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ORIGINAL PAPER

Soybean Physiological Properties and Grain Quality Responses to Nutrients, and Predicting Nutrient Deficiency Using Chlorophyll Fluorescence

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Abstract

Managing plant nutrition is crucial to getting optimum yield quantity and quality. Soybean is an important plant for oil and protein production, as well as N biological fixation. The current experiment aims were to study soybean responses to some macronutrient and micronutrient deficiency and diagnosing nutrient deficiency using the chlorophyll fluorescence. A 2-year field experiment during the 2019–2020 growing seasons was conducted. Treatments were N, P, Fe, and Mo, accompanied with and without humic acid. N and P were applied in the soil, but Fe, Mo, and humic acid were foliar applied at the final vegetative growth stage. Effect of fertilizer treatments was significant on all traits. N-P-Fe-Mo treatment improved photosynthesis rate and grain yield. However, their application accompanied with humic acid (HA) induced a synergistic effect. The maximum grain yield, oil and protein content, and photosynthesis rate were recorded in the N-P-Fe-Mo + HA. Fertilizer application decreased F_0 and F_m and increased F_v/F_m. Besides, there was a significant negative correlation between the leaf's N, P, Fe, and Mo content with F_m. The negative correlation between the leaf nitrogen and F_m was more potent than the other applied nutrients. Chlorophyll fluorescence technique as a valid non-destructive physiological indicator could be used to monitor N-P-Fe-Mo nutritional status in soybean plants. Findings showed that biologically fixing N is not sufficient to achieve high grain protein content. Although soybean is a nitrogen-fixing plant, it needs complementary N fertilizer to achieve maximum PSII efficiency, minimum chlorophyll fluorescence, and optimal yield.

Keywords *Glycine max* L. · Humic acid · Iron chelate · Molybdenum · Nitrogen · Nutrient monitoring

Abbreviations

Chl	Chlorophyll
F_0	Minimum fluorescence
Fe	Iron
F_m	Maximum fluorescence
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
HA	Humic acid
C_i	Internal CO_2
M	Molybdenum
N	Nitrogen
P	Phosphorus
P_N	Net photosynthesis rate
PS	Photosystem
QA	Quinone A
ROS	Reactive oxygen species

1 Introduction

Soybean (*Glycine max* L.) is one of the globally staple crops that plays a vital role in public health due to its oil and protein composition (Chen et al. 2005). Therefore, in developing countries, where the costs for import oils, oilseeds, and protein products are high, improving the efficiency of oilseed crops is of great importance (Akbari and Soltani 2018). The area under soybean cultivation in Iran in 2019 was 67,000 ha, with an average yield of 2388 kg ha^{-1}, while the total

The original version of this article was revised: Table citations in the section entitled 2.1 Experimental location and design, and plant material have been updated.

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cultivated area of soybean in the world was ~120 million ha, with an average production of 2769 kg ha^{-1} (FAO 2019).

Chemical fertilizers are typically used through soil or foliar application. However, leaching, runoff, and evaporation usually decrease nutrients available for the plants resulted in their deficiency. Therefore, using new technologies are necessary to meet the crop need for elements without wasting and polluting the environment. Research on fast and non-destructive methods of diagnosing plant nutritional deficiencies will be a significant help in improving crop yield, reducing production costs and environmental pollutions.

Nitrogen is one of the macronutrients that are essential for soybean growth and development (Gai et al. 2017). N deficiency induces changes in some physiological processes. For example, its deficiency meaningfully declines the carbon dioxide assimilation capacity, leading to decreases in light-saturated photosynthetic rates and photosynthetic quantum yields. This element affects photosynthesis and chlorophyll fluorescence. So increasing N levels increases F_v/F_m, but nitrogen deficiency decreases F_v/F_m and quantum yield of PSII electron transport (Jin et al. 2015). Although there is a report from a pot experiment on *Triticum aestivum* L. that shows F_v/F_m is insensitive to N treatment (Živčák et al. 2014).

P is a macronutrient required for completing the plants life cycle. This element is involved in photosynthesis energy store and transfer; produced energy is then used by plant's growth and reproduction stages. P shortage limits the plant's ability against environmental stresses. Root and shoot development will weak resulted in delayed maturity and yield reduction. Phospholipids, a major component of membranes, contain P, and also it is needed for DNA structure (Marschner 2011). It has been reported a strong correlation of leaf P concentration with CO_2 assimilation rate and chlorophyll fluorescence parameters in soybean; so, the P deficiency shows decreases in photosynthesis rate and quantum yield of photosystem II (Singh and Reddy 2015).

Micronutrients have many effects on plant performance. These nutrients are involved in many plant biochemical reactions while participating in the structure of some organs. The deficiency of these elements can sometimes inhibit the uptake of other nutrients and growth; therefore, more attention should be paid to their application. Fe is an important part of electron chain in photosynthesis. Fe deficiency reduces cell's size; however, cell death does not occur. Also, photochemical reactions in the PSII reduce remarkably, and donor and acceptor parts of PSII are equally damaged. Additionally, the fluorescence emission maxima increase due to impaired energy transfer from PSII to PSI. Furthermore, the most of the proteins of reaction center and light-harvesting antenna are reduced in Fe-depleted cells. The super complexes of PSI and PSII are destabilized from thylakoids under Fe-deficient condition showing that Fe is an important element in photosynthesis mechanism (Devadasu et al. 2016).

Mo is a cofactor in the active center of plant enzymes catalyzing key steps of nitrogen, carbon, and sulfur metabolisms, making them essential for efficient growth under the varied environmental conditions. Moreover, legumes similarly need Mo for symbiotic nitrogen fixation relying on the bacterial Mo-dependent enzyme nitrogenase (Manuel et al. 2018). Iron and molybdenum increase the soybean grain yield quantity and quality by improving growth indices (Rahman et al. 2010). Iron is an essential nutrient for all organisms; its deficiency exists in many crops. The soil iron content is usually high, but a large part is fixed in the soil as Fe^{3+}, especially at high pH, which is not available for the plants (Mimmo et al. 2014).

Iron compounds are the best solution to eliminate iron chlorosis in all plants, especially in alkaline soils, which iron deficiency is most severe. The role of Fe in nitrogen fixation in legumes has been well established (Brear et al. 2013). Joorabi et al. (2020) reported that nano-zinc chelate (ZnO) significantly increased proline content, catalase, peroxidase activities, and soybean oil yield under drought. Fe chelate foliar application affects the soybean's leaf area, the number of pods per plant, seeds per pod, and 100-seed weight under drought stress (Vaghar et al. 2020). It has been reported that molybdenum foliar application on soybean increased seed Mo content but did not significant effects on crud protein and lipids (Cardoso et al. 2021).

Photosynthesis, as the primary plant metabolism process, is strongly influenced by environmental conditions. It consists of four stages: light perception, electron transfer, energy fixation, photo-assimilate biosynthesis, and transfer (Blankenship 2014). It is a significant determinant of plant growth and yield; photosynthesis permanence under environmental stresses is essential for yield stability. The higher nutrient availability increased the photosynthesis rate because of the improvement of plant growth conditions (Guo et al. 2019). Conversely, nutrient deficiency strongly affects the structure and function of the photosynthetic apparatus (Schlau-Cohen and Berry 2015). According to air temperatures during the current experiment (Table 1), high temperature was prevalent. High temperature close to or above 40°C can be considered as a negative factor for growth rate and pod retention in soybean (Avila et al. 2013). Heat stress decreases photosynthesis rate, stomatal conductance, internal CO_2 concentration (Chovancek et al. 2019), maximal quantum yield of PSII photochemistry, efficiency of PSII in the light-adapted state, and the grain yield (Eisvand et al. 2018).

Chlorophyll fluorescence is used as a method to study disorders in photosynthetic systems. It is a non-destructive methodology for estimating plant photochemical

Table 1 Temperature, precipitation, and relative humidity for 2 years (2019–2020) of the research farm of Lorestan University during the experimental period

Month	Mean of air temperature (°C)						Precipitation (mm)		Relative humidity (%)		Average Sunshine (h)	
	Maximum		Minimum		Average							
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
May	35.8	38.2	4.6	6.7	20.2	21.8	5.5	6.6	51.0	40.0	10.0	10.8
June	41.6	40.5	14.0	13.6	28.2	26.6	0.0	0.0	27.0	23.0	12.3	12.2
July	45.4	45.4	17.0	15.7	30.2	31.3	0.0	1.8	24.0	22.0	12.0	11.1
August	44.6	41.6	18.3	14.2	31.3	28.7	0.0	0.0	21.0	23.0	11.0	12.0
September	39.7	39.4	12.1	11.7	25.8	26.9	0.0	0.1	22.0	22.0	11.0	10.3

efficiency and photosynthetic status. It has been widely used to evaluate the plant response to environmental stresses. It is also a reliable method for studying photosynthetic processes under environmental stresses (Jin et al. 2015). Using this chlorophyll fluorescence technique is rapid and non-destructive, representing thylakoid membrane integrity and the relative efficiency of electron transfer from PSII to PSI (Kalaji et al. 2017).

Analysis of chlorophyll fluorescence of soybean leaves under the flooding period and different nutritional diets showed that maximal quantum yield of PSII photochemistry (F_v/F_m) decreased during flooding stress and nitrogen deficiency (Khadempir et al. 2015). Li et al. (2021) reported that foliar application of iron nano-particles stimulated the photochemical activity of PSII and relieved photoinhibition by enhancing the photochemical use of excess excitation energy in *Pseudostellaria heterophylvia.*

Optimal plant nutrition promotes the achievement of maximum quantitative and qualitative yield. Using organic fertilizer besides finding nondestructive methods to detect nutrient deficiency will be very useful in precision and organic agriculture, aiming optimal nutrition management of soybean. So far, no research with such treatment combinations (applied in the current research) has been done on soybean and there is no report about prediction of nutrient deficiency using chlorophyll fluorescence under such treatments. Therefore, the objectives of this experiment were to study soybean photosynthesis, chlorophyll fluorescence, grain yield, and quality in response to some essential nutrients' deficiencies as well as detecting plant nutritional stress using the chlorophyll fluorescence technique.

2 Materials and Methods

2.1 Experimental Location and Design, and Plant Material

The experiment was carried out for two consecutive years (2019 and 2020) in the research farm of Lorestan University, with a coordination of 33° 26′ 15″ N, 48° 15′ 39″ E, and an altitude of 1117 m above sea level. Before starting the experiment, 15 random soil samples were taken (0–30 cm in depth) by an auger, and the samples were combined. Then the combined sample was analyzed (Table 2). Some of the critical climatic parameters were recorded during the experiment period (Table 1).

In each year, the experiment was performed as a randomized complete block design with 12 treatments (Table 3) and 3 replications. The required amount of fertilizers was determined based on the soil test results (Table 2). Nitrogen 150 kg ha^{-1} (75 kg at tilling and 75 kg ha^{-1} as top dressing at V5) of urea fertilizer (NPK, 46-0-0) and phosphorus 80 kg ha^{-1} of triple superphosphate (46% P2O5) were applied before planting and incorporated into

Table 2 Soil (0–30 cm in depth) physico-chemical analysis for 2 years (2019–2020) of the research farm of Lorestan University

Soil texture	pH	EC (dsm^{-1})	C (%)	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Fe (mg kg^{-1})	Mo (mg kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
The first year (2019)											
Clay loam	7.73	0.64	1.04	0.039	7.6	352	3.4	0.032	0.3	2.8	0.7
The second year (2020)											
Clay loam	7.42	0.57	1.02	0.036	7.3	368	3.9	0.041	0.28	0.23	0.6

EC, electerical conductivity; *C*, carbon; *N*, nitrogen; *P*, phosphorus; *K*, potasium; *Fe*, iron; *Mo*, molibdenium; *Mn*, magnesium; *Zn*, zinc

Table 3 Fertilizer treatments and their abbreviations

Abbreviation	Full treatment name
C	Control (no fertilizer)
N-P-Fe-Mo	Nitrogen + phosphorus + iron + molybdenum
P-Fe-Mo (N deficiency)	Phosphorus + iron + molybdenum
N-Fe-Mo (P deficiency)	Nitrogen + iron+ molybdenum
N-P-Mo (Fe deficiency)	Nitrogen + phosphorus + molybdenum
N-P-Fe (Mo deficiency)	Nitrogen + phosphorus + iron
HA	Humic acid
N-P-Fe-Mo + HA	Nitrogen + phosphorus + iron + molybdenum + HA
P-Fe-Mo (N deficiency) + HA	Phosphorus + iron + molybdenum + HA
N-Fe-Mo (P deficiency) + HA	Nitrogen + iron + molybdenum + HA
N-P-Mo (Fe deficiency) + HA	Nitrogen + phosphorus + molybdenum + HA
N-P-Fe (Mo deficiency) + HA	Nitrogen + phosphorus + iron + HA

C, control (no application of N, P, Fe, and Mo fertilizers); *N*, nitrogen; *P*, phosphorus; *Fe*, iron; *Mo*, molybdenum; *HA*, humic acid

the soil by tilling. These amounts of N and P fertilizers were the treatments that applied in the experiment.

Each experimental plot area was 15 m^2, including five furrows. The distance between the main plots was 3 m, and the distance between the blocks was 5 m. The soybean seeds (*Glycine max* L. CV. Kosar) of 58 kg ha^{-1} were inoculated by *Bradyrhizobbium japonicum* and planed at 4–5-cm soil depth on 28 May each year. The plant density was adjusted at 35 plants per m^2 (Plant × Plant = 5.5 cm and Row × Row = 60 cm) after seedling establishment.

A tape irrigation system was used, and the plant water requirement was calculated based on the lack of soil moisture relative to field capacity. Irrigation was restarted when the soil moisture reached to 82% of field capacity. The weed control was done manually if needed.

Kosar soybean cultivar was released in 2015 as an early maturing and indeterminate cultivar with average of 1000-seed weight of 135 g, oil content 22%, protein content 37%, growth period 110 days, grain yield 3300 kg ha^{-1}, and resistant to *Phytophthora*, lodging, and grain shedding.

Iron chelate from Sequestrene 138 (Fe-EDDHA) with the dose of 2 g L^{-1} in water, molybdenum chelate (of Khazra Molybdenum chelate 5%) with the dose of 1 g L^{-1} in water, and humic acid (15 L ha^{-1}) were foliar sprayed at the pre-flowering stage (V7). Spraying was done at 8 am for three consecutive days, i.e., molybdenum on the first day, iron chelate on the second day, and finally, humic acid on the third day. Plastic guards were used between the plots to prevent diffusion to other plots.

2.2 Chlorophyll Fluorescence

Chlorophyll fluorescence parameters were measured at the R1 stage by a fluorometer (Pocket PEA, Hansatech Ltd. U.K.; light intensity 3000 µmol photon m^{-2} s^{-1}) from 9:30 to 11:30 a.m. At each plot, two plants and from each plant, three developed leaves (in the upper, middle, and lower part of the plant) were randomly selected and labeled for the both chlorophyll fluorescence parameters and photosynthesis measurements (photosynthesis measurement is described in details in section 2.3). The air temperature was 32.4–36.8 °C. The sampled leaves were placed in the dark for 20 min using a leaf clip (Kalaji et al. 2014). The minimum fluorescence (F_0) with all PSII open reaction centers and the maximum fluorescence (F_m) with all PSII closed reaction centers were determined on leaves adapted in the dark (Genty et al. 1989).

The F_v/F_m parameter was calculated according to the following equations:

$$F_v/F_m = \left(F_m - F_0\right)/F_m$$

where F_v/F_m is the maximum quantum efficiency of PSII in the dark-adapted state; F_m is the maximum fluorescence (dark); F_0 is the minimum fluorescence (dark); and F_v is the variable fluorescence (dark) ($F_m - F_0$).

2.3 Photosynthesis Rate

Net photosynthesis rate (P_N) was recorded at the R1 stage using IRGA photosynthesis device (model LCA4 made by ADC BioScientific Ltd. UK). P_N was measured in a 6.25 cm^2 of full-expanded leaves, at 9:30–11:30 a.m. Light intensity, air flow rate, leaf temperature, relative humidity at boundary layer, and atmospheric CO_2 during measurement were 850–950 mol quanta m^{-2} s^{-1} PAR, 0.3 mol m^{-2} s^{-1}, 24–26 °C, 58–62%, and 380–400 ppm, respectively.

2.4 Chlorophyll Contents

According to the Maclachlan and Zalik (1963) method, leaf chlorophyll content was measured at the R1 stage from the

leaves which previously had been used for the measurements of fluorescence and photosynthesis. First, half a gram of fresh leaf was crushed and ground in a Chinese mortar using liquid nitrogen. Then 20 mL of 80% acetone was added to it and centrifuged at 10000 rpm for 10 min. The supernatant was used for spectrophotometry, and the absorbance was read separately at 663 and 645 nm for Chl a and Chl b, respectively. Chlorophyll content was calculated by the following equations.

$$\text{Chl a (mg/gFW)} = [12.3(A663) - 0.86(A645)] \times V/1000 \times W$$
$$\text{Chl b (mg/gFW)} = [19.3(A645) - 3.6(A663)] \times V/1000 \times W$$

where V is the final volume of chlorophyll extracted in 80% acetone, and W is the fresh weight.

2.5 Leaf Analysis for N, P, Fe, and Mo

One day after chlorophyll fluorescence, photosynthesis, and chlorophyll measurements, a number of the leaves which were used for fluorescence and photosynthesis measurement were sampled. First, leaves were washed with distilled water to remove probable dust, then with hydrochloric acid (0.1 M), and finally with distilled water again, and oven-dried at 65 °C for 48 h and then powdered by a mixer.

Leaf dry weight (0.2 g) was used for N measuring by Kjeldahl method according to Bremner (1996) using an auto Kjeldahl distiller instrument (K9840 model, Hanon, China).

Half of a gram of dry weight was used for dry combustion (550 °C, 4h). A 10 mL of 2 M HCl was added to ash and filtered by filter paper. Then its volume increased to 100 mL by distilled water. An atomic absorption spectrometer determined Fe and Mo concentration in the extraction (Agilent 240FS AAS, USA). According to Chapman and Pratt (1961), phosphorus was measured using a UV-VIS spectrophotometer (MPADA CO. China) at 640 nm.

2.6 Grain and Biological Yields

At harvest maturity (22–25 September each year), a square meter including four middle rows of each plot was harvested. At first, their grains (with 13% moisture content) were weighed and considered grain yield. Then, the plant samples were oven (24 h at 75 °C) dried to obtain biological yield.

2.7 Grain Analysis for Oil and Protein

The grains were analyzed using a NIR instrument (DA 7250, Perten Co., Sweden). The oil and protein percent, as well as the primary fatty acids, were measured.

2.8 Data Analysis

MSTAT-C (1991) and Excel software were used for data analysis and drawing charts, respectively. Bartlett's test was used for testing homogeneity of variances, and means comparison was made by Duncan's multiple range test.

3 Results

3.1 Chlorophyll Fluorescence

The results showed that fertilizer treatments significantly affected the chlorophyll fluorescence parameters (Table 4, $p \leq 0.01$). The highest and lowest F_0 and F_m were observed in control (nutritional deficiency) and N-P-Fe-Mo + HA treatment, respectively (Tables 5). The plant has shown higher F_0 and F_m under nutrient deficiency. However, applying all considered nutrients decreased these parameters, which indicated that the nutrition stress was relieved. There were negative and significant correlations between the leaf's N, P, Fe, and Mo content with F_0 and F_m (Table 6). The highest F_v and F_v/F_m was observed in N-Fe-Mo + HA treatment and the lowest in the control treatment (Table 5). F_v/F_m was more sensitive to N availability than the other nutrients (Table 5).

3.2 Photosynthesis Rate (P_N)

The effect of fertilizer treatments on the P_N was significant (Table 4, $p \leq 0.01$). The maximum photosynthesis rate of 9.72 µmol CO_2 m^{-2} s^{-1} was observed in N-P-Fe-Mo + HA while the minimum photosynthesis rate (2.62 µmol CO_2 m^{-2} s^{-1}) was recorded in the control treatment. The photosynthesis rate was most sensitive to nitrogen deficiency (Table 7).

3.3 Chlorophyll Content

Chl a and Chl b contents were significantly affected by fertilizer treatments (Table 4, $p \leq 0.01$). The highest Chl a (11.13 mg g^{-1} FW) and Chl b (4.44 mg g^{-1} FW) contents were recorded in N-P-Fe-Mo + HA treatment, and the lowest ones in control, respectively (Table 7).

3.4 Leaf Nutrients (N, P, Fe, and Mo) Content

Fertilizer treatments significantly affected leaf N, P, Fe, and Mo (Table 4, $p \leq 0.01$). The complete nutrition (N-P-Fe-Mo + HA) treatment induced maximum leaf N, P, Fe, and Mo contents. The lowest amounts of these nutrients were observed in the control treatment (Table 7). The application of each nutrient resulted in increased content of the same

Table 4 Compound analysis of variance (mean of squares) of the effects of fertilizer treatments on soybean's chlorophyll fluorescence parameters, P_N, chlorophylls, leaf's N, P, Fe, and Mo contents, grain yield, biological yield, grain protein, grain oil, and grain fatty acids

Source of variation	df	F_0	F_m	F_v	F_v/F_m	P_N	Chl a	Chl b	Leaf N	Leaf P	Leaf Fe	Leaf Mo	Grain yield	Biological yield	Grain protein	Grain oil	Oleic acid	Linoleic acid	Palmitic acid	Stearic acid
Year (*Y*)	1	351401^{ns}	17973010^{ns}	4190030^{ns}	0.003^{ns}	0.93^{ns}	0.84^{ns}	0.10^{ns}	0.00^{ns}	78.0^{ns}	119.7^{ns}	27.1**	3570.12^{ns}	144632.34 ^{ns}	0.029^{ns}	0.130^{ns}	0.008^{ns}	0.246^{ns}	0.050^{ns}	0.001^{ns}
Error	4	250804	6095912	1660531	0.004	0.55	1.75	0.15	0.03	429.2	384.8	0.30	31802.36	115757.19	3.146	1.323	0.052	1.171	0.117	0.068
Fertilizer (*F*)	11	1390940**	6808727*	5912103**	0.022**	33.79**	11.26**	2.13**	0.75**	16081.6**	216263**	54.6**	358836.86**	1160233.85**	2.509**	1.806**	3.591**	3.232**	0.992**	0.734**
*Y***F*	11	1670^{ns}	3031995^{ns}	458152^{ns}	0.006**	0.36^{ns}	0.103^{ns}	0.05^{ns}	0.01^{ns}	0.37^{ns}	1.64^{ns}	0.44^{ns}	27309.97^{ns}	148021.74^{ns}	0.843^{ns}	0.169^{ns}	0.401^{ns}	1.270^{ns}	0.063^{ns}	0.038^{ns}
Error	44	26486	2653347	548822	0.002	0.41	0.179	0.07	0.01	41.2	6131.92	0.40	25383.02	225859.80	0.719	0.482	0.299	1.095	0.163	0.035
Total	71																			
CV (%)		2.63	5.48	3.11	4.96	9.29	5.38	7.78	3.63	2.22	12.42	5.07	10.95	11.00	2.47	2.91	2.42	2.86	3.40	3.97

*, **, and ns represent significant at 0.05, 0.05 probability levels, and not significant, respectively. Abbreviations: *df*, degrees of freedom; F_0, minimum fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence; F_v/F_m, maximal quantum yield of PSII photochemistry; P_N, net photosynthesis rate; *Chl a*, chlorophyll a; *Chl b*, chlorophyll b; *N*, nitrogen; *P*, phosphorus; *Fe*, iron; *Mo*, molybdenum

element in the leaf. Besides, the application of some of these elements had an indirect effect on the others. For example, Fe deficiency decreased N and Mo content, even under the application of N and Mo fertilizers (Table 7). There was a strong negative correlation (r= −0.806**, Table 6) between P content and F_m.

3.5 Grain and Biological Yields

Fertilizer application improved the grain yield, so the highest (1772 kg ha^{-1}) and lowest (933 kg ha^{-1}) grain yield were observed in N-P-Fe-Mo + HA treatment and control, respectively (Table 7). Although complete fertilizer treatment (N-P-Fe-Mo) increased the grain yield significantly, however, removing iron, phosphorus, and molybdenum from the N-P-Fe-Mo treatment composition did not cause a significant change in the yield. However, grain yield was more sensitive to N, Fe, P, and Mo, respectively. The use of humic acid alone did not significantly affect grain yield, but it improved the effect of nutritional treatments on grain yield (Table 7). Furthermore, humic acid compensated for the deficiency of phosphorus, iron, and molybdenum in treatments that did not have these nutrients, but could not substitute for nitrogen (Table 7).

The results showed that fertilizer treatments significantly increased biological yield (Table 4, p≤0.01). The highest (5157 kg ha^{-1}) and the lowest (3660 kg ha^{-1}) biological yields were observed in N-P-Fe-Mo and control, respectively (Table 7). Among the applied nutrients, Mo deficiency had the minimum effect on biological yield. The HA had no significant effect on biological yield; meanwhile, it was more effective on the grain yield when Mo was removed from the treatment combination (Table 7).

3.6 Grain Protein

Grain protein was significantly affected by fertilizer treatments (Table 4, p≤0.01). Application of N-P-Fe-Mo treatment produced maximum grain protein content. N application was most effective in the improvement of grain protein content. There was no significant effect of P, Fe, Mo, and HA on protein content (Fig. 1).

3.7 Grain Oil and Fatty Acids

The effect of fertilizer treatments on grain oil content was significant (Table 4, p≤0.01). The N-P-Fe-Mo and N-P-Fe-Mo + HA treatments significantly increased grain oil percent. However, HA did not affect oil content when used alone (Fig. 1). The affected fatty acids by fertilizer treatments were linoleic, oleic, palmitic, linolenic, and stearic acids, respectively (Table 4, p≤0.01). The variations in their contents in response to fertilizer treatments are shown in

Table 5 Effects of fertilizer treatments on soybean's chlorophyll fluorescence parameters

Fertilizer treatments	F_0	F_m	F_v	F_v/F_m
Control (no fertilizer)	5227 ± 191a*	25340 ± 1625a	16220 ± 348d	0.64 ± 0.05d
N-P-Fe-Mo	3886 ± 49.9h	19430 ± 1204f	18260 ± 527ab	0.94 ± 0.03a
P-Fe-Mo (N deficiency)	4636 ± 218cd	24390 ± 776ab	16550 ± 1272cd	0.68 ± 0.05d
N-Fe-Mo (P deficiency)	4719 ± 31c	23240 ± 464bc	17970 ± 324abc	0.77± 0.03c
N-P-Mo (Fe deficiency)	4490 ± 217de	22670 ± 567cd	17920 ± 374abc	0.79 ± 0.02c
N-P-Fe (Mo deficiency)	4403 ± 335ef	22320 ± 720cd	18720 ± 235a	0.84 ± 0.04bc
HA	4901 ± 64b	25570 ±1029a	16980 ± 272bcd	0.67 ± 0.02d
N-P-Fe-Mo + HA	3667 ± 82i	19700 ± 1398f	18290 ± 1004ab	0.93 ± 0.05a
P-Fe-Mo (N deficiency) + HA	4420 ±23ef	21830 ± 673cde	17070 ± 1866bcd	0.78 ± 0.08c
N-Fe-Mo (P deficiency) + HA	4283 ± 152f	21260 ± 969de	18950 ± 859a	0.89 ± 0.07ab
N-P-Mo (Fe deficiency) + HA	4015 ± 63gh	20530 ± 805ef	18940 ± 1422a	0.92 ± 0.05a
N-P-Fe (Mo deficiency) + HA	4093 ± 86g	20680 ± 749ef	18620 ± 1799a	0.90 ± 0.08ab

*In each column, means ± standard deviation with at least one common letter do not significantly different according to Duncan's multiple range test ($p<0.05$). Abbreviations: F_0, minimum fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence; F_v/F_m, maximal quantum yield of PSII photochemistry; *N*, nitrogen; *P*, phosphorus; *Fe*, iron; *Mo*, molybdenum; *HA*, humic acid

Fig. 2. Maximum linoleic and oleic acids were in N-P-Fe-Mo and N-P-Fe-Mo + HA treatments. The highest percent of palmitic acid was observed in N-P-Mo treatment. For the linolenic acid, the N-P-Fe-Mo was the best treatment. All fertilizer treatments improved the stearic acid content, but maximum percent was observed in N-P-Fe-Mo and N-P-Fe-Mo + HA treatments (Fig. 2).

4 Discussion

Fluorescence reflects the efficiency of the photosynthetic system. F_0 indicates the fluorescence level that the QA acceptor is at its highest oxidation state (PSII center is open). The lower the F_0, the better the photosynthetic activity is. However, a higher F_0 value indicates damage to the PSII electron transfer chain due to decreased QA capacity and lack of complete oxidation. Under nutrient deficiency, PSII is not working correctly because of the importance role of N, P, Fe, Mo, and HA in plant metabolism. Reaching chlorophyll fluorescence to F_m means the closure (saturation) of all reaction centers. At this situation, a gradual increase in fluorescence and a decrease in the rate of photochemical reactions occur (Maxwell and Johnson 2000). The F_v indicates the reduction situation of QA. Chlorophyll fluorescence is high when the electron acceptors are in a full reduction state, so F_v is high, but when the electron acceptors are oxidized, the fluorescence value is minimal and the F_v value decreases (Zlatev and Yordanov 2004). According to correlation coefficients, our finding indicates that F_0, F_m, and F_v parameters may be useful indicators of the nutrient content in the soybean leaf.

The F_v/F_m is an effective tool to detect damages in the photosynthetic apparatus before these damages are apparent in plant morphology; furthermore, it is a good indicator of detecting photoinhibition (Kalaji et al. 2014). The photochemical efficiency of PSII and the activity of PSII reaction centers decrease, and photoinhibition of PSII occurs due to

Table 6 Pearson correlation coefficients between soybean's chlorophyll fluorescence parameters and soybean leaf's N, P, Fe, and Mo content

	Leaf N	Leaf P	Leaf Fe	Leaf Mo	F_0	F_m	F_v	F_v/F_m
Leaf N	1							
Leaf P	0.552**	1						
Leaf Fe	0.735**	0.465**	1					
Leaf Mo	0.611**	0.721**	0.608**	1				
F_0	−0.776**	−0.806**	−0.653**	−0.712**	1			
F_m	−0.767**	−0.741**	−0.670**	−0.642**	0.869**	1		
F_v	0.558**	0.268*	0.395**	0.218ns	−0.465**	−0.486**	1	
F_v/F_m	0.806**	0.639**	0.645**	0.542**	−0.801**	−0.851**	0.713**	1

*, **, and ns represent significant at 0.05, 0.05 probability levels, and not significant, respectively. Abbreviations: *N*, nitrogen; *P*, phosphorus; *Fe*, iron; *Mo*, molybdenum; F_0, minimum fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence; F_v/F_m, maximal quantum yield of PSII photochemistry

Table 7 Effect of fertilizer treatments on soybean's P_N, chlorophylls, leaf's N, P, Fe, and Mo content, and grain and biological yields

Fertilizer treatments	P_N (μmol CO_2 m^{-2} s^{-1})	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Leaf N (%)	Leaf P (mg kg^{-1})	Leaf Fe (μg g^{-1})	Leaf Mo (μg g^{-1})	Grain yield (kg ha^{-1})	Biological yield (kg ha^{-1})
Control (no fertilizer)	2.62 ± 0.26i*	6.34 ± 0.2g	2.38 ± 0.08g	2.11 ± 0.02g	217.0 ± 3.9j	340.7 ± 8.9d	7.65 ±0.6g	933 ± 127d	3660 ± 155d
N-P-Fe-Mo	9.61 ± 0.39a	9.38 ± 0.74b	4.18 ± 0.12ab	3.09 ± 0.02a	343.6 ±11.2b	774.3 ± 55ab	15.52 ±0.9b	1494 ± 151bc	5157 ± 172a
P-Fe-Mo (N deficiency)	4.65 ± 0.54gh	6.72 ± 0.27fg	2.99 ± 0.27ef	2.32 ± 0.04ef	276.3 ± 3.6f	515.6 ± 5.9c	12.78 ± 1.5d	1482 ± 134bc	3913 ± 260cd
N-Fe-Mo (P deficiency)	5.35 ± 0.53fg	7.04 ± 0.50efg	3.10 ± 0.09ef	2.86 ± 0.04c	231.9 ± 6.4i	665.6 ± 69b	12.81 ± 1.3d	1386 ± 111c	4296 ± 353bcd
N-P-Mo (Fe deficiency)	6.12 ± 0.26ef	7.29 ± 0.65ef	3.33 ± 0.13cde	2.69 ± 0.05d	308.9 ± 4.4e	428.6 ± 8.7cd	11.14 ± 0.5e	1379 ± 148c	4247 ± 565bcd
N-P-Fe (Mo deficiency)	6.77 ± 0.84de	7.26 ± 0.49ef	3.30 ± 0.24de	2.68 ± 0.02d	265.0 ±10.4g	686.0 ± 21.9b	9.77 ± 0.5f	1451 ± 117bc	4271 ± 410bcd
HA	4.00 ± 0.4h	6.47 ± 0.42g	2.68 ± 0.11fg	2.21 ± 0.03fg	227. 8 ± 3.5i	389. 9 ± 4.6cd	8.42 ± 0.8g	1091 ± 85d	3668 ± 344d
N-P-Fe-Mo + HA	9.72 ± 0.26a	11.13 ± 0.47a	4.44 ± 0.14a	3.17 ± 0.07a	371.3 ± 9.1a	894.7 ± 3.6a	17.78 ± 0.7a	1772 ± 146a	4842 ± 268ab
P-Fe-Mo (N deficiency) + HA	7.47 ± 1.05cd	7.68 ± 0.57de	3.43 ± 0.37cde	2.43 ± 0.04e	328.5 ± 3.5cd	729.9 ± 10.3b	15.28 ± 0.8b	1465 ± 81bc	4376 ± 351abcd
N-Fe-Mo (P deficiency) + HA	8.31 ± 0.95bc	8.08 ± 0.61cd	3.72 ± 0.07cd	3.04 ± 0.03ab	247.2 ± 5.5h	860.7 ± 7.5a	13.19 ± 1.1cd	1679 ± 132ab	4375 ± 337abcd
N-P-Mo (Fe deficiency) + HA	9.29 ± 0.19ab	8.31 ± 0.70cd	3.78 ± 0.40bc	2.80 ± 0.11cd	334.2 ± 3.8bc	493.7 ± 2.7c	13.89 ± 0.5c	1619 ± 54abc	4378 ± 298abcd
N-P-Fe (Mo deficiency) + HA	8.84 ± 1.06ab	8.59 ± 0.44c	3.76 ± 0.53bc	2.90 ± 0.02bc	321.5 ± 2.9d	786.2 ± 29.3ab	10.61 ± 0.8ef	1706 ± 116ab	4672 ± 398abc

*In each column, means ± standard deviation with at least one common letter do not significantly different according to Duncan's multiple range test ($p<0.05$). Abbreviations: P_N, net photosynthesis rate; *Chl a*, chlorophyll a; *Chl b*, chlorophyll b; *N*, nitrogen; *P*, phosphorus; *Fe*, iron; *Mo*, molybdenum

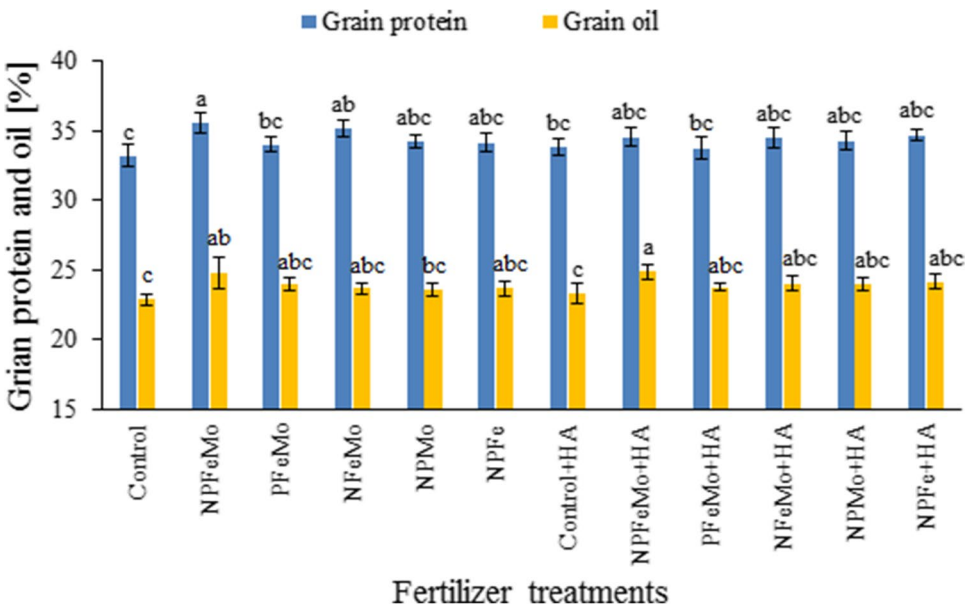

Fig. 1 Effects of fertilizer treatments on grain soybean's protein and oil percent. In each series, means with at least one common letter do not significantly different according to Duncan's multiple range test ($p<0.05$). N, P, Fe, Mo, and HA represent nitrogen, phosphorus, iron, molybdenum, and humic acid fertilizers respectively. Error bars indicate the standard error

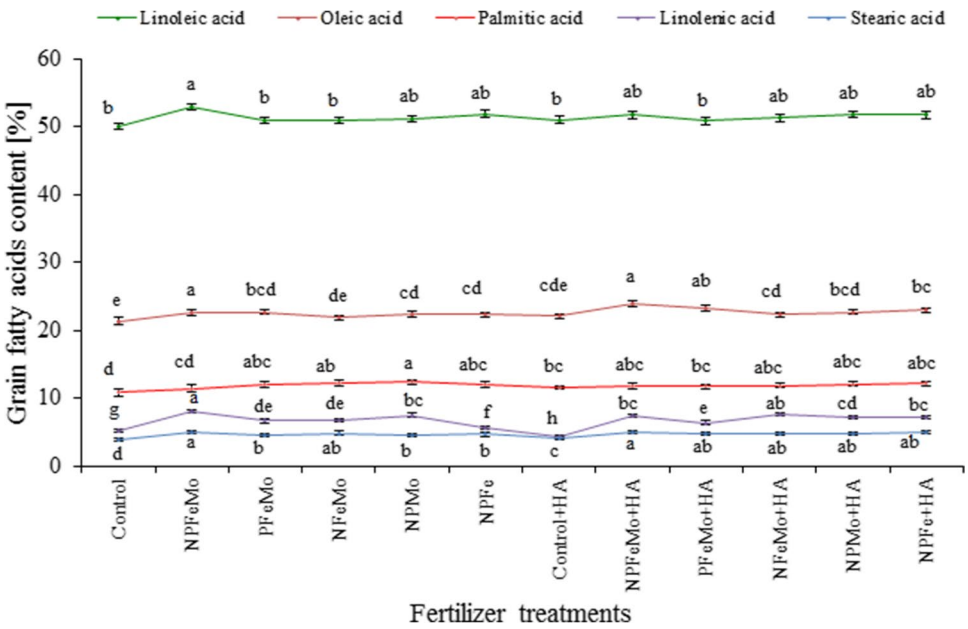

Fig. 2 Effects of fertilizer treatments on percent of five major fatty acids in soybean's grain oil. In each series, means with at least one common letter do not significantly different according to Duncan's multiple range test ($p<0.05$). N, P, Fe, Mo, and HA represent nitrogen, phosphorus, iron, molybdenum, and humic acid fertilizers respectively. Error bars indicate the standard error

nitrogen starvation (Zhao et al. 2017). The decrease in F_v/F_m index can be due to photooxidation and damage to the PSII reaction centers. Kalaji et al. (2018) reported that the F_v/F_m ratio was 0.8 in the non-stress conditions, and values less than 0.8 indicated the existence of biotic and abiotic stresses in plants. Eisvand et al. (2018) reported that using phosphate bio-fertilizer in the soil + foliar application of Zn improved F_v/F_m under late-season heat stress and normal conditions, resulting in increased wheat grain yield. Our findings confirm these results and suggest the F_v/F_m as an indicator of nutritional stress in soybean. However, correlation between this parameter and leaf content of each applied nutrients in the current research is not the same; more related with N and less related with Mo. Any stress can inhibit electron transfer in the PSII, thus reducing photosynthetic efficiency and increasing chlorophyll fluorescence. Nutrient shortages impair the function of the photosynthetic apparatus, increase F_0 and F_m parameters, which result in reduced PSII quantum efficiency (Kalaji et al. 2018). Nitrogen deficiency reduces the PSII quantum yield and maximal efficiency. Nutrient deficiencies such as P, K, Ca, Mg, S, and Fe also impair the function of the photosynthetic apparatus and reduce the PSII efficiency (Kalaji et al. 2017). Also, reduced PSII efficiency due to insufficient N may be related to decreased chlorophyll content. In addition, the chlorophyll molecule contains N, making this element an essential factor in the development

of the photosynthetic apparatus and prevent leaf senescence (Bassi et al. 2018).

The soybean's F_v/F_m has been studied using nitrogen (urea fertilizer) and bacterial inoculation (*Bradyrhizobium japonicum*) treatments under waterlogging conditions. Results showed that in normal conditions, the highest F_v/F_m belonged to the bacterial inoculation treatment; however, nitrogen fertilizer application caused the highest efficiency of PSII under waterlogging stress (Khadempir et al. 2015). Thus, reduction of F_v/F_m under N deficiency may be related to the positive role of N in photosynthesis, which is linked to nitrogen (N) partitioning in photosynthetic enzymes, pigment content, and total number and composition of chloroplasts (Bassi et al. 2018; Marschner 2011; Taiz and Zeiger 2010).

Decreased photosynthesis is mainly due to stomatal (reduced stomatal conductance) and non-stomatal (structure and function of photosystems and Calvin cycle) factors. Our results (Supplementary Table 1) confirm the role of stomatal conductance and sub-stomatal CO_2 on P_N. So, the N-P-Fe-Mo+HA treatment induced the highest P_N as well as the highest stomatal conductance and sub-stomatal CO_2 concentration. The lowest of stomatal conductance and P_N both were observed in the control and HA treatment. N limitation decreases photosynthesis because of reducing stomatal conductance which influences intercellular CO_2 concentration, reducing the content of bioenergetics and light-harvesting protein which inhibits electron transport rate and increases the light energy dissipated as heat, and reducing the content and/or activity of photosynthetic enzymes (Mu and Chen 2021). The nutrient availability in the rhizosphere has remarkable effects on the rate of photosynthesis. It has been reported that humic acid could improve the millet growth by increased net photosynthetic rate and decreased ROS content under drought stress (Shen et al. 2020). This finding is consistent with our results; however, we found a synergistic effect when a combination of HA and chemical fertilizer was applied. Humic substances are detected by receptors located in the cell membrane and increase NO, H_2O_2, and H_2S signaling molecules (Jannin et al. 2012); these molecules initiate pathways which trigger various homeostatic mechanisms in the plant cell which resulted in speed up the photosynthesis rate (Orsi 2014). Furthermore, humic substances upregulate the genes of the Calvin cycle, such as genes coding RUBPCs, G3PdHs, and GPATs, therefore stimulate photosynthetic activity (Shah et al. 2018).

Chlorophyll is the most important pigment in photosynthesis process. Plant nutrition improves chlorophyll biosynthesis. Our results showed an improvement in chlorophyll content under nutrient utilization. Although the use of nitrogen had a more effect on chlorophyll concentration than other elements, using complete nutrition plus humic acid produced the highest amounts of chlorophylls (a and b). Cendrero-Mateo et al. (2015) results reported the positive role of N on chlorophyll concentration. Foliar application of Fe-chelate on soybean under alkaline soil resulted in increased chlorophyll level and mineral accumulation (Santos et al. 2021). Chlorophyll content is balanced by its production in the one hand and by its degradation on the other hand. Stresses can reduce chlorophyll index (SPAD number) through its degradation and finally decrease net photosynthesis (Liu et al. 2018). Nitrogen directly and indirectly affects chlorophyll biosynthesis. Also, other elements are indirectly involved in chlorophyll biosynthesis; P through ATP and phospholipid synthesis, increases magnesium uptake; and Mo via improvement in the plant N content by involving in the nitrogen fixation process (Mo-dependent nitrogenase) and as a cofactor in the enzyme catalyzing key steps of nitrogen, carbon, and sulfur metabolisms, making them essential for efficient growth under the varied environmental conditions (Manuel et al. 2018; Marschner 2011). One of the highlighted roles of Fe in the Chl biosynthesis relates to Chl precursor biosynthesis, where particular emphasis is placed on the involvement of iron in the formation of δ-aminolevulinic acid (ALA), the initial committed step in chlorophyll formation (Pushnik et al. 1984).

The amount of leaf's elements is a function of the plant nutrition and the application of nutrients resulted in increased their content in the leaf. Application of some of these elements had an indirect effect on the other element absorption. Fe deficiency decreased leaf's N and Mo, even under the application of N and Mo. This may be explained by Fe role as a metal enzyme cofactor of the nitrogen reductive assimilatory pathway, such as nitrate reductase, and increased its activity (Borlotti et al. 2012; Marschner 2011).

There was a strong negative correlation between P content and F_m. Therefore, soybean phosphorus status can be monitored by fluorescence chlorophyll. Singh and Reddy (2015) reported that leaf P concentration applies key control over soybean's photosynthetic performances. Because P is involved in the transformation of energy, regulation of several enzymatic activities (Schulze et al. 2006), biosynthesis of nucleic acids, proteins, lipids, sugars, and adenylates (Zhang et al. 2014), its deficiency will affect photosynthesis reaction, which is reflected in Chl fluorescence.

Iron chelate foliar application on soybean increased grain yield (Joorabi et al. 2020). In addition, Caliskan et al. (2008) reported that N and Fe fertilizers had a positive effect on growth parameters and soybean grain yield. This is related to crucial role of these elements in plant physiological process such as phyotosynthesis, respiration, and antioxidant defense system (Taiz and Zeiger 2010; Marschner 2011).

There is a cross-talk between nutrient uptake and metabolism in plants. Therefore, we observe the highest grain yield in complete treatment. In the one hand, this is due to the

positive function of these nutrients that improve the effectiveness of one another, and also because of Liebig's Law of the Minimum. Nitrogen increases yield by developing leaf area, increasing chlorophyll content, biosynthesis of essential enzymes involved in photosynthesis, and preventing leaf aging (Marschner 2011). During reproductive phase, N deficiency induces senescence and N components degradation, especially photosynthetic enzymes and thylakoid N, and thus reduces photosynthesis (Mu and Chen 2021), so will results in yield reduction. Phosphorous improved the grain yield via improved biosynthesis of nucleic acids, proteins, lipids, sugars, and adenylates (Zhang et al. 2014). Iron is a vital constituent of electron chains and a cofactor of many enzymes. It is involved in metabolic processes such as photosynthesis, respiration, DNA synthesis, and nitrogen fixation (Schmidt et al. 2020). Mo is essential for plants as required by several enzymes that catalyze critical reactions in nitrogen assimilation, purine degradation, phytohormone synthesis, and sulfite detoxification. Moreover, a tight connection between molybdenum and iron metabolisms is presumed (Bittner 2014).

Iron chelate increases plant biological function due to its ease of uptake by plants (foliar application) and its essential role in plant physiology (Taiz and Zeiger 2010). Iron deficiency will lead to the yellowing of young leaves and a significant reduction in photosynthetic activity, resulting in reduced biomass production. The biological yield was increased due to the application of these elements. They develop a root system and energy transfer (P roles); promote protein and enzyme synthesis, photosynthesis, and plant growth (N roles), which play a vital role in enzymatic reactions and nitrogen metabolism (Fe roles); and finally improve nitrogen fixation (Mo role) (Marschner 2011).

Deficiencies of nitrogen, phosphorus, iron, and molybdenum significantly limit legume vegetative growth and fertility. Azizi et al. (2017) reported that the biological yield of the *Cicer arietinum* L. was improved by application of 4 mg kg^{-1} molybdenum without using calcium nano-oxide. Lenssen et al. (2019) reported that two times formulated HA foliar application increased soybean yield, but did not affect grain oil and reduced grain protein in one environment. We did not observe a significant effect of HA on yield, which could be due to the low dose and frequency of spraying or time of application (V7, end of vegetative growth); however, our results about effect of HA on grain oil are consistent with report of Lenssen et al. (2019).

In current research, deficiency of each studied elements individually did not affect biological yield; however, their complete combination (N-P-Fe-Mo) with or without HA improved biological yield. This result may be attributed to synergistic effect of these elements as well as Liebig's Law of the Minimum.

Heat stress was not considered as a treatment in this study, but due to the high temperature more than the optimum for soybean during growing seasons, it seems this phenomenon has prevented maximization of photosynthesis, increased chlorophyll fluorescence (Avila et al. 2013; Chovancek et al. 2019), and finally may be an important factor for production a grain yield lower than its global average.

5 Conclusion

Plant nutrition management is essential for enhance soybean grain quality (protein and oil) and quantity. Nitrogen fertilizer noticeably increases grain protein content. Therefore, it seems that biologically fixed nitrogen is not sufficient to supply nitrogen demand for producing high protein content grains. Chlorophyll fluorescence monitoring can be a good indicator of changes in the photosynthetic apparatus before general morpho-physiological symptoms. We find that chlorophyll fluorescence parameters, i.e., F_0 and F_m at the R1 stage of soybean increased due to nutrient deficiency. Meanwhile, fertilizers, especially nitrogen, improved PSII photochemical performance. There was a relationship between the leaf element (i.e., nitrogen, phosphorous, iron, and molybdenum) content with chlorophyll fluorescence parameters, which can be a basis for using these parameters as a valid, non-destructive, and rapid physiological indicator for detecting nutrient deficiencies in soybeans in precision agriculture, leading to an optimum yield. However, this project requires further research on the integration and modeling of fluorescence parameters at multiple growth stages, soil properties, and other plant morpho-physiological characteristics. Also, we suggest it be considered the possibility of using drone or satellite pictures in this field of study.

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Declarations

Conflict of Interest The authors declare no competing interests.

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