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Effect of iron, zinc and manganese shortage-induced change on photosynthetic pigments, some osmoregulators and chlorophyll fluorescence parameters in lettuce

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Abstract

Although the beneficial role of Fe, Zn, and Mn on many physiological and biochemical processes is well established, effects of each of these elements on chlorophyll (Chl) *a* fluorescence and photosynthetic pigment contents is not well studied. The objective of this study was to evaluate effects of Fe, Zn, and Mn deficiency in two lettuce cultivars. The parameters investigated could serve also as physiological and biochemical markers in order to identify stress-tolerant cultivars. Our results indicated that microelement shortage significantly decreased contents of photosynthetic pigments in both lettuce cultivars. Chl *a* fluorescence parameters including maximal quantum yield of PSII photochemistry and performance index decreased under micronutrient deficiency, while relative variable fluorescence at J-step and minimal fluorescence yield of the dark-adapted state increased under such conditions in both cultivars. Micronutrient deficiency also reduced all parameters of quantum yield and specific energy fluxes excluding quantum yield of energy dissipation, quantum yield of reduction of end electron acceptors at the PSI, and total performance index for the photochemical activity. Osmoregulators, such as proline, soluble sugar, and total phenols were enhanced in plants grown under micronutrient deficiency. Fe, Zn, and Mn deficiency led to a lesser production of dry mass. The Fe deficiency was more destructive than that of Zn and Mn on the efficiency of PSII in both lettuce cultivars. Our results suggest that the leaf lettuce, which showed a higher efficiency of PSII, electron transport, quantum yield, specific energy fluxes, and osmoregulators under micronutrient deficiency, was more tolerant to stress conditions than crisphead lettuce.

Additional key words: chlorophyll fluorescence transients, micronutrient, *Lactuca sativa*, quantum yield.

Introduction

Lettuce is one of the important plants for human health because of various vitamins and essential ingredients. Lettuce also is important to facilitate intestinal peristalsis because of high amount of cellulose and fiber (Xue *et al.* 2001). Due to its high contents of folate and minerals, lettuce is beneficial for human body. Potassium is another important mineral of lettuce, which helps controlling heart rate and blood pressure, while manganese has also a wide range of functions in our body, especially as a component of metalloenzymes (Xue *et al*. 2001).

Due to different environmental stresses, planting lettuce in hydroponic systems has expanded widely all over the world. One of the major problems in hydroponic systems is the nutrient management (Petrazzini *et al.* 2014). Although iron, manganese, zinc, and other micro-elements are essential for plant physiological reactions, their role in electron transport chain is less understood. Fe is a part of cytochrome, which plays an important role

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Abbreviations: Area – area above the OJIP curve, it expresses the size of the reduced PQ pool; Chl – chlorophyll; DM - dry mass; ET_0/RC – electron transport flux per RC; FeD – iron deficiency; FM – fresh mass; F_m – maximal fluorescence of the dark-adapted state; F_o – minimal fluorescence yield of the dark-adapted state; F_v – maximal variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; MnD – manganese deficiency; PI – performance index; PI_{abs} – performance index for the photochemical activity; PI_{tot} – total performance index for the photochemical activity; RC – reaction center; SM – normalized area related to the number of electron carriers per electron transport chain; TChl – total chlorophyll; TR_0/RC – trapped energy flux per RC; V_i – relative variable fluorescence at time 30 ms I-step after start of actinic light pulse; V_j – relative variable fluorescence at J-step; ZIP – zinc transporters proteins; Φ_{do} – quantum yield of energy dissipation; φET_{20} – quantum yield of electron transport from Q_A to Q_B in PSII; Φ_{po} – maximum quantum yield of primary PSII photochemistry; Φ_{re10} – quantum yield of reduction of end electron acceptors at the PSI; Ψ_o – trapped exiton moves an electron in to the electron transport chain beyond Q_A; ZnD – zinc deficiency.

in electron transport chain. Likewise, ferredoxin contains Fe and participates in electron transport, oxidation, and reduction reactions (Barker and Pilbeam 2015). Zinc is another essential element in plants and animals, is a part of enzymatic redox reactions, and is essential for energy transport, protein synthesis, protection of protein structure and cellular membrane structures (Aravind and Prasad 2004). Manganese also is one of the microelements that enables water splitting in PSII.

In hydroponic systems, micronutrient-deficiency symptoms appear due to changes in pH and temperature, therefore recognizing these signs could help to a better feeding management (Bityutskii *et al.* 2014). Microelements shortage disarranges photosynthetic systems and reduces amount of Chl. Meanwhile, lack of Fe, Zn, and Mn leads to decomposition of D1 protein in the reaction center of PSII (Bertamini *et al.* 2001). Fe deficiency (FeD) causes reduction in chloroplast proteins, concentration of Chl, and photochemical efficiency (Morales *et al.* 1991). This reduction in Chl and protein content changes ultrastructure of the thylakoid membrane (Terry and Abadía 1986). Timperio *et al*. (2007) reported that FeD significantly reduced amount of PSI proteins, while PSII proteins were less affected by this stress. Zn deficiency (ZnD) reduced plant growth by decreasing photosynthetic rate (Henriques 2001), disorganization of chloroplast thylakoids, degeneration of chloroplast membrane, and reducing photochemical efficiency of PSII (Donnini *et al.* 2013). Nutrient deficiency increases electron excitation energy and reduces number of carried electrons by electron transport chain (Evans and Terashima 1987). On the other hand, Mn deficiency (MnD) causes significant reduction in transported electrons between two photosystems. MnD can reduce Mg^{2+}-ATPase and Ca^{2+}-ATPase activities, and rate of photophosphorylation in thylakoid membranes of maize chloroplast (Qu *et al.* 2012).

Recently, Chl fluorescence analysis has become one of the most quick, reliable, powerful and simple methods to collect information about PSII and electron transport (Kalaji *et al.* 2014, Osório *et al.* 2014). The OJIP-test is common for evaluating the response of plant species under stress conditions (Strasser *et al.* 2000, Percival and Henderson 2003). Chl fluorescence, produced by excited Chl molecules, is a nondestructive biomarker for evaluating effects of stress on PSII (Strasser *et al.* 2000, Kalaji and Loboda 2007, Tuba *et al.* 2010, Oukarroum *et al.* 2015). PSII sensitivity is considered as a basic parameter to estimate plant response under micronutrient changes. Donnini *et al.* (2013) reported that FeD decreased maximal variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) ratio in cucumber. Increasing Chl fluorescence under micronutrient deficiency was previously reported in sunflower (Ciompi *et al.* 1996), wheat (Shangguan *et al.* 2000), maize, and tomato (Chaves *et al.* 2003).

Osmoregulation, is an important plant mechanism how to reduce negative effects of stresses. Osmoregulation leads to accumulating low-molecular-mass and nontoxic organic compounds, such as osmoregulators (Chaves *et al.* 2003). Biosynthesis and accumulation of osmoregulators in vacuole and cytosol, *e.g.,* soluble sugars, proline, phenolic compounds, and others compounds, may be involved in membrane stability, especially, of thylakoid membrane (Fu *et al.* 2015). Therefore osmotic adjustments could increase electron transport as a result of increasing membrane stability. Cultivars with the ability to produce better osmotic adjustments have more efficient photosynthesis under stress conditions. Sperotto *et al.* (2007) reported an increasing rate of soluble sugar accumulation in plants grown under FeD. In rice plants, ZnD induced accumulation of carbohydrates and starch molecules in roots and shoots (Suzuki *et al.* 2012).

Since, crisphead (*Lactuca sativa* var. *capitata*) and leaf lettuce (*Lactuca sativa* var. *crispum*) are the two most common lettuce cultivars planted in Iran greenhouses and micronutrient deficiency reduces the formation and growth of lettuce head, the objectives of our work were: *a*) to evaluate effects of Fe, Zn, and Mn on lettuce photosynthesis by analyzing Chl fluorescence parameters; *b*) to investigate effects of Fe, Zn, and Mn deficiency on photosynthetic pigment contents, proline, phenolic compounds, and soluble sugar concentration in lettuce plants; *c*) to compare micronutrient-stress tolerance of two commercial lettuce cultivar utilizing Chl fluorescence parameters as physiological and biochemical markers.

Materials and methods

Plant material and growth conditions: This experiment was conducted during the 2015 growing season at the experimental greenhouse of Vali-e-Asr University. The experiment was arranged as a factorial in the framework of completely randomized design with three replications. Two lettuce cultivars, including crisphead (*Lactuca sativa* var. *capitata*) and leaf lettuce (*Lactuca sativa* var. *crispum*), were treated by micronutrient deficiency, including control, FeD, ZnD, and MnD. Seeds were planted in trays containing perlite. After fifteen days, at four-leaf stage, seedlings were transferred to a 5-L buckets (upper diameter of 30 cm, lower diameter of 20 cm, and 25 cm high) containing a nutrient solution. Seedlings were grown under greenhouse conditions with relative humidity of 50 ± 10%, temperature of 25/15 ± 2ºC (day/night), and a 13/11 h photoperiod. During this period, seedlings were nourished with nutrient solutions containing 5 mM $Ca(NO_3)_2.4H_2O$, 0.2 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.3 mM $MgSO_4.7H_2O$, and 0.1 mM NaCl. Microelements were 7 μM $MnSO_4.H_2O$, 0.7 μM $ZnSO_4$, 0.8 μM $CuSO_4.5H_2O$, 2 μM H_3BO_3, 0.8 μM $Na_2MoO_4.2H_2O$, and 20 μM of Fe-ethylenediamine di-2-hydroxyphenyl acetate

(EDDHA) under control conditions (Roosta and Schjoerring 2007). The nutrient solution (pH 6.5 ± 0.1) was renewed every 3 d. Twenty days after transferring seedlings into pots, the composition of nutrient solution changed in each experimental plot, according to the treatment plan. In this step, Fe, Zn, and Mn were omitted from nutrient solution in each treatment for threee weeks. The solution in every pot was changed weekly and pH was checked daily. In this experiment, all parameters were measured after nutrient deficiency for three weeks.

Dry mass: At the end of experiment, the plants were harvested from each pot and divided to shoots and roots. Fresh mass (FM) was measured immediately after harvest and dry mass (DM) was obtained when samples were dried in oven for 72 h at 70ºC.

Total Chl and carotenoids (Car): Chl pigments (Chl *a*, Chl *b*, and TChl) and Car contents were measured at the end of the experiment according to Lichtenthaler (1987). Middle part of the youngest fully expanded leaf was collected and wrapped in aluminum foil to avoid degradation of pigments by light. One gram of fresh leaves was ground with 10 ml of 80% aqueous acetone in mortar and pestle. After filtering, absorbance of the centrifuged extracts was measured at 480, 510, 645, 652, and 663 nm using a spectrophotometer (*U-2000*, *Hitachi Instruments*, Tokyo, Japan).

Proline content: To determine the free-proline concentration, the youngest fully expanded leaf of sample plants (0.5 g) were homogenized with 5 ml of 95% ethanol. The insoluble fraction of the extract was washed with 5 ml of 70% ethanol. Extracts were centrifuged at 3,500 rpm for 10 min and the supernatant was preserved at 4ºC for the proline determination. An aliquot of this supernatant was taken, reactive ninhydrin acid reagent (ninhydrin, 6 M phosphoric acid and glacial acetic acid at 99%) was added, and then placed in a bath at 100ºC. After 45 min, samples were cooled and absorbance was determined at 520 nm using a spectrophotometer (*U-2000*, *Hitachi Instruments*, Tokyo, Japan). The proline concentration was calculated according to a standard curve and expressed as μg g^{-1}(FM).

Soluble sugar content: The soluble sugars were extracted by adding 10 ml ethanol (alcoholic extracts, the same as for proline) and mixed with antron (200 mg of antron + 100 ml of 72% sulphuric acid). Tubes were heated in boiling water bath for 10 min. Then samples were cooled and absorbance was measured at 625 nm by the above spectrophotometer. The concentration of soluble sugars was calculated using the standard curve and the results were expressed as mg g^{-1}(FM).

Elemental analysis: Leaf and root contents of Fe, Zn, and Mn were quantified as described previously by Roosta and Mohsenian (2012). Leaf and root samples were ground and dry-ashed at 550ºC for 4 h. The ashes were dissolved with 5 ml of 2N HCl and made up to 50 ml with distillated water. The concentrations of Fe, Zn, and Mn were measured by atomic absorption spectrometry (*Version 1/33 GBC Avanta*, Australia). The results were expressed as mg kg^{-1}(DM).

Phenolic compounds: The extraction was carried out according to Isfendiyaroglu and Ozeker (2002). Fresh plant material was mixed with 95% ethanol (5 ml) and kept in dark condition for 48 h. Then 1 ml of ethanol was added to 1 ml of the supernatant and distilled water added to make a total volume of 5 ml. Folin reagent (0.5 ml of 50%) and 1 ml of 5% calcium carbonate were added to samples which changed their color to black. In this step, samples were placed in dark place for 1 h and the phenolic compound content was measured at 725 nm by the above spectrophotometer. Phenolic compound concentration was calculated according to a standard curve and expressed as μg g^{-1}(FM).

Chl fluorescence parameters were measured and calculated at the end of nutrient-deficiency period, 41 d after planting, by portable photosynthetic efficiency analyzer (*PEA*, *Hansatech Inc. Co*., UK). The parameters included variable fluorescence (F_v), maximal fluorescence of the dark-adapted state (F_m), minimal fluorescence yield of the dark-adapted state (F_o), F_v/F_m, Area (area above the OJIP curve, which expresses the size of the reduced PQ pool), relative variable fluorescence at time 30 ms at the I-step after start of actinic light pulse (V_i), relative variable fluorescence at the J-step (V_j), F_v/F_o, the relative number of electron carriers per electron transport chain (S_m), and performance index (PI). Fully expanded leaves were collected from each pot and adapted to a dark period for 15 min by fixing special tags on each upper leaf blade before taking measurements. After 15 min of dark adaptation, the sensor cup was fitted on the leaf for measurement. The Chl fluorescence transients were induced by a red light up to 3,500 μmol(photon) m^{-2} s^{-1} and recorded from 10 μs up to 1 s with a peak wavelength of 627 nm. The fluorescence transients were analyzed according to the equations of the JIP-test (Strasser *et al.* 2000).

Statistical analysis: All data were analyzed *via SAS* software, *SAS Institute*, Cary, NC, USA. When analysis of variance (*ANOVA*) showed significant treatment effects, the least significant differences (LSD) test was applied to compare means at $P<0.05$. The biophysical parameters were calculated with the help of the ''*PEA Plus*'' software package, *version 1.02*.

Results

Ion concentrations: As we expected, micronutrient deficiency, caused by Fe, Zn, and Mn had significant effect on Fe, Zn, and Mn contents (Table 1S, *supplement available online*). The Fe concentration was severely reduced in the plants grown under FeD and MnD conditions and the highest Fe concentration was observed in the plants grown under ZnD (Table 1). FeD and MnD treatments significantly increased Zn concentration, while ZnD treatment decreased Zn concentration significantly. The highest Zn concentration were observed in crisphead under FeD condition and the lowest Zn concentrations were observed in leaf lettuce under ZnD condition. Under MnD, Mn concentration in leaves of leaf lettuce and crisphead cultivar decreased by 26.7% and 18.4%, respectively. By contrast, after FeD and ZnD treatments, the Mn concentration in leaves was stable in both cultivars (Table 1).

Osmotic regulating compounds: Proline and total phenolic components were significantly affected by micronutrient deficiency, cultivar, and their interaction. The soluble sugar content was not significantly affected by interaction of micronutreint deficiency and cultivar (Table 1S). Proline and total phenol components in lettuce leaves increased by micronutrient deficiency. The highest proline and total phenol contents in leaves were observed in leaf lettuce under FeD and the lowest proline and lowest total phenol contents were observed in crisphead lettuce under control conditions (Table 1). In both cultivars, soluble sugar contents were enhanced by increasing micronutrient deficiency.

Pigment content: In both cultivars, reduction of Fe, Zn and Mn caused a decrease in photosynthetic pigment concentrations. Chl *a* and TChl significantly decreased, compared with control, under micronutrient deficiency in both cultivars. Maximum reduction of Chl *a* and TChl occurred in crisphead lettuce, by 35 and 28% under FeD, respectively. Zn and Mn shortage showed a lesser impact on the amount of Chl *a* and TChl than that of FeD (Fig. 1). Regardless of lettuce cultivars, omitting micronutrients from the nutrient solution significantly decreased the

Table 1. Effect of micronutrient deficiency on ion concentration (Fe^{2+}, Zn^{2+}, Mn^{2+}) in leaves of lettuce cultivars. Values are means ± SE of three replicates. *Different letters* in each column show significant differences at $P \leq 0.05$ (LSD). DM – dry mass; FM – fresh mass.

Micronutrient deficiency	Cultivar	Fe [mg kg^{-1}(DM)]	Zn [mg kg^{-1}(DM)]	Mn [mg kg^{-1}(DM)]	Proline [µg g^{-1}(FM)]	Soluble sugar [µg g^{-1}(FM)]	Total phenol [µg g^{-1}(FM)]
Control	Leaf lettuce	71.6 ± 1.8^{b}	28.3 ± 0.8^{d}	51.6 ± 1.3^{a}	82 ± 1.5^{d}	2.50 ± 0.05^{a}	83.0 ± 1^{f}
	Crisphead	66.3 ± 0.8^{c}	32.2 ± 0.8^{c}	52.3 ± 1.3^{a}	81.6 ± 2.8^{d}	2.00 ± 0.05^{a}	72.6 ± 1.2^{g}
Fe (-)	Leaf lettuce	56.7 ± 1.5^{e}	38.0 ± 1.6^{b}	52 ± 1.6^{a}	99 ± 3.6^{a}	3.53 ± 0.10^{a}	121.7 ± 0.8^{a}
	Crisphead	47.0 ± 2.6^{f}	41.0 ± 0.6^{a}	58 ± 1.6^{a}	94 ± 4.6^{b}	3.40 ± 0.05^{a}	115.7 ± 0.9^{bc}
Zn (-)	Leaf lettuce	80.0 ± 4.8^{a}	21.6 ± 0.8^{f}	50.6 ± 1.8^{a}	93 ± 2.5^{c}	3.50 ± 0.03^{a}	116.6 ± 0.9^{b}
	Crisphead	72.0 ± 1.6^{b}	25.0 ± 2.6^{e}	52.6 ± 1.3^{a}	96 ± 5.5^{b}	3.13 ± 0.03^{a}	113 ± 0.5^{c}
Mn (-)	Leaf lettuce	68.3 ± 2.3^{c}	33.0 ± 0.6^{c}	36.3 ± 3.3^{c}	91 ± 3.6^{c}	2.86 ± 0.03^{a}	103 ± 0.8^{d}
	Crisphead	62.3 ± 3.1^{d}	32.0 ± 3.3^{c}	42.7 ± 2.5^{b}	92 ± 2.1^{c}	2.60 ± 0.10^{a}	97.3 ± 0.1^{e}

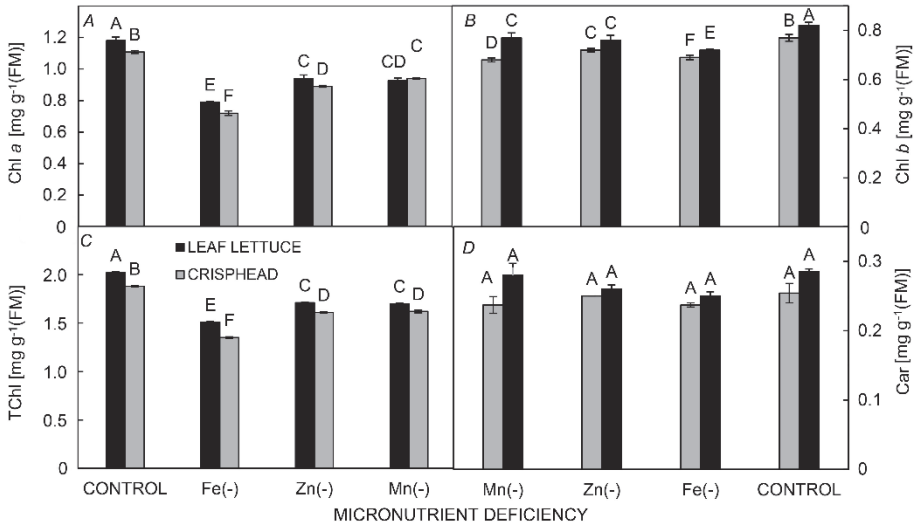

Fig. 1. Effect micronutrition deficiency on chlorophyll (Chl) *a* (*A*), Chl *b* (*B*), TChl (*C*), and carotenoids (Car) (*D*) of two lettuce cultivars: leaf and crisphead lettuce. Bars indicate standard error. Columns with *different letters* are significantly different at $P \geq 0.05$.

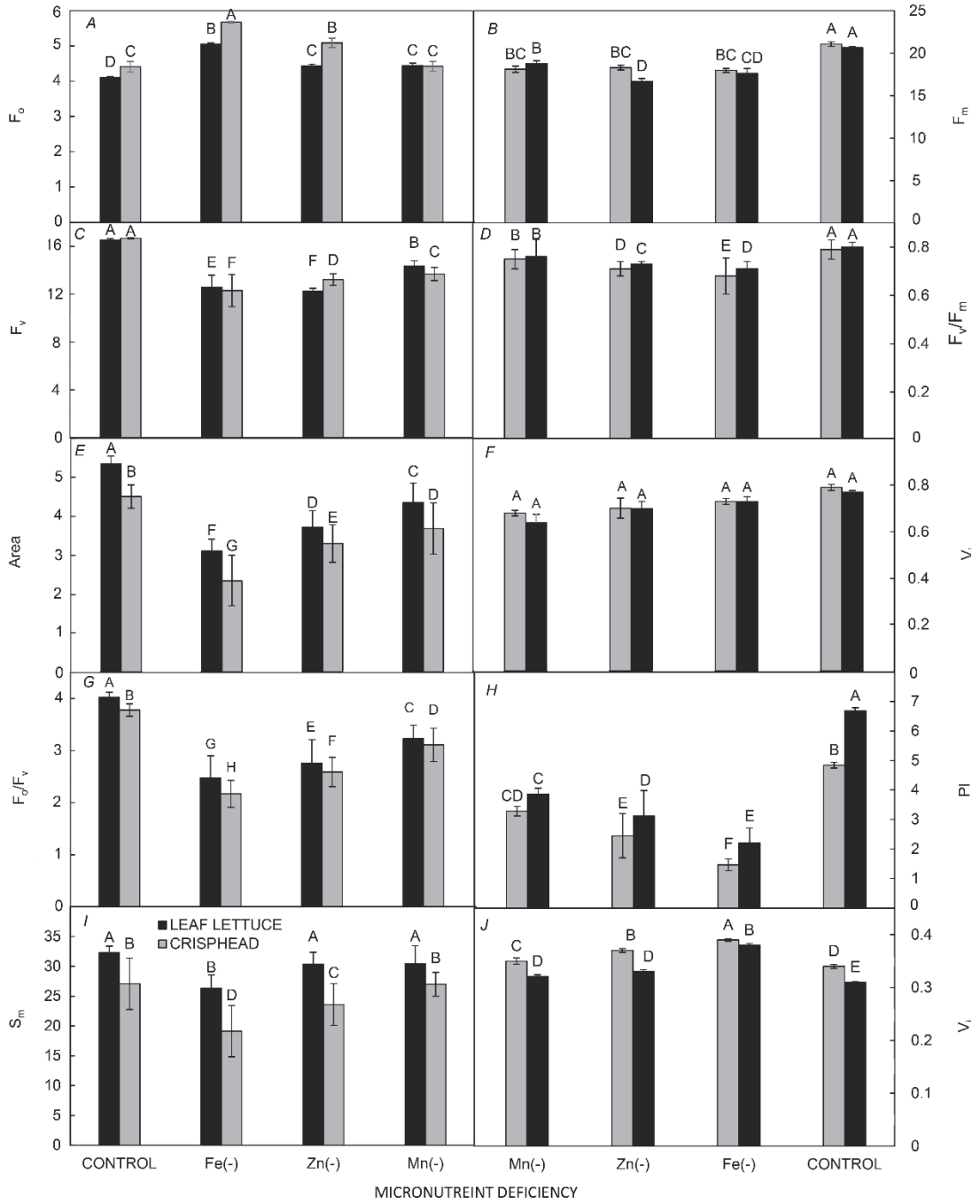

Fig. 2. Effect of micronutrient deficiency on chlorophyll fluorescence parameters of two lettuce cultivars: leaf lettuce and crisphead lettuce. Bars indicate standard error. Columns with *different letters* are significantly different at $P \geq 0.05$.

amount of Chl *b* in comparison with control. The maximum reduction of Chl *b* was recorded in the plants grown under FeD, while there was no significant change in the plants grown under MnD and ZnD. In general, Chl *b* reduction in crisphead lettuce was higher than that in leaf lettuce (Fig. 1). Data also indicated that micronutrient deficiency had no significant effect on the Car content (Table 2S, *supplement available online*). But the Car contents after FeD, ZnD, and MnD treatments slightly decreased in comparition with control treatment (Fig. 1*D*).

Chl fluorescence parameters including F_v, F_m, F_o, F_v/F_m, Area, V_i,V_j, F_v/F_o, S_m, and PI were significantly affected by microelement deficiency (Table 3S, *supplement available online*). As indicated in Fig. 2, F_v, F_m, F_o, F_v/F_m, Area, F_v/F_o, S_m, and PI decreased by microelement deficiency, especially by FeD. FeD in crisphead cultivar caused 13, 56, and 69% reduction in F_v/F_m, Area, and PI,

Table 2. Effect of micronutrient deficiency on quantum yield and specific energy fluxes (per Q_A-reducing PSII reaction center) of lettuce genotypes. Values are means ± SE of three replicates. *Different letters* in each column show significant differences at $P \leq 0.05$ (LSD), Ψ_o – trapped exiton moves an electron in to the electron transport chain beyond Q_A, Φ_{do} – quantum yield of energy dissipation, φET_{20} – quantum yield of electron transport from Q_A to Q_B in PSII, Φ_{re10} – quantum yield of reduction of end electron acceptors at the PSI, PI_{abs} – performance index for the photochemical activity; PI_{tot} – total performance index for the photochemical activity; TR_0/RC – trapped energy flux per RC; ET_0/RC – electron transport flux per RC.

Micronutrient deficiency	Cultivars	Ψ_o	Φ_{do}	φET_{20}	Φre_{10}	PI_{abs}	PI_{tot}	Tr_0/RC	Et_0/RC
Control	Leaf lettuce	0.69 ± 0.006^{a}	0.20 ± 0.02^{h}	0.54 ± 0.03^{a}	$0.18 \pm 0.03de$	2.65 ± 0.018^{a}	0.88 ± 0.023^{c}	2.48 ± 0.02^{a}	1.67 ± 0.05^{a}
	Crisphead	0.66 ± 0.002^{b}	0.21 ± 0.03^{g}	0.52 ± 0.006^{b}	0.17 ± 0.02^{e}	2.43 ± 0.03^{ab}	0.77 ± 0.01^{def}	2.77 ± 0.03^{b}	1.71 ± 0.02^{ab}
Fe (-)	Leaf lettuce	0.62 ± 0.003^{d}	0.29 ± 0.02^{b}	0.44 ± 0.008^{f}	0.19 ± 0.02^{d}	1.68 ± 0.008^{f}	0.73 ± 0.02^{f}	1.71 ± 0.05^{e}	1.06 ± 0.08^{d}
	Crisphead	0.61 ± 0.006^{e}	0.31 ± 0.01^{a}	0.41 ± 0.02^{g}	0.18 ± 0.15^{de}	1.68 ± 0.008^{f}	0.74 ± 0.03^{f}	1.36 ± 0.11^{f}	0.83 ± 0.16^{d}
Zn (-)	Leaf lettuce	0.67 ± 0.002^{b}	0.27 ± 0.01^{d}	0.49 ± 0.03^{cd}	0.21 ± 0.01^{bc}	1.94 ± 0.03^{de}	0.87 ± 0.06^{d}	1.72 ± 0.04^{e}	1.16 ± 0.07^{d}
	Crisphead	0.64 ± 0.003^{d}	0.28 ± 0.01^{c}	0.47 ± 0.15^{e}	0.20 ± 0.008^{c}	1.76 ± 0.04^{ef}	0.80 ± 0.03^{de}	1.78 ± 0.08^{e}	1.15 ± 0.08^{d}
Mn (-)	Leaf lettuce	0.68 ± 0.03^{b}	0.24 ± 0.01^{f}	0.50 ± 008^{e}	0.27 ± 0.02^{a}	2.25 ± 0.04^{bc}	1.19 ± 0.02^{a}	2.38 ± 0.05^{c}	1.63 ± 0.07^{b}
	Crisphead	0.65 ± 0.008^{c}	0.25 ± 0.01^{e}	0.49 ± 0.07^{cd}	0.24 ± 0.02^{b}	2.09 ± 0.04^{cd}	1.03 ± 0.012^{bc}	2.21 ± 0.02^{d}	1.46 ± 0.2^{c}

respectively. Leaf lettuce cultivar showed lesser sensitivity compairing to the crisphead cultivar (Fig. 2*D*,*E*,*H*). In addition, data showed that ZnD affected more Chl fluorescence parameters in comparison with MnD. According to our data, V_j and F_o parameters increased by microelement deficiency treatments. Regardless of lettuce cultivars, FeD, ZnD, and MnD, comparing with control, increased F_o value by 26, 12, and 4%, respectively (Fig. 2*A*). The V_j value increased under micronutrient deficiency. The maximum increase was recorded in leaf lettuce grown under FeD and the minimum increase was observed in crisphead lettuce grown under MnD (Fig. 2*J*). Leaf lettuce cultivar showed higher tolerance towards micronutrient deficiency according to Chl fluorescence parameters, comparing to crisphead lettuce.

Quantum yields and specific energy fluxes: Lack of micronutrients had a significant effects on quantum yield parameters of PSII including trapped exiton (which moves an electron in to the electron transport chain beyond Q_A, Ψ_o), quantum yield of energy dissipation (Φ_{do}), quantum yield of electron transport from Q_A to Q_B in PSII (φ_{ET20}), quantum yield of reduction of end electron acceptors at the PSI (Φ_{re10}), performance index for the photochemical activity (PI_{abs}), total performance index for the photochemical activity (PI_{tot}), trapped energy flux per reaction center (TR_0/RC), electron transport flux per reaction center (ET_0/RC) (Table 4S, *supplement available online*). In both lettuce cultivars, all parameters of the quantum yield and specific energy fluxes excluding Φ_{do}, Φ_{re10}, and PI_{tot} decreased significantly with FeD, ZnD, and MnD. The Φ_{do} value increased in the plants grown under micronutrient deficiency condition. The highest Φ_{do} value was recorded in crisphead cultivar, which was grown under FeD (Table 2). Results were completely different for Φ_{re10} and PI_{tot}. Plants grown under MnD showed the highest increase in Φ_{re10} and PI_{tot} and plants under ZnD showed the least increase in Φ_{re10} and PI_{tot} compared with control. FeD had no significant effect on Φ_{re10} compared with the control treatment but PI_{tot} decreased remarkably under FeD (Table 2).

Dry mass: Plant growth was determined by DM. DM showed statistically significant variations caused by effects of variety and micronutrient deficiency (Table 4S). As expected, the highest DM was recorded under control conditions and leaf lettuce was significantly superior to crisphead. Regardless of lettuce cultivars, the reduction rate of DM under FeD, ZnD, and MnD conditions were 43, 22, and 11%, respectively (Fig. 3).

Discussion

Ion concentrations: Different response to micronutrient shortage was observed in both lettuce cultivars. As Fe, Zn, and Mn are divalent elements, they show antagonistic behavior during absorption. Therefore, a high concentration of each element in a nutrient solution could affect absorption of other elements. Generally, especial transporters, *i.e.*, zinc transporters proteins (ZIP), are involved in absorbing these elements. Therefore amount of ZIP transporters is essential for the metal homeostasis in the plant (Milner *et al.* 2013). Some studies reported antagonistic effects of microelements in soybean (Izaguirre-Mayoral and Sinclair 2005), wheat (Zhao *et al.* 2011), and cucumber (Bityutskii *et al.* 2014), which is in agreement with our results.

Increasing Zn and Mn absorption through roots by iron-regulated transporter 1 (IRT1) can increase Zn and Mn concentration in lettuce leaves. In FeD conditions, IRT1 plays an important role as a regulator protein (Pavlovic *et al.* 2013). On the other hand, at ZnD, ZIP transporters increase amount of Fe and Mn (Sinclair and Krämer 2012). Zhao *et al.* (2011) suggested three reasons for antagonistic effects of Fe and Zn including: (*1*) a competition for uptake by transporters located on root cells, (*2*) disorder in metal chelation process in roots, (*3*) a competition for entering to xylem cells.

Osmotic pressure-regulating compounds: Micronutrient shortage increased amount of osmoregulators significantly, *e.g.,* proline, soluble sugars, and phenolic compounds. Both lettuce cultivars showed higher concentrations of osmoregulators under the FeD treatment, comparing to ZnD and MnD treatments. The osmoregulators had positive effects on preventing plants from environmental stresses and improving stress tolerance in plants (Cesco *et al.* 2010). Phenolic compounds are also considered as an osmolite, which increases under FeD conditions (Valentinuzzi *et al.* 2015). Increasing phenolic compounds as a result of FeD conditions caused: (*1*) increasing Fe movement in apoplast, (*2*) improving Fe solubility in rhizosphere, (*3*) increasing available Fe by auxin and siderophores synthesis. Previous studies reported more proline under FeD condition in strawberry (Heidari and Sarani 2012, Valentinuzzi *et al.* 2015), chamomile (Heidari and Sarani 2012), and barley (Arias-Baldrich *et al.* 2015). Proline production may increase as a result of higher amount of NADH compared to NAD^+ which causes disorganization in the electron transport chain (Saradhi *et al.* 1993). On the other hand, proline reduces the disorganization of electron transport chain. Effects of MnD and ZnD on amount of osmoregulators was not well studied. Hajiboland and Amirazad (2010) reported a significant reduction of water-soluble carbohydrates in leaves and roots of cabbage under ZnD. A significant increase of the proline content under ZnD was also reported in plants and it confirms our results (Jamalomidi *et al.* 2006). Mn and Fe shortage cause an increase in amounts of free radicals, which can be explained by the role of Mn and Fe in electron transport chain and enzymatic activities. Production of osmotic regulators, *e.g.,* phenolic compounds, water soluble carbohydrates, and proline, is a defense strategy against free radicals (Hayat *et al.* 2012).

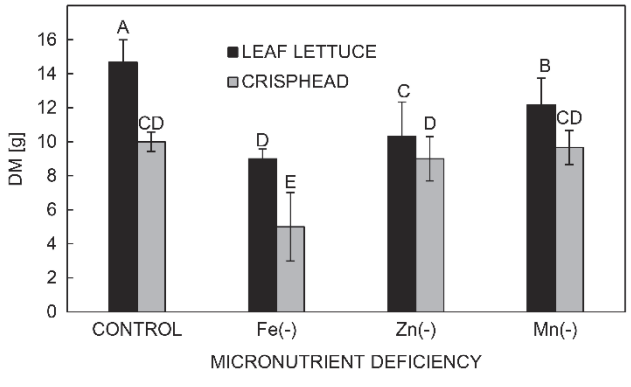

Fig. 3. Effect of micronutrient deficiency on dry mass (DM) of two lettuce cultivars: leaf lettuce and crisphead lettuce. Bars indicate standard error. Columns with *different letters* are significantly different at $P \geq 0.05$

Photosynthetic pigments: Amount of Chl *a*, *b,* and TChl decreased significantly under micronutrient-shortage conditions. Leaf lettuce cultivar showed higher amounts of Chl *a* and TChl, comparing to the crisphead cultivar. Reducing Chl content under microelement-shortage treatments could be explained by their effects on disruption of photosynthetic pigment production process, especially of Chl. Several factors are responsible for pigment decline under micronutrient-deficiency conditions. Data showed that FeD treatment was more effective in the reduction of photosynthetic pigments comparing to ZnD and MnD treatments. The important role of Fe for Chl and chloroplast functions can be the main reason. Fe plays a key role in oxidation and reduction mechanism in hem of cytochrome (Marschner 2011). Decreasing Chl pigments under FeD conditions was previously reported in pepper (Roosta and Mohsenian 2012), tomato (Machold and Stephan 1969), pea (Mahmoudi *et al.* 2005), and strawberry (Pestana *et al.* 2012). Zn is necessary for enzymatic activities and electron transport chain, so its shortage increases free radicals and damages of chloroplast membranes. ZnD also causes changes in Chl structure and a reduction in photosynthesis capacity. Reduction in the Chl content as a result of ZnD treatment was previously reported by Fu *et al.* (2015). Damages to thylakoid membranes and LHC proteins might be the main reason of Chl reduction. Effects of MnD on the Chl content is not well understood. Singh *et al.* (2001) reported significant decline in Chl in mint plants as a result of MnD treatment.

Chl fluorescence: All Chl fluorescence parameters, except V_i, changed significantly under FeD, ZnD, and MnD conditions. The changes were smaller in leaf lettuce cultivar than those in crisphead cultivar. As PSII is located in thylakoid membranes, reducing thylakoid membrane stability under stress condition causes lower efficiency of PSII reaction centers. Changes in Chl fluorescence are useful parameters for measuring stability and efficiency of thylakoid membranes (Weng *et al.* 2008). Kalaji *et al.* (2014) have used Chl fluorescence to evaluate the performance of PSII and PSI under nutrient-deficiency condition (K, Mg, Ca, N, and S) in tomato and maize. Fu *et al.* (2015) also utilized this method for apple trees under ZnD conditions. These reports confirm that Fe, Zn, and Mn shortage could affect different parts of photosynthetic apparatus. Increasing V_j in such a situation is a result of reduction in quinone a and plastoquinone (PQ), which reduces electron transport (Kalaji *et al.* 2014). F_v/F_m decline under FeD, ZnD, and MnD may occur as a result of damages to PSII reaction centers. Increasing amounts of free electrons and radicals under micronutrient-deficiency conditions are one of the main reason of such a damage (Roháček and Barták 1999, Breštič *et al*. 2015). F_v/F_m reduction under ZnD treatment was previously reported by Khan and Khan (2014) in coward and under FeD by Osório *et al.* (2014) in strawberry. Generally, after decreasing Q_A or after PQ oxidation, Chl fluorescence increases, which causes reaction center inactivation, and increases F_o and decrease F_m (Duysens and Sweers 1963). Based on the O-J-I-P curve, reduction in PQ capacity and other receptors, in both reaction centers, is the main reason of decreasing Chl fluorescence parameters (Strasser *et al*. 2010). Therefore a rapid increase in fluorescence parameters from J to I requires high PQ capacity, otherwise, process can result in a decrease of F_m and then the Area and other Chl fluorescence parameters start declining.

Quantum yields and specific energy fluxes: FeD, ZnD, and MnD reduced quantum yield parameters, excluding Φ_{do}, Φ_{re10}, and PI_{tot}. Reduction in quantum yield of PSII and electron transport has been reported in sunflower (Ciompi *et al.* 1996), maize, and tomato (Kalaji *et al.* 2014). They suggested that this decline occurrs due to decrease of LHC proteins in PSII. Decreasing photosynthetic pigments combined with lower transported energy and damages to D1 protein can cause φ_{Po} reduction under stress conditions (Qu *et al.* 2012). Less transported electrons from PHQ to PC could be another reason for the reduction in the quantum yield of PSII (Strasser *et al.* 2010). Beauchemin *et al.* (2007) suggested that micronutrient, especially FeD, deficiency reduces Q_A re-oxidation and inhibits transported electrons from Q_A to Q_B. According to the important role of Fe, Zn, and Mn in electron transport chain, reduction in these microelements also decreases efficiency of electron acceptors in the electron transport chain which causes decline in transported electrons between PSI and PSII (Strasser *et al.* 2010). Wasted energy in a form of heat causes Φ_{do} raise under microelement-scarcity conditions (Kalaji *et al.* 2014). Decreasing PI_{abs} and PI_{tot} could be also explained by a lowered number of transferred electrons.

Conclusion: We showed that Fe, Zn and Mn shortage significantly reduced photosynthetic pigments (Chl *a*, Chl *b*, and TChl), Chl fluorescence parameters (F_v/F_m, Area, V_i, V_j, F_v/F_o, S_m, and PI) and quantum yield of PSII (Ψo, ET_{20}, PI_{abs}, TR_0/RC, and ET_0/RC) in both lettuce cultivars. Reduction in chlorophyll fluorescence parameters, photosynthetic pigments, and quantum yield under Fe, Zn and Mn deficiency conditions confirmed their importance for photosynthesis and the electron transport chain. In addition, micronutrients can act as a cofactor for some enzymatic activities, especially for enzymes involved in pathways producing photosynthetic pigments.

Results also showed that osmoregulators, *e.g.,* proline, soluble sugars, and phenolic compounds, were enhanced under Fe, Zn, and Mn deficiency treatment. Higher amount of osmoregulators in leaf lettuce confirmed the higher membrane stability and PSII efficiency in this cultivar. Eventually, we can recommend chlorophyll fluorescence parameters as a useful and accessible markers to determine tolerant cultivars for environmental stresses.

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Appendix

(Breštič and Živčák 2013)

Ab		Calculation
Basic parameters derived from the extracted data		
F_o	Minimal fluorescence, when all PSII RCs are open	$F_o = F_{50\mu s}$
F_m	Maximal fluorescence, when all PSII RCs are closed	
F_v	Maximal variable fluorescence	$F_v = F_m - F_o$
F_v/F_m	Maximal quantum yield of PSII photochemistry	
V_j	Relative variable fluorescence at the J-step	$V_j = (F_{2ms} - F_o)/(F_m - F_o)$
V_i	Relative variable fluorescence at time 30 ms (I-step) after start of actinic light pulse	$V_i = (F_{30ms} - F_o)/(F_m - F_o)$
Area	Area above the OJIP curve; it express the size of the reduced PQ pool	
Sm	Normalized area; it is related to the number of electron carriers per electron transport chain	Sm = (Area/($F_m - F_o$)
Quantum yields		
Ψ_o	Probability that a trapped exiton moves an electron in to the electron transport chain beyond Q_A	$\Psi_o = (1 - V_j)$
φ_{Po}	Maximum quantum yield of primary PSII photochemistry	$\varphi_{Po} = 1 - (F_o/F_m)$
Φ_{do}	Quantum yield of energy dissipation	$\Phi_{do} = F_o/F_m$
φET_{20}	Quantum yield of electron transport from Q_A to Q_B in PSII	$\varphi ET20 = \varphi P_o(1 - V_j)$
φRE_{10}	Quantum yield of reduction of end electron acceptors at the PSI acceptor side	$\varphi RE_{10} = \varphi P_o(1 - V_i)$
PI_{ABS}	Performance index for the photochemical activity (basic formula on absorption basis)	$PI_{ABS} = [(1 - (F_o/F_m)]/(M_o/V_j).[(F_m - F_o)/F_o]\ [(1 - V_j)V_i]$
PI_{tot}	Total performance index for the photochemical activity (including the flow beyond PSI)	$PI_{tot} = PI_{ABS}(1 - V_i)/(1 - V_j)$
Specific energy fluxes (per QA-reducing PSII reaction center)		
TR_0/RC	trapped energy flux per RC (at t = 0)	Mo $(1/V_j)$
ET_0/RC	electron transport flux per RC (at t = 0)	Mo $(1/V_j)\ \psi_o$