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Influence of single and dual deficits of nitrogen and iron on growth, yield, and nitrogen metabolism of bread wheat

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Abstract
Wheat one of the widely grown food crop, contributes around 27% of the world's grain production. Due to the lack of protein, and essential mineral nutrients in their diets, almost 40% of the global population are malnourished. Improving the nutritional value of wheat genotypes while maintaining high production is important. To address this issue, scientists, breeders, and the food industry employ a variety of strategies. Bio-fortification is a successful way to improve wheat's nutritional content. HD3298, a promising bio-fortified wheat genotype with high yield and high nutrient (Fe, Protein) content was developed recently. Two essential mineral elements required for plant growth and development are iron (Fe) and nitrogen (N). There seems to be relatively little understanding of the crosstalk between different nutrients and their effects in the shifting nutritional environment. In this study, we aimed to understand the effects of individual nutrient stress (-Fe, -N) and combination nutritional stress (-N-Fe) in hydroponically grown bread wheat. The information gained from the current study will be helpful for engineering plants' adaption to a fluctuating nutritional environment.

Keywords: Bread wheat, Nitrogen, Iron, Photosynthesis, Nutrient deficiency, fluorescence, hydroponic.

Introduction
Among food grains consumed in the world, bread wheat (*Triticumaestivum*) contributes 20% of calories and protein (Dixon *et al*., 2018) [7]. Around two billion people worldwide are malnourished, and 815 million are undernourished (Bulti *et al*., 2017) [4]. Based on the National Family Health Survey-4 (Barman *et al*., 2020) [1], 35.7% of children under the age of five in India are underweight, and 38.4% of children are malnourished. An even more severe health problem is anemia, which affects 53% of adult women between the ages of 15 and 49 and 58.4% of Indian children between the ages of 6 and 19 months (Bulti *et al*., 2017) [4]. The percentage among adult males is equally concerning because 22.7% of them have been reported to be anemic. Worldwide, two billion individuals experience "hidden hunger," which is brought on by a regular diet that is insufficient in vital micronutrients. Twelve of the 17 global sustainable development goals have a strong association with nutrition iron (Fe) and zinc (Zn) deficiencies are prevalent in wheat and can negatively impact growth and productivity. A study was conducted to investigate the effects of Fe and Zn dual deficiency on wheat growth and metabolism (Barman *et al*., 2020) [1]. The results showed that Fe and Zn dual deficiency significantly reduced plant height, leaf area, and biomass. The study also revealed that the dual deficiency led to changes in the expression of genes related to Fe and Zn uptake and transport, as well as those involved in photosynthesis and stress response (Barman *et al*., 2020) [1]. Additionally, the study found that the dual deficiency led to a decrease in the concentration of chlorophyll and carotenoids, indicating a reduction in photosynthetic activity. Overall, the study demonstrated that Fe and Zn dual deficiency has a significant impact on wheat growth and metabolism, highlighting the importance of combined nutrient deficits on wheat productivity. Wheat plants exposed to nitrogen (N) deficiency show a variety of changes in their growth and development. These changes include reduced vegetative growth, smaller leaves, and a decrease in the number of tillers. Additionally, N deficiency leads to a decrease in the number of grains per spike, as well as a decrease in grain weight and protein content. These changes ultimately result in lower crop yields and a decrease in the overall quality of the wheat. It is well known that a deficiency of Fe, zinc, and protein in the diet brings more than 40% of the world's population at serious risk of malnutrition (Yi & Guerinot, 1996) [45].

By 2050, wheat production would have to double to assist in ensuring the security of the global supply of food. As wheat is the basis of important staple foods in both developing and developed countries across the world and is consumed more and more in developing countries, replacing traditional foods, and improving the nutritional properties of wheat varieties is another crucial challenge faced by wheat breeders along with the exponential rise in wheat production. Increasing the Fe and protein content of wheat plants with bio-fortification has proven to be successful. With a high yield (55–60 quintals) and excellent nutritional (high Fe and Protein) value, HD3298 is a bio fortified wheat variety. As a component of coenzymes, nucleic acids, amino acids, proteins, and countless other secondary metabolites, N is an important macro element. As a result, it plays a significant role in the growth and development of plant species. Crop response to applied N and the use efficiency is a key factor to maximize the economic yield. Therefore, increasing N use efficiency (NUE) is highly desired to improve crop yield, reduce production costs, and preserve ecological integrity. To improve N efficiency in agriculture, integrated strategies for N management, and crop management practices are required. According to Thoiron *et al*., (1997) [40], Fe is one of the essential microelements needed by plants in the form of heme and components for cellular respiration, photosynthesis, N-fixation, and other hemoprotein-driven metabolic activities (Yi & Guerinot, 1996) [45]. Fe is also a part of all the redox systems that exist in plants, thus it is critical for the biosynthesis of the porphyrin ring that comprises the structure of the chlorophyll molecule. According to (Msilini *et al*., 2011) [27], Fe plays a crucial role in plants by promoting the structural integrity of the Light Harvesting Complex (LHC) subunits and photosynthetic reaction centers (Yadavalli *et al*., 2012) [44]. Analysis of soil samples has revealed that 49% of soils in India are inadequate in Fe (Masto *et al*., 2008) [22]. Lacking Fe and N causes crops to grow slow and make them vulnerable to diseases (Chatterjee *et al*., 2006) [5]. Different adaptation strategies, such as altering morphology or physiological responses, have been developed by plants to survive the deficiency of N and Fe. Reports on the important signalling interactions between various nutrients and their effects in a changing nutritional environment are extremely rare. In the current work, we are attempting to comprehend the effects of individual nutrient stress (-Fe, -N) and their combined (-N-Fe) starvation response in the wheat HD3298 wheat variety.

Materials and Methods

Plant growth under hydroponics conditions

For the study, seeds of the bread wheat genotype HD 3298 stored at the Division of Plant Physiology, ICAR-IARI, New Delhi, were used. Genotype HD3298 is renowned for its bio-fortified properties and high levels of protein (12.12%) and Fe (43.1%), respectively content (ICAR Report 2022, Prabhjyot Kaur, *et al*., 2021) [31], wheat seedlings were grown under hydroponic conditions according to Sathee *et al*., (2018) [34]. The seeds were surface sterilized for 5 minutes with 0.1% mercuric chloride after being washed twice with distilled water. To remove any $HgCl_2$ traces, seeds were again thoroughly washed five to six times with double-distilled water. After uniform seedlings had grown for 5 to 6 days in Petri plates lined with wet germination paper, they were transferred onto trays with N and Fe-free Hoagland solution (Hoagland & Arnon, D. I., 1950) [13]. The plants were kept in plastic trays with 5 L of the nutrient solution and four levels of treatment condition Control (N: 7.5mM; Fe-III EDTA: 100 µM), Fe deficient (N: 7.5mM; Fe-III EDTA: 1 µM), N and Fe deficient (N: 0.05mM; Fe-III EDTA: 1 µM) and N deficient (N: 0.05mM; Fe-III EDTA: 100 µM) made in sterile deionized water on Styrofoam sheets. Constant aeration of the solution was accomplished using aquarium pumps. The growing material was changed every two to three days in order to maintain cultural conditions and assure a steady supply of nutrients. Two independent biological replications were kept for each treatment, and all parameters were collected in triplicate.

Assessment of yield parameters

At physiological maturity, the total number of productive and vegetative tillers was counted. The measurement of the ear's length was recorded in centimeters. Spikes (ears) were dried at 60 °C to maintain a steady weight that was measured in grams. The number of grains in each ear was counted, and the weight of 100 grains from each treatment was recorded. Each plant's total grain yield was measured and expressed in grams. Three different plants were used to replicate each parameter.

Leaf greenness measurement

In this work, the Chlorophyll content index (CCI) was measured in four replications at vegetative phases using the CCM-200 equipment (Opti-Sciences Inc., USA).

Measurement of photosynthetic rate, stomatal conductance, and transpiration rate (IRGA: Infrared gas analyzer)

Stomatal conductance, transpiration rate, and rate of photosynthesis were all measured in nine replications using the LI-6400XTPortable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA).

Biochemical parameters

Estimation of Nitrate reeducates activity in plant tissues (Nair and Abrol, 1973) [37]

Estimation of *in vivo* nitrate reductase activity was done by estimating the nitrite formed by the enzyme present in cells and the nitrite formed was then diazotized using sulphanilamide in an acidic medium and NEDD using the method of (Klepper *et al*., 1971) [16] and modified by (T. V. R. Nair & Y. P. Abrol, 1973) [37]. Nitrite was estimated by the method of Evans and (Evans & Nason, 1953) [9]. Absorbance was measured using a UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany) at 540 nm. The calibration curve was prepared using a standard sodium nitrite solution. The enzyme activity was expressed as µmol nitrite formed g^{-1} DW h^{-1}.

Estimation for GS, GOGAT, and GDH activity in plant tissue

Enzyme extraction

Extraction of enzyme and assays of GS, GOGAT, and GDH was done following the method of (Mohanty & Fletcher, 1980) [26]. Leaf and root samples were extracted in tris-HCl buffer, which contains 100mM Tris-HCl, 100mM sucrose, 10mM EDTA, and 10mM $MgCl_2$. Tissue samples were ground in a chilled pestle and mortar with an extraction buffer. Ground samples were centrifuged (sigma 3K30) at

5000g for 10min at 4^0C. The supernatant was collected and re-centrifuged at 12000g for 15min at 4^0C. The supernatant was collected in separate Eppendorf tubes and used for the estimation of GS and GOGAT. The pellet was dissolved in 1ml of 50mM phosphate buffer with 2.14g/100ml sucrose with p^H 7.5 and was used for estimation of GDH.

Estimation of glutamine synthetase (GS) activity in plant tissue

Estimation of glutamine synthetase activity was done by measuring the γ-glutamylhydroxamate formed g^{-1} protein hr^{-1}, All the reagents viz., 0.35ml of 200mM Tris buffer, 0.25ml of 200mM $MgSO_4$, 0.1ml of 50mM cysteine, 0.25ml of 500mM α-glutamate, 0.1ml of 50mM ATP and 0.25ml of 40mM hydroxylamine were pipetted out along with 0.2ml of aliquot and kept at 37^0C for 30minute. To stop the reaction 0.5ml $FeCl_3$ reagent was added and then centrifuged (sigma 3K30) at 1500 -2000g for 10 minutes. Absorbance was measured using a UV-visible spectrophotometer (Specord Bio-200, AnalytikJena, Germany). The enzyme activity was expressed as γ-glutamylhydroxamate formed g^{-1} protein hr^{-1}.

Estimation of glutamate synthase (GOGAT) activity in plant tissue

Estimation of glutamate synthase activity was done by measuring µmol NADH oxidized g^{-1} protein $hr^{-1,}$ All the reagents viz., 1ml of 75mM Tris-HCl, 0.2ml of 50mM α-ketoglutaric acid and 0.2ml of 200mM L-glutamine were pipetted out with 0.1ml of aliquot and volume made to 2.8ml with double distilled water. To it, 0.2ml of 1.5mM of NADH was added into a cuvette with a reaction mixture just before taking absorbance reading mixed well and absorbance was measured using a UV-visible spectrophotometer (Specord Bio-200, AnalytikJena, Germany) at 340nm for 60 seconds. The enzyme activity was expressed as µmol NADH oxidized g^{-1} protein hr^{-1}.

Estimation of glutamate dehydrogenase (GDH) activity in plant tissue

Estimation of glutamate dehydrogenase activity was done by measuring µmol NADH oxidized g^{-1} protein $hr^{-1,}$ All the reagents viz., 1ml of 75mM phosphate buffer, 0.2ml of 100mM α-ketoglutaric acid and 0.4ml of 750mM NH_4Cl were pipetted out with 0.2ml of aliquot and volume made to 2.8ml with double distilled water. To it, 0.1ml of 1.5mM of NADH was added into a cuvette with a reaction mixture just before taking absorbance reading mixed well and absorbance was measured using a UV-visible spectrophotometer (Specord Bio-200, AnalytikJena, Germany) at 340 nm for 60 seconds. The enzyme activity was expressed as µmol NADH oxidized g^{-1} protein hr^{-1}

Superoxide dismutase (SOD)

Superoxide dismutase activity was estimated by recording the decrease in optical density of formazone made by superoxide radical and nitro-blue tetrazolium dye by the enzyme (Kono, 1978) [18] (Dhindsa *et al*., 1981) [6]. Enzyme extract for superoxide dismutase, ascorbateperoxidase, glutathione reductase and catalase was prepared by first freezing the weighed amount of leaf samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). The extract was centrifuged for 20 min at 15,000 g and the supernatant was used as an enzyme (Dhindsa *et al*., 1981) [6]. Total SOD activity was estimated by the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) by the enzyme (Dhindsa *et al*., 1981) [6]. The reaction was started by adding 2 mM riboflavin (0.1 ml) and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as a control. Switching off the light and putting the tubes into the dark stopped the reaction. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

Visualization of Indole-3-acetic acid (IAA)

The colourimetric assay of Indole-3-acetic acid (IAA) was done using the Salkowski reagent method (Shraddha Gang, 2019) [35], which can be used to screen for the presence of Indole compounds. Salkowski reagent was prepared by mixing 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$). 1-2 ml Salkowski reagent was put in watch glasses. Plant root tips were cut and dipped into it for 1 hour under completely dark conditions by covering them with black cloth. The whole plant root was dipped and placed separately for the same duration. The pinkish brown color was developed where the IAA concentration is more in the root. Roots were visualized under the EVOS XL microscope (AMG, Bothell, WA), and images were taken and analyzed in "ImageJ" to determine the relative intensity.

***In vitro* staining for histochemical detection of iron in root tissue (Perl's staining)**

Iron staining of tissue was performed as described in the literature (Meguro *et al*., 2007) [25]; (Roschzttardtz *et al*., 2009) [33] one of the relatively simple yet powerful techniques is the classical Perls blue stain (M. Perls, 1867) [21]. The technique is based on the conversion of ferrocyanide to insoluble crystals of Prussian blue in the presence of Fe^{3+} under acidic conditions.

Statistical analysis

The One-way analysis of variance (ANOVA) was conducted using GraphPad Prism version 8 (La Jolla, California, USA) to determine the adjusted P values and level of significance.Graph construction and the Tukey's multiple comparisons test for mean separation were both completed in GraphPad.

Results

Effect of –N, –Fe, and dual deficiency treatments on biomass and yield parameters

The variation in total biomass, grain yield, plant height, number of tillers per plant, number of ears per plant, ear length, ear weight, number of spikelets per ear, 100-grain weight, and number of grains per ear is shown in Fig.1 and 2. The mean value of plant height significantly decreased in the -N-Fe dual deficiency treatment, by 28.81% in comparison to the control condition, and in the -N treatment, by 4.86%.The number of vegetative tillers per plant were 17.64% less in -N and -Fe dual plants. When compared to the control, the effective number of ears exhibited a similar trend for both N and Fe deficiency (a reduction of 29.41%). In contrast to the

control, the plant in the -Fe condition showed an overall 17% decrease in the number of ears. The number of spikes on the main shoot varied significantly in all treatments, with -N-Fe showing substantial reductions (64.47%), next to -N (64.84%), and -Fe showed a decrease of 53.94% from control. Nutrient deficiencies and its interactions greatly affected the total biomass accumulation (at harvest) (dual our single deficiency). The mean value of biomass was considerably different between -N and -N-Fe, and there was a 72.28% reduction in total plant dry biomass in -N-Fe combined deficiency. In comparison to the -N, -Fe, and -N-Fe treatments, the average biomass was increased in the -Fe treatment. Among all treatments, the control plant had shown maximum biomass accumulation, whereas -N-Fe showed the lowest. In -N, -Fe, and -N-Fe, respectively, there were significant differences in total ear weight and grain number per ear. In comparison to the control, there was a considerable reduction in -N-Fe (51.61%), followed by (45.16%), in the total ear weight. The trend in grain production per ear is the same as the 43.52% reduction in N-Fe comparison to control. In comparison to the control, there was a non-significant increase in the weight of 100 grains in -N (3%). There was a 25% and a 32.02% reduction in grain yield in -N and -N-Fe, respectively. In-N grain yield differed significantly between the control and dual deficit conditions. The treatment with the least grain yield, all in all, was -N-Fe. Based on nutrient deficiency, ear height, and weight varied significantly among all treatments (single or dual). Ear height was lowest in dual nutrient deficit as compared to the other treatments and control. In comparison to the control, -N-Fe reflected a 54.67% reduction in ear height. Depending on the treatments, root length varied significantly. The most substantial decrease in plant length was observed in the scenario of dual nutrient starvation; with dual N and Fe deficiency, there is a 72.85% reeducation of plant length. As we absorb an increase of 129.65% in -N treatment, root biomass drastically changes in plants under nutrient starvation situations.

Effect of –N, –Fe and dual deficiency treatments on leaf greenness, photosynthetic and biochemical parameters

The CCM-200 device (Opti-Sciences Inc., USA) was used in this experiment to measurethe Chlorophyll content index (CCI) in four replications. In hydroponic conditions, CCI was reduced in –N-Fe (84.44%), followed by –N (68.68%) and –Fe (30.02%). compared with control plant leaf Chlorophyll CCI. The rate of photosynthesis, stomatal conductance, water use efficiency, intercellular CO_2, and transpiration rate were measured using the LI-6400XTPortable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA). Intercellular CO_2 was dramatically increased in all treatments in comparison with control, and the highest increment was seen in -Fe (11.79%) deficiency, followed by –N (9.59%) and –N-Fe (4.86%) (Fig. 3). The rate of photosynthesis varied significantly based on treatment and deficiency levels. In -N deficiency, the mean value of plant photosynthesis rate was significantly decreased by 28.89% compared with the control condition, and in –N-Fe dual deficiency, an increase of 60.37 % in photosynthesis rate was noticed with regards to the control. Photosynthesis rate was lower in –Fe treatment with a 39.99 % reduction (Fig 3, B). There was a non-significant increase in -N–Fe stomatal conductance by 137.29% compared to the control. However, 36.58% up-regulation is noticed in –N treatment. Conversely, the rate of transpiration was significantly different according to the treatment, and an overall increment is noticed in all treatments. – N, -N-Fe, and –Fe (25.33%, 74.43%, 40.12%). Depending on treatment and deficiency levels, there were significant variations in the plant's biochemical parameters. Glutamate syntheses (GOGAT) activity showed non-significant differences under different treatments. GOGAT activity was increased by 94.26% under –N-Fe dual deficiency treatment compared to control. Under all the treatments, minimum GOGAT activity was recorded during the -Fe stage. A significant difference was observed in GOGAT activity compared to the control treatment. Glutamate synthase (GOGAT) is an enzyme that catalyzes the synthesis of glutamate, an amino acid, in plants. Under Fe deficiency conditions, plants activate a mechanism called the "Fe-deficiency response" to acquire more Fe from the soil. This response includes the upregulation of genes involved in Fe uptake, such as those encoding for Fe transporters, as well as the down regulation of genes involved in Fe-containing protein synthesis, such as those encoding for GOGAT. The decrease of GOGAT activity under Fe deficiency is an adaptive mechanism that reduces the demand for Fe in the synthesis of non-essential amino acids and channels it towards essential Fe-containing proteins (Plaitakis *et al*., 2017) [30]. Estimation of *in vivo* nitrate reductase activity was done by estimating nitrite formed by the enzyme present in cells and nitrite formed was then diazotized using sulphanilamide in an acidic medium and NEDD using the method of Klepper *et al.* (1971) [16] and modified by Nair and Abrol (1973) [37]. Depending on treatment and deficiency levels, the percent of Nitrate reductase (NR), glutamate dehydrogenase (GDH), and Superoxide dismutase (SOD) content is varied in the plant tissue. Glutamate dehydrogenase (GDH) is an enzyme that catalyzes the reversible oxidation of glutamate to alpha-ketoglutarate and the reduction of NADP+ to NADPH. In plants, GDH is involved in the metabolism of N and carbon and can play a role in stress responses (Plaitakis *et al*., 2017) [30]. Under Fe deficiency, plants have been shown to increase their GDH activity as a response to stress. This increased GDH activity is thought to help the plant acquire more Fe from the soil through the synthesis of the Fe chelator, nicotinamide. Additionally, GDH can also generate NADPH which is essential for the production of antioxidant molecules, which can help protect the plant from the oxidative damage caused by Fe deficiency (Labboun *et al*., 2009) [19]. NR activity showed nonsignificant differences under various levels of Treatments. It increased by 48.54% under –N-Fe dual deficiency and decreased by 15.85% in -N treatment compared to control. Under all the treatments, maximum GDH activity was recorded during the -N-Fe stage (285.76%) non significantly, under –N treatment GDH increased by 74.71% compared to control plants. A significant difference was observed in plant SOD activity compared to the control treatment. We absorb an overall reeducation of 7.37% to 91.86% in plant SOD activity during nutrient starvation. The highest reduction is seen in –Fe treatment (91.86%) when compared to the control (Fig. 4).

In vitro staining for histochemical detection of pearls staining and indole-3-acetic acid (IAA)

Iron staining of tissue was performed as described in the literature (Meguro *et al*., 2007) [25]; (Roschzttardtz *et al*., 2009) [33]. One of the relatively simple yet powerful technique is the classical Perls blue stain (M. Perls, 1867) [21]. The

technique is based on the conversion of Ferro cyanide to insoluble crystals of Prussian blue in the presence of Fe^{3+} under acidic conditions. The variation in blue color can be observed under a light microscope. Wheat seedlings receiving high +Fe treatment displayed stronger Prussian blue stains, compared with –Fe and –N-Fe treatments. Color development in –Fe and –N-Fe treatment is reduced respectively (50.56%; 37.10%) when compared with control.-N deficiency treatment has a 24.69 % decrease compared to control treatment (Fig.5). The Salkowski reagent method was used for the detection of Indole-3-acetic acid (IAA) which can be used to screen for the presence of Indole compounds (Gang *et al*., 2019) [11]. IAA relative abundance is higher in –N,-Fe, and –N-Fe (99.97%, 48.60%, and 65.90% respectively) as compared with the control treatment (Fig.5).

Discussion

Different adaptation mechanisms, including altering morphology and physiological and biochemical responses, have been developed by plants to survive nutrient scarcity. Under N and Fe dual deficiency, wheat plants exhibit several changes, including reduced growth, chlorosis, reduced tillering, and lowered grain yield (EL Sabagh *et al*., 2021) [8]. The objective of the current study was to investigate the physiological and biochemical impacts of -Fe, N, and dual -N-Fe deficiency response on bread wheat variety HD3298. Leaf Chlorophyll content index (CCI) in the field and hydroponic conditions showed a serial reduction in –N-Fe, -N, and –Fe plants which are aligned with previous studies (Terry & Low, 1982) [29].

According to Teixeira *et al*., (2020) [38], under conditions of combined N and Fe deficiency, the chlorophyll content of wheat leaves may show an overall decrease. This can lead to yellowing or chlorosis of the leaves, as well as a decrease in photosynthetic efficiency and plant growth. Additionally, the plant may try to compensate for the deficiency by increasing the number of accessory pigments such as carotenoids, which can result in a change in leaf color. Overall, the plant may struggle to survive and grow in the presence of both N and Fe deficiency. Different treatments were found to have a substantial impact on the photosynthetic rate compared and the control plant had the highest photosynthetic rate of all the treatments (Fig 1). Prior research by Mathur & Vyas, (1995) [23] suggested that increasing leaf area and chlorophyll could be responsible for the photosynthetic rate; our findings support this. The global supply of nutrients is expected to decrease by 14-20% in the case of protein, Fe, and Zn relative by 2050 (Beach *et al*., 2019) [2]. Deprecation in Zn in rice, wheat, maize, sorghum, soybeans, and field pea is a global nutritional security issue affecting 17% of the world population (Myers *et al*., 2017) [28]. Similarly, deprecation in Fe availability will involve 10–15% of the worldwide population (Myers *et al*., 2017) [28]. The N availability for plant root absorption is a critical and limiting factor determining plant growth, development, and economic production (Richardson *et al*., 2009) [32]; (McAllister *et al*., 2012) [24]; Xu *et al*., 2012 [43]. Another aim was to understand the effects of nutrient deficiencies on the HD 3298 wheat genotypes in vitro histochemical level. In hydroponic conditions, the effects of -N, -Fe, and -N-Fe levels on growth, yield parameters, and plant biochemical parameter were investigated. Field and hydroponic leaf chlor measurements indicated a sequential reduction in -N-Fe, -N, and -Fe plants, which is in line with previous results Terry & Low, (1982) [39]. The root length has increased in the wheat genotype grown with nutrient deficiency compared to the control. The highest increase is seen in –N treatment which is reported by previous researchers also. Under N and Fe deficiency, the levels of Indole 3-acetic acide (IAA) the endogenous auxin in the wheat root may increase. IAA has been shown to have a positive effect on the root system, promoting root growth and development, which may also be beneficial for the plant under these conditions (Pascale *et al*., 2020) [29]. Under N deficiency, the levels of IAA in wheat roots increases, that may also lead to changes in the root architecture and growth patterns of the wheat plant, as well as changes in the expression of genes involved in N uptake and utilization. Additionally, the increase in IAA may also lead to changes in the composition of the root microbiome, which may further impact the plant's ability to cope with N deficiency (Koevoets *et al*., 2016) [17]. According to Broberg *et al*. (2019) [3], the number of grains subjected to environmental change was the major cause of a 26% rise in wheat grain yields. Similar results were reported by Högy *et al*. (2013) [14], who noted an increase in the number of grains per ear despite no change in the number of ears, which was similar to previous data. The results of this investigation revealed that dual deficiency (-N-Fe) had the highest reduction in grain yield per ear and weight of 100 seeds, by 43.52% and 32.02%, respectively. The amount of grains per ear of wheat often falls in N-deficient conditions. This is due to the fact that N is a crucial ingredient for plant growth and development, and a deficiency might impact a plant's ability to produce and fill grains. Lower yields and less productive crops may result from this decrease in the number of grains. Additionally, the grains that do develop could be small and of lower quality (Y. Wang *et al*., 2021) [41]. In this study, nutrient deficiency drastically lowered the number of spikelets on the main shoot spike (Galindo *et al*., 2017) [10]. In comparison to control plants, the total ear weight of plants under the -N-Fe, -N, and -Fe conditions is lower. Similar cases of N and Fe starvation were described by Gregory *et al*., (1979) [12] and Spiertz and Ellen, (1978) [36]. In comparison to control treatments, -N and -Fe treatments had less grains number. Grain production under nutrient deficiency varies significantly depending on the growth conditions (J. Wang *et al*., 2013) [41]. Plants that are deficient in both N and Fe experiences reduced growth and overall yield, including smaller ear size and weight. This is because both N and Fe play important roles in plant growth and development, including photosynthesis, cell division and expansion, and chlorophyll formation. Without adequate levels of these nutrients, the plant may not be able to produce as much biomass, leading to smaller ears and lower overall yield (Galindo *et al*., 2017) [10]. Physiological and molecular responses and combined nutrient deficiency homeostasis reports in the crop are very much limited. The current work aimed to fill this knowledge gap by providing insights into the wheat responses to single and dual nutrient deficiency stresses that could help cope with varying soil-environmental conditions and unravel new and unexpected aspects of the response of plants to stress combinations.

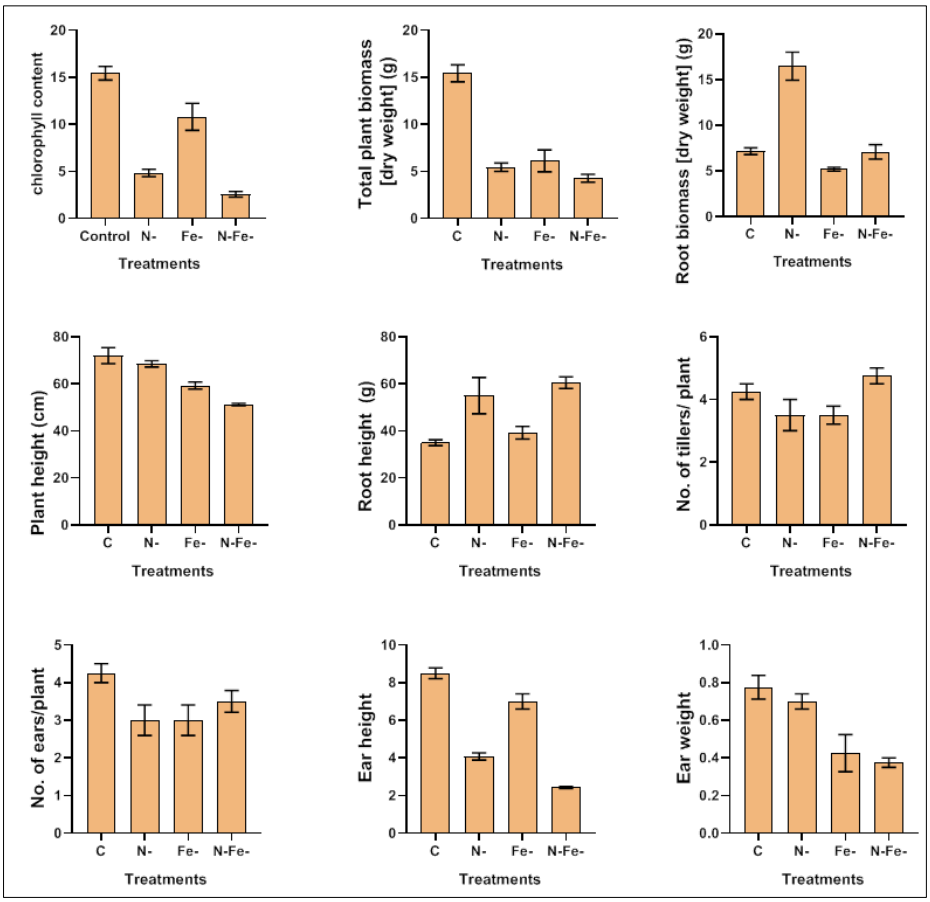

Fig 1: Variation in A. Chlorophyll content index (CCI); B. Total plant biomass; C. Root biomass; D. Plant length; E. Root length; F. No. of tillers/plant; G. No. of ears/plant; H. Ear length; and I. Ear weight of wheat genotype HD2398 grown under control, nitrogen deficiency (-N), iron deficiency (-Fe) and N and Fe combined deficiency (-N - Fe) in hydroponic conditions. Values are mean (±SE) of four biological replicates.

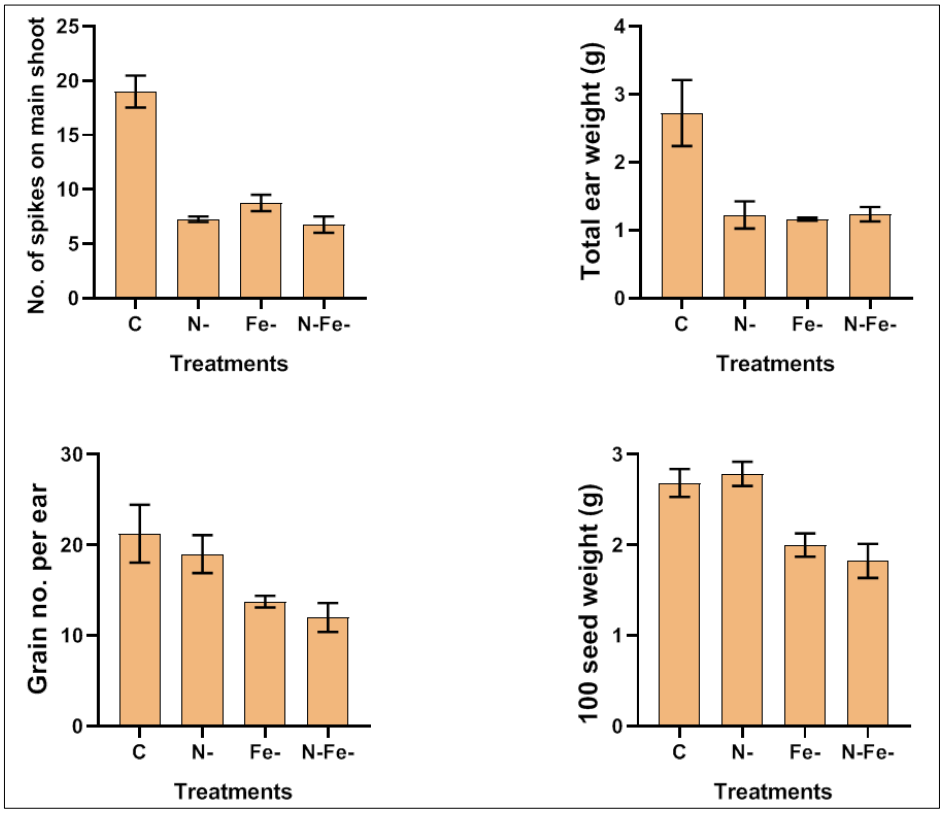

Fig 2: Variation in A. No. of spikes on main shoot; B. Total Ear weight; C. Grain no. per ear; and D. 100 seed weight of wheat genotype HD2398 grown under control, nitrogen deficiency (-N), iron deficiency (-Fe) and N and Fe combined deficiency (-N - Fe) in hydroponic conditions. Values are mean (±SE) of Five biological replicates.

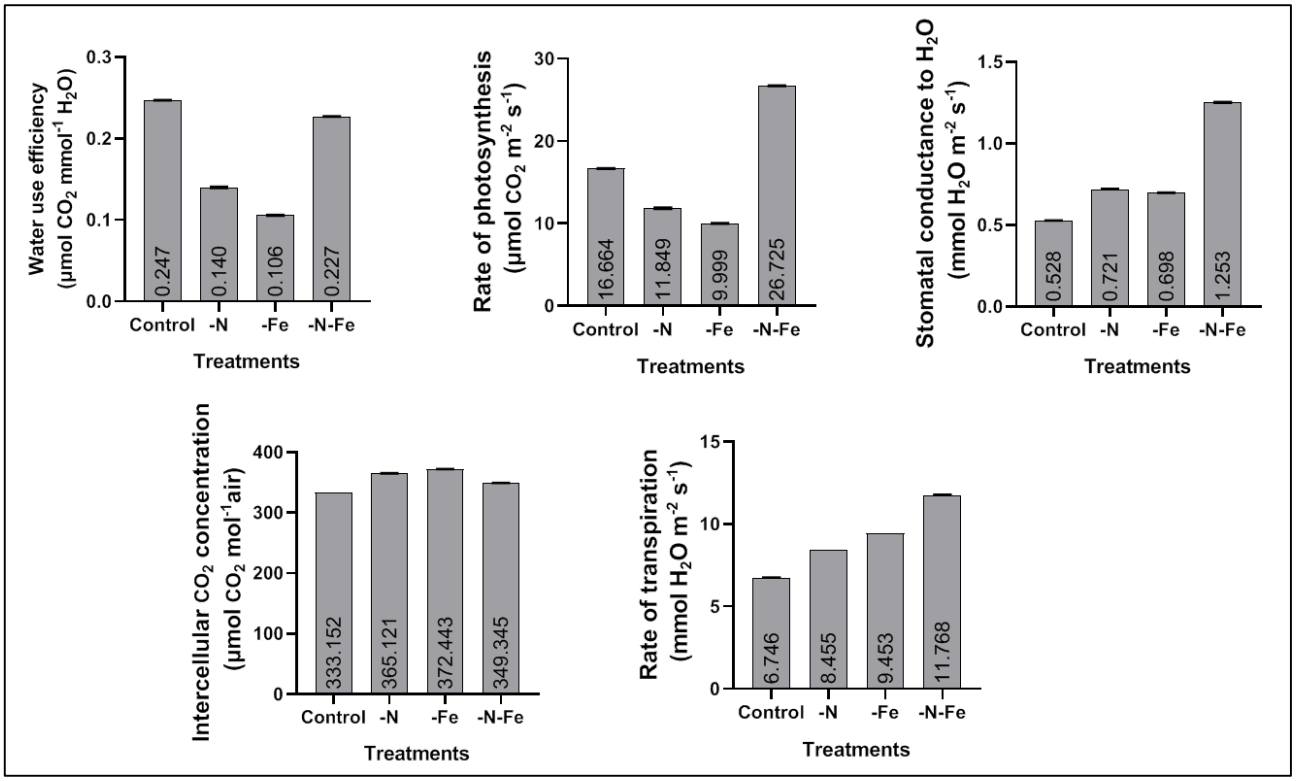

Fig 3: Variation in photosynthetic parameters A. Water use efficiency; B. Rate of photosynthesis; C. Stomatal conductance ; D. Intercellular CO2 concentration; and E. Rate of transpiration of wheat genotype HD2398 grown under control, nitrogen deficiency (-N), iron deficiency (-Fe) and N and Fe combined deficiency (-N - Fe) in hydroponic conditions. Values are mean (±SE) of nine biological replicates.

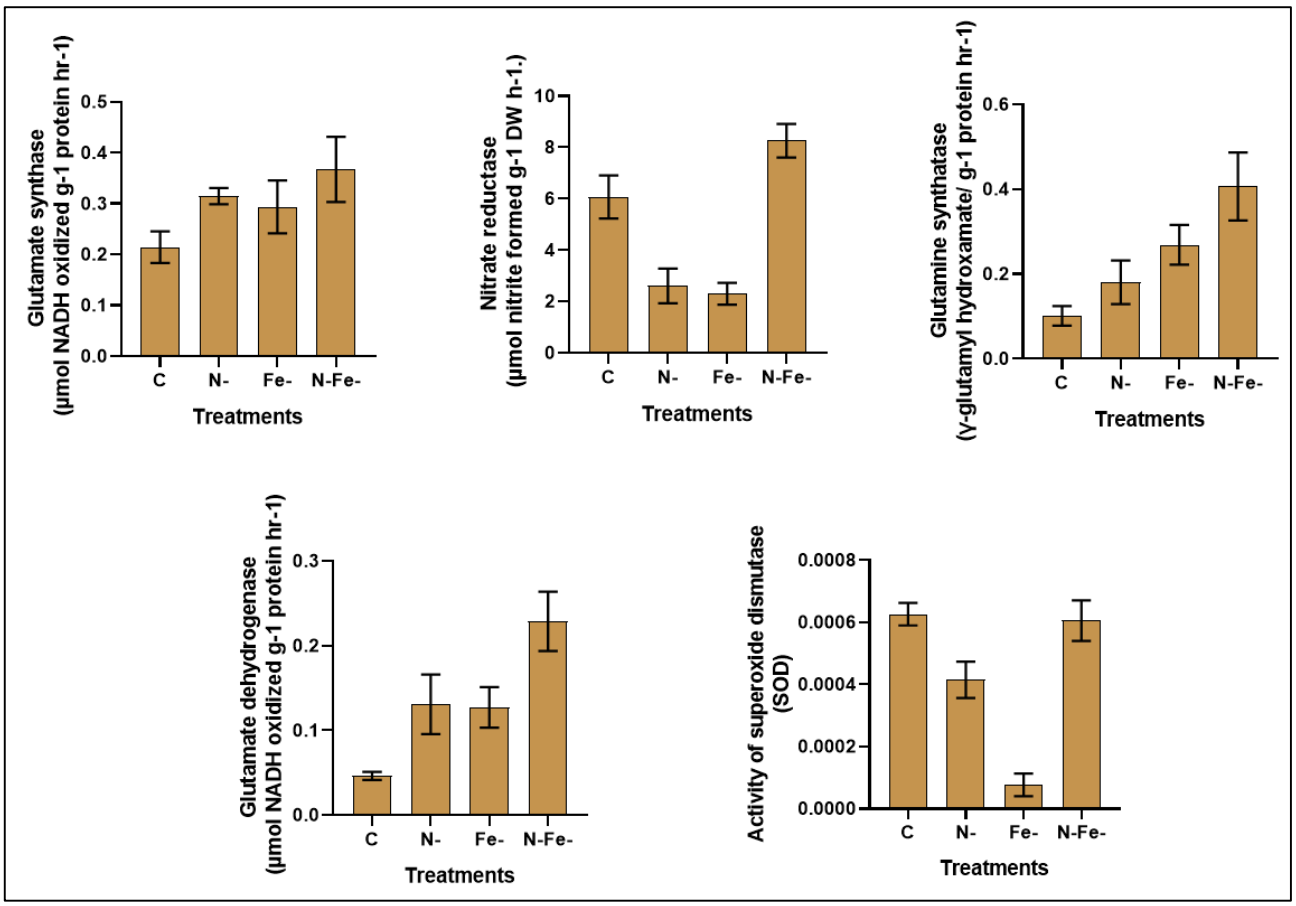

Fig 4: Variation in biochemical parameters A. Glutamate synthase ; B. Nitrate reductase ; C. Glutamine synthatase ; D. Glutamate dehydrogenase ; and E. Activity of superoxide dismutase (SOD) in wheat genotype HD2398 grown under control, nitrogen deficiency (-N), iron deficiency (-Fe) and N and Fe combined deficiency (-N - Fe) in hydroponic conditions. Values are mean (±SE) of three biological replicates.

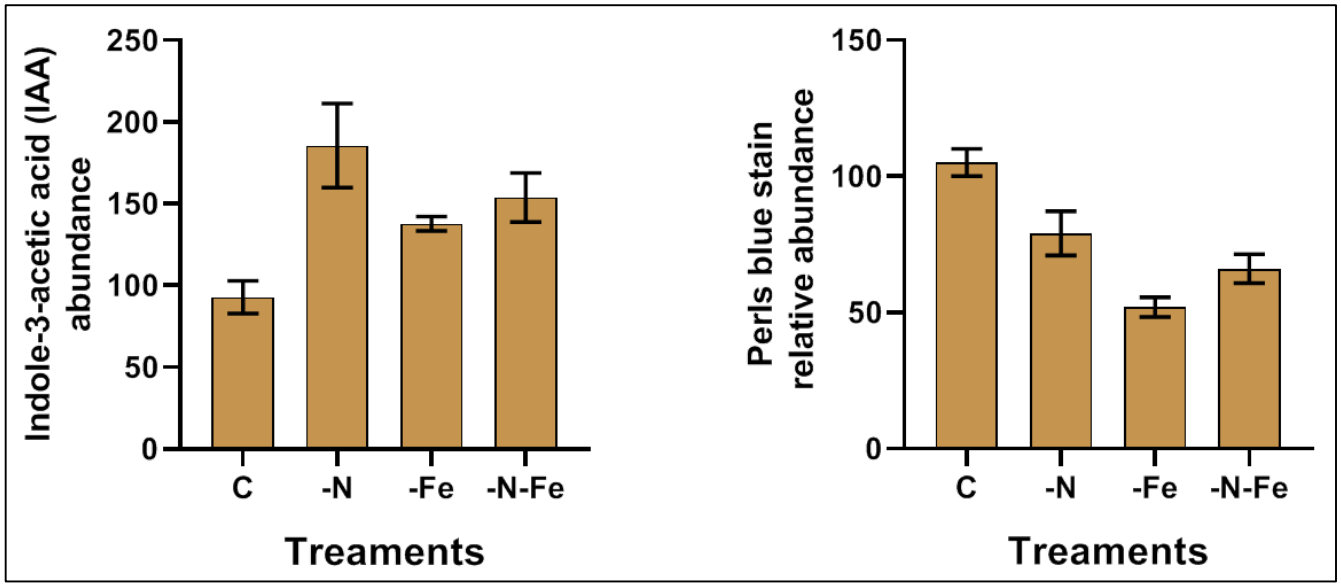

Fig 5: Variation in accumulation of A. IAA and B. Fe in roots of wheat genotype HD2398 grown under control, nitrogen deficiency (-N), iron deficiency (-Fe) and N and Fe combined deficiency (-N - Fe) in hydroponic conditions. Values are mean (±SE) of nine biological replicates.

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