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Research Article

Physiological and biochemistry response of mustard greens (*Brassica juncea* var. Tosakan*)* to iron and zinc biofortification

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Abstract
Iron (Fe) and zinc (Zn) agronomically biofortification is required on mustard greens, as their nutritional value is still insufficient to meet human needs. This biofortification is anticipated to meet the dietary requirements of Zn and Fe to prevent the stunting phenomenon. This investigation aimed to examine the consequences of foliar Fe and Zn with varying concentrations (T_1 to T_{16}) on the physiology and biochemistry of biofortified mustard. The research was carried out in a greenhouse located in Karang-anyar, Central Java, Indonesia using a randomized block design (RBD) with two factors, namely Fe-EDTA and Zn-EDTA, each concentration of 0; 0.2; 0.4; 0.6 gl^{-1} with three replications. Fe and Zn were applied on the 21st day after sowing with foliar spray. The results showed that Fe biofortification up to a concentration of 0.6 g L^{-1} with or without a combination of Zn can increase physiological and biochemical parameters in mustard greens, namely photosynthetic rate up to 6.747 µmol m^{-2} s^{-1} (T_{15}) transpiration rate 0.653 µmol m^{-2} s^{-1} (T_5) stomatal conductance 18.854 µmol m^{-2} s^{-1}(T_5) number of stomata by 283.7 (T_{13}), stomatal opening width by 7.777 µm (T_{16}), iron content by 56.109 mg kg^{-1} (T_{14}), but Zn content can only increase when Zn is added alone without Fe, an increase in Zn up to 18.127 mg kg^{-1} (T_4).This study showed that Fe and Zn biofortification up to a concentration of 0.6 g L^{-1} was able to improve the biochemicals of mustard greens, especially their Fe and Zn contents and also the physiology of mustard greens.

Keywords: Biofortification, Foliar, Iron, Mustard greens, Zinc

INTRODUCTION

Stunting is a form of malnutrition closely linked to insufficient nutrient conditions (Nasriyah *et al*., 2023). The failure of human growth and development is viewed as a condition that occurs, accumulating both pre and post -birth as a result of insufficient nutrient intake (Awwalin *et al*., 2023; Rohman *et al*., 2023). A contributing factor to stunting is the lack of micronutrients like iron (Fe) and zinc (Zn), posing a health concern that impacts half of the worldwide population (Kiran *et al*., 2022).

Iron and zinc are vital minerals for the human body; people who suffer from significant and persistent iron and Zn deficiency can suffer physiological disorders, illness, and mortality (Clemens, 2014; Di Gioia *et al*., 2019). Iron is an important mineral for the body, participating in the synthesis of hemoglobin, the formation of myoglobin, and the synthesis of collagen and enzymes (Kristin *et al*., 2022). Zinc is crucial in numerous biological activities, as it is integral to essential enzyme activities like RNA polymerase, superoxide dismutase, and cellular signaling proteins (Chasapis *et al*., 2020).

According to the Ministry of Health Regulation of the Republik of Indonesia No. 28 of 2019 recommended nutritional adequacy for the Indonesian population, humans require Fe ranging from 7 mg/day^{-1}- 27 mg/day^{-1},

Zn in the human body is needed in a smaller amount than iron, approximately 3 mg/day^{-1} – 13 mg/day^{-1}. Humans need to meet the nutritional requirements for these nutrients to avoid malnutrition. Green vegetables are a common food source containing Fe and zinc frequently found in communities (Purwaningsih, 2020).

Mustard greens contain several nutrients, including Fe and Zn (Elsi *et al*., 2018).Although mustard greens contain Fe and Zn, there is still a need for nutritional enrichment with Fe and Zn (Buturi *et al*., 2021). This is because the amount of Fe and Zn in mustard greens is still insufficient to meet the nutritional needs of humans, with Fe content at 1.5 mg 100g^{-1} and Zn at 0.2 mg 100g^{-1} (Latifah *et al*., 2014).

Biofortification is an effort to increase the mineral content or other compounds in plants, particularly in parts that are consumed as food (Buturi *et al*., 2021). Agronomic biofortification becomes an alternative that can be pursued because, through this method, plants can respond quickly due to the high bioavailability of the microelements provided and it can be implemented through foliar fertilization (Stangoulis and Knez, 2022).The foliar spray technique is considered one of the effective techniques in agronomic biofortification, being evaluated as the best technique with cost efficiency (Consentino *et al*., 2023; Stangoulis and Knez, 2022).

Fe and Zn in the form of EDTA chelate represent a fertilizer with improved solubility compared to other forms, facilitating enhanced absorption through foliar application, as noted in prior research (Buturi *et al*., 2021). Studies investigating Fe and Zn biofortification consistently demonstrate that the EDTA form effectively increases Fe and Zn content compared to other formulations (Buturi *et al*., 2023; Zhao *et al*., 2019). Furthermore, EDTA chelate application minimises the risk of plant toxicity (Szerement *et al*., 2022). Hence, EDTA was selected for research on the biofortification of Fe and Zn thorugh foliar spray in mustard greens.Previous research on biofortification has been conducted on various leafy vegetable commodities such as arugula (Rugeles-Reyes *et al*., 2019), lettuce (de Moraes *et al*., 2022), and meanwhile, Di Gioia *et al*. (2019) successfully increased nutrients in some kinds of brassica vegetables such as microgreens and mustard, and other leafy vegetables. Based on this information, there is a need for biofortification in the mustard greens because there is no research related to biofortification in mustard greens. Not only that, mustard greens vegetables have a short lifetime (28 days) so have the advantage can be harvested faster and avoid losses during the growing period.

Hence, there is a need to research the biofortification of Fe and Zn in mustard greens. The objective is to augment the Fe and Zn content in mustard greens through foliar fertilization, utilizing the appropriate concentration. This study also seeks to assess the effect of biofortification from varying Fe and Zn concentrations on the elements' physiology and nutrition content. Thus, the optimal concentration for boosting or supplementing mustard greens' Fe and Zn nutrients can be determined.

MATERIALS AND METHODS

Research location

The research was carried out from July until August 2023 within the screen house at the Laboratory of the Faculty of Agriculture, Universitas Sebelas Maret, in Sukosari Village, Jumantono Subdistrict, Karanganyar Regency. The experimental site is geographically located at 7°37'48 "N 110°56'52 "E at an altitude of 170 metres above sea level. Meanwhile, plant physiology and biochemistry analysis also occurred at the Laboratory of Plant and Physiology and the Soil Chemistry Laboratory of Universitas Sebelas Maret.

Treatment and experimental design

The initial soil used as media had pH of 6.71, a total Fe content of 19200 mg.kg^{-1} and total Zn content of 85.44 mg.kg^{-1}. The experimental area used was 33 m^2 (3 m x 11 m) and the research employed a factorial randomized complete block design (RCBD) with two factors as the experimental design. The first factor is Fe-EDTA and the second factor is Zn -EDTA, Fe and Zn EDTA used in this study were purchased from a farm shop located around the study area. Fe EDTA and Zn EDTA concentrations were 0, 0.2, 0.4, 0.6 g L^{-1}, respectively. There were 16 treatment combinations and 3 replications, resulting in 48 experimental units. The specifics of the experiment treatments (T_1 to T_{16}) are shown in Table 1. The spraying was conducted on the 21st day after sowing (DAS).

The observed variables include gas exchange (photosynthesis and transpiration rate), stomatal conductance, number of stomata, stomata aperture width, and biochemistry content such as chlorophyll, Fe, and Zn.

Analysis of the parameters

Gas exchange

Measurement of these parameters utilized a gas exchange analysis tool, such as a photosynthesis meter, and was performed at 28 DAS. The measured variables included photosynthesis, transpiration, and stomatal conductance. These measurements were conducted on the third leaf from the shoot, specifically between 10 AM and 12 PM (Zhang *et al*., 2017).

Number of stomata and stomata aperture width measurement of these parameters was carried out at 28 DAS with the method of making replicas of stomata using the nail polish method (Forrest, 1962). The un-

Table 1. Treatment details of the field experiment

Treatments	Details of Treatment
T_1	0 g L^{-1} Fe EDTA + 0 g L^{-1} Zn EDTA (Control)
T_2	0 g L^{-1} Fe EDTA + 0.2 g L^{-1} Zn EDTA
T_3	0 g L^{-1} Fe EDTA + 0.4 g L^{-1} Zn EDTA
T_4	0 g L^{-1} Fe EDTA + 0.6 g L^{-1} Zn EDTA
T_5	0.2 g L^{-1} Fe EDTA + 0 g L^{-1} Zn EDTA
T_6	0.2 g L^{-1} Fe EDTA + 0.2 g L^{-1} Zn EDTA
T_7	0.2 g L^{-1} Fe EDTA + 0.4 g L^{-1} Zn EDTA
T_8	0.2 g L^{-1} Fe EDTA + 0.6 g L^{-1} Zn EDTA
T_9	0.4 g L^{-1} Fe EDTA + 0 g L^{-1} Zn EDTA
T_{10}	0 .4 g L^{-1} Fe EDTA + 0.2 g L^{-1} Zn EDTA
T_{11}	0.4 g L^{-1} Fe EDTA + 0.4 g L^{-1} Zn EDTA
T_{12}	0.4 g L^{-1} Fe EDTA + 0.6 g L^{-1} Zn EDTA
T_{13}	0.6 g L^{-1} Fe EDTA + 0 g L^{-1} Zn EDTA
T_{14}	0.6 g L^{-1} Fe EDTA + 0.2 g L^{-1} Zn EDTA
T_{15}	0.6 g L^{-1} Fe EDTA + 0.4 g L^{-1} Zn EDTA
T_{16}	0.6 g L^{-1} Fe EDTA + 0.6 g L^{-1} Zn EDTA

derside of the leaf was evenly coated with nail polish and allowed to dry for approximately 3–5 minutes. Next, the dried nail polish layer was carefully peeled off using adhesive tape and attached to a microscope slide to be counted under a microscope at 10x magnification. The number of stomata was counted using computer software called Image Raster. Observations of the width of stomatal openings were made using a microscope with 40x magnification, and measurements of stomatal openings were made using Image Raster software on a computer.

Chlorophyll Content (mg/g)

Chlorophyll content was measured by extracting leaf samples collected 28 DAS, using acetone as the extracting agent. The measurement of chlorophyll content refers to the method outlined by Arnon (1949).

Iron (Fe) and zinc (Zn) nutrient content

Iron and zinc content in mustard greens were determined on the 28th day after sowing using leaf and stem samples. The methodology followed Eviati and Sulaeman (2009) protocols for chemical analyses. Plant samples were digested with nitric acid and perchloric acid, then heated gradually up to 200°C. After cooling and dilution, the extract was analyzed using Atomic Absorption Spectrophotometry for Fe and Zn concentrations.

Data analysis

The data collected were analyzed using the variance analysis (ANOVA) method using SPSS version 25.0 statistical software. In the event of significant differences between groups, continued analysis using Duncan's multiple range test, with the significance threshold set at α = 5%.

RESULTS AND DISCUSSION

Photosynthesis rate

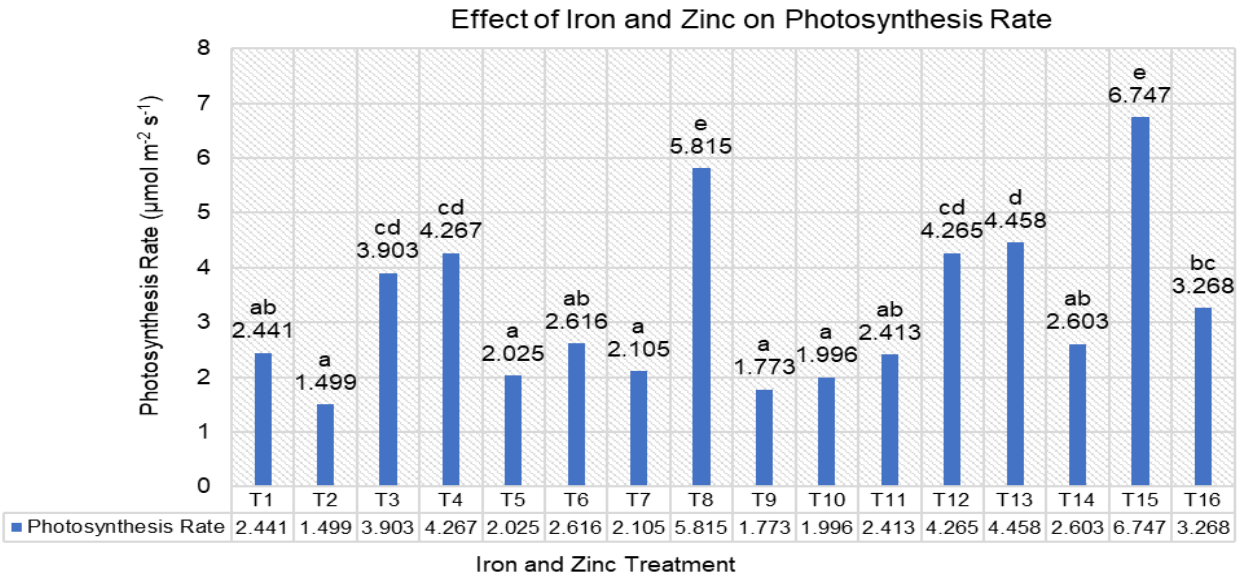
Photosynthesis is the process of light uptake by plants to be converted into chemical energy and stored as carbohydrates. Photosynthesis is influenced by several factors, such as water availability, temperature, leaf age, carbohydrate translocation, and CO2 availability (Wimalasekera, 2019). Fe and Zn spraying on mustard greens significantly affected plants' photosynthetic rate (Table 2). It is evident that the foliar spraying of Fe and Zn on mustard greens displays a significant impact on enhancing the plant's photosynthetic rate (Fig. 1). The highest photosynthetic rate is observed in the treatment T_{15} (Fe 0.6 gl^{-1} + Zn 0.4 gl^{-1}), reaching 6.747 µmol m^{-2} s^{-1}. This treatment also exhibited a statistically significant difference when compared to the control plants, showcasing a remarkable 176.4% increase in the photosynthetic rate. Fe and Zn are necessary micronutrients that are engaged in metabolic and catalytic processes and play an important function in photosynthetic processes (Bhatla and Lal, 2023). Iron serves as a key component of ferredoxin and cytochrome, playing a vital role in photosynthesis (Sharma *et al*., 2023), while Zn is a nutrient supporting the formation of NADPH or NADH, which are involved in converting radiant energy for the subsequent use in the plant's photosynthetic process (Ayyar *et al*., 2020).

An elevation in the photosynthetic rate in plants is also noted in corn plants subjected to micro-fertilizer $FeSO_4.7H_2O$ + $ZnSO_4.7H_2O$ at different concentrations. The most substantial increase in the photosynthetic rate was observed with the treatment of 20% $FeSO_4.7H_2O$ + $ZnSO_4.7H_2O$, leading to a 41% augmentation compared to the control plants (Mugenzi *et al*., 2018). Plants that are biofortified with Fe and Zn exhibit an enhanced photosynthetic rate.This is be-

Table 2. ANOVA (mean of squares) for physiological parameters affected by biofortification of iron and zinc

Source	DF	Photosynthesis rate	Transpiration rate	Stomatal conductance	Number of stomata	Stomata opening
Replication	2	0.300^{ns}	0.007^{ns}	7.561*	3278.896^{ns}	1.093^{ns}
Iron Concentrations (Fe)	3	6.026*	0.030^{ns}	42.949*	4964.306*	6.773*
Zinc Concentrations (Zn)	3	12.420*	0.078*	103.765*	1554.028^{ns}	1.191^{ns}
Fe*Zn	9	5.373*	0.063*	35.428*	1580.102^{ns}	1.938*
Error	30	0.390	0.013	1.943	997.14	0.474

ns: non-significant, *: significant at 5%, respectively



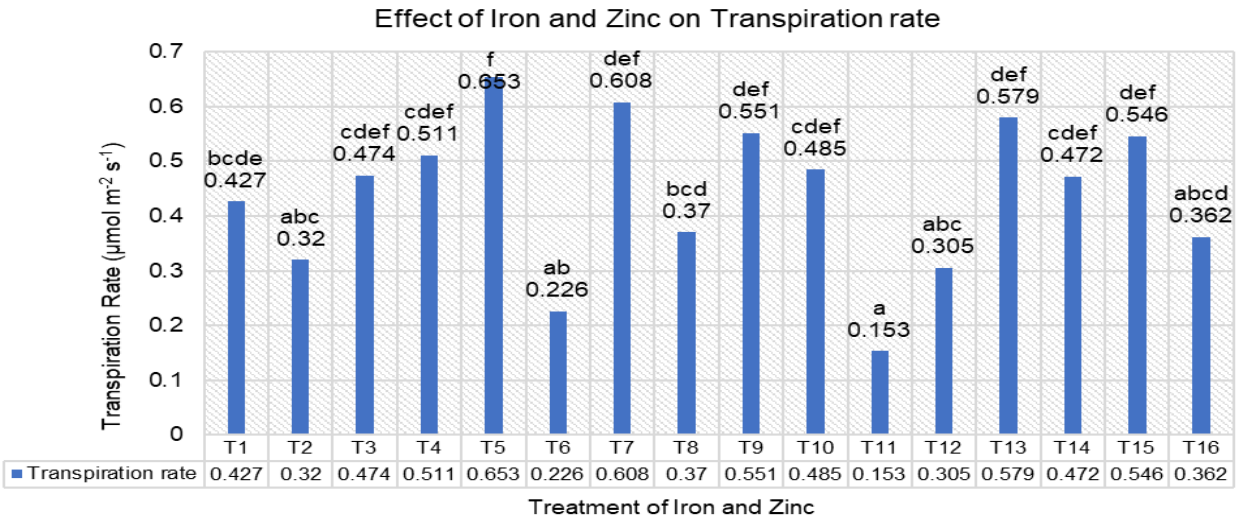
Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 1. *Effect of iron and zinc biofortification on photosynthesis rate*

cause Fe and Zn are crucial micronutrients for plants, playing vital roles in photosynthesis and respiration processes for various life-sustaining (Marian and Tuhuteru, 2019; Sharma *et al*., 2023). Therefore, when plants are supplied with Fe and Zn, their metabolic activities, including photosynthesis, are optimized, leading to an increased photosynthetic rate (Ma *et al*., 2017; Ma *et al*., 2019). Through respiration, the products of photosynthesis are converted into energy, which is then used by plant cells to perform functions like cell division and enlargement, so that this will have a postitive impact on plants. (Yunus et al., 2018).

Transpiration rate

Transpiration is the process of water loss in the form of water vapour that occurs in plant tissues and transpiration occurs right in the stomata. Transpiration is also the release of H_2O and CO_2 vapour, and the faster a plant's transpiration rate is, the faster the transport of water and dissolved nutrients will be (Silaen, 2021). This study showed that Fe and Zn spraying significantly affected the transpiration rate of plants (Table 1) and based on the graph (Fig. 2), it was observed that there was an increase in the transpiration rate in plants sprayed with Fe and Zn, namely in the T_5 treatment . The T_5 treatment (Fe 0.2 gl^{-1} + Zn 0 gl^{-1}) exhibits the highest recorded transpiration rate, measuring 0.653 µmol m^{-2} s^{-1}. This result indicates a significant difference compared to the plants without Fe and Zn treatment, as there was a notable increase of 52.9% in the transpiration rate. The transpiration rate in plants exposed to the combined treatment of Fe and Zn also showed an increase. Specifically, in the T_7 treatment (Fe 0.2 gl^{-1} + Zn 0.4 gl^{-1}), the transpiration rate



Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 2. *Effect of iron and zinc biofortification on transpiration* ***rate***

reached 0.608 µmol m^{-2} s^{-1}. However, this increase does not exhibit a statistically significant difference compared to the control plants.

Transpiration occurring at stomata is the major contributor to plant water loss, although some evaporation also occurs on the leaf cuticle and young stem (Creek *et al*., 2020; Díaz-Pérez, 2019). Leaves are crucial in transpiration (Ningsih and Entin Daningsih, 2022). Foliar spraying of Fe and Zn on leaves increases stomatal opening, improving enzymatic activities (Hafiz *et al*., 2021; Hussein and Alva, 2014). Additionally, Zn foliar spraying on plants can elevate stomatal activity, ultimately leading to an increased transpiration rate (Mahmoud *et al*., 2022; Saboor *et al*., 2021; Subba *et al*., 2014).

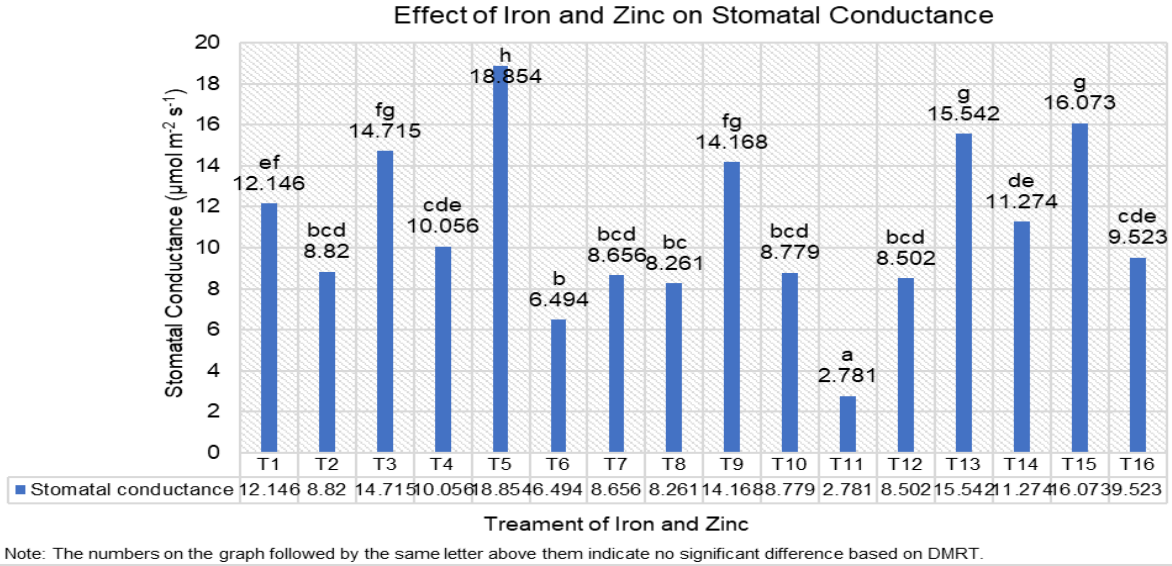
The transpiration rate has also been augmented in potato plants treated with foliar Fe EDTA, showing a 118% increase from the control plants (Zhang *et al*., 2022). The reported increase in transpiration rate due to the simultaneous application of Fe and Zn was documented by Zaheer *et al*.(2020), specifically in Japanese spinach plants treated with10 mgl^{-1} Zn-lysine + 5 mgl^{-1} Fe-lysine, resulting in a 31% increase in the transpiration rate compared to plants without Fe and Zn treatments.

Stomatal conductance

Stomatal conductance refers to the stomata's ability to release gas or water vapour, and it is intricately linked to the flexibility of the guard cells. A higher stomatal conductance value signifies an optimal state for photosynthesis, reflecting the ease with which stomata exchange CO2 gas (Herawati *et al*., 2022; Soleh *et al*., 2020). Fe and Zn treatments on mustard green plants significantly affected stomatal conductance (Table 2). Referring to the data presented in Fig. 3, an upsurge in stomatal conductivity was noticed in plants treated with Fe and Zn. This treatment also showed that the treatment labelled as T_5 (Fe 0.2 gl-1 + Zn 0 gl-1) displayed the highest stomatal conductance among all the treatments, measuring 18.854 µmol m^{-2} s^{-1}. The stomatal conductance in the T_5 treatment (Fe 0.2 gl^{-1} + Zn 0 gl^{-1}) showed a remarkable 55.2% increase upon comparison with control plants, and the results of this treatment were also significantly different. Significant increases in stomatal conductance were also observed in the T_{13} (Fe 0.6 gl^{-1} + Zn 0 gl^{-1}) and T_{15} (Fe 0.6 gl^{-1} + Zn 0.4 gl^{-1}) treatments, measuring 15.542 and 16.073 µmol m^{-2} s^{-1}, respectively. Compared with control plants, these represent a 28% and 32.3% rise in stomatal conductance parameters. Stomatal conductance refers to the ability of stomata to open and close or the ease with which stomata exchange CO2 gas (Pertiwi and Soverda, 2012). A higher stomatal conductance value indicates that the plant is in an optimal state for photosynthesis (Soleh *et al*., 2020).

Fe can increase stomatal conductance in plant leaves by reason of its function in plant physiology (Kobayashi *et al*., 2019). Iron has a crucial function in the production of chlorophyll and participates in numerous enzymatic functions; when photosynthetic activity intensifies, there is a greater need for carbon dioxide, resulting in the opening of stomata to facilitate increased carbon dioxide absorption (Zewide and Sherefu, 2021). This is congruent with El-Desouky *et al*. (2021), which shows an increase in stomatal conductance in tomato plants treated with Fe chelate at 50 mg kg^{-1} and 100 mg kg^{-1}. Specifically, they reported a 7.73% increase with the 50 mg kg^{-1} dose and a 1.37% increase with the 100 mg kg^{-1} dose.

Fe and Zn serve as catalysts for enzymes involved in the transfer of oxygen and electrons, further contributing to the observed rise in stomatal conductance (Nandal and Solanki, 2021; Zewide and Sherefu,



Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 3. *Effect of iron and zinc biofortification on stomatal conductance*

2021). An increase in stomatal conductance has also been reported by Iqbal *et al*. (2019) stomatal conductance as a result of Fe and Zn treatments in drought-stressed wheat plants, showing a 53.6% increase compared to plants without treatment of Fe and zinc. The increased stomatal conductance is thought to be attributed to the production of auxins triggered by the presence of these elements, enabling the plants to withstand drought stress (Ghaffar *et al*., 2011).The increased stomatal conductance is thought to be attributed by auxin production triggered by the presence of these elements, the hormone auxin also plays a role in morphogenesis so that crop plants are able to withstand drought stress (Ghaffar *et al.,* 2011; Prabowo *et al.,* 2018)

Number of stomata

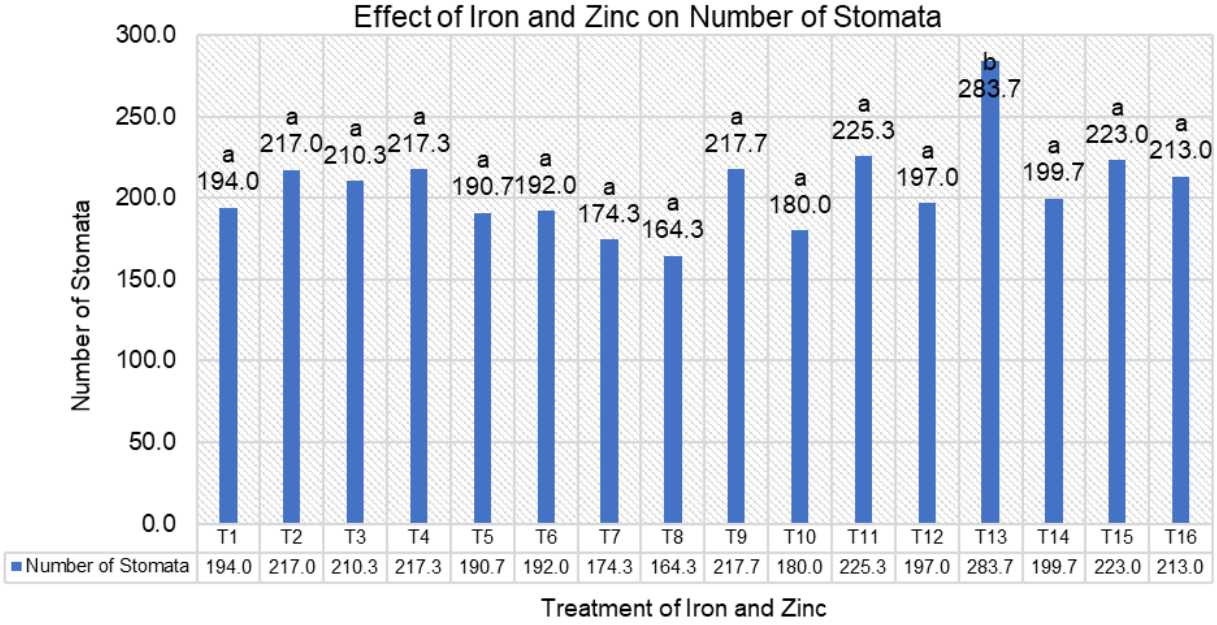
The stomata on leaves are essential to the physiology of plants because they allow for the exchange of CO2 during photosynthesis and the transpiration of water vapour. Both internal and external variables can affect a leaf's stomata count (Dama *et al*., 2020). In this study, Fe and Zn sprayed on mustard greens did not give significant results on the number of stomata (Table 2), although the graph shows an increase in the number of stomata from the control plant (Fig. 4).The treatment labelled as T_{13} (Fe 0.6 gl^{-1} + Zn 0 gl^{-1}) displayed the highest number of stomata, totalling 283.7 stomata. However, the remaining treatments did not exhibit statistically significant differences compared to the control treatment (T_1). The increase in the number of stomata, which is 46% of the control, may be attributed to the possibility that Fe plays a more direct or significant role in influencing stomatal density than Zn in green mustard plants. This finding aligns with Feng *et al*. (2022), who investigated the application of Fe_3O_4 on wheat plants. In contrast, applying varying concentrations of Zn through spraying did not significantly increase stomatal density.

The finding that Fe and Zn had no effect on the number of stomata in mustard greens may be because plant stomata are influenced by other variables, such as plant genetics and the surrounding environment, not solely dependent on plant nutrition. This is in line with Aryandhita and Kastono (2021); Dama *et al*. (2020), who stated that stomatal density in plants is generally influenced by both environmental and genetic factors and the increase in stomatal plants represents an adaptation to the surrounding environment, which occurs as a response to increased evaporative demands.

For instance, plants may be more vulnerable to the development of diseases like fungus or bacteria in highly humid environments (Dinda *et al.,* 2020). Plants can increase the number of stomata on their leaves in an effort to lower the danger of infection by pathogens through increased air circulation along the leaf surface. this increase in stomatal number seeks to lower humidity levels and inhibit the growth of diseases that need high humidity to proliferate (Aung *et al.,* 2018).

Stomatal aperture width

In this study, Fe and Zn spraying on mustard greens significantly affected the width of the stomatal aperture (Table 2). The graph in Fig. 5 also shows an increase in stomatal aperture width in green mustard plants under Fe and Zn treatments. The largest stomatal aper-



Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 4. *Effect of iron and zinc biofortification on number of stomata*

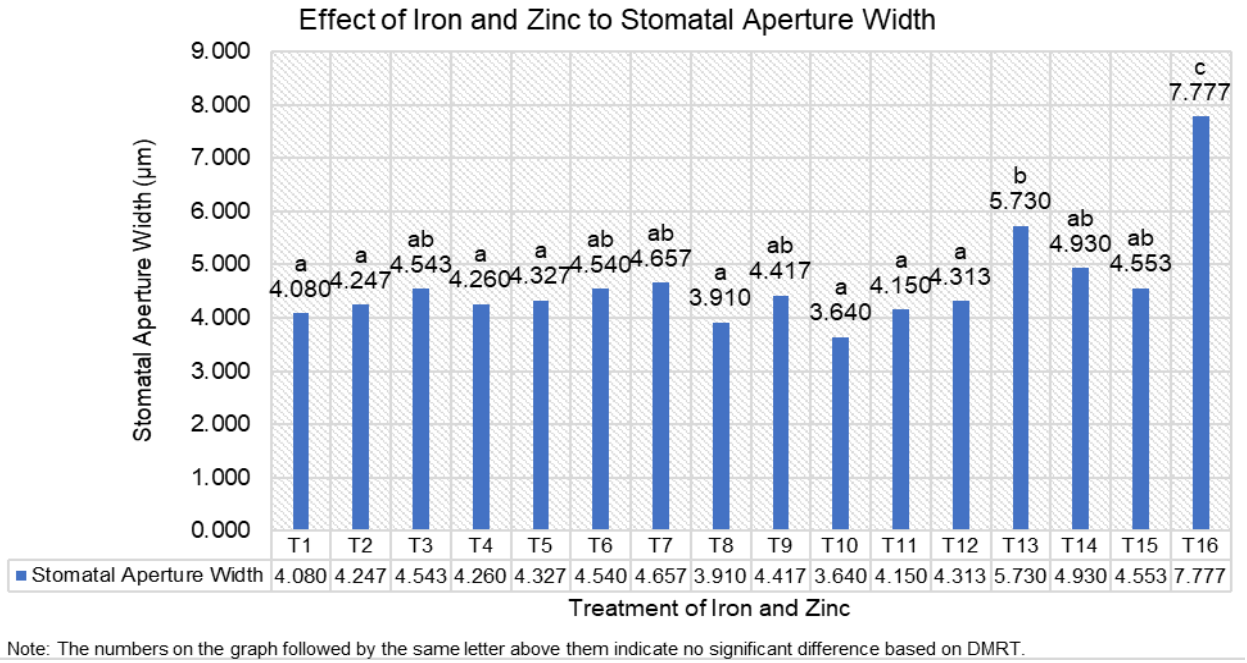


Fig. 5. *Effect of iron and zinc biofortification on stomatal aperture width*

ture width was observed in the T_{16} treatment (Fe 0.6 gl^{-1} + Zn 0.6 gl^{-1}), measuring 7.777µm and showed significant differences compared with control plants. The significant increase in the number of stomata was also found in treatment T_{13}, where the stomatal count increased by 40.4% compared to its control plants. Based on these results, it can be inferred that the stomatal aperture width can be increased by spraying a combination of Fe and Zn and by applying either Fe or Zn alone, in comparison with the control.

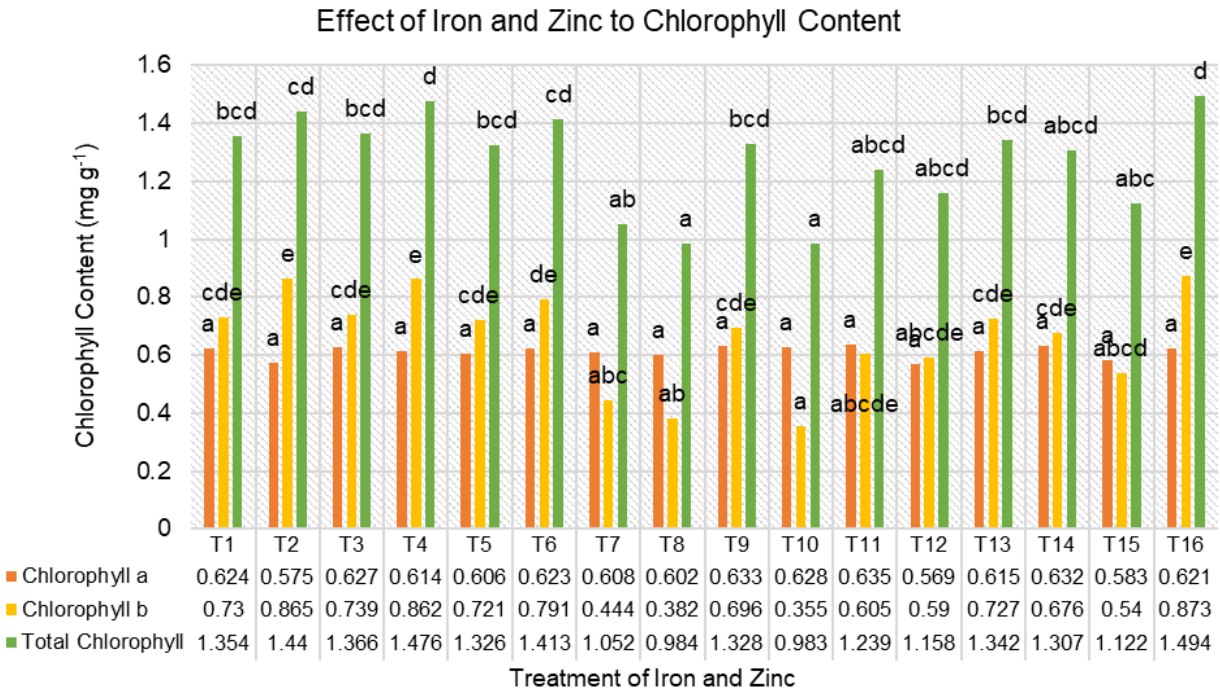
The spraying of Fe and Zn on plants can increase the stomatal aperture width in green mustard plants. This finding corroborates the statement made by Mannan *et al*. (2022), who stated that Zn contributes as a function in maintaining potassium levels in guard cells, as well as Fe, which can increase the concentration of osmoregulatory in plant cells, leading to higher osmotic changes. The increased activity of plasma membrane H+-ATPase, which promotes stomatal opening in the leaves, can be attributed to the widening of the stomatal aperture in plants treated with Fe spraying (Khan *et al*., 2020).

H+-ATPase has a function in regulating stomatal aperture and utilizes energy from ATP hydrolysis to generate a proton concentration gradient along the plasma membrane (Li *et al*., 2022); when plasma membrane H+-ATPase is active, protons are pumped out of the guard cells, creating a higher proton concentration gradient outside those cells consequently, the influx of potassium ions (K+) into the guard cells triggers an increase in water osmosis, enhancing cell turgor pressure (Kabała and Janicka, 2023; Weng *et al*., 2020). This process ultimately results in the swelling of the guard cells and a wider opening of the stomata.

Chlorophyll content

According to the data shown in Fig. 6, iron and zinc treatments increased chlorophyll content compared to the control treatment. However, the increase in all chlorophyll, such as chlorophyll a, b, and total chlorophyll, showed no statistically significant differences compared to a control plant because, based on the analysis of variance, Fe and Zn did not significantly affect the chlorophyll content in mustard greens (Table 3). The T_{11} (Fe 0.4 gl^{-1} + Zn 0.4 gl^{-1}) treatment exhibited the highest chlorophyll a content at 0.635 mg/g, while the T_{16} (Fe 0.6 gl^{-1} + Zn 0.6 gl^{-1}) treatment displayed the highest chlorophyll b content at 0.873 mg g^{-1}, this treatment also demonstrated the highest total chlorophyll content at 0.494 mg g^{-1}.

Iron and zinc have roles as catalytic enzymes and proteins, thereby promoting chlorophyll synthesis (Wani *et al*., 2022). According to Jalal *et al*. (2020), Fe has a role in various physiological processes, including chlorophyll formation. A comparable enhancement in chlorophyll content has also been observed in peppermint plants sprayed with 0.5 gl^{-1}, 1 gl^{-1} and 1.5 gl^{-1} concentrations of Fe chelated, resulting in a 44.4%, 36.11% and 19.4% increase in total chlorophyll content from the control plants (Lafmejani *et al*., 2018). Similar findings were reported in a study of wheat plants sprayed with Fe EDTA by 1%, 1.5% and 1.5%, experiencing a 33.9%, 51.9% and 75.6% increase in chlorophyll content compared to control treatment (Ru *et al*., 2018). Zinc can boost the functionality of enzymes and other vital components in the chlorophyll synthesis process (Kavian *et al*., 2022). Zinc enhances the activity of catalase and tryptophan synthesis enzymes and other enzymes involved in chlorophyll synthesis (Jha, 2019).



Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 6. *Effect of iron and zinc biofortification on chlorophyll content*

Consistent with these findings, Ru *et al*. (2018) revealed an increase in total chlorophyll content ranging from 6% to 35% due to Zn EDTA application at concentrations of 0.5-1.5%. %. Further supporting evidence is provided by Tolay (2021), who reported a substantial 26% increase in total chlorophyll content in basil plants through Zn spraying at a concentration of 5 mg kg^{-1}, compared with control.

Contrary to expectations, the total chlorophyll content of biofortified mustard greens supplemented with Fe and Zn did not show any significant difference. This finding is consistent with a study on wheat by Ali *et al*. (2022). It is worth noting that factors other than Fe and Zn, such as environmental conditions, may affect determining the total chlorophyll content, as Nikitovic *et al*. (2021) suggested.

Iron content

Biofortification of Fe and Zn in mustard greens showed significant results on the iron content of mustard greens (Table 3). Based on the graphic (Fig.7), it is evident that the highest iron content was found in the T_{14} treatment (Fe 0.6 gl^{-1} + Zn 0.2 gl^{-1}) with an iron content of 56.109 mg kg^{-1}. This treatment significantly differed from T_1 treatment (control), with a 137.3% increase in iron content. Significant differences in iron content are also observed in the T_9 (Fe 0.4 gl^{-1} + Zn 0 gl^{-1}) and T_{13} (Fe 0.6 gl^{-1} + Zn 0 gl^{-1}) treatments, with Fe contents of 44.888 and 54.859 mg kg^{-1}, respectively. These represent a 90% and 137% increase in Fe content compared with control plants. The spraying of Fe on plants can enhance their ability to absorb and utilize Fe (Y. Zhang *et al*., 2023). After Fe is translocated to all parts of the plant, it will accumulate in edible parts (Giordano *et al*., 2019).

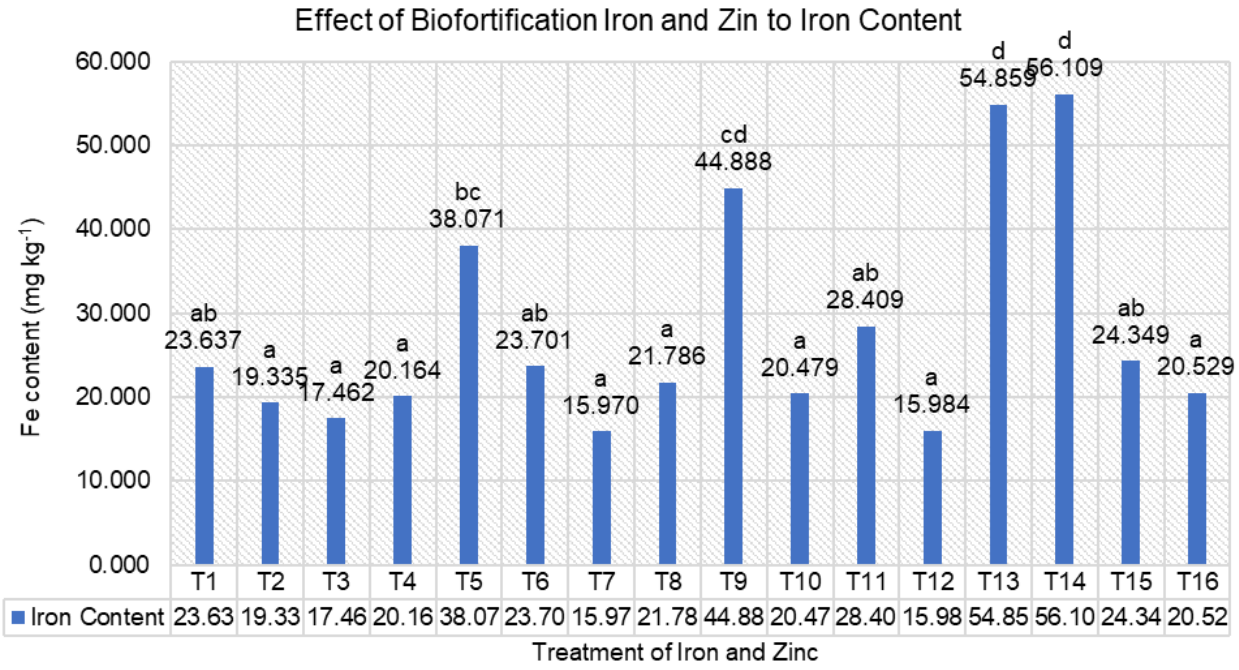
Indeed, the study conducted by Taskin and Gunes (2022) observed a similar trend of increased Fe content in wheat when foliar spraying of Fe chelate was employed. Their research revealed a remarkable 89% increase in Fe content compared with the control group. The findings closely resembled the results obtained by Giordano *et al*. (2019), who explored the impact of adding Fe chelate at concentrations of 1.0 mM and 2.0 mM to the nutrient solution used for lettuce cultivation. They reported a significant rise in Fe content, with increases of 20.5% and 53.7%, respectively.

The enhanced bioavailability of these micronutrients in plants may be ascribed to the enhancement in Fe lev-

Table 3. ANOVA (mean of squares) for biochemistry parameters affected by biofortification of iron and zinc

SOV	DF	Chl a	Chl b	Chla+b	Iron content	Zinc content
Replication	2	0.003^{ns}	0.012^{ns}	0.012^{ns}	91.994^{ns}	7.426^{ns}
Iron Concentrations (Fe)	3	0.000*	0.147*	0.143*	766.973*	74.12*
Zinc Concentrations (Zn)	3	0.001^{ns}	0.040^{ns}	0.042^{ns}	1073.367*	39.226*
Fe*Zn	9	0.909^{ns}	0.073*	0.078*	265.375*	4.777^{ns}
Error	30	0.002	0.028	0.031	69.182	12.5

ns: non-significant, *: significant at 5%, respectively



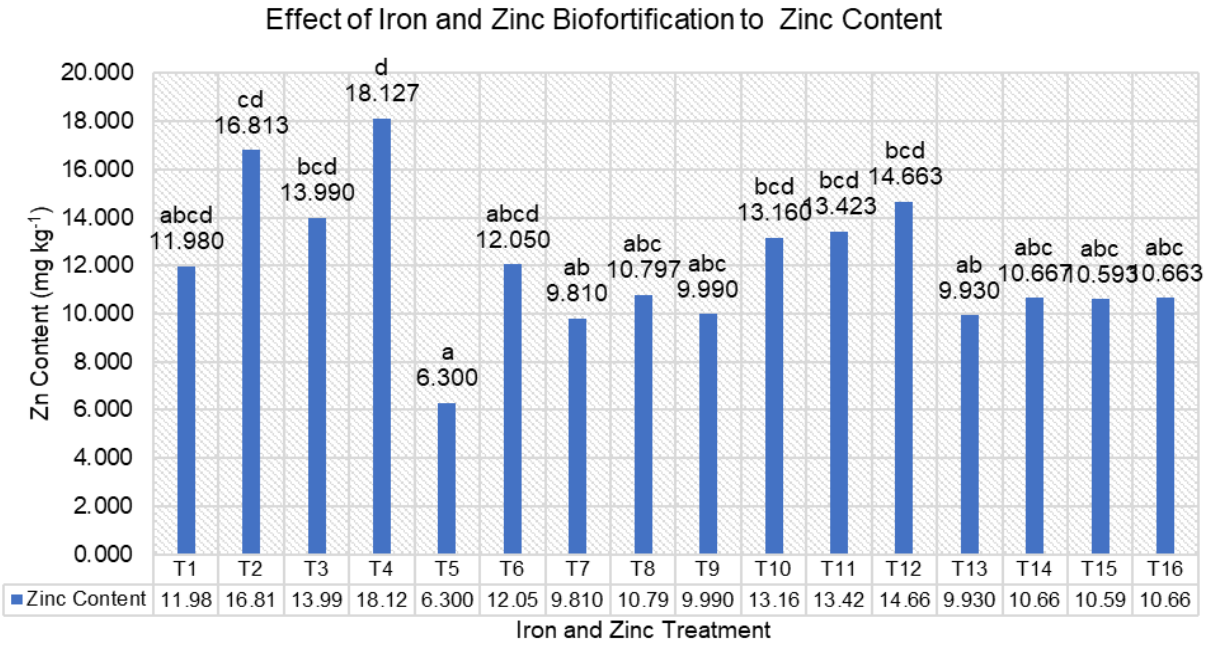
Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 7. *Effect of iron and zinc biofortification on iron content*

els observed in plants with Fe and Zn spraying treatments.This is consistent with Dhaliwal *et al*. (2021), where foliar spraying of Fe combined with Zn enhanced average Fe content from 4% to 11% compared to the plants without Fe and Zn treatment. In this context, Zn is also important in plants for various physiological and bio-physiochemical processes, which helps absorb Fe that may already be present in the plant or the growing medium (Rahayu *et al.,* 2021; Saleem *et al.,* 2022). The integrated treatment of Fe and Zn can synergistically enhance the plants' uptake and utilization of these micronutrients, leading to increased Fe content.

Zinc content

Fe and Zn spraying on mustard greens did not demonstrate any interaction concerning the Zn content; however, each treatment of Fe and Zn independently significantly affected the mustard greens' Zn content (Table 3). The graph (Fig. 8) showed that the highest Zn content in green mustard is found in the T_4 treatment (Fe 0



Note: The numbers on the graph followed by the same letter above them indicate no significant difference based on DMRT.

Fig. 8. *Effect of iron and zinc biofortification on zinc content*

g L^{-1} + Zn 0.6 g L^{-1}), with a Zn content of 18.127 mg/ kg^{-1} . This treatment shows a 51.3% increase in Zn content compared to the control plants, but it does not demonstrate a significant difference. There was also a decrease in Zn content in almost all treatments added with Fe, which indicated that foliar spraying of Fe on green mustard does not fully enhance the Zn content in the plants. The foliar spraying of Fe on green mustard lead to a decrease in Zn content. This occurrence is likely a result of the antagonistic relationship between Fe and Zn in plants, where Fe hinders the translocation of Zn (Rai *et al*., 2021). Similar findings to Félix *et al*. (2021) in a study on black beans, where foliar spraying of Fe showed a decrease in Zn content, as the applied Fe concentration increased, the Zn content decreased by 28.8% to 55.6% compared to the plants without Fe spraying. This is also believed to result from the antagonistic interaction between minerals (Niyigaba *et al*., 2019).

Spraying Zn on green mustard can enhance plants' ability to absorb Zn, which can lead to increased Zn accumulation in plants. This is consistent with Xia *et al*. (2019) in a study on maize, where the spraying of 0.2% $ZnSO_4.7H_2O$ showed a 47.8% increase in Zn content if compared with plants without Zn treatment.

Conclusion

Agronomic biofortification of Fe by foliar spray up to a concentration of 0.6 g L^{-1} with or without a combination of Zn can increase physiological and biochemical parameters in mustard greens, namely photosynthetic rate up to 6.747 µmol m^{-2} s^{-1} (T_{15}), transpiration rate 0.653 µmol m^{-2} s^{-1} (T_5), stomatal conductance 18.854 µmol m^{-2} s^{-1} (T_5), number of stomata by 283,7 (T_{13}), stomatal opening width by 7.777 µm (T_{16}), Fe content by 56.109 mg kg^{-1} (T_{14}), but Zn content could only increase when Zn was added alone without Fe, an increase in Zn up to 18.127 mg kg^{-1} (T_4).The biofortification of Fe and Zn through agronomic approaches could also increase the chlorophyll content by 0.6 g L^{-1} Fe and 0.6 g L^{-1} Zn. In addition, Fe content increased with a foliar spray of 0.6 g L^{-1} Fe + 0.2 g L^{-1} Zn, while Zn content increased in the foliar spray treatment of 0.6 g L^{-1} Zn without a Fe combination. Overall, it can be concluded that biofortification with agronomic method, especially by foliar of Fe and Zn can enhance physiological activities and biochemical in green mustard, particularly the increase in Fe and Zn content by 137.3% and 51.3%, respectively.

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Conflict of interests

The authors declare that they have no conflict of interest.

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