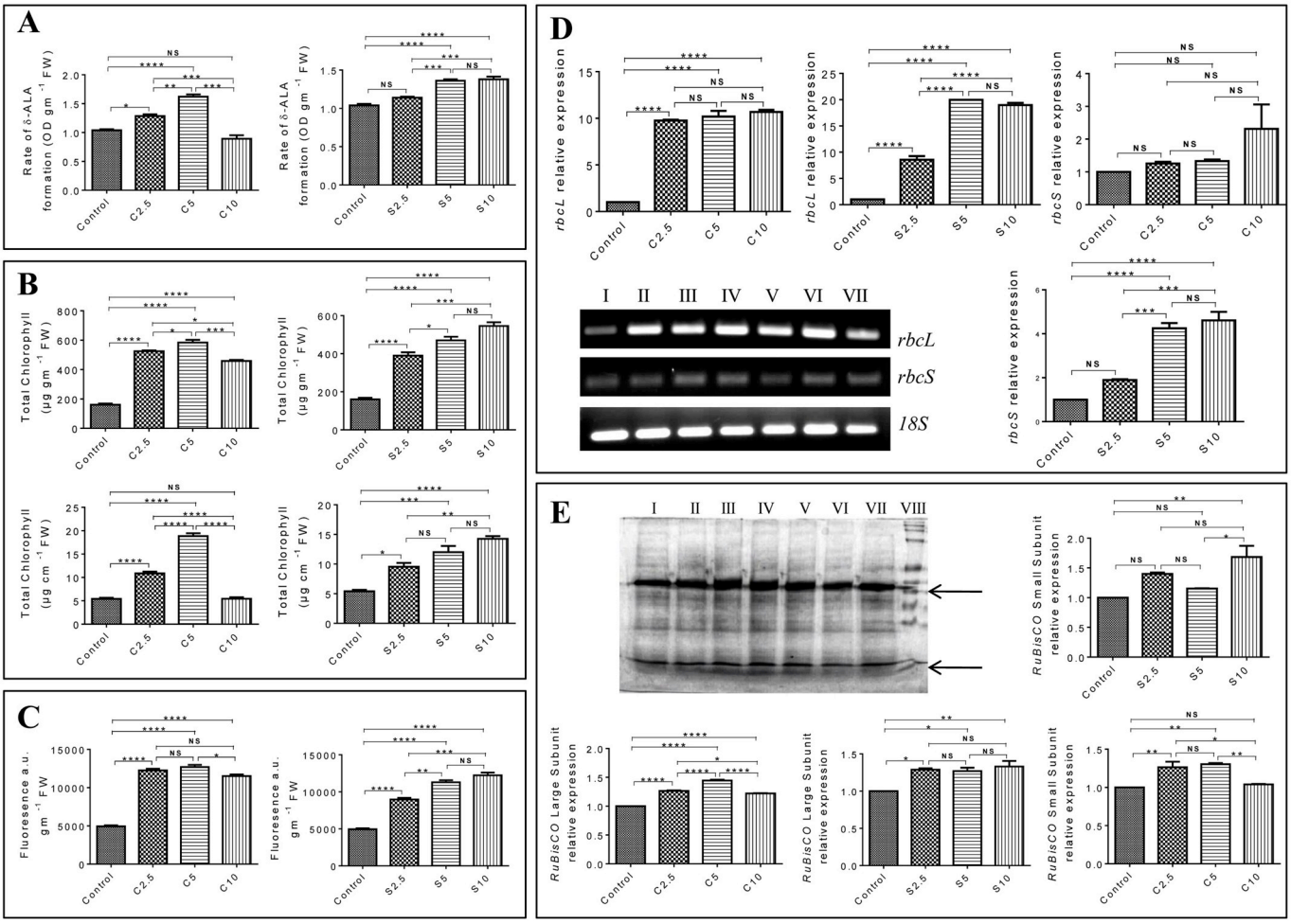

Fig. 5. Photosynthetic efficiency (A) δ-amino levulinic acid formation (B) Total chlorophyll and carotenoid content per gm and per cm, (C) Chlorophyll fluorescence (D) *rbcL* and *rbcS* expression with respective real time PCR results, [lanes I: Control, II: C2.5, III: C5, IV: C10, V: S2.5, VI: S5, VII: S10] (E) *RuBisCO* expression [arrows showed large and small subunits] [lanes I: Control, II: C2.5, III: C5, IV: C10, V: S2.5, VI: S5, VII: S10 VIII: Ladder] with densitometric analysis. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

control set. $FeSO_4$ treated plants were still better than $FeCl_3$ treated ones. S5 and S10 were the best in this respect.

It has been reported earlier that, treatment of soil with $FeSO_4$ inorganic salt has helped combat chlorosis better than only elemental iron treatment because of the fact that sulfur in $FeSO_4$ salt can undergo oxidation to form mineral acid H_2SO_4 which in turn makes the pH of the soil acidic thereby facilitating translocation of iron (http://www.soilmanagement, 2848). Thus it is evident that $FeSO_4$ treatment yields better and promising results than $FeCl_3$ treated plants in terms of plant growth and vigor.

Nitrogen assimilation and amino acid biosynthesis is governed by several enzyme catalyzed reactions involving many important intermediates. Nitrate is the predominant form of nitrogen source absorbed by plant roots either via Low Affinity Transport System (LATS) or via High Affinity Transport System (HATS) involving gene family NRT1 and NRT2 respectively (Williams and Miller, 2001). In the cytosol nitrate is reduced to nitrite by enzyme Nitrate Reductase (NR) having three prosthetic groups FAD, heme and MoCo. NR is the rate limiting enzyme in the assimilation of nitrate to amino acids thus its activity is positively related with grain yield and protein content of cereals (Hageman et al., 1979). Our treatment has increased the amount of nitrate as well as its associated enzyme NR. Heme being an integral component of NR enzyme, iron treatment empowers the enzyme activity in the treated sets.

Nitrite formed by NR activity immediately enters chloroplasts/plastids and gets reduced to ammonia by the action of Nitrite Reductase (NiR) enzyme (Masclaux-Daubresse et al., 2010). Nitrite is highly reactive and toxic ion so plants do not accumulate it, rather readily converts them to ammonia. NiR enzyme catalyzing this reaction also contains a heme prosthetic group. Thus iron pulsing plays a significant role in elevating nitrite content as well as NiR enzyme activity in treated sets. Ammonium ions are primarily assimilated in the plastid/chloroplast by the GS-GOGAT cycle. Glutamine synthetase (GS) incorporates ammonium in a glutamate molecule yielding glutamine which in turn reacts with 2-oxoglutarate to form two molecules of Glutamate by the action of Glutamine -2-oxoglutarate amino transferase (GOGAT) or Glutamate synthase (Lea and Miflin, 1974). GS has two isoforms viz GS1 (cytosolic) mainly associated with recycling of ammonium during developmental stages and GS2 (chloroplastic) that helps in assimilation of ammonium formed by nitrite reduction. Similarly GOGAT has two isoforms: NADH GOGAT that uses NADH as an electron donor, located in plastids and other non photosynthetic tissues like roots and FD GOGAT that uses Ferredoxin as electron donor occurring predominantly in chloroplasts (Masclaux-Daubresse et al., 2010). Attempts have been made to over express GS with the goal of superior crop nitrogen efficiency (Vanoni et al., 2005). High concentrations of ammonium ions are toxic to plants which highlight the important role of GS-GOGAT enzyme system to alleviate stress due to

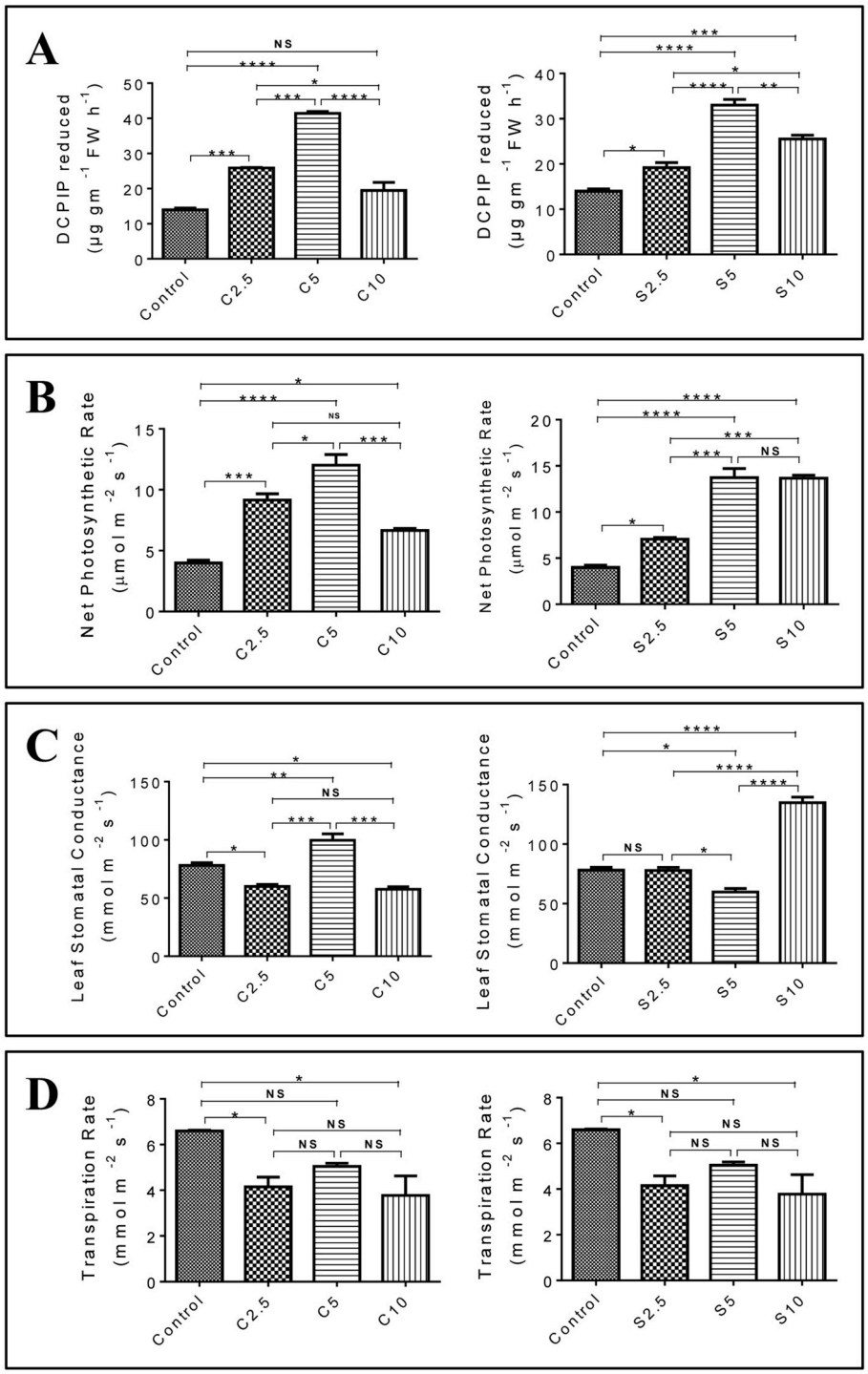

Fig. 6. Rate of photosynthesis at 14 day plants (A) Hill activity (B) net photosynthetic rate (C) leaf stomatal conductance (D) transpiration rate. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

ammonium ion accumulation. NADH Glutamate Dehydrogenase (GDH) also helps to incorporate ammonium ion into glutamate as an alternative pathway when ammonium ions are increased in the system (Skopelitis et al., 2006).Both GS- GOGAT and GDH has increased significantly in the treated plants where as the amount of dissolved ammonia has decreased when compared with control plants. Final product of nitrogen assimilation is amino acids which are building blocks for proteins. Increase in free amino acid content and total protein of the plants under iron pulsing treatment substantially supports the efficacy of the treatment in enhancing nitrogen assimilation of the treated plants than the control set. Moreover the total nitrogen and soluble nitrogen content has increased with treatment which makes above results more evident.

Studies have revealed that majority of Nitrogen (N) assimilated in plants is invested in photosynthetic machinery which includes photosynthetic enzymes, pigment content and the size, number and composition of chloroplasts. Leaf nitrogen content has a positive effect on photosynthetic rate. It has been observed that photosynthesis increases

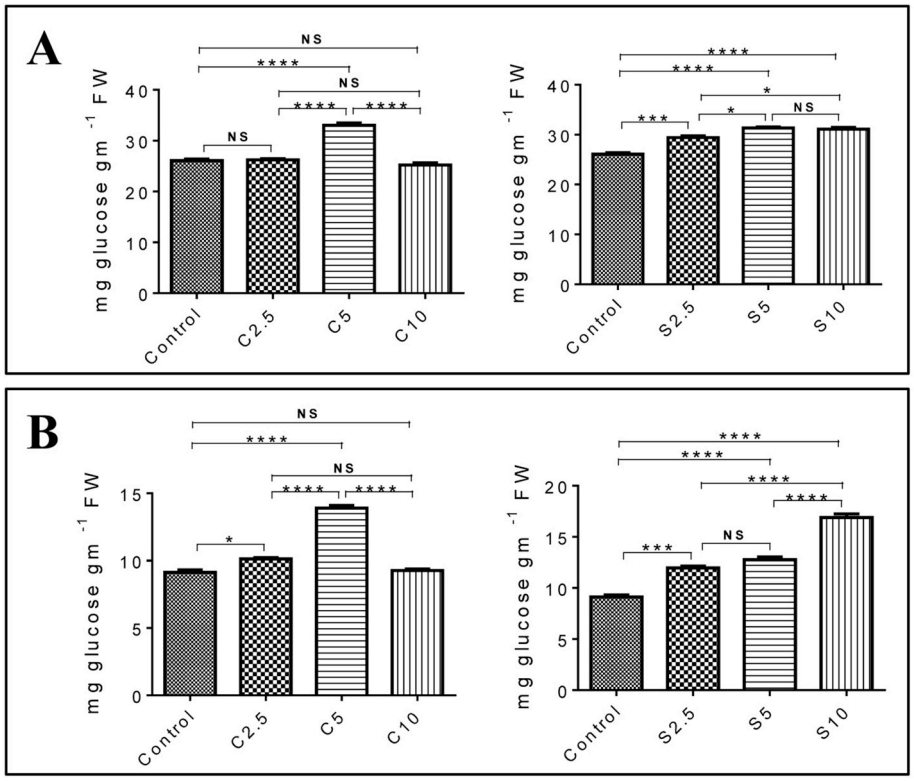

Fig. 7. Soluble (A) and reducing sugar (B) in 14 day plants. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

linearly with the increase in leaf N content in sugarcane (Bassi et al., 2018). Iron pulsed plants had a higher photosynthetic rate as compared to normal wild type which is analogous to the previously reported data. Role of N in stimulating leaf growth through the synthesis of proteins involved in cell growth, cell division and cell wall and cytoskeleton synthesis, increasing the photosynthetic area has been reported (Lawlor, 2002). N is a part of chlorophyll molecule which makes it a crucial factor in the development of the photosynthetic apparatus in plants. Increased N supply has led to subsequent increase in leaf chlorophyll content in sugarcane as reported by several workers (Bassi et al., 2018; Rhein et al., 2016). Our treatment also yielded similar results where chlorophyll content increased considerably in treated plants than control plant. δ-Amino levulinic acid (δ-ALA), the first compound in the porphyrin synthesis pathway, an endogenous non-proteinogenic amino acid, leads to biosynthesis of chlorophyll in plants (Von WettsteinDitet and GaminiKannangara, 1995). A subsequent enhancement in the δ-ALA content was also noticed in iron pulsed sets. Nitrogen assimilation may also be related with increment of δ-ALA because glutamate, an important intermediate of nitrogen assimilation pathway, is the precursor of δ-ALA. Glutamate in glutamyl-tRNA and glutamate-1-semialdehyde pathway may help in synthesizing δ-ALA (Beale, 1990). Besides directly participating in photosynthesis, N also holds a positive correlation with stomatal conductance (Tosens et al., 2012). Stomatal conductance of treated samples was quite high in C5 and S5 samples. Optimum rates of CO_2 assimilation requires sufficient amounts of light harvesting chloroplast protein complexes (LHCP), *RuBisCO*, electron transport enzymes, NADP + -reducing components of thylakoids and several other enzymes associated with chloroplast. These conditions were best maintained at greater N conditions whereas CO_2 assimilation was affected when nitrogen was cut down (Lawlor, 2002). As derived from SDS-PAGE, *RuBisCO* content in treated sets had also increased. Both large and small subunits showed a distinct difference with normal set. This is supported by the upregulation of nuclear multigene family *rbcS* which encodes the eight small subunits of *RuBisCO* and chloroplast single gene *rbcL* which encodes the 8 large subunits, as depicted from real time PCR data.

To establish impact of our treatment in yielding better photosynthetic efficiency we also ventured into estimating chlorophyll fluorescence and hill activity. Chlorophyll fluorescence is an effective approach to determine the efficiency of photosystem II and Hill activity reveals the optimum functioning of light dependent reactions of photosynthesis which contains a series of redox reactions (Murchie and Lawson, 2013; Jayashantha, 2015). While chlorophyll fluorescence was quite high in all the treated sets, hill activity was maximum in C5 and S5 sets. Thus there exist an important cross talk between N and C assimilation and metabolism where photosynthesis provide carbon skeletons, a prerequisite for ammonium condensation into amino acids and also supply ATP, NADH and Fd, whereas nitrogen assimilation helps in biosynthesis of chlorophyll and other photosynthetic enzymes.

Plants tend to accumulate an array of metabolites when exposed to stress. Amino acid proline has been reported to have a positive relationship with stress. Besides functioning as an osmolyte, proline also helps to chelate metal, serve as an anti oxidant molecule and also as a signaling molecule. Plants subjected to stress produce proline in order to combat electrolytic leakage, stabilize membranes, maintain cell turgor and osmotic balance and also prevent oxidative burst by checking ROS limits (Hayat et al., 2012). Drought stress has resulted in increase of proline content in N22 genotype of rice (Samota, 2017). Uncontrolled oxidative stress can cause damage of cells, tissues and can also inflict damage to lipids by lipid peroxidation. The most mutagenic product of lipid peroxidation is MDA and it has been successfully utilized as a biomarker to measure stress. Thus MDA content is an important tool to measure the degree of damage due to lipid peroxidation and higher MDA content indicates severity of damage. MDA content has been reported to increase considerably under salt stress in rice (Yamane et al., 2009). Our treatment does not cause any detectable stress to the treated plants and it is evident from lower levels of proline and MDA in the root and shoots of treated sets compared to the normal one. Proline

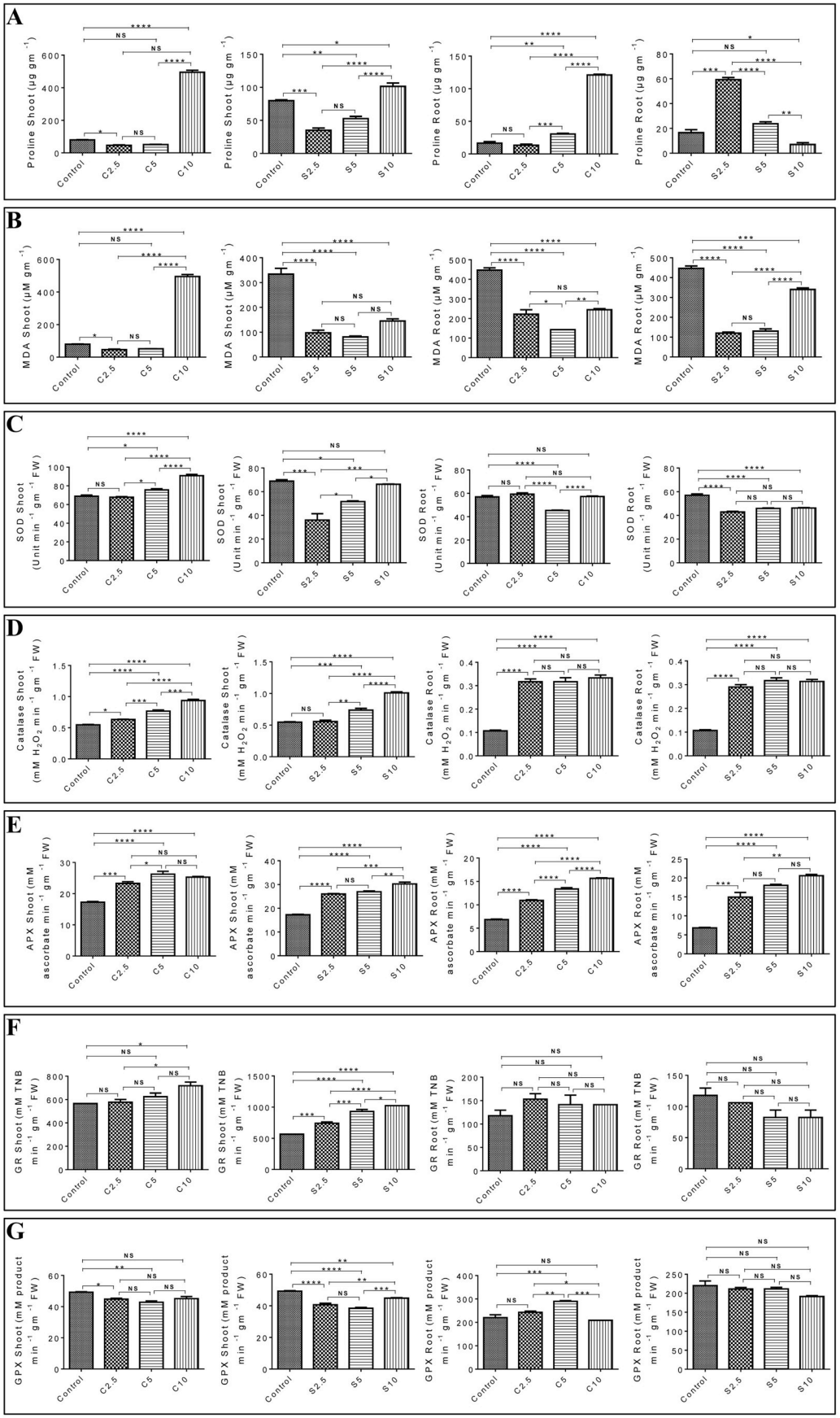

Fig. 8. Stress responses in 14 day plants. (A) Proline (B) MDA (C) SOD (D) Catalase (E) APX (F) GR (G) GPX. In histograms, columns represent the average values while bars represent standard deviations [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

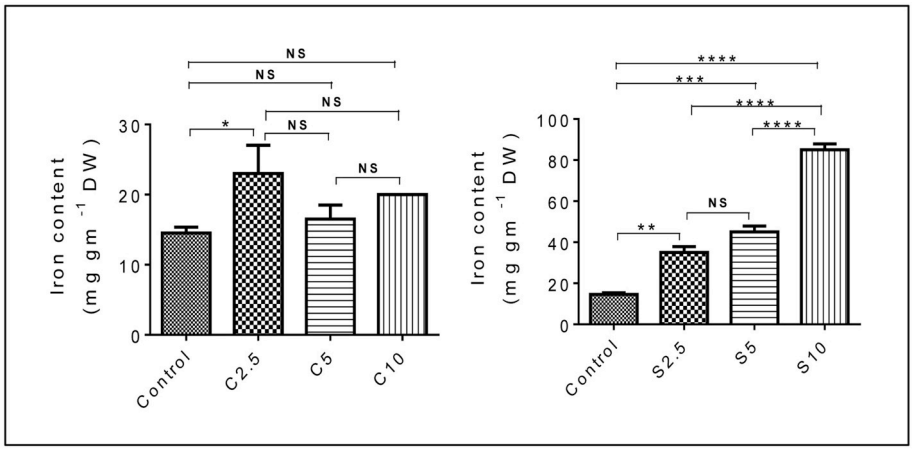

Fig. 9. Iron content in 14 DPG plants. In histograms, columns represent the average values while bars represent standard deviations. [One way ANOVA + Bonferroni's multiple comparison test - GraphPad Prism6, NS: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****$P \leq 0.0001$].

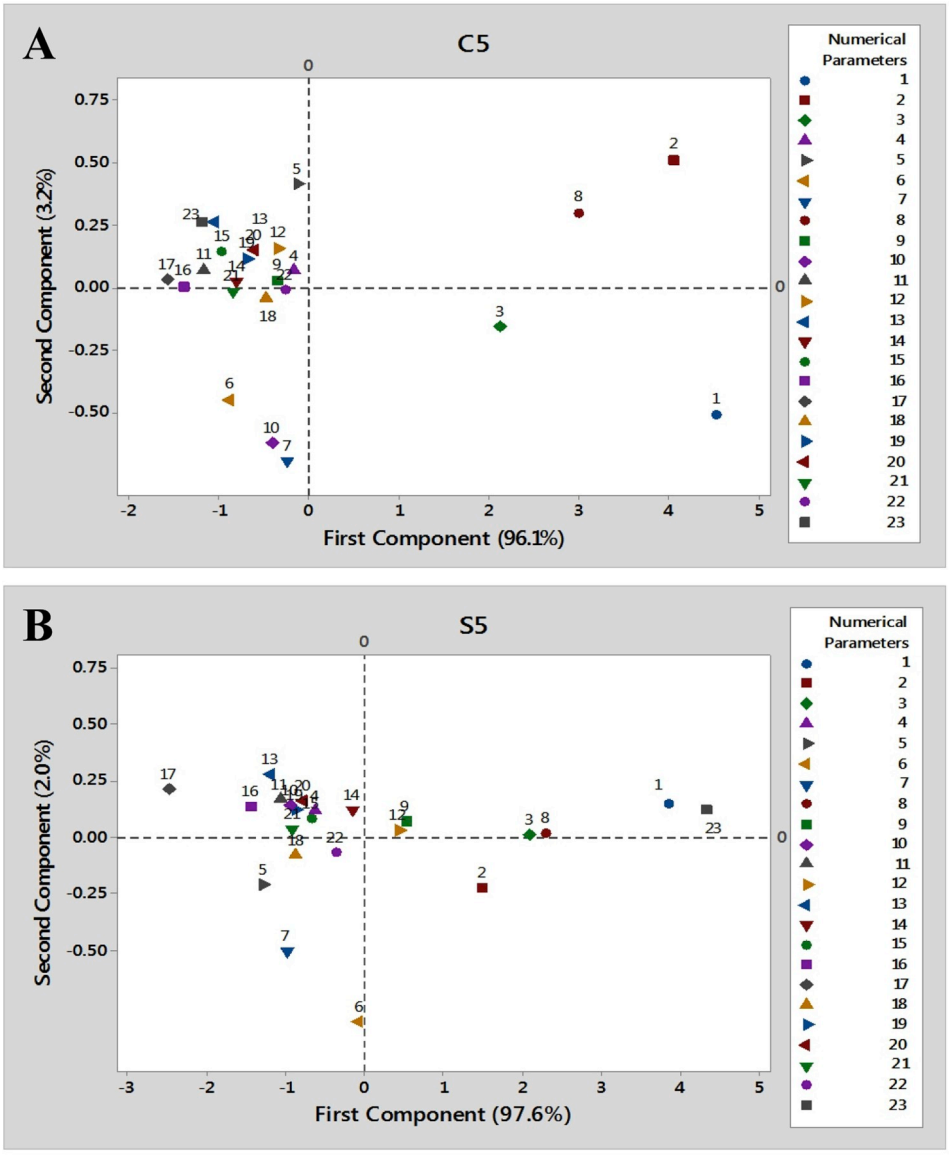

Fig. 10. PCA analysis to correlate photosynthesis and nitrogen assimilation in the two optimum treatment conditions: (A) C5 set, (B) S5 set. [1. Chlorophyll content per gm; 2. Chlorophyll content per cm; 3. Chlorophyll Fluorescence; 4. δ-ALA; 5. Net Photosynthetic Rate; 6.Transpiration rate; 7.Leaf stomatal conductance; 8.Hill activity; 9.Free Amino acid; 10.Total Protein; 11.Total nitrogen; 12.Soluble nitrogen; 13.Nitrate content; 14.Nitrite content; 15.Nitrate Reductase; 16.Nitrite Reductase; 17.Dissolved Ammonia; 18.GS; 19.GDH; 20.GOGAT; 21.Soluble Sugar; 22.Reducing Sugar; 23.Iron content.].

content in C10 is very high, suggesting stress condition which supports our other morphological observations as well. Plants protect themselves from the cytotoxic effects of ROS by action of antioxidant enzymes such as SOD, APX, GR and CAT (Agarwal et al., 2006). Iron has a structural role as prosthetic group of enzymes peroxidases and catalases. Iron also interacts with SOD which has an iron sulphide group (Barrs and Weatherley, 1962). Plants treated with iron resulted an increase in SOD activity whereas, iron deficiency was found to bring down SOD in wheat (Agarwal et al., 2006). Iron deficit conditions also reduced APX and CAT activities. Increase in activity of APX and GR in rice leaves due to iron supplementation was also recorded (Fang et al., 2001). Amount of GR tends to increase in the S sets while remains almost unchanged in the C sets where as APX increases in all the treated sets with maximum increase in S sets suggesting that iron absorption is better in S sets which in turn effects the enzyme activities. CAT and SOD activities are also high in treated sets especially in the roots (iron absorption takes place by root transporters) justifying the role of iron in the functioning of these enzymes. Enhancement in the activity of these antioxidant enzymes in treated sets does not indicate stress conditions but better mechanism to alleviate stress as iron is a key element responsible for up regulation of these enzyme activities.

AAS data reveals that iron content in 14 day plants have increased with our treatment than the control plant. It can also be noted that iron content of the plants treated with $FeSO_4$ has increased linearly with our treatment with S10 accumulating the maximum amount of iron. This data strongly supports our above findings where S sets plants tend to be better in every aspect compared to the C sets with few exceptions. Higher iron content in treated plants at 14 day of growth makes our study more evident and efficient.

5. Conclusion

Based on our present study it can be concluded that, iron pulsing can effectively enhance growth of plants at 14 days by elevating the physiological activities of plant (photosynthesis & nitrogen assimilation) as iron is intricately associated with several intermediates and enzymes of these processes. The treatment was found to enhance chlorophyll content and activity which contributed to better photosynthetic efficiency of the plants. Along with that, the nitrogen assimilation in the treated plants was escalated as revealed from the nitrogen cycle intermediates and associated enzymes (NR, NiR, GS, GOGAT, GDH). Efficient nitrogen and carbon fixation were finally translated into higher protein and sugar levels in iron pulsed plantlets. However, statistical analysis (PCA) reveals that C5 and S5 among the other sets are best suitable concentrations for this treatment, as all the concerned parameters lie around the same cluster, indicating a significant correlation, which is in agreement with the findings of this study. Iron pulsing technique is a novel and simplified approach that have great potential in agricultural application specially in order to increase growth and vigor of plants without any stress or cytotoxicity in the treated plants. This work shows highly promising outcome if applied in rice cultivation in order to maximize yields from the existing plants.

Author's contribution

SD performed the experiments, analyzed the data and helped in writing the work; **RK** conceptualized the work and reviewed the manuscript; **GG & AM** analyzed the iron concentration by AAS; **AN** performed the statistical analysis; **SP** conceptualized, designed, analyzed and drafted the work. All authors have read and approved the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

Acknowledgment

The research was supported by CAS, Dept. of Botany, University of Calcutta; Department of Biotechnology, West Bengal [project no. 76 (Sanc.) – BT/P/Budget/RD-14/2017, Date 27.03.2017] and FRPDF grant of Presidency University. The authors are grateful to CAS, Department of Botany, University of Calcutta and Department of Life Sciences, Presidency University for providing institutional and infrastructural support. Special gratitude is given to Prof. S. Bhattacharyya, Bidhan Chandra Krishi Vishwavidyalaya, India, for providing the handheld photosynthesis meter and Ms. S. Ganguly for helping in the experiment. Authors are grateful to Dr. Shantanu Chakraborty, Dr. M. Ganesan, Dr. Jayonti Jodder and Ms. Madhurima Ghosh, Presidency University for their generous help in real time PCR experiments. The authors are indebted to Prof. A.K. Biswas, University of Calcutta for providing key insights of the research problem.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.037.

References

India: A Country Study:Crop Output, 1995. Library of Congress, Washington D.C. September Retrieved March 21, 2009.
Abdul-Baki, Aref A., Anderson, James D., 1973. Vigor determination in soybean seed by multiple criteria 1. Crop Sci. 13 (6), 630–633.
Adhikari, Tapan, Kundu, S., Subba Rao, A., 2013. Impact of SiO2 and Mo nano particles on seed germination of rice (*Oryza sativa* L.). Int. J. Agric. Food Sci. Technol. 4 (8), 809–816.
Agarwal, Sarika, et al., 2006. Effect of excess and deficient levels of iron and copper on oxidative stress and antioxidant enzymes activity in wheat. Plant Sci. 1, 86–97.
agricoop.nic.in/sites/default/files/Annual_rpt_201617_E.pdf.
Barrs, H.D., Weatherley, P.E., 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust. J. Biol. Sci. 15 (3), 413–428.
Bassi, Denis, Menossi, Marcelo, Mattiello, Lucia, 2018. Nitrogen supply influences photosynthesis establishment along the sugarcane leaf. Sci. Rep. 8 (1), 2327.
Bates, L.S.ᵻ, Pᵻ Waldren, R., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39 (1), 205–207.
Bauer, Helmut, Senser, Margot, 1979. Photosynthesis of ivy leaves (Hedera helix L.) after heat stress II. Activity of ribulose bisphosphate carboxylase, Hill reaction, and chloroplast ultrastructure. Z. Pflanzenphysiol. 91 (4), 359–369.
Beale, Samuel I., 1990. Biosynthesis of the tetrapyrrole pigment precursor, δ-aminolevulinic acid, from glutamate. Plant Physiol. 93 (4), 1273–1279.
Catada, Merivic, Campos, Jehson, Zamora, Arjay, 2016. Utilization of naturally occurring seed priming agents in enhancing seed germination and seedling emergence of rice (*Oryza sativa* L.). Prism 21 (1).
Dubois, Michel, et al., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.
Elavarthi, S., Martin, B., 2010. Spectrophotometric Assays for Antioxidant Enzymes in Plants. Plant Stress Tolerance Methods and Protocols, vol. 639. Sunkar R. Humana Press, pp. 386.
Fang, Wei-Ching, et al., 2001. Iron induction of lipid peroxidation and effects on antioxidative enzyme activities in rice leaves. Plant Growth Regul. 35 (1), 75–80.
Farooq, Muhammad, et al., 2006. Enhancing the performance of direct seeded fine rice by seed priming. Plant Prod. Sci. 9 (4), 446–456.
Ghosh, Srijita, Saha, Jayeeta, Biswas, Asok K., 2013. Interactive influence of arsenate and selenate on growth and nitrogen metabolism in wheat (Triticumaestivum L.) seedlings. Acta Physiol. Plant. 35 (6), 1873–1885.
Guha, Titir, et al., 2018. Nanopriming with zero valent iron (nZVI) enhances germination and growth in aromatic rice cultivar (*Oryza sativa* cv. Gobindabhog L.). Plant Physiol. Biochem. 127, 403–413.
Hageman, R.H., 1979. Nitrogen assimilation of plants. In: Hewitt, E.J., Cutting, C.V. (Eds.), Sixth Long Ashton Symposium. Academic Press.
Hayat, Shamsul, et al., 2012. Role of proline under changing environments: a review. Plant Signal. Behav. 7 (11), 1456–1466.
Heath, Robert L., Packer, Lester, 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125 (1), 189–198.
Hoagland, Dennis Robert, Arnon, Daniel Israel, 1950. The water-culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. 347 second ed.
http://www.soilmanagementindia.com/nutrient-elements-in-soil/importance-of-sulphur-in-plant-physiology/2848).
https://www.un.org/en/development/desa/publications/mdg-report-2015.html.
Jayashantha, Eranga, 2015. Why Is the Hill Reaction Important in the Elucidation of the Mechanism of Photosynthesis. https://doi.org/10.13140/RG.2.1.1311.9525.
Kar, Saradia, Kumar Panda, Sanjib, 2018. Iron homeostasis in rice: deficit and excess.

Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 1–9.
Lawlor, David W., 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. J. Exp. Bot. 53, 773–787 370.
Lea, P.J., Miflin, B.J., 1974. Alternative route for nitrogen assimilation in higher plants. Nature 251, 614 5476.
Lichtenthaler, Hartmut K., 1987. [34] Chlorophylls and Carotenoids: Pigments of Photosynthetic biomembranes." Methods in Enzymology, vol. 148. Academic Press, pp. 350–382.
Lowry, O.H., et al., 1951. Measurement of protein with the Folin phenol reagent. J. Biol. Chem. 193 (1), 265–275.
Lutts, Stanley, et al., 2016. "Seed Priming: New Comprehensive Approaches for an Old Empirical technique." New Challenges in Seed Biology-Basic and Translational Research Driving Seed Technology. IntechOpen.
Mahakham, Wuttipong, et al., 2017. Nanopriming technology for enhancing germination and starch metabolism of aged rice seeds using phytosynthesized silver nanoparticles. Sci. Rep. 7 (1), 8263.
Marschner, Horst, 1995. Mineral Nutrition of Higher Plants, 2nd. Edn. Academic Pres.
Masclaux-Daubresse, Céline, et al., 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann. Bot. 105 (7), 1141–1157.
Masoner, M., Kasemir, H., 1975. Control of chlorophyll synthesis by phytochrome. Planta 126 (2), 111–117.
Miller, G.L., 1972. Estimation of reducing sugar by dinitrosalicylic acid method. Anal. Chem. 31, 426–428.
Murchie, Erik H., Lawson, Tracy, 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J. Exp. Bot. 64 (13), 3983–3998.
Rhein, Andressa FL., et al., 2016. Technological quality and yield of sugarcane grown under nitrogen doses via subsurface drip fertigation. Rev. Bras. Eng. Agrícola Ambient. 20 (3), 209–214.
Rout, Gyana R., Sahoo, Sunita, 2015. Role of iron in plant growth and metabolism. Rev. Agric.Sci. 3, 1–24.
Samota, Sasiand Singh, 2017. Impact of seed priming on proline content and antioxidant enzymes to mitigate drought stress in rice genotype. Int. J. Curr. Microbiol. Appl. Sci. (2319-7706) 6 (5), 2459–2466.
Singh, Akanksha, Gupta, Rupali, Pandey, Rakesh, 2016. Rice seed priming with picomolar rutin enhances rhizospheric Bacillus subtilis CIM colonization and plant growth. PloS One 11 (1), e0146013.
Sinha, Rajiv K., 1997. Embarking on the second green revolution for sustainable agriculture in India: a judicious mix of traditional wisdom and modern knowledge in ecological farming. J. Agric. Environ. Ethics 10 (2), 183–197.
Skopelitis, Damianos S., et al., 2006. Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 18 (10), 2767–2781.
Soltys-Kalina, Dorota, et al., 2016. The effect of drought stress on the leaf relative water content and tuber yield of a half-sib family of 'Katahdin'-derived potato cultivars. Breed Sci 66 (2), 328–331.
Theerakulpisut, Piyada, Nantawan, Kanawapee, Bunika, Panwong, 2016. Seed priming alleviated salt stress effects on rice seedlings by improving $Na+/K+$ and maintaining membrane integrity. Int. J. Plant Biol. 7, 1.
Tosens, Tiina, et al., 2012. Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in Populustremula: how structure constrains function. Plant Cell Environ. 35 (5), 839–856.
Vanoni, Maria A., et al., 2005. Structure–function studies on the complex iron–sulfur flavoprotein glutamate synthase: the key enzyme of ammonia assimilation. Photosynth. Res. 83 (2), 219–238.
Von Wettstein, Ditet, Simon Gough, GaminiKannangara, C., 1995. Chlorophyll biosynthesis. Plant Cell 7 (7), 1039.
White, P.J., Brown, P.H., 2010. Plant nutrition for sustainable development and global health. Ann. Bot. 105 (7), 1073–1080.
Williams, L.E., Miller, A.J., 2001. Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu. Rev. Plant Biol. 52 (1), 659–688.
Wray, John L., Fido, Roger J., 1990. "Nitrate Reductase and Nitrite reductase." Methods in Plant Biochemistry, vol. 3. Academic Press, pp. 241–256.
Yamane, Koji, et al., 2009. Antioxidant capacity and damages caused by salinity stress in apical and basal regions of rice leaf. Plant Prod. Sci. 12 (3), 319–326.