

Foliar-applied iron and zinc nanoparticles improved plant growth, phenolic compounds, essential oil yield, and rosmarinic acid production of Lemon balm (*Melissa officinalis* L.)

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Research Article

Keywords: Foliar nutrition, Metal nanoparticles, Plant performance, Secondary metabolites

Posted Date: March 13th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3924433/v1>

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Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on May 17th, 2024. See the published version at <https://doi.org/10.1007/s11356-024-33680-5>.

Abstract

Metal nanoparticles (NPs) have been highlighted to improve plant growth and development in the recent years. Although positive effects of some NPs have been reported on medicinal plants, the knowledge for stimulations application of iron (Fe) and zinc (Zn) NPs is not available. Hence, the present work aimed to discover the effects of Fe NPs at 10, 20, and 30 mg L⁻¹ and Zn NPs at 60 and 120 mg L⁻¹ on growth, water content, photosynthesis pigments, phenolic content, essential oil (EO) quality, and rosmarinic acid production of lemon balm (*Melissa officinalis* L.). The results showed that Fe NPs at 20 and 30 mg L⁻¹ and Zn NPs at 120 mg L⁻¹ significantly improved biochemical attributes. Compared with control plants, the interaction of Fe NPs at 30 mg L⁻¹ and Zn NPs at 120 mg L⁻¹ led to noticeable increases in shoot weight (72%), root weight (92%), chlorophyll (Chl) a (74%), Chl b (47%), rosmarinic acid (66%), proline (1.8-fold), glycine betaine (GB, 3.3-fold), protein (3.8-fold), relative water content (8%), EO yield (3.1-fold), total phenolic content (63%), and total flavonoid content (57%). The agglomerative hierarchical clustering represented three different clusters for Zn NPs levels and three clusters for Fe NPs concentrations so that NPs at 10 and 20 mg L⁻¹ were placed in one cluster. Heat map analysis revealed that protein, GB, EO yield, shoot weight, root weight, and proline possessed the maximum changes upon Fe NPs. Totally, the present study recommended the stimulations application of Fe NPs at 20–30 mg L⁻¹ and Zn NPs at 120 mg L⁻¹ to reach the optimum growth and secondary metabolites of lemon balm.

Introduction

Through a reduction in nutrient losses to the environment, the application of nanotechnology in plant nutrients regulates the release of fertilizers (Fincheira et al. 2021). Nanomaterials are coated on nanofertilizers, enabling them to control the release of nutrients and enhance the effectiveness of their delivery to plants. Nanofertilizers play a significant role in agricultural sustainability by supplying nutrients that increase plant productivity and lower production costs. Nanomaterials have the potential to greatly increase plant production when compared to conventional fertilizers (Rossi et al. 2019; Afshari et al. 2021). These gains may be attributable to nanofertilizers' increased involvement in plant nutrition and their superior particle penetration and transportation capabilities in plant tissues (Afshari et al. 2021; Jan et al. 2021). A particle of substance with a diameter of between 1 and 100 nm is referred to as a nanoparticle (NP). Because they are less than 100 nm in size and have a high surface-to-volume ratio, engineered NPs can interact and penetrate plant cells more effectively (Jan et al. 2011). These specific characteristics explain why synthetic NPs are being employed more frequently in a variety of fields, including the food and agricultural industries (Hu and Xianyu 2021).

Metals NPs have been recently used in agriculture to improve plant performance. Iron (Fe) is part of the chlorophyll molecule and plays a role in the reduction of nitrate to ammonia nitrogen as a part of an enzyme (Briat et al. 2015). Other enzyme systems such as catalase and peroxidase also require Fe. This element is absorbed through an active process in the form of Fe²⁺ or through iron chelates, which are organic molecules (Briat et al. 2015; Santos et al. 2021). The main symptom of Fe deficiency is chlorosis,

which is usually seen in the young leaves (Santos et al. 2021). In addition, enzymatic activities of zinc (Zn) are critical for plants, which directly influences plant growth. This element has a specific effect on leaf falling, and its deficiency causes a delay in the timely opening of leaves and flowers and ultimately limits the growth of plants (Basnet et al. 2018). By interacting with other minerals, excessive Zn can cause phytotoxic symptoms that reduce photosynthesis or lead to nutritional imbalance (Sharifan et al. 2019).

Lemon balm (*Melissa officinalis*), belonging to the Lamiaceae family, is medicinally important because of critical compounds such as phenolics and essential oils (EOs) with valuable pharmaceutical properties viz. antibacterial, spasmolytic, antiviral, and analgesic (Ghasemian et al. 2021). Lemon balm is extremely eminent in terms of an economic aspect due to its similar smells to lemon. Most of the medicinal effects of this plant have been attributed to its active ingredient, rosmarinic acid (RA). It is one of the main active phenolic compounds of several medicinal plants in the Lamiaceae and Boraginaceae families which is an ester of caffeic acid and 3,4-dihydroxyphenyllactic. Rosmarinic acid is a defensive compound against herbivores, pathogens, and UV with characteristic features such as antimicrobial, antibacterial, antiviral and anti-inflammatory, antioxidant, anti-depressant, and anti-rheumatic effects (Albergaria et al. 2020). Two parallel pathways are involved in RA biosynthesis; L-phenylalanine and L-tyrosine, both deriving from the tyrosine biosynthetic pathway are precursors of RA (Trócsányi et al. 2002).

The use of NPs to enhance plant growth and active compounds is becoming more widespread. The improved growth, antioxidant capacity, and effective materials like EOs and phenolic compounds have been reported by different metal NPs (Afshari et al. 2021; Memari-Tabrizi et al. 2021; Shankar et al. 2021; Ojagh et al. 2022). Accordingly, Rizwan et al. (2019) have shown the positive effects of the combined application of Fe and Zn NPs on the growth and antioxidant capacity of wheat plants. However, little information is available on the use of Fe and Zn NPs in medicinal plants. The research question is how Fe and Zn NPs can improve growth and biochemical status of lemon balm, and which one is superior. Hence, the present work was carried out to discover the changes in growth, water status, phenolic compounds, EO yield, and RA content of lemon balm under foliar-applied Fe and Zn NPs.

Materials and methods

Plant Materials and treatments

Seeds of *M. officinalis* (Pakan bazr, Isfahan) were cultivated on the sand culture media. Pots containing seeds were transferred to the greenhouse with a 16/8 light/dark cycle (irradiance: $120\text{--}150\ \mu\text{M m}^{-2}\text{ s}^{-1}$). Seeds of *M. officinalis* (Pakan bazr, Isfahan) were cultivated in 4-liter volume pots, filled with a mixture of perlite, coco peat, and sand. Germinated seedlings were irrigated daily with standard Hoagland solution (EC 1.7 dS m⁻¹, pH 6.0-6.5, 750 mL/pot/day) for 30 days. The plants were treated with 20 ml of each treatment weekly for three weeks. Iron NPs were used in the form of Fe₃O₄ with CAS NO: 1309-38-2, purity: 97%, average size: 10–20 nm, and density: 8.9 g cm⁻³, and Zn NPs were applied as ZnO with

CAS NO: 1314-13-2, purity: 99%, average size:10–30 nm, and density: 5.6 g cm⁻³ made by US-NANO. The Fe NPs were foliar applied at 10, 20, and 30 mg L⁻¹ and Zn NPs were sprayed on the leaves at 60 and 120 mg L⁻¹ as a factorial based on completely randomized design (CRD) with five replicates. The NPs were sprayed on leaves weekly (20 mL for each plant) for 28 days. At the end of the experiment, the plants were harvested for physiological and biochemical analyses.

Root and shoot weight

The plants were harvested from the culture media and were cut from the bottom of the stem. A digital scale was then used to measure the weight of the root and shoot. Samples were carefully cleaned with water, dried at 70 °C for 48 h in an oven, then weighed to determine dry weight (Memari-Tabrizi et al. 2021).

Chlorophyll (Chl) Content

The Chl contents of leaves were determined according to the method described by Lichtenthaler (1987). Fresh samples (0.1 g) were ground to powder in acetone (85%, v/v). Afterward, the homogenate was centrifuged at 10000 rpm for 15 min at 4°C, and absorbance of supernatants was determined at three wavelengths of 470, 646.8, and 663.2 nm by spectrophotometer (Varian Cary 50, Australia) (Lichtenthaler 1987). The Chl Concentrations were calculated by following formulas:

$$\text{Chlorophyll a} = (12.25 \times \text{OD}_{663.2 \text{ nm}} - 2.79 \times \text{OD}_{646.8 \text{ nm}})$$

$$\text{Chlorophyll b} = (21.21 \times \text{OD}_{646.4 \text{ nm}} - 5.1 \times \text{OD}_{663.2 \text{ nm}})$$

$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

Relative water content (RWC) measurement

The Dhopte and Manuel procedure was utilized to estimate the RWC of leaves as a percentage (2002). The fresh leaves were weighed (FW), soaked (SW) for 24 h at room temperature (FW), and then dried (FW) for the next 24 h at 75 ° C. RWC was finally calculated as follows:

$$\text{RWC} = \frac{(\text{FW} - \text{DW})}{(\text{SW} - \text{DW})} \times 100$$

Proline concentration

To measure proline content, a 0.5 g fresh leaf sample was mixed with 10 ml of sulfosalicylic acid (3% w/v). The mixture containing the sample was centrifuged at 4000 × g for 20 min, and then 2 ml of ninhydrin acid and 2 ml of glacial acetic acid were added and vortexed. Simultaneously, 2 ml of standard 0, 4, 8, 12, 16, 20 mg L⁻¹ proline and 2 ml ninhydrin acid, and 2 ml acetic acid were mixed and vortexed. All samples were heated in a hot water bath for 60 min and then placed on ice to be cooled completely. 4 ml of toluene was added to the solution and stirred with Vertex for 20 sec. Using 0, 4, 8, 12, 16, and 20

mg L⁻¹ proline standards, the standard curve regression equation was determined spectrophotometrically at 520 nm. The toluene soluble proline was sufficiently was measured at 520 nm expressed as $\mu\text{mol proline g}^{-1} \text{FW}$ (Bates et al. 1973).

Protein Content

Protein extraction was done by homogenizing 0.5 g of fresh samples in 50mM potassium phosphate buffer (pH = 7.0) containing 1% (w/v) Polyvinylpyrrolidone (PVP) and 1 mM EDTA. Then the homogenates were centrifuged at 11000 rpm for 20 min at 4°C. The supernatants, as a crude enzyme had been stored at -80 °C until they were used. The concentration of protein was determined according to Bradford's method using Bovine Serum Albumin (BSA) as a standard (Bradford 1976).

RA Quantification

The dried sample (100mg) was ground into powder and mixed with 25mL ethanol/water (30:70, v/v) solution and then sonicated for 10 min. The resulting mixture was centrifuged at 4500 rpm for 5 min at 4°C. The volume of the supernatant reached 50mL by using sterile distilled water. The extract solution was filtered by using a 0.2 μm syringe-headed filter before being injected into a column of High-Performance Liquid Chromatography (HPLC, ZORBAX SB-C18 column, Agilent 1100 series, USA) (Wang et al. 2004; Yan et al. 2006). The mobile phase containing; 40% solvent A (orthophosphoric acid in water, 1.0% v/v) and 60% solvent B (orthophosphoric acid in methanol, 1.0% v/v), was run at 1.0 mL/min at room temperature. Identification of RA was achieved by comparing retention time with an authentic standard that was detected at 330 nm.

Total phenolic content (TPC) measurement

Folin-Ciocalteu reagent was chosen to measure leaf TPC spectrophotometrically to quantify TPC. The Folin-Ciocalteu reagent was dissolved in 100 l of the MeOH solution and let to stay at 22°C for 5 min. 0.75 ml of NaHCO₃ was mixed into the mixture. After 90 min at 22 ° c., an absorbance of 725 nm was measured using a UV-VIS spectrophotometer (Varian Cary 50). Gallic acid was used to calibrate the standard curve (GA). The outcome was expressed as mg GA g⁻¹ Dry weight (Afshari et al. 2021).

Total flavonoid content (TFC) measurement

Aluminum chloride colorimetric method was followed to measure leaf TFC. The 0.5 mL of extract solution with 1.5 ml of 95% ethanol, 0.1 ml of aluminum chloride 10%, 0.1 mL of 1 M potassium acetate were mixed with 2.8 ml of distilled water. The mixture vortexed for 10 s and left to stand at 25 ° C for 30 min. The absorbance of the mixture was read at 415 nm. The results were represented as mg QE g⁻¹ dry weight (Khosropour et al. 2021).

Essential oil (EO) assay

EO content of flowering branches was quantified using the method described by the European Pharmacopoeia for oil production. Briefly, 100 g of dried aboveground plant parts were subjected to

hydro-distillation for 3 h using a Clevenger-type apparatus (Afshari et al. 2021).

Statistical analysis

All experiments were carried out as a full factorial experiment with a completely randomized design (CRD) with three replicates. Duncan's multiple range tests were used to compare the treatments' means in the statistical package SAS Version 2.3. The significance level was set at 5%. The multivariate analysis was done by XLSTAT software.

Results

Plant weight

Shoot and root weight of *M. officinalis* L. significantly ($P \leq 0.05$) increased by Fe and Zn NPs. Although Fe NPs at 20 and 30 mg L⁻¹ represented no significant differences, all levels significantly increased shoot weight relative to control plants. Fe NPs at 10, 20, and 30 mg L⁻¹ remarkably improved plant weight by 12, 18, and 21%, respectively, as compared with non-Fe NPs without Zn application. The interaction of Zn and Fe NPs at 120 mg L⁻¹ yielded 44 and 45% increases in shoot weight in comparison with the alone Fe and Zn, respectively (Fig. 1a). Like shoot weight, the improvement in root weight was observed upon Zn and Fe NPs. Plants sprayed with Zn NPs at 120 mg and Fe NPs at 20 or 30 mg showed higher root weights. In non-Zn NPs treatments, Fe NPs at 10, 20, and 30 mg L⁻¹ enhanced root weight by 14, 46, and 85%, respectively, compared with non-Fe NPs. Zn NPs at 120 mg L⁻¹ had positive effects on root weight, particularly when used with Fe NPs at 20 mg L⁻¹ to be 0.82 g (Fig. 1b).

Proline and glycine betaine (BG) content

Proline and BG noticeably ($P \leq 0.05$) increased upon Zn and Fe NPs. Under non-Zn application, Fe NPs at 10, 20, and 30 mg L⁻¹ raised proline concentration by 26, 45, and 76%, respectively, when compared with non-Fe NPs. The interaction of Fe NPs at 30 mg L⁻¹ and all levels of Zn NPs represented the higher proline content relative to other treatments (Fig. 2a). Moreover, BG showed an increased pattern under the treatments of Zn and Fe NPs. Interestingly, it increased by progressing the concentration of Zn and Fe NPs, with the maximum amount in the interaction of Fe NPs at 30 mg L⁻¹ and Zn NPs at 120 mg L⁻¹ to be 3.94 mg g⁻¹ (Fig. 2b).

Protein and relative water content (RWC)

Foliar application of Fe and Zn NPs led to noticeable increases in protein content. The highest protein content (3.26 mg g⁻¹) was observed in plants nourished by Fe NPs at 60 mg L⁻¹ without Zn application, while the lowest protein (0.062 mg g⁻¹) was obtained in control plants (without Fe and Zn NPs) (Fig. 3a). Additionally, RWC increased upon foliar application of Fe and Zn NPs, ranging from 84% in control plants to 93% in plants sprayed with Fe and Zn NPs at 120 mg L⁻¹. At high concentrations of Fe NPs (20 and 30

mg L⁻¹), there were no significant differences between the levels of Zn NPs, but the increased RWC upon Zn NPs was observed when plants were nourished only by Zn (Fig. 3b).

Photosynthesis pigments and Rosmarinic acid content

The Chl contents greatly increased by Fe and Zn NPs. Chlorophyll a ranged from 0.58 mg g⁻¹ in control to 1.48 mg g⁻¹ in Fe NPs at 30 mg g⁻¹. In addition, the maximum Chl b (0.62 mg g⁻¹) was obtained in plants sprayed with Fe NPs and Zn NPs at 120 mg L⁻¹, with a 63% increase relative to the control. Total Chl was found in a range of 1.4–2.19 mg g⁻¹, with the highest amount in the infraction of Fe NPs at 30 mg L⁻¹ and Zn NPs at 120 mg L⁻¹ (Table 1). The main polyphenol in *M. officinalis* L. is rosmarinic acid, ranging from 6.33 mg g⁻¹ in the Fe and Zn NPS at 60 mg L⁻¹ to 12.59 mg L⁻¹ in Fe NPs at 60 mg L⁻¹ without Zn application. Fe NPs at 10, 20, and 30 mg L⁻¹ yielded 95, 20, and 56% increases when compared with the non-Zn application. Interestingly, the highest rosmarinic acid was observed in plants sprayed with only Fe NPs at 60 mg L⁻¹. Accordingly, in non-Fe treatments, Zn NPs at 60 and 120 mg L⁻¹ increased rosmarinic acid by 10 and 52%, respectively, as compared with the non-Zn application (Table 1).

Table 1
Iron and zinc nanoparticles on chlorophyll (Chl) and rosmarinic acid content of Lemon balm

Fe NPs	Zn NPs	Chl a	Chl b	Total Chl	Rosmarinic acid
Non-Fe	Non-Zn	0.85 ± 0.02^f	0.38 ± 0.02^g	1.41 ± 0.02^f	6.45 ± 0.4^g
	60 mg L ⁻¹	0.86 ± 0.06^f	0.39 ± 0.02^g	1.43 ± 0.08^{ef}	7.06 ± 0.23^f
	120 mg L ⁻¹	0.9 ± 0.02^{ef}	0.45 ± 0.02^{ef}	1.53 ± 0.04^{de}	9.84 ± 0.3^c
10 mg L ⁻¹	Non-Zn	0.93 ± 0.02^e	0.42 ± 0.01^{fg}	1.53 ± 0.05^{de}	12.59 ± 0.23^a
	60 mg L ⁻¹	0.89 ± 0.02^{ef}	$0.52 \pm 0.04^{b-d}$	1.58 ± 0.06^{cd}	6.33 ± 0.13^g
	120 mg L ⁻¹	1 ± 0.05^d	$0.51 \pm 0.03^{b-d}$	1.67 ± 0.02^c	9.08 ± 0.13^d
20 mg L ⁻¹	Non-Zn	0.94 ± 0.02^{de}	$0.49 \pm 0.01^{c-e}$	1.59 ± 0.01^{cd}	7.75 ± 0.24^e
	60 mg L ⁻¹	1.21 ± 0.02^c	0.55 ± 0.01^b	1.94 ± 0.06^b	8.1 ± 0.4^e
	120 mg L ⁻¹	1.41 ± 0.02^b	0.62 ± 0.01^a	2.14 ± 0.03^a	8.88 ± 0.22^d
30 mg L ⁻¹	Non-Zn	1.24 ± 0.04^c	0.48 ± 0.03^{de}	1.86 ± 0.07^b	10.08 ± 0.18^c
	60 mg L ⁻¹	1.37 ± 0.02^b	0.54 ± 0.02^{bc}	2.12 ± 0.1^a	9.97 ± 0.26^c
	120 mg L ⁻¹	1.48 ± 0.03^a	0.56 ± 0.01^b	2.2 ± 0.03^a	10.72 ± 0.34^b
Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.					

Essential oil (EO) content and EO yield

Foliar application of Fe and Zn NPs led to increased EO and EO yield. The EO content differed from 0.16% in control plants to foliar-applied Fe and Zn NPs at 120 mg L⁻¹. Like EO content, the positive effects of Fe and Zn NPs were observed in EO yield (Fig. 4a). Under non-Zn application, Fe NPs at 10, 20, and 30 mg L⁻¹ increased EO yield by 35, 39, and 49%, respectively, when compared with non-Fe NPs. In addition, the 53 and 63% increases in EO yield were observed in plants sprayed by 60 and 120 mg L⁻¹ relative to control plants (Fig. 4b).

Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC were significantly ($P \leq 0.05$) improved when plants nourished by Fe and Zn NPs. The maximum TPC was observed by using Fe NPs at 30 mg L⁻¹ and Zn NPs at 60 and 120 mg L⁻¹. However, the minimum amount was recorded in control plants as 19.63 mg GA g⁻¹ DW (Fig. 5a). Interestingly, Zn NPs had more effects on TFC than Fe NPs. In non-Zn treatments, Fe NPs at 10, 20, and 30 mg L⁻¹ led to

16, 20, and 30% raises in TFC, respectively, relative to control, while Zn NPs at 60 and 120 respectively increased TFC by 35 and 44% relative to control plants (Fig. 5b).

Agglomerative hierarchical clustering (AHC) and heat map

The AHC represented three different clusters for Zn NPs levels and three cluster for Fe NPs concentrations. Accordingly, all levels of Zn NPs were significantly different from each other (Fig. 6a). Fe NPs at 10 and 20 mg L⁻¹ were placed in a cluster; therefore, these concentrations had the same effects on the studied traits of *M. officinalis* (Fig. 6b).

According to heat map analysis, protein, GB, EO yield, shoot weight, root weight, and proline were determined as the more sensitive (higher variability) traits upon Fe NPs, while RWC and Chl contents showed the minimum variability when experienced Fe NPs. On the other hand, EO yield, Chl a, and total Chl represented the maximum changes under Zn NPs (Fig. 7).

Discussion

Nanoparticles with their small size have the ability to penetrate the biological membrane and play a critical role in the ion transfer in the plant cell by affecting the cellulose wall (Afshari et al. 2021). Nanoparticles are involved in stimulating vegetative growth and facilitating the uptake of microelements into plant roots; thus, they increase the growth and yield of plants (Nasirzadeh et al. 2022). According to the current results, remarkable increases were observed in the weights of roots and aerial parts. This can be expressed by the fact that Fe and Zn NPs, due to their very small size and high specific surface area, are easily absorbed by the capillary roots and by transferring these absorbed elements to the aerial part of the plant and involving them in the process of photosynthesis, more carbohydrates are produced, some of which are transferred back to the meristematic tissues of the roots, which result in increased length and volume. Foliar-applied Fe and Zn NPs have been shown to enhance plant growth by improving biochemical pathways. Fe NPs enhance enzyme activity involved in photosynthesis, respiration, and nitrogen fixation, leading to increased photosynthetic efficiency and plant biomass (Adrees et al. 2020). Zn NPs regulate hormone synthesis, enzyme activity, and DNA synthesis, promoting plant growth and development. Additionally, both iron and zinc nanoparticles improve nutrient uptake and utilization, further enhancing plant growth (Rizwan et al. 2020). Similarly, the increased weight in strawberry (Havas et al. 2017) and wheat (Adrees et al. 2020) with Fe NPs and wheat (Zahra et al. 2020) and lettuce (Sharifan et al. 2019) with Zn NPs. Interestingly, Rizwan et al. (2020) represented that the simultaneous application of Fe and Zn NPs noticeably increased the plant growth of wheat.

The increasing amount of protein, which is also directly related to increasing the concentration of the Fe and Zn NPs, is also consistent with the findings of Mousavi Kouhi et al. (2015), who found higher soluble sugars and proteins under Fe treatment. Due to the diameter of NPs, the rate of their adsorption, transport, and accumulation is much higher than ordinary particles, and perhaps this high adsorption efficiency and specific surface area of NPs compared to the size of conventional particles justifies the greater effectiveness of these particles (Afshari et al. 2009). In addition, the results released increased

RWC upon Fe and Zn NPs. Water content is the critical factor for evaluating plant survival (Khosropour et al. 2021). Foliar application Fe and Zn NPs improves the RWC in plants. Fe and Zn are NPs that play important roles in plant growth and development. When applied to the leaves, Fe and Zn NPs release ions that activate biochemical pathways related to water regulation. Fe ions enhance photosynthesis and respiration, leading to increased water uptake and better water management, improving RWC (Bidabadi et al. 2023). Zn ions regulate stomatal behavior and hormone synthesis, reducing water loss and improving water retention in plant tissues (Azmat et al. 2022; Pandya et al. 2023). It had been reported that increased RWC leads to better photosynthesis rate and improved plant growth (Memari-Tabrizi et al. 2021).

Foliar-applied Fe and Zn NPs resulted in noticeable increases in proline and GB, which are the most important organic osmolytes accumulating in plant species in response to environmental stresses. Both compounds seem to have positive effects on enzymes and membrane strength along with adaptive roles through osmotic regulation in plants (Ahmad et al. 2021). Most studies displayed a positive relationship between BG and proline accumulation in plants. The use of these compounds in plants can lead to a significant increase in the growth and final yield of the product. These compounds prevent the absorption of toxic ions and stabilize cell membranes by helping to inhibit ROS (Chrysargyris et al. 2018). When Fe and Zn NPs are applied to the leaves of plants, they can be absorbed and transported to different tissues. Inside the cells, these nanoparticles release Fe and Zn ions, which act as cofactors for enzymes involved in amino acid synthesis. Fe is a crucial component of enzymes involved in proline synthesis, while Zn is involved in GB biosynthesis (Ahmad et al. 2021). By supplying plants with these essential micronutrients in NPs form, the availability of Fe and Zn for amino acid synthesis increases. Accordingly, the increased proline amount has been addressed by Noohpishah et al. (2021) upon Zn NPs and the elevated proline and BG were reported by Ahmad et al. (2021) when plants were sprayed with Si and Fe NPs. These metal NPs are effective in stimulating proline and GB production to improve the growth and development of plants. The heat map showed that these osmolytes mainly changed under Fe NPs, which can be considered in further studies.

Photosynthetic pigments specify the physiological status of the plants. As the photosynthetic capacity increases, it will play an effective role in increasing plant performance (Khataee et al. 2020). It should be noted that Fe depletion can have adverse effects on chloroplasts among the organs. In this study, the interaction of Fe NPs at $20\text{--}30\text{ mg L}^{-1}$ and Zn NPs at 120 mg L^{-1} led to enhanced Chl content. Morsy et al. (2017) on snap bean plants and Moazam et al. (2017) on rice. Foliar applied Fe and Zn can improve the Chl content of plants, because of the promotive effects on protein and enzymes biosynthesis as well as play cofactor roles in the accumulation of photosynthesis pigments (carotenoids and chlorophylls) (Lingyun et al. 2016). The use of Fe and Zn NPs release ions that serve as cofactors for enzymes involved in chlorophyll synthesis. Fe ions help form the central structure of chlorophyll, while zinc ions enhance enzyme activity for pigment synthesis. This increased availability of micronutrients stimulates the biochemical pathways, resulting in more Chl and improved photosynthesis (Moazam et al. 2017; Bidabadi et al. 2023). In line with present results, the increased photosynthesis content has been reported on wheat upon Fe and Zn NP (Rizwan et al. 2020). Accreting to heat map results, Chl content

has the maximum variability under Zn NPs, which can be due to the critical role of Zn NPs in activating enzymes involved in the photosynthesis process.

Essential oil content and EO yield increased by NPs. The EO gland can be changed under fertilization (Mirzaie et al. 2020). Fe and Zn can improve EO production due to their positive role on secondary metabolism in plants by providing essential nutrients and activating the enzymes involved in EO pathways. Fe and Zn NPs enhances EO content and yield in plants through biochemical pathways. Fe and Zn NPs release ions that activate enzymes involved in EO synthesis. Adequate iron availability promotes the production of terpenes, while Zn regulates enzyme activity and gene expression. The nanoparticle form of these micronutrients improves their availability, leading to increased EO yield and potentially enhanced aroma and medicinal properties (Shahhoseini et al. 2020). The increased EO yield under metal NPs has been previously reported by Afshari et al. (2021) and Memari-Tabrizi et al. (2021) and Ojagh and Moaveni (2022). Metals NPs can modify the pathways of EO production and especially EO yield can be improved by NPs because they can significantly improve the plant weight as the main component of EO yield (Afshari et al. (2021).

Rosmarinic acid is the main active ingredient of the Lamiaceae family with antioxidant activity and numerous therapeutic properties (Esmaeilzadeh-Salestani and Riahi-Madvar 2014). The production of secondary metabolites in *vitro* cultures might be enhanced by elicitors (Trocsanyi et al. 2020). Recently, several studies have been published, which explore the effect of elicitation on the production of main secondary metabolites like RA and the expression of genes involved in its biosynthetic pathway. For example, Ghasemian et al. (2021) showed increased RA production under selenium NPs in *M. officinalis*. This increase is due to the effect of Fe and Zn NPs on the phenylpropanoide pathway. It seems that the stimulation of the above pathway is due to the motivation of the expression of genes involved in this biosynthetic pathway.

There is a highly efficient antioxidant defense system, which contains antioxidant enzymes and a non-enzymatic antioxidant system such as carotenoids, flavonoids, and proline (Khataee et al. 2019). The increased TPC and TFC upon Fe and Zn NPs can be due to the significant role of these metal NPs in improving the biochemical pathways of TPC and TFC. Phenolic compounds have a significant role in scavenging reactive oxygen species (ROS) generated upon abnormal conditions (Tungmunthum et al. 2018). In this regard, Afshari et al. (2021) represented that silicon NPs were more effective than their conventional form in improving TPC and TFC in coriander plants.

Conclusions

Foliar application of iron and zinc nanoparticles has been found to be beneficial for Lemon balm (*Melissa officinalis* L.) in terms of plant growth, phenolic compounds, essential oil yield, and rosmarinic acid production. The use of these nanoparticles provides a more efficient and readily available source of iron and zinc ions, which are important for various biochemical processes in plants. This enhanced availability of micronutrients leads to improved plant growth, higher levels of phenolic compounds,

increased essential oil yield, and enhanced production of rosmarinic acid. The co-application of lead and zinc nanoparticles are effective in improving lemon balm yield via increasing essential oil yield, phenolic content, rosmarinic acid, and expression of corresponding genes. Overall, application of iron nanoparticles ($20\text{--}30\text{ mg L}^{-1}$) and zinc nanoparticles (120 mg L^{-1}) offers a promising approach to enhance the quality and quantity of lemon balm plants.

Declarations

Ethical Approval Ethical approval was not required for this work

Consent to Participate The author agreed to submit the manuscript to *Environmental Science and Pollution Research*

Consent to Publish The author approved the final manuscript to publish in *Environmental Science and Pollution Research*

Author Contribution The data were provided by Samaneh Farnosh, Nahid Masoudian, Akbar Safipour Afshar, Fatemeh Saeid Nematpour, Bostan Roud; The initial draft was prepared by Samaneh Farnoush and revised by the others.

Funding Not applicable

Competing Interests There is no competing interest

Availability of data and materials The data are available on request

Acknowledgements Not applicable.

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Figures

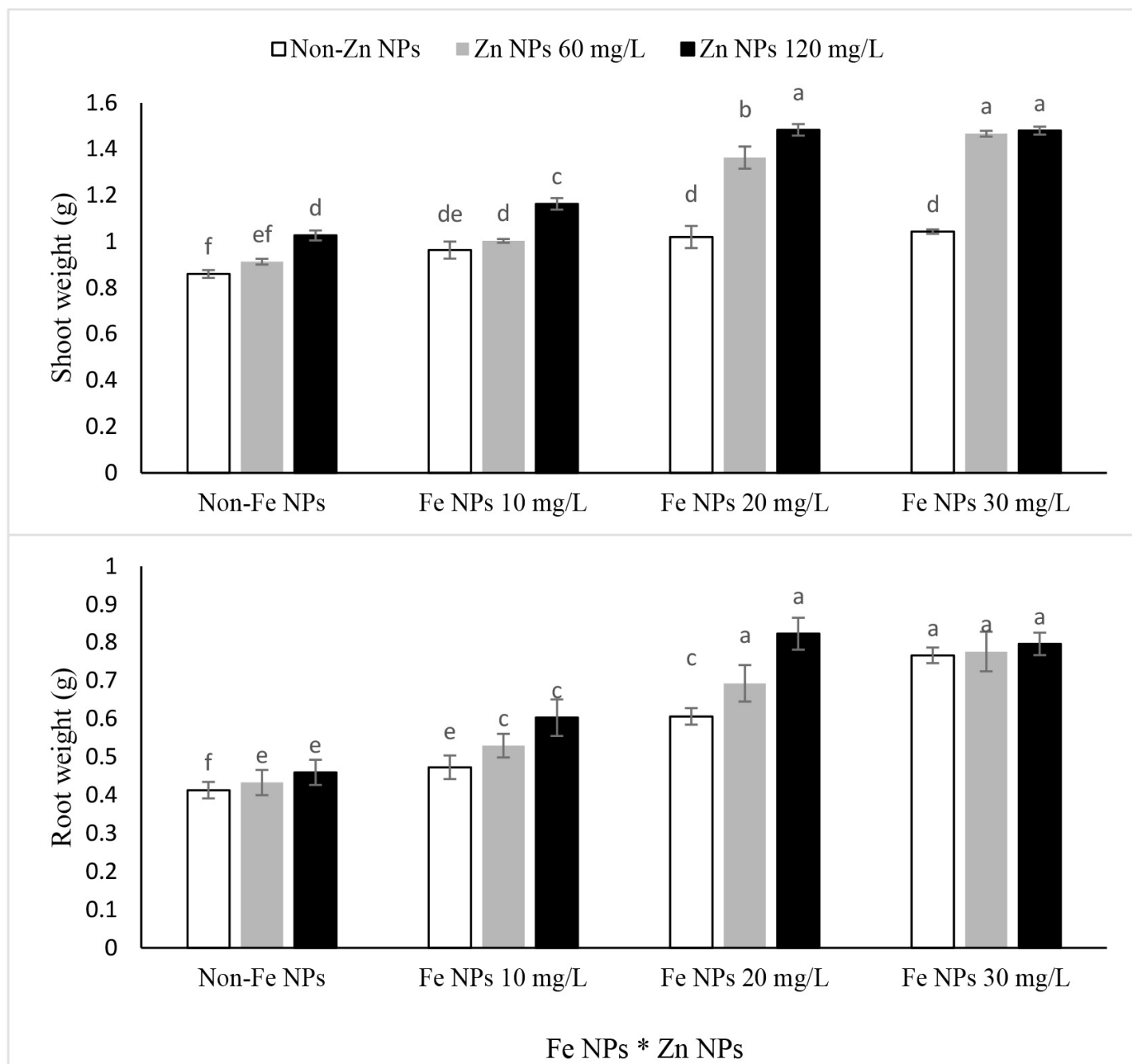


Figure 1

Iron (Fe) and zinc (Zn) nanoparticles on shoot weight (a) and root weight (b) of lemon balm plants. Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.

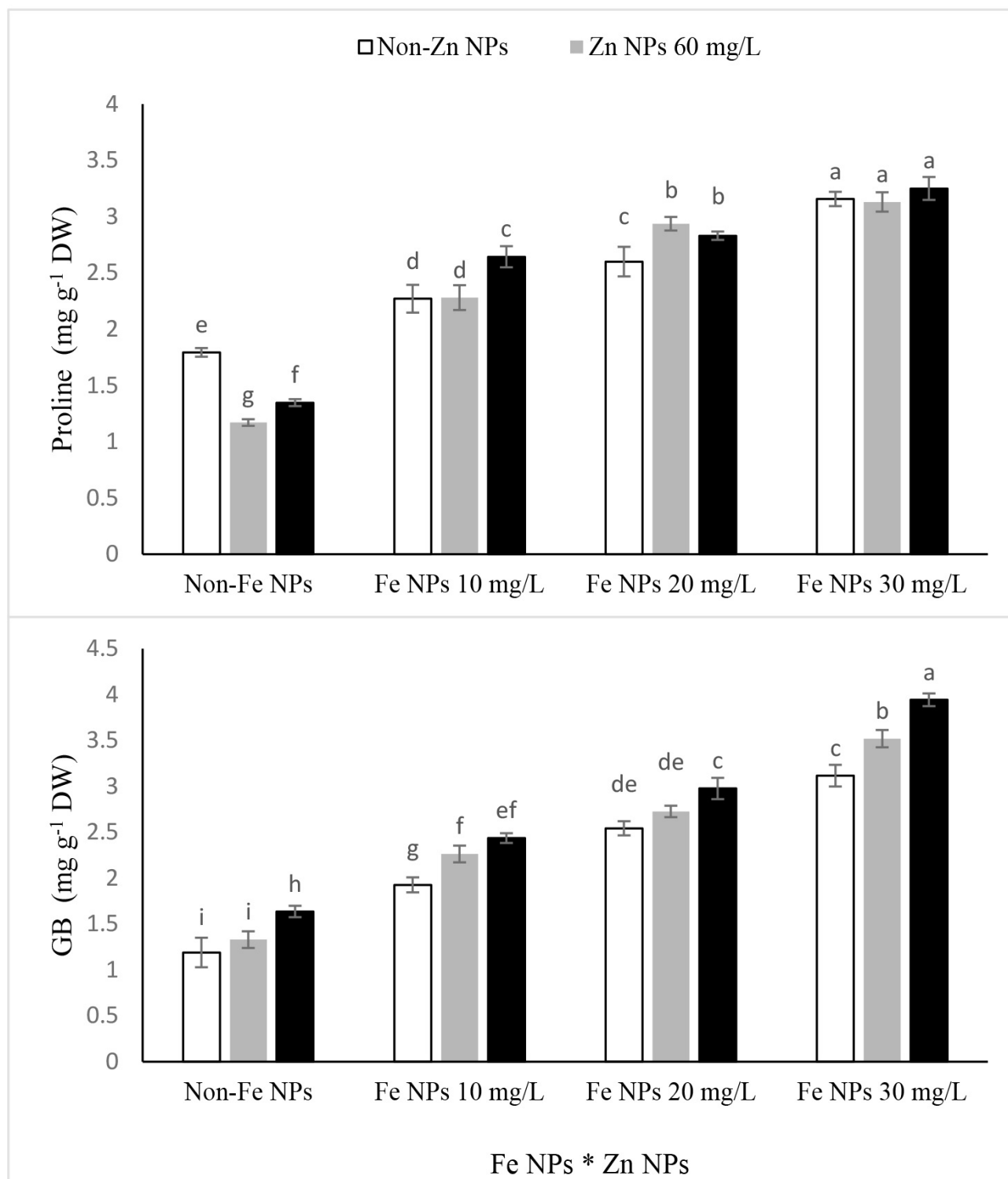


Figure 2

Iron (Fe) and zinc (Zn) nanoparticles on proline (a) and glycine betaine (GB, b) of lemon balm plants. Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.

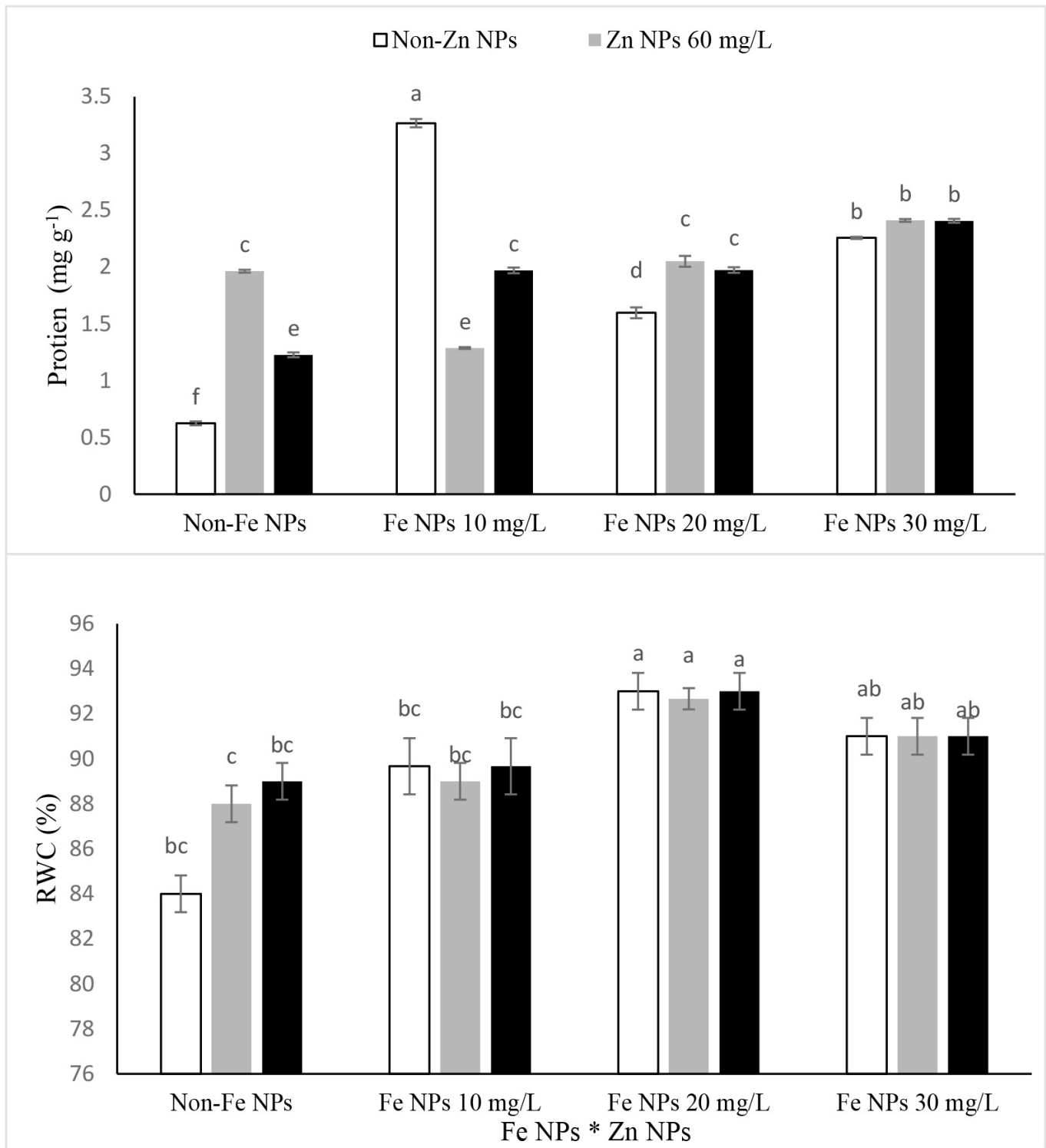


Figure 3

Iron and zinc nanoparticles on protein (a) and relative water content (RWC, b) of lemon balm plants. Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.

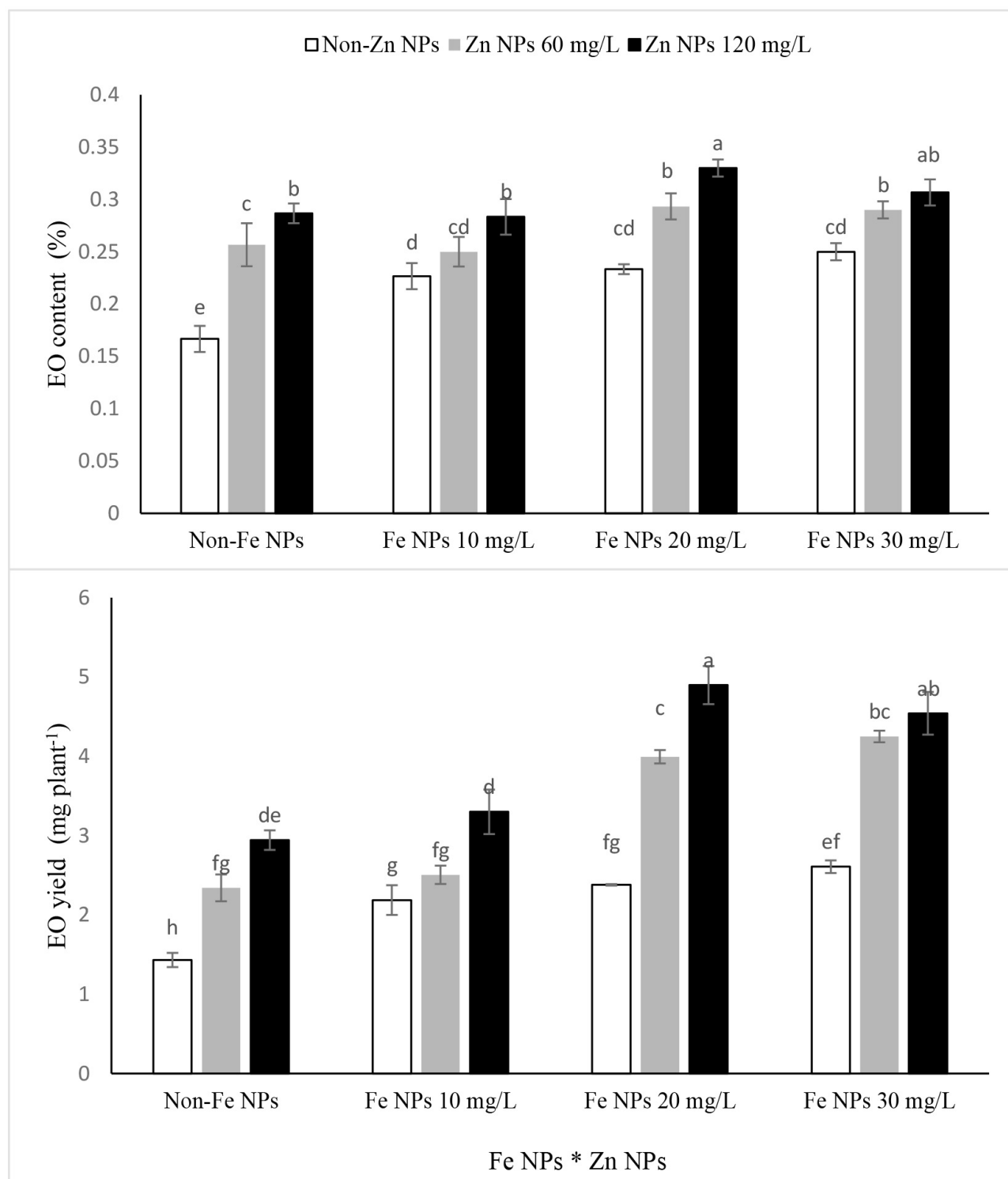


Figure 4

Iron and zinc nanoparticles on essential oil (EO) content (a) and EO yield (b) of lemon balm plants. Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.

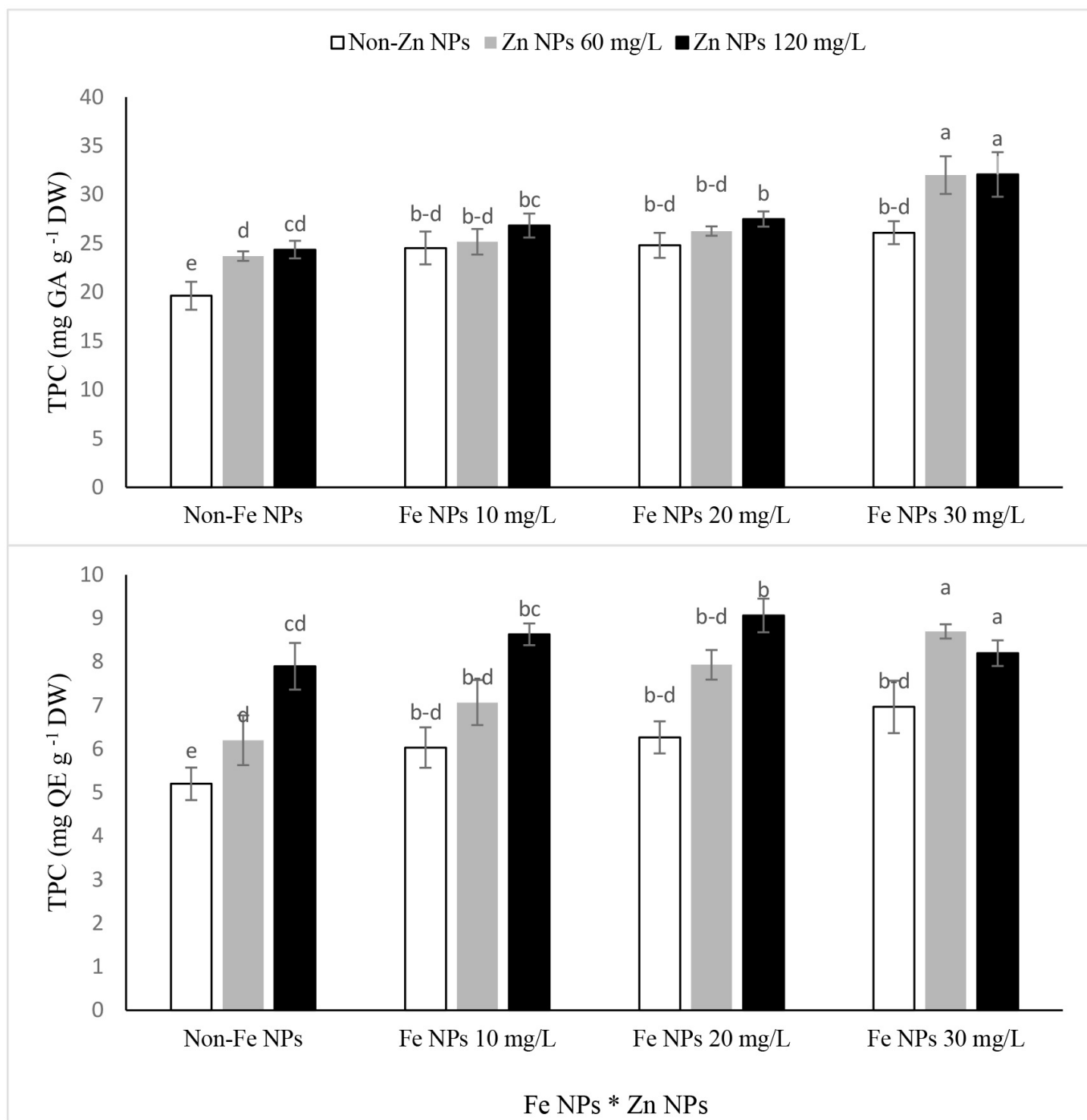


Figure 5

Iron and zinc nanoparticles on total phenolic content (TPC, a) and total flavonoid content (TFC, b) of lemon balm plants. Values are mean $\pm$ standard deviation in three replicates. Letters show significant difference between treatments at $P \leq 0.05$.

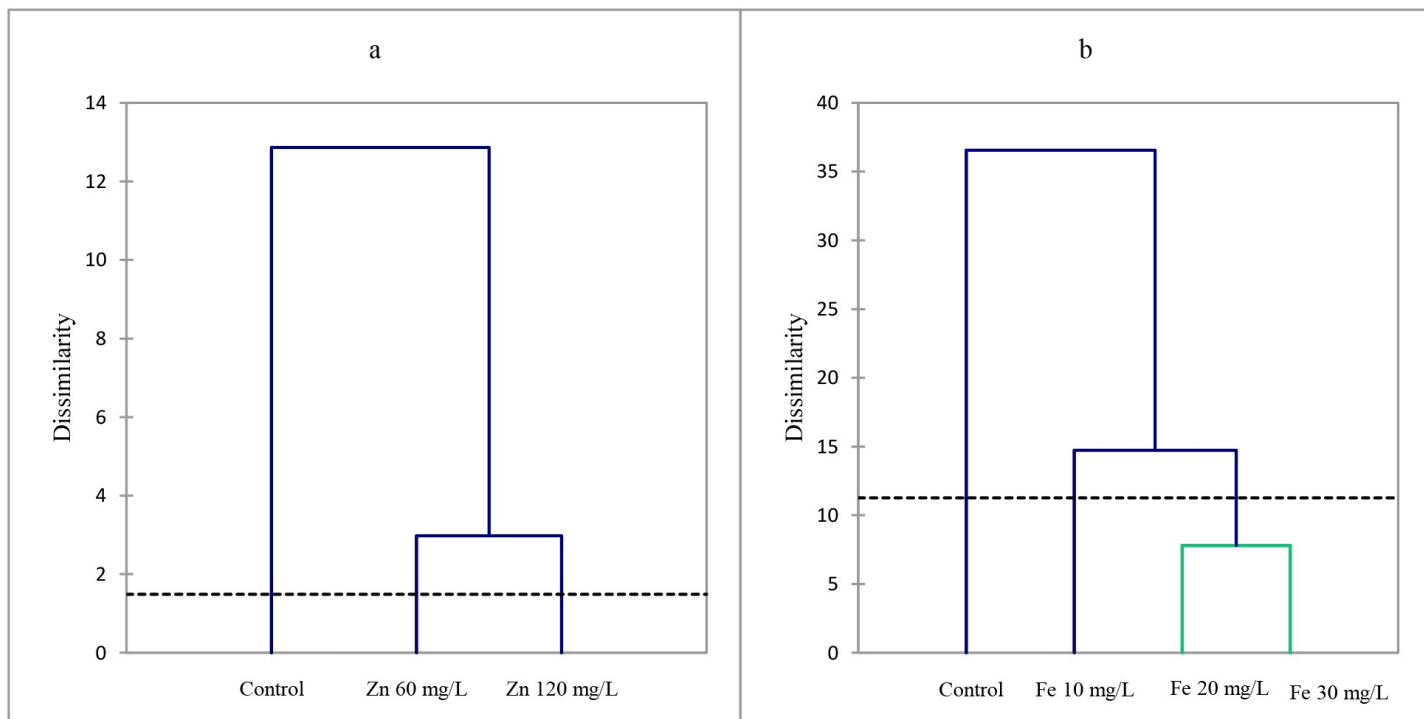


Figure 6

Agglomerative hierarchical clustering for zinc (a) and iron (b) nanoparticles

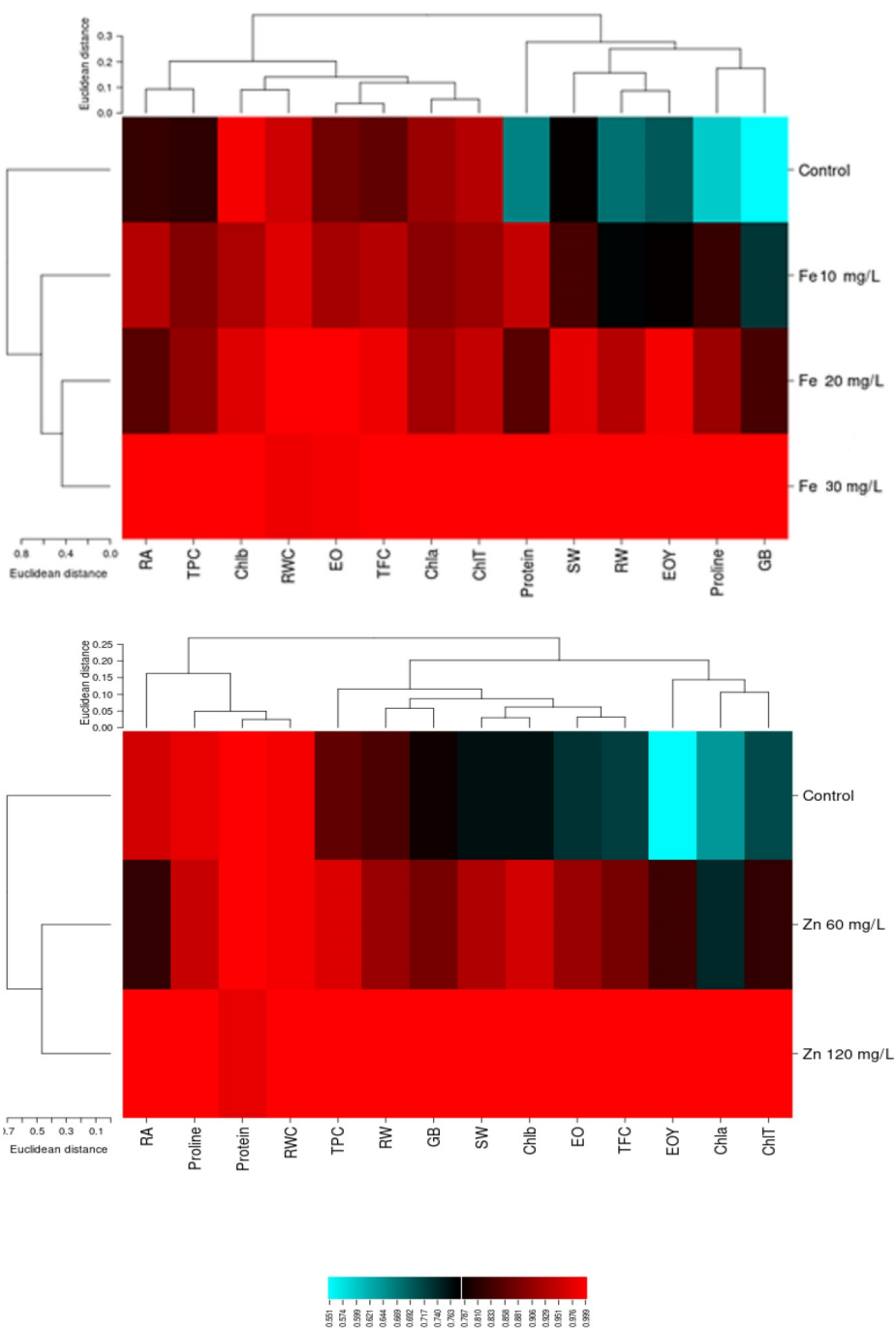


Figure 7

Heat map analysis for the studied traits. RA: rosmarinic acid, RWC: relative watwr content, TPC: total phenolic content, TFC: total flavonoid conetnet, EO: essential oil, EOY; essential oil yield, Chl: chlorophyll, BG: glycine betaine.