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Iron-induced effect on the chemical composition of *Moringa oleifera* sprouts

Efecto inductor del hierro sobre la composición química de germinados de *Moringa oleifera*

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ABSTRACT

To increase the levels of phenolic compounds, flavonoids, and glucosinolates, *Moringa oleifera* seeds were treated with iron, acting as a stress trigger, during germination. The treatments consisted of adding the elicitor at concentrations of 200, 600, and 1000 ppm directly to the germination substrate, including a control group. We harvested the sprouts at 6, 9, 12, and 15 days after germination and quantified their phenolic compounds, flavonoids, and glucosinolates as well as analysed their antioxidant activity using DPPH, ABTS, and FRAP assays. Overall, the concentrations of these phytochemicals increased compared to the control group, and we observed a similar trend regarding antioxidant capacity. We found that the most

favourable results for phenolic compounds came from sprouts harvested on day 15 under the 600 and 1000 ppm iron treatments. We also observed that the highest flavonoid production occurred with the 1000 ppm treatment after 12 days of germination. Across all three methods used, antioxidant capacity yielded the best results with the 1000 ppm treatment at 15 days of germination. All sprouts exhibited higher glucosinolate concentrations than seeds. Iron has the potential to act as a stress-inducing agent in reusable substrates, demonstrated by stimulating phytochemical synthesis during germination.

Keywords: Germination, glucosinolates, iron, *Moringa oleifera*, phytochemicals, polyphenols.

RESUMEN

Con el fin de aumentar la cantidad de compuestos fenólicos, flavonoides y glucosinolatos se trataron semillas de *Moringa oleifera* durante su germinación con hierro como detonador de estrés. Los tratamientos consistieron en adicionar el elicitor en concentraciones de 200, 600 y 1000 ppm directamente en el sustrato de germinación. Un control también fue conducido. Los germinados se recolectaron a los 6, 9, 12 y 15 días de germinación. Se cuantificaron los compuestos fenólicos, flavonoides y glucosinolatos totales; además se analizó su actividad antioxidante mediante los ensayos de DPPH, ABTS y FRAP. De manera general, los fitoquímicos incrementaron su concentración con respecto al control y el mismo comportamiento se

observó para la capacidad antioxidante. Los mejores resultados para compuestos fenólicos correspondieron a los germinados colectados el día 15 de los tratamientos 600 y 1000 ppm de hierro. La mayor producción de flavonoides fue con 1000 ppm y 12 días de germinación. En los tres métodos empleados, la capacidad antioxidante mostró los mejores resultados con el tratamiento 1000 ppm a los 15 días de germinación. Todos los germinados presentaron concentraciones de glucosinolatos superiores a las semillas. El hierro posee potencial para emplearse como agente estresor en sustratos reutilizables ya que estimula la síntesis de fitoquímicos durante la germinación.

Palabras clave: Fitoquímicos, germinación, glucosinolatos, hierro, *Moringa oleifera*, polifenoles.

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1. INTRODUCTION

There are currently dietary options that offer a significant nutritional alternative; however, in some cases, they remain inaccessible due to factors as diverse as socioeconomic status or geographic location. This constitutes a problem, particularly in developing countries such as Mexico, severely contributing to malnutrition among people of all ages (Espinoza-Ramos & Rodríguez, 2018). Similarly, the food production industry is severely affected by biotic and abiotic factors that limit crop production (Reyes-Palomino & Cano, 2022). Nowadays, it is critical to develop alternative food sources that provide new sources of nutrients and bioactive compounds, helping to mitigate malnutrition (Miramontes-Escobar *et al.*, 2020).

Moringa oleifera (moringa) is one of 13 species within the genus *Moringa*, which encompasses a highly diverse range of growth habits or forms, ranging from herbs and shrubs to trees (Olson & Fahey, 2011). *Moringa* is a fast-growing evergreen tree that can reach a height of 10-12 m. The plant is native to the northwestern region of India and has been domesticated in tropical and subtropical regions. *Moringa* is well-known for its bioactive and nutritional properties, widely used in sectors such as cosmetics, health, food, agriculture, and pharmaceuticals (Jikah & Edo, 2023). Among the bioactive compounds and constituents that make it so appealing to the market are glucosinolates, oils, proteins, and carbohydrates (Dzuovor *et al.*, 2022).

Edible sprouts have been incorporated into human nutrition for a long time. *Moringa* sprouts represent an unconventional source of nutrients, having the advantage of needing minimal cultivation and reduced transport and storage costs (Núñez-Gastélum *et al.*, 2023). Additionally, the nutrient and secondary metabolite content of sprouts can be enhanced through the application of stress-inducing agents.

Iron (Fe) is a transition metal present in most organisms. It participates in biochemical processes, such as oxygen transport, electron transfer reactions, and DNA biosynthesis and repair, to mention a few. However, when present in excess within cells and tissues, iron disrupts redox homeostasis and catalyzes the propagation of reactive oxygen species, thereby inducing oxidative stress (Galaris *et al.*, 2019). Furthermore, it has been observed that plants chelate metal ions to enhance the production of antioxidant compounds as a means of detoxification. Thus, stress-induced defense mechanisms significantly influence the synthesis of secondary metabolites (Zielińska-Dawidziak, 2021). Consequently, this study aims to evaluate the effect of iron on the composition of bioactive compounds and the antioxidant capacity of *M. oleifera* sprouts.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds were collected from different plants of similar age growing in southern Sonora, northwestern Mexico (26°54'39" N, 109°37'31" W). Surface disinfection was then performed by immersion in a 1% sodium hypochlorite solution for 3 min; after this soaking period, they were washed three times with distilled water and then air-dried. Subsequently, the seeds were placed in a substrate (Nutrigarden® Tierra Negra, Queretaro, Mexico) containing treatments at concentrations of 200 (Fe200), 600 (Fe600), and 1000 (Fe1000) ppm of iron (Fe); and were allowed to germinate at room temperature under artificial light with a 14:10 h photoperiod.

Watering was performed daily using distilled water until saturation, and the sprouts were harvested at 6, 9, 12, and 15 days (Cota-Ruiz *et al.*, 2018). A control group (Fe0), which was not subjected to stress induction, was also included. The sprouts were harvested on the corresponding days and dried in an oven (Fisher Isotemp 637 G Oven, MA, USA) at 40 °C for 72 h. Finally, they were ground (Nutribullet® NB-100, CA, USA) and stored at -20 °C for subsequent analysis.

2.2. Total phenolic content

Reagents were purchased from Sigma-Aldrich (Estado de Mexico, Mexico), unless otherwise indicated in the text. The total content of phenolic compounds, total flavonoid content, and antioxidant capacity were determined spectrophotometrically according to Núñez-Gastélum *et al.* (2018). For quantification of total phenolic compounds (TPC), gallic acid (J.T. Baker, PA, USA) was used as a calibration standard, and the reactions were performed in the dark. A 100 mg sample was weighed, and 5 mL of 80% methanol was added; the mixture was then subjected to sonication (Vevor JPS-10A, CA, USA) for 30 min and subsequently centrifuged (ZENROE LCS-20H, Japan) at 4,000 rpm for 10 min. 100 µL of 7.5% sodium bicarbonate solution was added to 25 µL of the resulting supernatant (or the standard solution) and allowed to stand for 2 min; then, 125 µL of 10% Folin-Ciocalteu reagent was added, and the mixture was incubated at 50 °C for 15 min. Finally, 300 µL of the reaction mixture was transferred to each well of a microplate at room temperature, and absorbance was measured at 760 nm. TPC was expressed in milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

2.3. Total flavonoid content

In this case, a calibration curve was constructed using catechin as the calibration standard in 80% aqueous methanol. For the extraction, the procedure described in the previous section was employed, except that this time, 50 mg of each sample was weighed. From the supernatants, 124 µL aliquots were taken, to which 500 µL of distilled water and 33.2 µL of 5% sodium nitrite were added; the mixture was then allowed to stand for 5 min. After this time had elapsed, 37.2 µL of 10% aluminum chloride (Fagalab, Sinaloa, Mexico) were added, and the mixture was allowed to react for 3 min. Subsequently, 500 µL of 0.5 M sodium hydroxide were added, and the solution was incubated for 30 min in the absence of light. Finally, 300 µL of the solutions were transferred to the wells of a microplate, and the absorbance was measured at 520 nm. The total flavonoid content (TFC) was expressed as milligrams of catechin equivalents per gram of sample (mg CE/g) (Núñez-Gastélum *et al.*, 2018).

2.4. DPPH assay

For all antioxidant capacity analyses, Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as the calibration standard, and the same procedure was employed for the extraction of antioxidants. First, a 60 mM solution of the DPPH radical (2,2-Diphenyl-1-picrylhydrazyl) in methanol was prepared. For the extraction, 25 mg of the sample were weighed and extracted with 5 mL of 80% methanol. From the extract or standard, 50 µL were taken and placed into a microplate well along with 200 µL of the 0.190 mM DPPH reagent, after which the absorbance was measured at 520 nm. The percentage of inhibition was calculated, and the data

were expressed as micromoles of Trolox equivalents per gram of sample ($\mu\text{mol TE/g}$) (Núñez-Gastélum *et al.*, 2018).

2.5. ABTS method

The ABTS^{•+} radical cation (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) was prepared by oxidation with potassium persulfate. A 7 mM ABTS solution was diluted in 50 mM phosphate buffer (pH 7.4), and 2.5 mM potassium persulfate was added, followed by incubation in the dark for 16 h. After incubation, the radical solution was adjusted by dilution with ethanol to an absorbance of 0.7 at 750 nm. 12 μL of extract or standard were mixed with the ABTS^{•+} radical solution, and the absorbance was monitored for 30 min at 734 nm. The percentage of inhibition was calculated, and the results were expressed as $\mu\text{mol TE/g}$ (Núñez-Gastélum *et al.*, 2018).

2.6. Ferric reducing antioxidant power assay

The Ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 20 mM ferric chloride, 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), and 0.3 M acetate buffer (pH 3.6) in a ratio of 1:1:10. For the extract analysis, 24 μL of extract or standard were placed in a well together with 180 μL of the FRAP reagent, and the absorbance was measured at 620 nm. The results are expressed as millimoles of Trolox equivalents per gram of sample (mmol TE/g) (Núñez-Gastélum *et al.*, 2018).

2.7. Quantification of total glucosinolates

Glucosinolates (GSL) were estimated using the spectrophotometric method proposed by Mawlong *et al.* (2017). A 100 mg sample of moringa sprouts was suspended in a 2 mL vial containing 80% methanol (J.T. Baker, PA, USA), incubated overnight at room temperature, and subsequently centrifuged at 3,000 rpm for 4 min. The supernatant was separated from the pellet and resuspended in 2 mL of 80% methanol. Then, 100 μL of the extract along with 0.3 mL of distilled water and 3 mL of 2 mM sodium tetrachloropalladate were combined with the same solvent. The mixture was incubated at room temperature for 1 h. Immediately, the absorbance was measured at 425 nm using a microplate spectrophotometer (Thermo Scientific Multiskan FC, MA, USA). The concentration was expressed as micromoles of total glucosinolates per gram of sample ($\mu\text{mol GSL/g}$).

2.8. Statistical analysis

All experiments were conducted using a completely randomized factorial design with two fixed factors: iron concentration (Fe0, Fe200, Fe600, and Fe1000) and germination time (6, 9, 12, and 15 days), with three biological replicates per treatment combination. Data are presented as mean $\pm$ standard deviation (SD). Prior to statistical analysis, model assumptions were evaluated by assessing the normality of residuals using the Shapiro–Wilk test and the homogeneity of variances using Levene's test. Data were analyzed by two-way analysis of variance (ANOVA) to determine the main effects of iron concentration, germination time, and their interaction. Whenever a significant interaction was detected ($p \leq 0.05$), simple effects were examined through Tukey's honestly significant difference (HSD) multiple comparison test to evaluate

differences among iron treatments within each germination day and among germination days within each iron treatment. Statistical significance was established at $p \leq 0.05$. All statistical analyses were performed using IBM SPSS Statistics version 31.0.0.0(117) (IBM Corp., Armonk, NY, USA).

3. RESULTS

3.1. Total phenolic content

Statistical variability was observed among the treatments; all sprouts yielded a higher TPC content compared to the seeds (7.1 ± 0.1 mg GAE/g) (Table 1). Statistically, the highest values were obtained from sprouts harvested on day 15 under the Fe600 and Fe1000 treatments. However, the results for day 15 under treatment Fe200 were only 9% and 14% lower than those of the two treatments, respectively. TPC was significantly influenced by iron concentration, germination time, and their interaction. Significant differences were observed among iron treatments within each germination day and among germination days within each treatment.

Table 1. Total phenolic content (mg GAE/g) in sprouts of *M. oleifera* treated with iron.

Days	Fe0	Fe200	Fe600	Fe1000
6	28.9 ± 0.09^{cA}	19.6 ± 0.18^{aA}	27.8 ± 0.06^{bA}	37.5 ± 0.16^{dA}
9	19.3 ± 0.34^{aB}	36.5 ± 0.11^{bD}	46.9 ± 0.22^{dB}	41.2 ± 0.21^{cB}
12	16.0 ± 0.17^{aC}	37.5 ± 0.09^{cC}	20.8 ± 0.33^{bC}	48.5 ± 0.21^{dC}
15	43.3 ± 0.03^{aD}	48.2 ± 0.09^{bD}	53.1 ± 0.07^{cD}	56.1 ± 0.13^{dD}

Values are expressed as mean $\pm$ SD ($n = 3$). Different lowercase letters within the same row indicate significant differences among iron treatments at the same germination day according to Tukey's HSD test ($p \leq 0.05$). Different uppercase letters within the same column indicate significant differences among germination days within each iron treatment according to Tukey's HSD test ($p \leq 0.05$).

3.2. Total flavonoids content

The results of the flavonoid quantification (Table 2) indicated that the highest production of these compounds occurred with Fe1000 and 12 days of germination. In general, the values increased until day 12. It is worth noting that flavonoids constitute an important class of phenolic compounds, and that their proportion is an intrinsic characteristic of plant species; in this case, approximately 15% of the TPC corresponds to TFC. Similarly, the flavonoid content was significantly influenced by iron concentration, germination time, and their interaction. Significant differences were observed among iron treatments within each germination day and among germination days within each treatment.

Table 2. Total flavonoids content (mg CE/g) of *M. oleifera* sprouts elicited by iron.

Days	Fe0	Fe200	Fe600	Fe1000
6	3.1 ± 0.08^{aA}	3.2 ± 0.09^{aA}	4.7 ± 0.20^{bA}	3.6 ± 0.16^{cA}
9	3.7 ± 0.17^{aB}	3.9 ± 0.06^{aB}	4.1 ± 0.06^{bB}	3.8 ± 0.23^{aA}
12	5.5 ± 0.16^{bD}	4.0 ± 0.04^{aB}	4.4 ± 0.16^{cA}	7.1 ± 0.14^{dB}
15	4.5 ± 0.03^{aC}	4.5 ± 0.35^{aC}	4.7 ± 0.39^{bA}	6.6 ± 0.39^{cC}

Values are expressed as mean $\pm$ SD ($n = 3$). Different lowercase letters within the same row indicate significant differences among iron treatments at the same germination day according to Tukey's HSD test ($p \leq 0.05$). Different

uppercase letters within the same column indicate significant differences among germination days within each iron treatment according to Tukey's HSD test ($p \leq 0.05$).

3.3. Antioxidant capacity

For all three methods utilized, the treatment that demonstrated the best results regarding antioxidant capacity was Fe1000 at 15 days of germination (Figure 1). Significant differences were observed among the germination days across all treatments. Overall, antioxidant capacity increased throughout germination regardless of the assay employed, although the magnitude of the response depended on iron concentration. DPPH, ABTS, and FRAP consistently demonstrated that iron supplementation enhanced the antioxidant potential of *Moringa oleifera* sprouts, particularly at the highest concentrations and advanced germination stages.

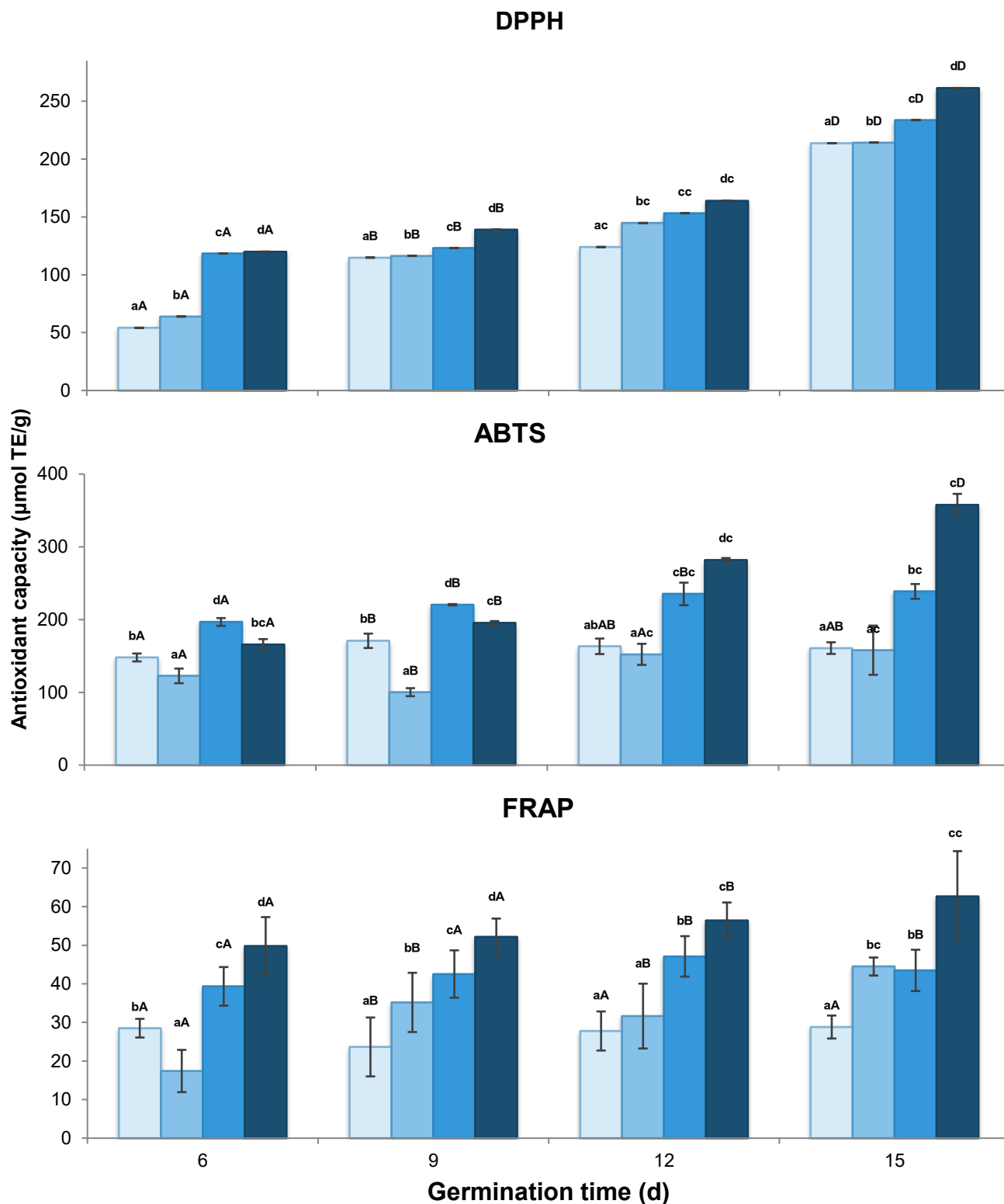


Fig. 1. Antioxidant capacity of *Moringa oleifera* sprouts evaluated by DPPH, ABTS, and FRAP assays during germination. Iron treatments are represented by light blue (Fe0), medium-light blue (Fe200), medium blue (Fe600), and dark blue (Fe1000) bars. Values are expressed as

mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences among iron treatments within the same germination day, whereas different uppercase letters indicate significant differences among germination days within each treatment (Tukey's HSD, $p \leq 0.05$).

3.4. Total glucosinolates

Statistically significant differences were observed among the means of each treatment (Table 3). All sprouts exhibited GSL concentrations higher than those of the seeds ($80.7 \pm 2.2 \mu\text{mol/g}$). The highest value was obtained with Fe200 at 15 days, although it did not differ statistically from the other treatments during the same period. It is worth noting that Fe600 was statistically equivalent to the highest recorded value, but at 9 days. These results suggest that, in order to influence the levels of these compounds in a shorter timeframe, this concentration represents the optimal choice. GSL exhibited significant effects of iron concentration, germination time, and their interaction. During the early germination stages (days 6 and 9), only limited differences were observed among treatments, whereas more pronounced treatment-dependent variations became evident by day 12, particularly under Fe600 and Fe1000. By day 15, glucosinolate contents converged, and no significant differences were detected among iron treatments, indicating that the influence of germination time became more pronounced at the final stage of the process.

Table 3. GSL content ($\mu\text{mol/g}$) in sprouts of *M. oleifera* treated with iron.

Days	Fe0	Fe200	Fe600	Fe1000
6	$132.4 \pm 8.4^{\text{aB}}$	$123.0 \pm 10.7^{\text{aA}}$	$133.4 \pm 2.7^{\text{bA}}$	$129.3 \pm 1.3^{\text{abA}}$
9	$129.2 \pm 0.5^{\text{aA}}$	$128.9 \pm 0.9^{\text{aA}}$	$148.6 \pm 6.4^{\text{bB}}$	$128.4 \pm 1.8^{\text{aA}}$
12	$123.7 \pm 1.7^{\text{aA}}$	$131.1 \pm 8.0^{\text{abA}}$	$149.1 \pm 4.0^{\text{cB}}$	$150.8 \pm 0.6^{\text{cB}}$
15	$147.6 \pm 3.5^{\text{aB}}$	$153.1 \pm 5.9^{\text{aB}}$	$151.7 \pm 2.5^{\text{aB}}$	$149.0 \pm 1.9^{\text{aB}}$

Values are expressed as mean $\pm$ SD ($n = 3$). Different lowercase letters within the same row indicate significant differences among iron treatments at the same germination day according to Tukey's HSD test ($p \leq 0.05$). Different uppercase letters within the same column indicate significant differences among germination days within each iron treatment according to Tukey's HSD test ($p \leq 0.05$).

4. DISCUSSION

TPC recorded in this research was twofold greater than the concentrations previously reported for germinated seeds by Ijarotimi *et al.* (2013) and nearly seven times higher than those obtained by Servín de la Mora-López *et al.* (2018), León-López *et al.* (2019), and Coello *et al.* (2020). It is important to note that these values are even higher than those reported for moringa leaves (Castro-López *et al.*, 2017). Ijarotimi *et al.* (2013) and Nobossé *et al.* (2018), reported lower values for moringa sprouts and leaves, respectively. Flavonoid ranges spanning from 3.26 to 14.07 mg EC/g have been reported in moringa leaves originating from South Asia, West Africa, and South America (Pollini *et al.*, 2020).

Regarding DPPH, this study yielded better results compared to those reported by Castro-López *et al.* (2017) and Servín de la Mora-López *et al.* (2018) for moringa sprouts. The antioxidant activity results obtained via the ABTS assay were higher than those reported by Servín de la Mora-López *et al.* (2018). In the case of FRAP, the results obtained in this study were considerably higher than those reported by Castro-López *et al.* (2017), yet lower than those

obtained by Servín de la Mora-López *et al.* (2018). Overall, antioxidant capacity was higher than that reported in moringa leaves at various developmental stages (Coz-Bolaños *et al.*, 2018).

In addition to the advantages offered by sprouts, such as their high nutritional value, increased nutrient bioavailability, and reduced levels of antinutritional factors; high antioxidant activity is a desirable attribute expected in functional foods. As seeds initiate the germination process, they significantly increase their total antioxidant capacity compared to ungerminated seeds. Consequently, consuming sprouts may improve the ability to combat oxidative stress and prevent chronic diseases (Karaman *et al.*, 2024).

There are a few reports quantifying GSL in moringa sprouts. Coello *et al.* (2020) studied the effects of temperature on GSL content in moringa sprouts; their results showed nearly 1.5 times more GSL than those obtained in the present study. Liu *et al.* (2024) observed that glucomoringin, the characteristic glucosinolate of moringa, increased sharply under moderate heat during germination. Minerals are mainly involved in modifying human general nutrition and stress tolerance. However, when compared to temperature, they do not directly trigger the same stress-response pathways. The action of minerals occurs mainly at a general physiological level (e.g., water balance, antioxidants), rather than at the specific level of biosynthetic pathways (Bhat *et al.*, 2020).

5. CONCLUSION

This study provides evidence that iron functions as an effective abiotic elicitor capable of modulating phytochemical accumulation and antioxidant capacity during *Moringa oleifera* germination. These findings expand current knowledge on mineral-induced elicitation and provide a scientific basis for optimizing germination strategies to obtain sprouts with enhanced functional value. Future studies should elucidate the molecular mechanisms underlying iron-mediated regulation of phytochemical biosynthesis.

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AUTHOR CONTRIBUTION

J.A. Núñez-Gastélum: Conceptualization, Supervision, Investigation, Formal analysis, Writing – Reviewing and Editing. E. Durán-Rodríguez: Investigation, Methodology, Writing - Original draft preparation. A.G. Díaz-Sánchez: Formal analysis, Review and Editing. C. Figueroa-Batalla: Resources, Methodology. Y. Ortiz-Rivera: Methodology. C.P. Grijalva-Verdugo; Methodology. J.R. Rodríguez-Núñez: Formal analysis.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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