

Soybean responses to nutrients deficiency, and the possibility of detecting this deficiency using chlorophyll fluorescence technique

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Research Article

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Abstract

Aims Managing plant nutrition is a key factor to getting optimum yield quantity and quality. Soybean is an important plant as an oil and protein producer crop as well as a biological nitrogen fixing plant. The aims of current work were studying soybean's responses to some macro and micronutrients deficiency stress as well as the possibility of diagnosing this deficiency using chlorophyll fluorescence technique.

Methods The two-year field experiment during 2019 and 2020 growth seasons were conducted based on a randomized complete block design with three replications. Treatments were use and non-use of 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.

Results Results showed that the effect of fertilizer treatments was significant on all traits. N-P-Fe-Mo improved grain yield and photosynthesis rate, but their application accompanied with humic acid induced a synergistic effect, and the maximum grain yield and photosynthesis 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 leaf's N, P, Fe, and Mo content with F_m ; meanwhile, the negative correlation between leaf's nitrogen and F_m was stronger than the other applied nutrients ($r=-0.767$).

Conclusions Research findings show that, it is possible to use the chlorophyll fluorescence technique as a valid non-destructive physiological indicator and a quick way to monitor the nutritional status of soybean plant about N-P-Fe-Mo to timely fertilizing. Although soybean is a nitrogen fixing plant, but it needs complementary N fertilizer to achieve maximum PSII efficiency, minimum chlorophyll fluorescence, and optimal yield.

Introduction

Soybean (*Glycine max* L.) is one of the staple crops throughout the world that plays an important role in public health due to its oil and protein composition (Chen et al. 2005). In developing countries, where the costs for import oils, oilseeds and protein products are high, improving the efficiency of oilseed cultivars 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. Meanwhile, the total world cultivated area of this crop was 120501628 ha with an average production of 2769 kg/ha (FAO 2019).

Chemical fertilizers are typically used through soil or foliar application. Leaching, runoff and evaporation, usually decrease nutrients availability for the plants resulted in its deficiency. Therefore, using new technologies, the plant's need for elements should be met promptly and without wasting and polluting the environment (Ramesh et al. 2010).

Micronutrients have many effects on plant performance. These nutrients are involved in many plant's biochemical reactions while participating in the structure of some organs. Deficiency of these elements can sometimes inhibit the uptake of other nutrients and growth; therefore, it should be paid more attention to their application. Iron and molybdenum increase the soybean quantitative and qualitative yield 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 of it 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 soils, especially in alkaline ones, which can cure the most severe iron nutritional problems in plants. The role of Fe in nitrogen fixation and the activity of some

enzymes such as catalase, peroxidase and cytochrome oxidase has been well established (Askary et al. 2020). Joorabi et al. (2020) reported that nano zinc chelate (ZnO) significantly increased proline content, catalase and peroxidase activities and oil yield of soybean 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 at the 6-leaf stage could increase the mung bean's agronomic and quality indices (Shohlibor Rodgazi et al. 2021).

Photosynthesis, as the main plant metabolism process, strongly is influenced by the environmental conditions. It consists of four stages: light perception, electron transfer, energy fixation, photoassimilate biosynthesis and transfer (Blankenship 2014). It is a major determinant of plant growth and yield; photosynthesis permanence under environmental stresses is important for yield stability. The nutrients increased photosynthesis rate because of improvement of plant growth conditions (Guo et al. 2019). Nutrients deficiency strongly affect the structure and function of the photosynthetic apparatus (Schlau-Cohen and Berry 2015).

Chlorophyll fluorescence uses as a method to study disorders in photosynthetic systems. It is a non-destructive tool for estimating the photochemical efficiency and photosynthetic status of plants and has been widely used to evaluate the plant's response to environmental stresses. It is a reliable method for studying photosynthetic processes under environmental stresses (Jin et al. 2015). Using this chlorophyll fluorescence technique is rapid and non-destructive that represents 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). It has been reported that the application of 0.9 g/L iron nano-oxide at the boot stage reduced F_v/F_m of PSII in wheat under dry-land conditions (Narimani et al. 2018).

Optimal plant nutrition promote achievement of maximum quantitative and qualitative yield. The objectives of this experiment were study of soybean's photosynthesis, chlorophyll fluorescence and grain yield to deficiency of some important nutrients, as well as studying the relationship between leaf's nutrients contents with chlorophyll fluorescence parameters to finding possible detecting nutritional stress using chlorophyll fluorescence technique.

Materials And Methods

Experimental location and design plant material

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

Table 1
Physico-chemical analysis of farm's soil.

The first year											
Soil texture	pH	EC d _{sm} ⁻¹	C (%)	N (%)	P ppm	K ppm	Fe ppm	Mo ppm	Cu ppm	Mn ppm	Zn ppm
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											
Clay loam	7.42	0.57	1.02	0.036	7.3	368	3.9	0.041	0.28	0.23	0.6

Table 2

Temperature, precipitation and relative humidity during the experimental period.

Month	Mean of air temperature [°C]										Average	
	Maximum		Minimum		Average		Precipitation [mm]		Rh [%]		Sunshine (h)	
	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

Each year, the experiment was performed as a randomized complete block design with 12 treatments (Table 3) and 3 replications. The amount of fertilizers was determined based on the results of the soil test (Table 1). Nitrogen 150 kg/ha (75 kg at tilling and 75 kg/ha as top dressing at V2) of urea fertilizer (NPK, 46-0-0) and phosphorus 80 kg/ha of triple superphosphate (46% P₂O₅) were applied before planting and incorporating into the soil by tilling.

Each experimental plot area was 15 m² including five furrows. The distance between the main plots was 3 m and the distance between the blocks was 5 m. The soybean's seeds (*Glycine max* L. CV. Kosar) 58 kg/ha were inoculated by *Bradyrhizobium japonicum* and planed at 4–5 cm soil depth on 28 May each year. The plant density was adjusted at 35 plants per m² (P * P = 5.5 cm and R* R = 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. The weeds control was done manually if needed.

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

Iron chelate from Sequestrene 138 (Fe-EDDHA) with the dose of 0.002, molybdenum chelate (of Khazra Molybdenum chelate 5%) with the dose of 1 g/l and humic acid (15 l/ha) 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.

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

Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured at the R1 stage by a fluorimeter (Pocket PEA, Hansatech Ltd. UK; light intensity 3000 $\mu\text{mol photon m}^{-2}\text{S}^{-1}$) during 10 am to 12 pm. In a way, 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, then these parameters were measured. Air temperature was 32.4–36.8 °C. The leaves were placed in the dark for 20 minutes using a leaf clips (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 parameter F_v/F_m was calculated according to the following equations:

$$F_v/F_m = (F_m - F_0)/F_m$$

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); F_v is the variable fluorescence (dark) ($F_m - F_0$).

Chlorophyll contents

Leaf chlorophyll content was measured at the early R1 stage and from the youngest mature leaves according to the Arnon method (Arnon 1949). 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 minutes. 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 below equations.

$$\text{Chl a (mg/g FW)} = [12.3 (A_{663}) - 0.86 (A_{645})] \times V/1000 \times W$$

$$\text{Chl b (mg/g FW)} = [19.3 (A_{645}) - 3.6 (A_{663})] \times V/1000 \times W$$

Where V: final volume of chlorophyll extracted in 80% acetone; W: fresh weight.

Photosynthesis rate

Photosynthesis rate was recorded at flowering stage (R1) using IRGA photosynthesis device (model LCA4 made by ADC BioSciversity Ltd. UK) at 10 am to 12 pm.

Leaf analysis for N, P, Fe, and Mo

One day after chlorophyll fluorescence measurement, leaves that had been used for fluorescence measurement and some others leaves that were similar to them in position and development stage were sampled. Leaves were washed with distilled water to remove probable dusts, 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. Fe and Mo concentration in the extraction was determined by an atomic absorption spectrometer (Agilent 240FS AAS, USA). Phosphorus was measured according Chapman and Pratt (1961) using a UV-VIS spectrophotometer (MPADA CO. China) at 640 nm.

Grain yield

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 as grain yield. Then, the plant samples were oven (24 hours at 75°C) dried to obtain biological yield.

Data analysis

MSTAT-C software was used for data analysis. Bartlett's test was used for testing homogeneity of variances, and means comparison was done by Duncan's multiple range test.

Results

Chlorophyll fluorescence

The results showed that fertilizer treatments had a significant effect on 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 (Table 5). The plant has shown higher F_0 and F_m under nutrients deficiency. However, the applying of all considered nutrients decreased these parameters, which indicated that the nutrition stress was relieved. There were negative and significant correlations between leaf's N, P, Fe, and Mo content with F_0 and F_m (Table 10). The highest F_v and F_v/F_m were observed in N-Fe-Mo + HA treatment and the lowest ones in control (Table 5). F_v/F_m was more sensitive to N than the other nutrients (Table 5).

Table 4

Compound ANOVA (mean of squares) of the effects of fertilizer treatments on soybean's chlorophyll fluorescence parameters.

SOV	df	F_0	F_m	F_v	F_v/F_m
Year(Y)	1	351401.38 ^{ns}	17973010.12 ^{ns}	4190030.01 ^{ns}	0.003 ^{ns}
Error	4	250804.22	6095912.36	1660531.11	0.004
Fertilizer (F)	11	1390940.16 ^{**}	6808727.43 [*]	5912103.73 ^{**}	0.022 ^{**}
Y*F	11	16707.32 ^{ns}	3031995.88 ^{ns}	458152.83 ^{ns}	0.006 ^{**}
Error	44	26486.72	2653347.10	548822.82	0.002
Total	71				
CV (%)		2.63	5.48	3.11	4.96
*, ** and ns, represent significant at 0.05, 0.05 probability levels and not significant, respectively.					

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 ^{a*}	25340 ^a	16220 ^d	0.6428 ^d
N-P-Fe-Mo	3886 ^h	19430 ^f	18260 ^{ab}	0.9417 ^a
P-Fe-Mo (N deficiency)	4636 ^{cd}	24390 ^{ab}	16550 ^{cd}	0.6790 ^d
N-Fe-Mo (P deficiency)	4719 ^c	23240 ^{bc}	17970 ^{abc}	0.7733 ^c
N-P-Mo (Fe deficiency)	4490 ^{de}	22670 ^{cd}	17920 ^{abc}	0.7912 ^c
N-P-Fe (Mo deficiency)	4403 ^{ef}	22320 ^{cd}	18720 ^a	0.8397 ^{bc}
HA	4901 ^b	25570 ^a	16980 ^{bcd}	0.6648 ^d
N-P-Fe-Mo + HA	3667 ⁱ	19700 ^f	18290 ^{ab}	0.9303 ^a
P-Fe-Mo (N deficiency) + HA	4420 ^{ef}	21830 ^{cde}	17070 ^{bcd}	0.7818 ^c
N-Fe-Mo (P deficiency) + HA	4283 ^f	21260 ^{de}	18950 ^a	0.8933 ^{ab}
N-P-Mo (Fe deficiency) + HA	4015 ^{gh}	20530 ^{ef}	18940 ^a	0.9218 ^a
N-P-Fe (Mo deficiency) + HA	4093 ^g	20680 ^{ef}	18620 ^a	0.9008 ^{ab}
* In each column, means with at least one common letter do not significantly differ according to Duncan's multiple range test (p < 0.05).				

3.2 Photosynthesis rate

Effect of fertilizer treatments was significant (Table 6, $p \leq 0.01$) on photosynthesis rate. The maximum photosynthesis rate ($9.72 \mu\text{mol Co}_2 \text{ m}^{-2} \text{ S}^{-1}$) was observed in N-P-Fe-Mo + HA; however, there was no significant difference between this treatment and N-P-Mo (Fe deficiency) + HA, and N-P-Fe (Mo deficiency) + HA. The minimum photosynthesis rate ($2.62 \mu\text{mol Co}_2 \text{ m}^{-2} \text{ S}^{-1}$) was recorded in control. Photosynthesis rate was most sensitive to nitrogen deficiency (Table 7).

Chlorophylls content

Chl a and Chl b contents were affected by fertilizer treatments significantly (Table 6, $p \leq 0.01$). The highest Chl a (11.13 mg/gm FW) and Chl b (4.44 mg/g 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 affected leaf's N, P, Fe, and Mo meaningfully (Table 6, $p \leq 0.01$). Full nutrition (N-P-Fe-Mo + HA) induced maximum leaf's N, P, Fe, and Mo contents. The lowest contents of these nutrients were in control (Table 7). Application of each nutrient resulted in increased content of the same one in the leaf. Besides, the application of some of these elements had an indirect effect on the others. For example, Fe deficiency resulted in decreased N and Mo, even under application of N and Mo (Table 7). There was a strong negative correlation ($r = -0.806^{**}$, Table 10) between P content and F_m.

Table 6

Compound ANOVA (mean of squares) for the effects of fertilizer treatments on soybean's photosynthesis rate, chlorophylls and N, P, Fe, and Mo contents.

SOV	df	Photosynthesis rate	Chl a	Chl b	N	P	Fe	Mo
Year (Y)	1	0.929 ^{ns}	0.841 ^{ns}	0.097 ^{ns}	0.000 ^{ns}	78.00 ^{ns}	119.68 ^{ns}	27.09 ^{**}
Error	4	0.550	1.755	0.152	0.027	429.23	384.84	0.32
Fertilizer (F)	11	33.790 ^{**}	11.258 ^{**}	2.128 ^{**}	0.748 ^{**}	16081.63 ^{**}	216263.25 ^{**}	54.62 ^{**}
Y*F	11	0.358 ^{ns}	0.103 ^{ns}	0.050 ^{ns}	0.009 ^{ns}	0.37 ^{ns}	1.635 ^{ns}	0.44 ^{ns}
Error	44	0.410	0.179	0.071	0.010	41.18	6131.923	0.39
Total	71							
CV (%)		9.29	5.38	7.78	3.63	2.22	12.42	5.07
*, ** and ns, represent significant at 0.05, 0.05 probability levels and not significant, respectively.								

Table 7

Effect of fertilizer treatments on soybean's photosynthesis rate, chlorophylls, N, P, Fe, and Mo content.

Fertilizer treatments	Photosynthesis rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Chl a ($\text{mg g}^{-1} \text{ Fw}$)	Chl b ($\text{mg g}^{-1} \text{ Fw}$)	N %	P (mg/kg)	Fe ($\mu\text{g/g}$)	Mo ($\mu\text{g/g}$)
Control (no fertilizer)	2.62 ^{i*}	6.34 ^g	2.38 ^g	2.11 ^g	217.0 ^j	340.7 ^d	7.65 ^g
N-P-Fe-Mo	9.61 ^a	9.38 ^b	4.18 ^{ab}	3.09 ^a	343.6 ^b	774.3 ^{ab}	15.52 ^b
P-Fe-Mo (N deficiency)	4.65 ^{gh}	6.72 ^{fg}	2.99 ^{ef}	2.32 ^{ef}	276.3 ^f	515.6 ^c	12.78 ^d
N-Fe-Mo (P deficiency)	5.35 ^{fg}	7.04 ^{efg}	3.10 ^{ef}	2.86 ^c	231.9 ⁱ	665.6 ^b	12.81 ^d
N-P-Mo (Fe deficiency)	6.12 ^{ef}	7.29 ^{ef}	3.33 ^{cde}	2.69 ^d	308.9 ^e	428.6 ^{cd}	11.14 ^e
N-P-Fe (Mo deficiency)	6.77 ^{de}	7.26 ^{ef}	3.30 ^{de}	2.68 ^d	265.0 ^g	686.0 ^b	9.77 ^f
HA	4.00 ^h	6.47 ^g	2.68 ^{fg}	2.21 ^{fg}	227.8 ⁱ	389.9 ^{cd}	8.42 ^g
N-P-Fe-Mo + HA	9.72 ^a	11.13 ^a	4.44 ^a	3.17 ^a	371.3 ^a	894.7 ^a	17.78 ^a
P-Fe-Mo (N deficiency) + HA	7.47 ^{cd}	7.68 ^{de}	3.43 ^{cde}	2.43 ^e	328.5 ^{cd}	729.9 ^b	15.28 ^b
N-Fe-Mo (P deficiency) + HA	8.31 ^{bc}	8.08 ^{cd}	3.72 ^{cd}	3.04 ^{ab}	247.2 ^h	860.7 ^a	13.19 ^{cd}
N-P-Mo (Fe deficiency) + HA	9.29 ^{ab}	8.31 ^{cd}	3.78 ^{bc}	2.80 ^{cd}	334.2 ^{bc}	493.7 ^c	13.89 ^c
N-P-Fe (Mo deficiency) + HA	8.84 ^{ab}	8.59 ^c	3.76 ^{bc}	2.90 ^{bc}	321.5 ^d	786.2 ^{ab}	10.61 ^{ef}
* In each column, means with at least one common letter do not significantly differ according to Duncan's multiple range test ($P < 0.05$).							

Grain yield

Fertilizer application improved the grain yield, so the highest (1772 kg/ha) and lowest (933 kg/ha) grain yield were observed in N-P-Fe-Mo + HA treatment and control, respectively (Table 9). Although complete fertilizer treatment (N-P-Fe-Mo) increased the grain yield significantly, but, the one by one removal of iron, phosphorus and molybdenum from N-P-Fe-Mo treatment composition did not cause a significant change in yield, however, grain yield was more sensitive to N, Fe, P and finally to Mo, respectively. Interestingly, the use of humic acid alone did not have a significant effect on grain yield, but when used with other elements, it improved the effect of those treatments on grain yield (Table 9). Humic acid was able to compensate for the deficiency of phosphorus, iron and molybdenum in treatments that did not have these fertilizers, but could not be a substitute for nitrogen (Table 9).

Biological yield

The results showed that fertilizer treatments had a significant effect on biological yield (Table 8, $p \leq 0.01$). The highest (5157 kg/ha) and the lowest (3660 kg/ha) biological yield was observed in N-P-Fe-Mo and control, respectively (Table 9). Among the applied nutrients, Mo deficiency had a 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 9).

Table 8

Compound ANOVA (mean of squares) for the effects of fertilizer treatments on soybean's grain and biological yields.

SOV	Df	Grain yield	Biological yield
Year (Y)	1	3570.12 ^{ns}	144632.34 ^{ns}
Error	4	31802.36	115757.19
Fertilizer treatments (F)	11	358836.86 ^{**}	1160233.85 ^{**}
Y*F	11	27309.97 ^{ns}	148021.74 ^{ns}
Error	44	25383.02	225859.80
Total	71		
CV (%)		10.95	11.00
*, ** and ns, represent significant at 0.05, 0.05 probability levels and not significant, respectively.			

Table 9

Effect of fertilizer treatments on soybean's grain and biological yields.

Fertilizer treatments	Grain yield (kg/ha)	Biological yield (kg/ha)
Control (no fertilizer)	933 ^d	3660 ^d
NPF ₂ Mo	1494 ^{bc}	5157 ^a
PF ₂ Mo (N deficiency)	1482 ^{bc}	3913 ^{cd}
NFeMo (P deficiency)	1386 ^c	4296 ^{bcd}
NPMo (Fe deficiency)	1379 ^c	4247 ^{bcd}
NPF ₂ (Mo deficiency)	1451 ^{bc}	4271 ^{bcd}
HA	1091 ^d	3668 ^d
NPF ₂ Mo + HA	1772 ^a	4842 ^{ab}
PF ₂ Mo (N deficiency) + HA	1465 ^{bc}	4376 ^{abcd}
NFeMo (P deficiency) + HA	1679 ^{ab}	4375 ^{abcd}
NPMo (Fe deficiency) + HA	1619 ^{abc}	4378 ^{abcd}
NPF ₂ (Mo deficiency) + HA	1706 ^{ab}	4672 ^{abc}

* In each column, means with at least one common letter do not significantly different according to Duncan's multiple range test (p < 0.05).

Table 10

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

	Nitrogen	Phosphor	Fe	Mo	F0	Fm	Fv	Fv/Fm
Nitrogen	1							
Phosphor	0.552 ^{**}	1						
Fe	0.735 ^{**}	0.465 ^{**}	1					
Mo	0.611 ^{**}	0.721 ^{**}	0.608 ^{**}	1				
F0	-0.776 ^{**}	-0.806 ^{**}	-0.653 ^{**}	-0.712 ^{**}	1			
Fm	-0.767 ^{**}	-0.741 ^{**}	-0.670 ^{**}	-0.642 ^{**}	0.869 ^{**}	1		
Fv	0.558 ^{**}	0.268 [*]	0.395 ^{**}	0.218 ^{ns}	-0.465 ^{**}	-0.486 ^{**}	1	
Fv/Fm	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.

Discussion

F_0 indicates the fluorescence level that the Quinone A (QA) acceptor is at its highest oxidation state (PSII center is open). The lower the F_0 , the better the photosynthetic activity. However, a higher F_0 value indicates damage to the PSII electron transfer chain due to a decrease in QA capacity and lack of its complete oxidation. Therefore, under nutritional stress conditions (C), PSII is not working properly. Reaching chlorophyll fluorescence to F_m is caused by the photons absorption and the reduction of all electron carriers, and the closure (saturation) of all reaction centers. When all PSII reaction centers are closed, 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 the electron acceptor (QA). Chlorophyll fluorescence is high when the electron acceptors are in 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).

The F_v/F_m is an effective tool to detect damages in the photosynthetic apparatus before these damages have appeared in plant morphology; furthermore, it is a good indicator of detecting photoinhibition (Kalaji et al. 2014). It has been reported that the photochemical efficiency of PSII and the activity of PSII reaction centers decreased, and photoinhibition of PSII occurred due to nitrogen starvation (Zhao et al. 2017).

The decrease in this index can be due to photooxidation and damage to the PSII reaction centers. The researchers 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 (Kalaji et al. 2018). Eivsand 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 resulted in increased wheat grain yield.

Any stress can inhibit electron transfer in the PSII, thus reducing photosynthetic efficiency and increasing chlorophyll fluorescence. Nutrient deficiencies impair the function of the photosynthetic apparatus. This deficiency causes some damages to the photosynthetic apparatus by reducing the PSII quantum efficiency. Lack of nutrients causes stress and increases F_0 and F_m parameters, which results in reduced PSII quantum efficiency (Kalaji et al. 2018). Nitrogen deficiency reduces the PSII quantum yield and maximal efficiency. Nutrient limitations 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 the decreases in chlorophyll content (Table 7). In addition, the chlorophyll molecule contains N, making this element an important 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 has belonged to the bacterial inoculation treatment; however, nitrogen fertilizer application caused the highest PSII under waterlogging stress (Khadempir et al. 2015). 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 Kelvin cycle) factors. Restriction of photosynthesis will increase chlorophyll fluorescence (Taiz and Zeiger 2010).

The nutrients amount in the rhizosphere can have special effects on the rate of photosynthesis. It has been reported that the humic acid in both foliar and soil applications could improve the photosynthetic indices of sunflower (Heidari et al. 2020). Also, Zaremanesh et al. (2019) showed that the use of humic acid as a soil application in a pot experiment increased the photosynthesis rate in *Satureja Khuzestanica* under control and salinity stress. This finding is consistent with our results; however, we were finding a synergistic effect when HA and chemical fertilizer were applied together (Table 7).

Higher concentrations of chlorophylls were observed under nitrogen fertilizer treatment. This result is consistent with the findings of the other study (Cendrero-Mateo et al. 2015). However, other applied elements did not have a significant effect on chlorophylls content, and the use of humic acid alone was not significantly different from the control (Table 7). Nevertheless, iron and manganese foliar application on mung bean under dehydration resulted in increased chlorophyll and carotenoids (Izadi and Modares Sanavey 2018) .

Stresses can reduce chlorophyll index (SPAD number) through its degradation and finally decrease net photosynthesis (Liu et al. 2018). Our results showed an improvement in chlorophyll content under nutrients utilization (Table 7). Although the use of nitrogen had a greater effect on chlorophyll concentration than other elements, using all of them plus humic acid (N-P-Fe-Mo + HA) produced the highest amounts of chlorophylls (a and b). In addition to nitrogen, which directly and indirectly affects chlorophyll biosynthesis, other elements are also indirectly involved in its synthesis; P through ATP and phospholipid synthesis, also increasing magnesium uptake; and Mo via improvement the plant's N content by involving in the nitrogen fixation process (Marschner 2011). One of the highlighted roles of Fe in the biosynthesis of chlorophyll relates to Chl precursors biosynthesis, where special 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).

Application of each nutrient resulted in increased content of the same one in the leaf. Besides, the application of some of these elements had an indirect effect on the others. For example, Fe deficiency resulted in decreased N and Mo, even under application of N and Mo (Table 7). This may be related to Fe's role as an enzyme metal cofactor of the nitrogen's reductive assimilatory pathway such as nitrate reductase and increased its activity (Borlotti et al. 2012; Marschner 2011).

There was a strong negative correlation ($r = -0.806^{**}$, Table 10) between P content and F_m . Therefore, soybean phosphorus status can be monitored by fluorescence chlorophyll. 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 and is reflected in Chl fluorescence.

Iron chelate foliar application on soy increased grain yield (Joorabi et al. 2020). Caliskan et al. (2008) reported that nitrogen and Fe fertilizers had a positive effect on growth parameters and soybean's grain yield.

Mo chelate foliar application at the 6-leaf stage improved the yield and its components in mung bean. Also, it was observe that the nitrogen application reduced the loss of flowers and pods and increased the number of seeds per plant resulted in increased yield (Shohlibor Rodgazi et al. 2021), which is consistent with the results of other researchers.

There is a cross-talk between nutrients uptake and metabolism in plants. Therefore, we observe the highest grain yield in full treatment. This is due to the positive function of these nutrients that improve the effectiveness of each other. Nitrogen increases yield by developing leaf area, increasing chlorophyll content, biosynthesis of important enzymes involved in photosynthesis, and preventing leaf ageing (Marschner 2011). 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 some metabolism processes such as photosynthesis, respiration, DNA synthesis, and also nitrogen fixation (Schmidt et al. 2020). Mo is essential for plants as required by several enzymes that catalyze key reactions in nitrogen assimilation, purine degradation, phytohormone synthesis, and sulphite 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 important roles 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 (Marschner 2011).

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), 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 the vegetative growth and fertility of legumes. Azizi et al. (2017) reported that the biological yield of the *Cicer arietinum* L. was improved by applying 4 mg/kg molybdenum without using calcium nano-oxide.

In a water limitation regime, spraying 300 mg/l humic acid in periods improved physiological parameters and increased biological yield in wheat (Tourfi and Shokuhfar 2019). However, we did not observe a significant effect of molybdenum on biological yield, which could be due to low dose and frequency of spraying or time of application (V7, end of vegetative growth).

Conclusion

Chlorophyll fluorescence is an estimate of electron transport in photosynthesis, and reflects photosynthesis efficiency. Its monitoring can be a good indicator of changes in the photosynthetic apparatus. Plants show different photosynthetic characteristics under different environmental conditions such as nutrients availability and biotic and abiotic stresses. These stresses can be traced using chlorophyll fluorescence technique even before the onset of their general morphophysiological symptoms. In the current research, fertilizer treatment had a significant effect on all studied traits. Measurement of chlorophyll fluorescence parameters before flowering at the beginning of reproductive phase (R1) showed that F_0 and F_m parameters increase when fertilizers are not applied, and PSII photochemical performance is improved by fertilizers application, especially nitrogen.

There was a significant relationship between the content of these elements (N-P-Fe-Mo) in the leaves 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 resulting in optimum yield. Of course, this work requires further research on the integration and modelling of fluorescence parameters along with growth stages, soil properties and other plant appearance characteristics.

Abbreviations

Chl – chlorophyll; F_v/F_m – maximal quantum yield of PSII photochemistry; HA – humic acid; P – phosphorus; PS – photosystem; ROS – reactive oxygen species.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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