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Iron Biofortification of Hydroponically Grown Widely Consumed Leafy Greens in Sri Lanka

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Abstract

Iron (Fe) deficiency is a prevalent nutritional concern in Sri Lanka, compelling innovative approaches to enhance dietary iron intake. This research explores the biofortification of iron in hydroponically grown Green Thampala (*Amaranthus viridis*) and Mukunuwenna (*Alternanthera sessilis*), which are widely consumed leafy greens in Sri Lanka. The effects of varying Fe concentrations on growth parameters, fresh yield, nutrient contents, antioxidant activity, total phenolic, and flavonoid content of hydroponically grown leafy greens under controlled conditions by administering $FeSO_4$ in Albert's solution at concentrations of 162.5 ppm (control), 200 ppm, and 240 ppm were assessed in this study. The Phenol-Sulfuric assay, Lowry assay, Folin-Ciocalteu assay, $AlCl_3$ spectrophotometric method, and DPPH assay, were used to estimate the Total Carbohydrate Content (TCC), Total Protein Content (TPrC), Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and total antioxidant activity of Fe-fortified plants, respectively. Moreover, the Fe content of Fe-fortified plants was determined using the Atomic Absorption Spectrophotometry (AAS) technique. The plants were tested positive for saponins, tannins, polyphenols, terpenoids, and steroids and they were tested negative for anthraquinones. The 200 ppm of Fe showed the highest Fe content in both plants, along with growth parameters such as significantly increased height in both plants, the highest leaf count in Green Thampala, and optimized fresh yield in Mukunuwenna. In both plants, the 240 ppm of Fe had the highest nutritional content. Both 200 ppm of Fe and 240 ppm of Fe concentrations appeared as successful for fortifying Fe in Green Thampala and Mukunuwenna plants, since they showed a higher Fe content than control, which had a concentration of 162.5 ppm of Fe.

Keywords: Iron biofortification, Green Thampala, Mukunuwenna, Coco peat grow bags, Albert's solution

1. Introduction

Iron (Fe) is widely recognized as a necessary mineral element for humans, as it plays a role in the synthesis of both hemoglobin and myoglobin.[1] Beyond its role in oxygen transport, iron is essential for immune system function, neural systems, homeostasis, energy metabolism, exercise, and overall human health maintenance.[2] The Recommended Daily Allowance (RDA) for Fe is 8–18 mg, which is the amount that the human body needs each day; particularly, pregnant mothers necessitate 27 mg of iron per day. However, the tolerable Upper Intake Level (UL) of Fe in adults is 40 mg per day.[3] Insufficient iron consumption through diets can lead to iron deficiency anaemia, posing severe health risks, as heart and lung failure, restless legs syndrome, pregnancy complications, and developmental delays in children.[4] Two billion people worldwide are anaemic, and according to the World Health Organization (WHO), the major reason is Fe deficiency in human diet. 11.2% of 6 to 11month-old babies in Sri Lanka was reported to have an iron deficiency in 2022.[5] But occasionally, the diet falls short of the minimum requirements, leading to micronutrient deficiencies and the phenomenon referred to as "hidden hunger".[6]

Biofortification is a method of addressing hidden hunger by increasing the nutritional content of plant edible parts during their vegetative life cycle.[7] To accomplish biofortification, techniques including as breeding, mineral fertilization, and biotechnological approaches can be applied.

All these approaches, though, have drawbacks. For instance, excessive fertilizer application might contaminate the soil or precipitate insoluble mineral forms; high concentrations of minerals can stress the plants. An alternate strategy to these approaches is to use hydroponic technique to increase the target minerals in food crops through biofortification.[8]

In hydroponics, plants are grown without the use of soil. They are planted in inert growing media as mentioned in Figure 1, and given access to water, oxygen, and nutrient-rich solutions.

Figure 1. Inert growing media for hydroponics.[9]

Hydroponics offers quicker growth, larger yields, and superior quality. A plant's roots are always looking for the nutrients to survive when it is grown in soil. A plant does not require energy to survive when it gets water and nutrients straight through its roots. The way hydroponic systems function is by enabling fine control over environmental parameters such as pH and temperature balance, electrical conductivity (EC value), and optimal exposure to water and nutrients. The typical hydroponic pH range is 5.5 to 6.5, and ideal EC range is between 1.5 to 2.5 ds/m.[10]

Various hydroponics systems are available including Nutrient Film Technique (NFT), deep water culture, drip irrigation and aeroponics.[11] Each technique has unique features and advantages. The NFT system is a recirculating hydroponic system among hydroponic techniques. It suspends the plant above a continuously flowing stream of nutrient solution that covers the ends of the root system, and it reduces the nutrient loss and effectively increases the nutrient absorption.[12] The sterility of the coco peat grow bags set it apart from other growing media. Additionally, it effectively retains water, ensuring plant hydration and reducing the likelihood of dehydration.[13]Biofortification of leafy greens can be carried out in hydroponics by adding higher concentrations of target minerals in the nutrient solution.[14] Moreover, Fe, which presents a low solubility in the soil, a hydroponics system can be a good option to increase micronutrient availability, since it facilitates the pH management in the nutrient solution.[15] The effectiveness of biofortification depends on the chemical form of micronutrients. In the case of Fe absorption, roots absorb Fe^{2+} which is oxidized to Fe^{3+}, chelated, and transported to the plant top.[16] Fe is essential for respiration, photosynthesis, and enzyme reactions. The concentration of Fe in plant leaf tissues varies depending on the species of the plant, but it usually falls between 50-500 ppm. Toxic effects could be observed at concentrations of Fe greater than 500 ppm. In general, if the concentration is less than 50 ppm, there are signs of deficiency.[17]

The reduction of Fe deficiency in Sri Lanka can be achieved through the implementation of strategies designed to raise the Fe content of food. A balanced diet can be completed with the help of healthy foods like green leafy vegetables. They are often high in fibre and minerals and low in calories and fat. Leafy vegetables have long been used in traditional medicine for their medicinal and therapeutic properties.[18] Two of the mostly consumed leafy greens in Sri Lanka are Mukunuwenna (*Alternanthera sessilis*) and Green Thampala (*Amaranthus viridis*) (Figure 2). Biofortifying Fe in these leafy greens can be a solution to iron deficiency since Green Thampala contains 207.38 µg of Fe in 100 g of leaves and Mukunuwenna contains 55.16 µg of Fe in 100 g of leaves.[18]

Figure 2. A) *Alternanthera sessilis* B) *Amaranthus viridis*

Based on the background information, this study was designed to address iron deficiency in Sri Lanka by biofortifying Fe in hydroponically grown Mukunuwenna, and Green Thampala, as hydroponic biofortification has never been attempted in Sri Lanka before. In this study, we selected two different concentrations of Fe (200 ppm and 240 ppm) to increase the Fe content in those plants, and Fe was added to the Nutrient solution as $FeSO_4$, based on the plant tolerance level of Fe range of 50 ppm – 500 ppm. Moreover, this study aimed to evaluate the effects of varying Fe application rates within the nutrient solution on growth parameters, fresh yield, mineral composition, antioxidant activities, and the phenolic and flavonoid profiles of leafy greens in the NFT system and coco peat grow bags under controlled conditions.

2. Methodology

2.1 Selection of seeds and nutrient solution. Commercially available seeds of Green Thampala and stem cuttings of Mukunuwenna, were collected (Figure 3). To support plant growth in a hydroponic system, Albert's solution, a commercially available nutrient solution, was utilized (Figure 4, Table 1).

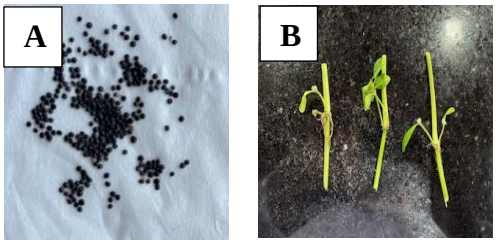

Figure 3. A) Green Thampala seeds, B) Mukunuwenna stem cuttings.

Figure 4. Albert's solution.

Table 1. Nutrient content in Albert's Solution

Macro nutrients	Percentage (%)	Micro nutrients	Percentage (%)
N	10.5	S	1.00
H_3PO_4	9.1	B	0.003
K	16.4	Zn	0.014
Mg	0.86	Cu	0.0004
Ca	9.5	Fe	0.065
		Mn	0.012
		Mo	0.0019

2.2 Germination of the plants. Coconut coir pellets were soaked in water for 10 minutes before placing the seeds of Green Thampala into the pellets. The plants were watered and kept in the dark for three days. Meanwhile, the stem pieces of Mukunuwenna were placed in water until new roots formed.

2.3 Preparation of hydroponics system.

2.3.1 Method 1: NFT System. According to the user manual, the parts of the NFT system were assembled (Figure 5A). A 50% concentration of Albert's solution was prepared and used as a control. After that, 0.25 g and 0.5 g of $FeSO_4$(s) were added to the 50% Albert's solution, creating iron concentrations of 325 ppm (control), 350 ppm, and 375 ppm. The pH and EC values of the nutrient solutions were measured. The motor was then installed in the system. Mukunuwenna stems, which had been kept in water for six days, were then placed into the holes of the system.

2.3.2 Method 2: Coco peat grow bag method. 10 kg of coco peat were soaked in water for 24 hours. The polythene bags were then filled with the coco peat. Green Thampala and Mukunuwenna stems were planted in the coco peat grow bags (Figure 5B). A 25% concentration of Albert's solution was prepared and used as a control. Subsequently, 0.375 g and 0.775 g of $FeSO_4$(s) were added separately to the 25% Albert's solution, resulting in iron concentrations of 162.5 ppm (control), 200 ppm, and 240 ppm. The pH and EC values of the nutrient solution were measured, and it was poured onto the plants in the morning, and water was sprayed on them in the evening on a daily basis.

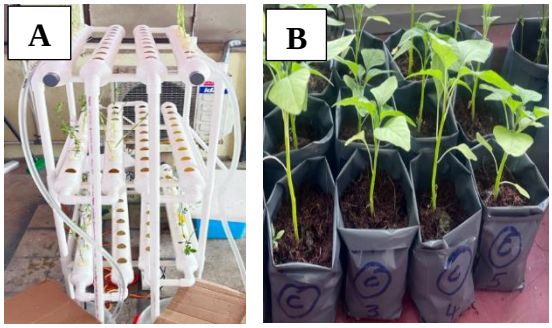

Figure 5. A) NFT system B) Coco peat grow bags

2.4 Homogenization and Preparation of aqueous plant extracts. Fully hydroponically grown plants were harvested after 5 weeks, and fresh weight was measured. Then the plants were shredded and kept in the hot air oven at 40°C for 48 hours. The dried plants were ground using a mortar and pestle. The powdered samples were diluted to 1:20 using water and the solutions were kept in the oven at 70°C for 10 minutes. After that the aqueous extracts were filtered, and the filtrates were refrigerated in falcon tubes for qualitative and quantitative analyses.

2.5 Determination of total carbohydrate content: Phenol- sulfuric acid method. A 0.1 g of dextrose powder was measured using an analytical balance and mixed with 2.5 N HCl. This mixture was placed in a water bath at 100°C for 3 hours. After cooling to room temperature, the mixture was neutralized with Na_2CO_3 and topped up to 100 ml in a volumetric flask to create a dextrose stock solution (1000 µg/ml). Using this stock solution, a dextrose standard series of known concentrations (50-250 µg/ml) was prepared. 1ml of diluted plant extract (1:20) was mixed with 5 ml of 96% H_2SO_4 and 1 ml of 5% phenol. The test tube was shaken for 10 minutes and then placed in a water bath at 25-30°C for 20 minutes until the solution turned green. Finally, the absorbance was measured at 490 nm using a UV-visible spectrophotometer.[19] A standard curve was plotted using the absorbance of the standard series. The total carbohydrate content of plants samples was derived by comparing their absorbance values to the standard curve.

2.6 Determination of total protein content: Lowry Assay. A BSA standard series (200-1000 µg/ml) was prepared. 1 ml of standard series and 1 ml of diluted (1:20) plant extract was added to the test tubes. 5 ml of Lowry A and B mixture was added to each test tubes and incubated at RT for 10 minutes. Then 0.5 ml of Lowry C was added to each tube and incubated at RT for 30 minutes. Finally, the absorbance was measured at 660 nm using a UV-visible spectrophotometer.[20] A standard curve was plotted using the absorbance of the BSA standard series. The total protein content of plant samples was derived by comparing their absorbance values to the standard curve.

2.7 Determination of total flavonoid content: $AlCl_3$ Spectrophotometric method. A quercetin standard series (20-100 µg/ml) were prepared. 1 ml of diluted Plant extract (1:10) and standards were mixed with 0.2 ml of 10% $AlCl_3$ and 0.2 ml of 1M potassium acetate. Then the mixture was incubated at RT for 30 minutes with intermittent shaking. Finally, the absorbance measured at 415 nm using a UV-visible spectrophotometer.[21] A standard curve was plotted using the absorbance of the quercetin standard series. The total flavonoid content of plant samples was derived by comparing their absorbance values to the standard curve.

2.8 Determination of total phenolic content: Folin-Ciocatleu assay. A standard series of gallic acid (20-100 µg/ml) was prepared. 0.3 ml of both standard and diluted sample (1:10) were mixed with 1.2 ml of 10% FC reagent and 1.5 ml of 7.5% Na_2CO_3. Then the mixture was shaken and incubated for an hour in the dark at RT. Finally, the absorbance was measured at 765 nm using UV-visible spectrophotometer.[21] The total phenolic content of plant samples was derived by comparing their absorbance values to the standard curve.

2.9 Determination of Fe content: Atomic Absorption Spectrophotometry (AAS). 10-20 g of the samples were dried at 100°C, and the samples were ashed using a programmable furnace. The temperature gradually increased to 450°C for8 hours. Ashing was repeated until the product turns white or grey. Then HCl was added to the ash, and it was allowed to evaporate. Then the residue was dissolved in HNO_3. The solution was transferred to a plastic

bottle, and blank was treated in the same way. Then Fe content of the samples was determined by flame AAS.[22]

2.10 Qualitative analysis of phytochemicals. Qualitative tests were carried out to check the presence of the phytochemicals as shown in Table 2.

Table 2. Qualitative tests for phytochemical screening.

Phytochemicals	Procedure
Saponins	2 ml of distilled water was added to 2 ml of extract. The mixture was then shaken for 15 minutes using a vortex until foam formed.[23]
Tannins	2 ml of 5% $FeCl_3$ solution were added to 1 ml of extract. The presence of tannins was confirmed if the color changed to greenish black.[23]
Polyphenols	3 drops of diluted iodine solution were added to 1ml of sample.[24]
Anthraquinones	2 ml of 10% Ammonia solution was mixed with 0.5 ml of plant, formation of red precipitate was indicative of anthraquinones.[24]
Terpenoids	0.5 ml of each plant sample was mixed with 2ml of chloroform and 2 ml of conc. H_2SO_4.[25]
Steroids	0.5 ml of each plant sample was mixed with 0.5ml of chloroform and 1 ml of conc.H_2SO_4.[25]

2.11 Determination of antioxidant activity: DPPH assay. 1 ml of 0.004% DPPH radical solution (100 µM in methanol) was mixed with 1ml of sample. Then the mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 517 nm using UV-visible spectrophotometer. Then DPPH radical scavenging activity was measured using the equation given below.[25]

Inhibition percentage (%) = $[(A_C-A_S)/A_C] \times 100$

(A_C= Absorbance of control, A_S = Absorbance of sample)

2.12 Statistical analysis. All graphs were generated using Microsoft Office 365 Excel. IBM SPSS Statistics Version 28.0.0.0 was used to perform the statistical analysis and all the values are expressed as mean ± SE. Statistical analysis was conducted by one-way ANOVA followed by LSD test for multiple comparison analysis. A p value less than 0.05 was considered to have a significant difference and $0.05 < p$ value < 0.1 was considered to have a tendency for a significant difference.

3. Results

3.1 Growth of plants (Plant height and Leaf number)

3.1.1 Growth of Green Thampala (GT). On the day of harvest (5^{th} week), the growth of GT, shown in Figure 6, was assessed under three conditions: A (200 ppm), B (240 ppm), and C (control).

Figure 6. Growth of GT on the day of harvest (5th week). A-200 ppm, B-240 ppm and C-control

3.1.2 Growth of Mukunuwenna (MK). On the day of harvest (5th week), the growth of MK was evaluated under three conditions: 200 ppm (A), 240 ppm (B), and control (C), as shown in Figure 7.

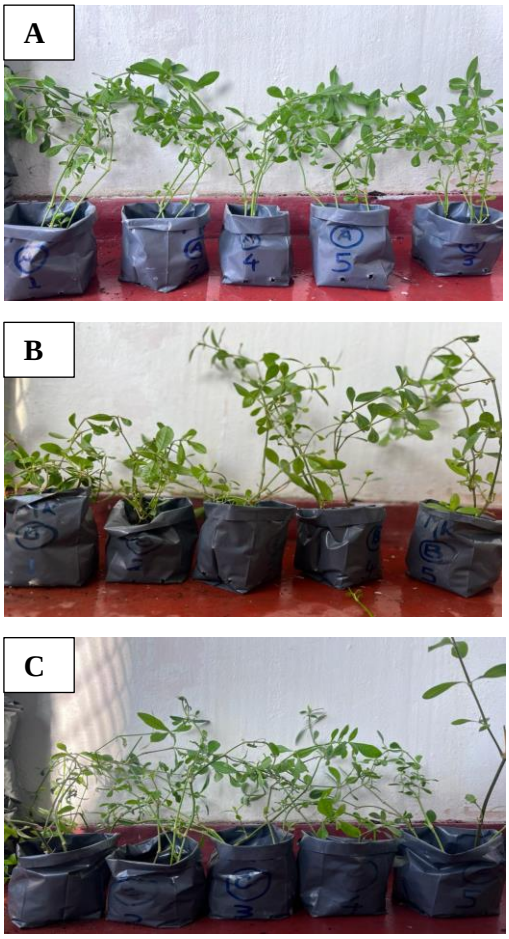

Figure 7. Growth of MK on the day of harvest (5th week), A-200 ppm, B-240 ppm and C-control.

3.2 Fresh yield of plants

3.2.1 Fresh yield of Green Thampala

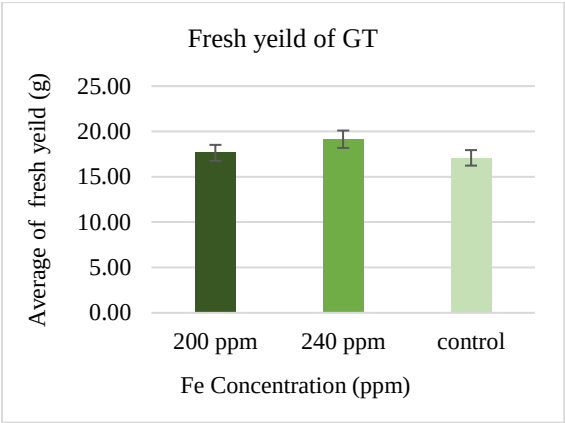

Figure 8. Average fresh yield of GT in different Fe concentrations.

GT grown at 240 ppm of Fe concentration had the highest fresh yield and control had the lowest fresh yield (Figure 8).

3.2.2 Fresh yield of Mukunuwenna

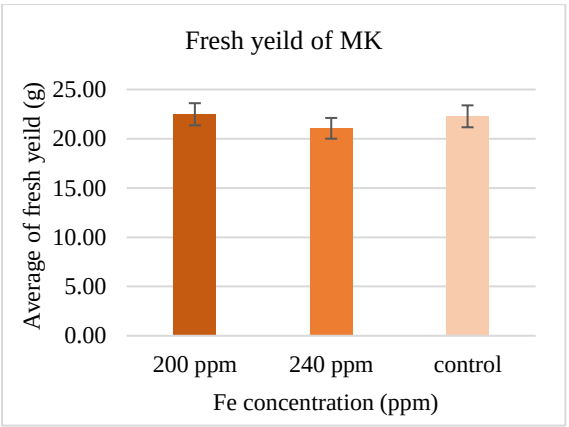

Figure 9. Average fresh yield of MK in different Fe concentration.

MK grown at 200 ppm of Fe concentration had the highest fresh yield and 240 ppm had the lowest fresh yield (Figure 9).

3.3 Height of plants

3.3.1. Height of Green Thampala

At the time of harvest, GT grown at 200 ppm of Fe concentration had the highest height during the growth period, and it was significantly high compared to GT grown at 240 ppm (Figure 10).

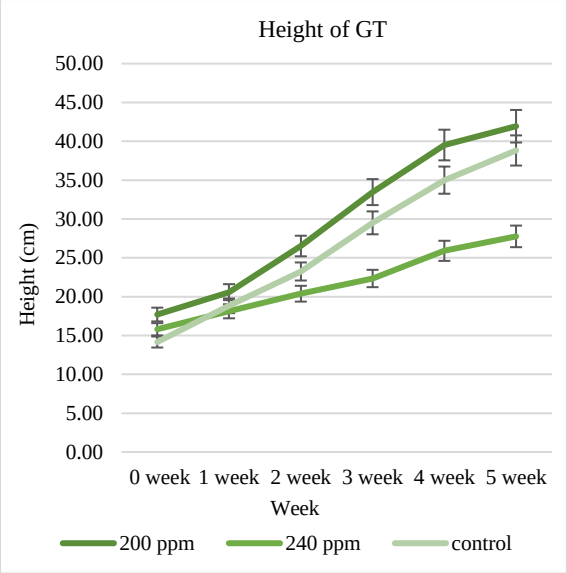

Figure 10. Average height of GT from week 0 to week 5.

3.3.2 Height of Mukunuwenna

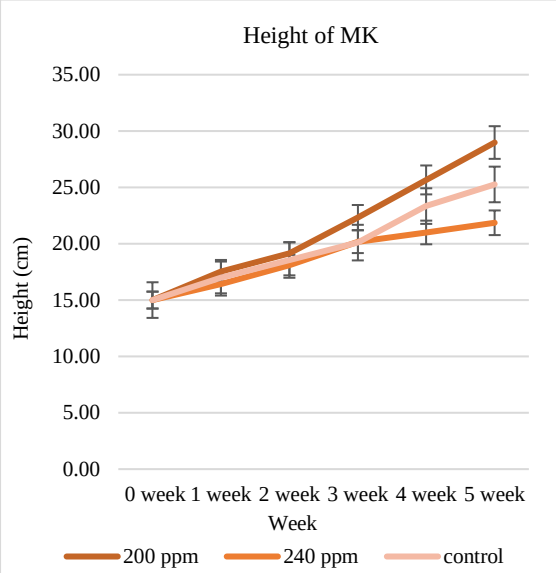

Figure 11. Average height of MK from week 0 to week 5

At the time of harvest, MK grown at 200 ppm of Fe concentration had the highest height during the growth period, and it was significantly high compared to MK grown at 240 ppm (Figure 11).

3.4 Leaf count of Plants

3.4.1. Leaf count of Green Thampala

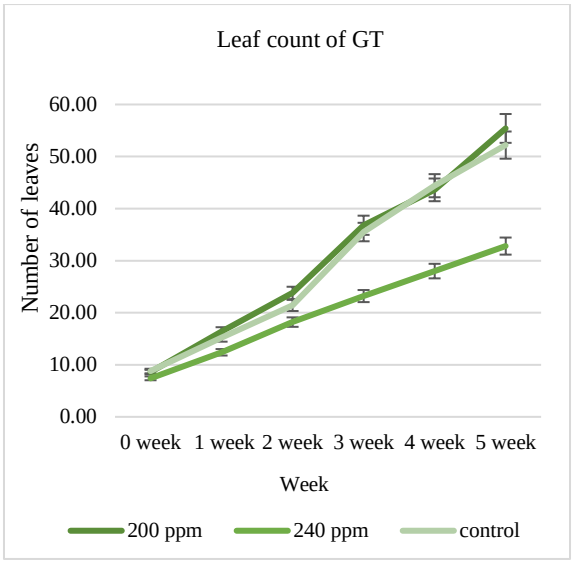

Figure 12. Average leaf count of GT from 0th week to 5th week.

GT grown at 200 ppm Fe concentration had the highest number of leaves during the growth period, and 240 ppm had the lowest number of leaves (Figure 12).

3.4.2 Leaf count of Mukunuwenna

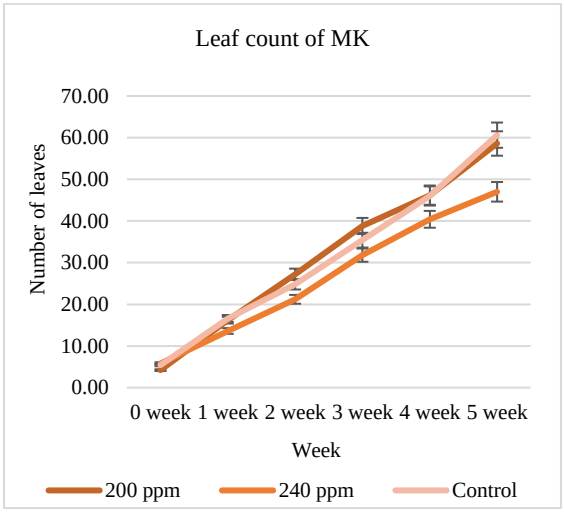

Figure 13. Average leaf count of MK from week 0 to week 5.

Mk grown at 200 ppm of Fe concentration, had the highest number of leaves during the growth period and 240 ppm had the lowest number of leaves (Figure 13).

3.5 Total Carbohydrate Content (TCC) of Plants

3.5.1. TCC of Green Thampala

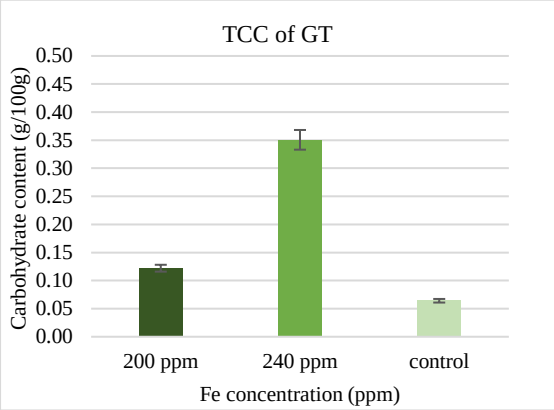

Figure 14. Total carbohydrate content of GT in different Fe concentrations.

GT grown at 240 ppm of Fe concentration had the highest content of carbohydrate and control had the lowest content of carbohydrate (Figure 14).

3.5.2 TCC of Mukunuwenna

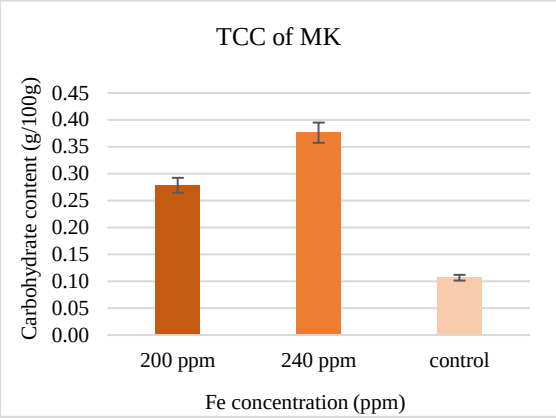

Figure 15. Total carbohydrate content of MK in different Fe concentrations.

MK grown at 240 ppm of Fe concentration had the highest carbohydrate content, while control had the lowest carbohydrate content (Figure 15).

3.6 Total Protein Content (TPrC) of Plants

3.6.1 TPrC of Green Thampala

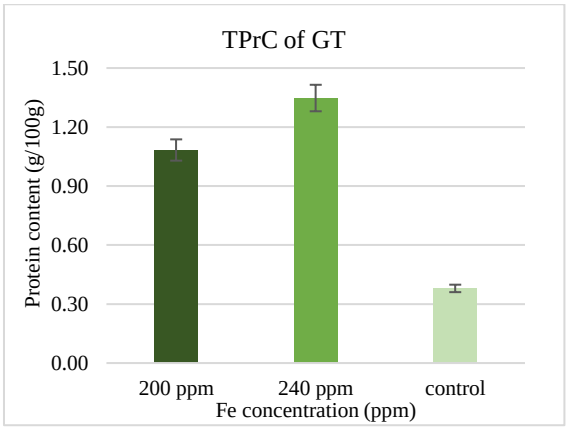

Figure 16. Total protein content of GT in different Fe concentration.

GT grown at 240 ppm of Fe concentration had the highest content of protein and control had the lowest content of protein (Figure 16).

3.6.2 TPrC of Mukunuwenna

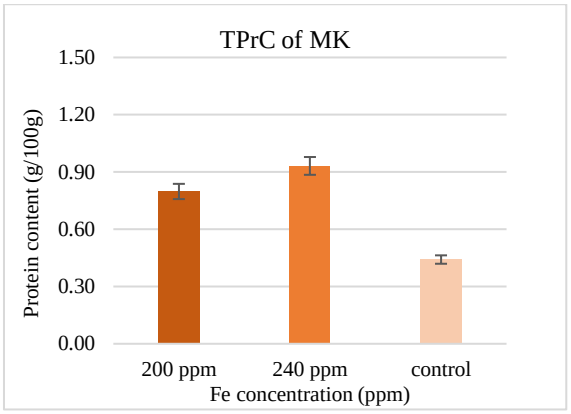

Figure 17. Total protein content of MK in different Fe concentration.

MK grown at 240 ppm of Fe concentration had the highest protein content, while control had the lowest protein content (Figure 17).

3.7 Total Flavonoid Content (TFC) of Plants

3.7.1 TFC of Green Thampala

GT grown at 240 ppm of Fe concentration had the highest content of flavonoid, and 200 ppm had the lowest content of flavonoid (Figure 18).

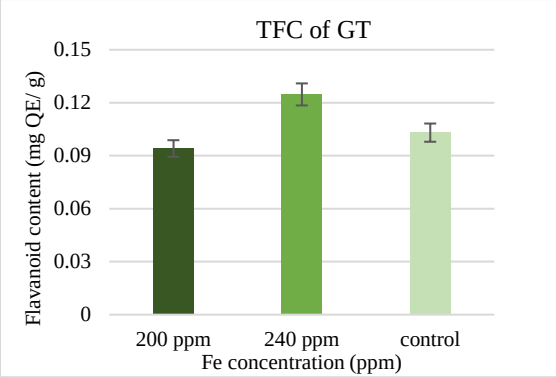

Figure 18. Total flavonoid content of GT in different Fe concentration.

3.7.2 TFC of Mukunuwenna

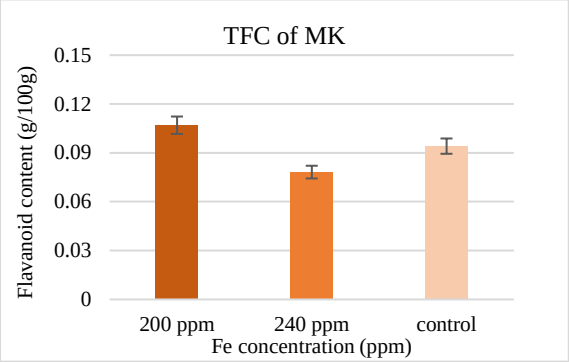

Figure 19. Total flavonoid content of MK in different Fe concentration.

MK grown at 200 ppm of Fe concentration had the highest content of flavonoid and, 240 ppm had the lowest content of flavonoid (Figure 19).

3.8 Total Phenolic Content (TPC) of Plants

3.8.1 TPC of Green Thampala

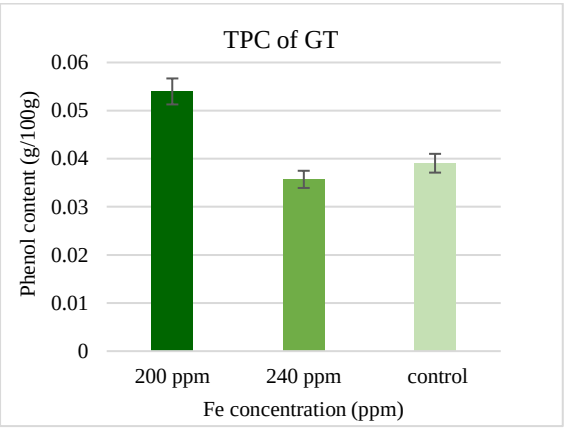

Figure 20. Total phenol content of GT in different Fe concentrations.

GT grown at 200 ppm of Fe concentration had the highest content of phenol, and 240 ppm had the lowest content of phenol (Figure 20).

3.8.2 TPC of Mukunuwenna

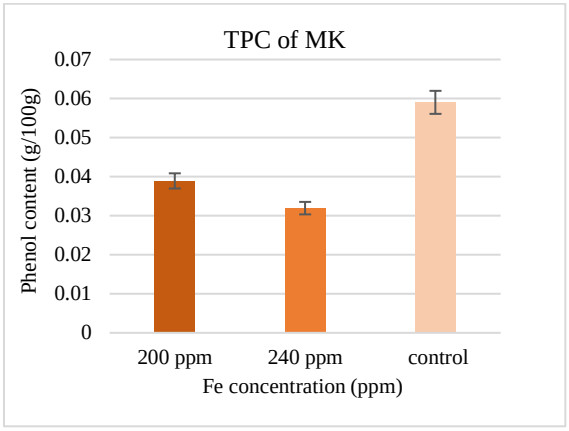

Figure 21. Total phenol content of MK in different Fe concentration.

MK grown in control of Fe concentration had the highest content of phenol, and 240 ppm had the lowest content of phenol (Figure 21).

3.9 Fe content of Green Thampala and Mukunuwenna

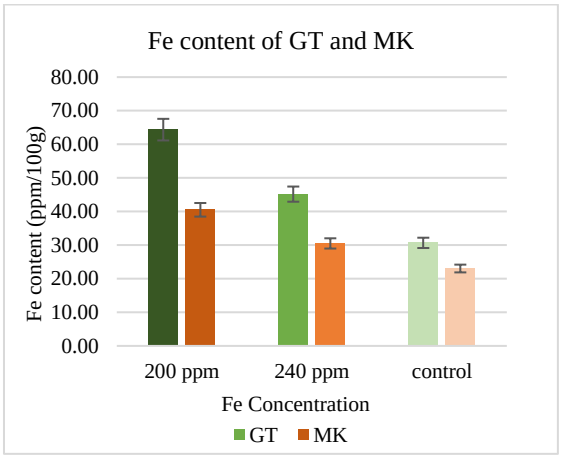

Figure 22. Fe content of GT and MK in different Fe concentration.

MK and GT grown at 200 ppm of Fe concentration had the highest content of Fe, and control had the lowest content of Fe (Figure 22).

3.10 Qualitative Analysis of Phytochemicals

Qualitative tests for phytochemicals were conducted across different Fe concentrations of Green Thampala and Mukunuwenna. The results of these tests are stated in Table 3.

Table 3. Results of the qualitative analysis (√- Present; x-Absent)

Bioactive compound	GT-200 ppm	GT- 240 ppm	GT- control	MK-200 ppm	MK-240 ppm	MK- control
Saponins	√	√	√	√	√	√
Polyphenols	√	√	√	√	√	√
Tannins	√	√	√	√	√	√
Anthraquinones	x	x	x	x	x	x
Steroids	√	√	√	√	√	√
Terpenoids	√	√	√	√	√	√

The results suggest that saponins, polyphenols, tannins, terpenoids, and steroids were present, while anthraquinones were not found in Green Thampala (GT) and Mukunuwenna (MK) across different Fe concentrations.

3.11 Antioxidant activity of plants

3.11.1 Antioxidant activity of Green Thampala

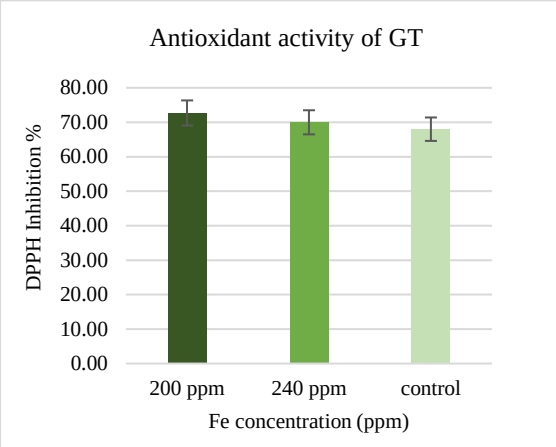

Figure 23. Antioxidant activity of GT in different Fe concentrations.

GT grown at 200 ppm of Fe concentration, had the highest percentage of inhibition, and control had the lowest percentage of inhibition (Figure 23).

3.11.2 Antioxidant activity of Mukunuwenna

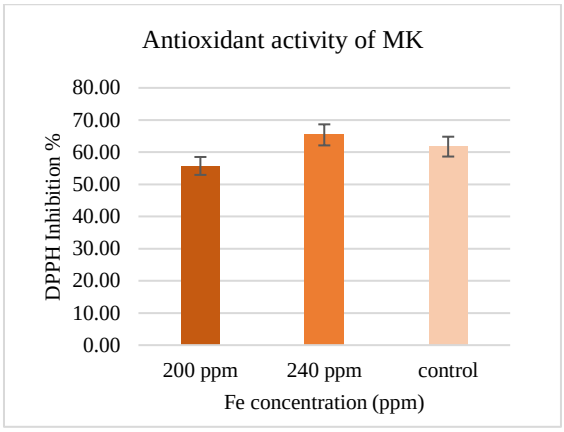

Figure 24. Antioxidant activity of MK in different Fe concentration.

MK grown at 240 ppm of Fe concentration had the highest percentage of inhibition, while 200 ppm of Fe concentration had the lowest percentage of inhibition (Figure 24).

4. Discussion

In this study, iron biofortification of Green Thampala and Mukunwenna was carried out using hydroponic techniques such as NFT and the coco peat method. Throughout the growth period, various parameters including height and leaf count, were recorded. Also, the total protein and carbohydrates of Fe-fortified plants were determined. Several qualitative tests were carried out to identify the presence of phytochemicals in GT and MK. Moreover, DPPH assay was carried out to determine the antioxidant activity. Furthermore, the TPC and TFC of plants were utilized to analyse the phenolic content and flavonoid content of Fe-fortified plants.

Albert's solution (AB solution) was used as a nutrient solution in the NFT system. It contains 650 ppm of Fe. Initially, 100% concentration of the AB solution was used as a control. MK plants were dead after 5 days of transferring to the NFT system due to the high concentrations of Fe. Normally, plants absorb 50–500 ppm of Fe; if it increases more than 500 ppm, it causes toxicity; if it is lower than 50 ppm, it will raise Fe deficiency in plants.[17] Moreover, 50% of the AB solution gives higher plant growth than 100% of the AB solution.[26]

Therefore, a 50% AB solution was used as a control in the next trial. However, this concentration was also not successful, possibly due to overwatering and over fertilizing. Overwatering may be prevented by submerging the tip of the root in a nutrient solution, and over fertilizing may be prevented by diluting the AB solution by 25%. Considering the drawbacks, the coco peat grow bag method was used as an alternative technique since it reduces overwatering and promotes plant growth.

When looking at the height and number of leaves of GT and MK in the 5th week, both showed the highest results in 200 ppm and the lowest in 240 ppm. Also, when comparing the fresh yield of GT at different Fe concentrations, the highest yield was obtained at 240 ppm, the lowest at control. These results agree with those of Buturi *et al*. 2022, where Fe deficiency stress inhibits the growth of plants while Fe efficiency induces the growth of plants.[14] Moreover, different parameters of the plant, such as height, weight, number of leaves, and root shoot ratio, increase with Fe concentration, but when the plant meets the threshold value of Fe, it suppresses the elevation of the parameters.[14] In this research, MK grown at 240 ppm of Fe concentration showed the lowest height, number of leaves, and fresh yield compared to MK grown at control and 200 ppm. Hence it can suggest that the threshold value of Fe for MK may fall between 200-240 ppm.

Carbohydrates produced during photosynthesis in plants are widely known as energy sources and carbon skeletons for organic molecules and storage components.[27] The phenol-sulfuric acid technique was employed in this investigation to identify TCC. In this method, disaccharides, oligosaccharides, and polysaccharides are broken down into monosaccharides by concentrated sulfuric acid. Following their reaction with phenol, these compounds provide a yellow-gold coloration. [28] When comparing the TCC of GT and MK in different concentrations of Fe, both showed the highest result at 240 ppm and the lowest in the control. These results were not in agreement with the findings of a previous study by Buturi *et al*.,2022, since excessive levels of Fe can increase Reactive Oxygen Species (ROS), causing cell damage and disrupting several metabolic events, including lowering the rate of photosynthesis. A reduction in the photosynthesis rate significantly reduces the carbohydrate content.[14]

Protein is a high-molecular weight bioactive compound. The Lowry method was used to determine the TPrC. Under alkaline conditions, cupric ions (Cu^{2+}) chelate with nitrogen atoms in peptide bonds, resulting in a reduction of Cu^{2+} to cuprous ions (Cu^{+}). Folin-Ciocalteau reagent reduces the Cu^{+} to produce tungsten blue.[29] When comparing the TPrC of GT and MK in different concentrations of Fe, both showed the highest protein content at 240 ppm and the lowest content in the control. The results were not in agreement with the findings of previous study by Ramzan *et al*., 2020. Protein content of maize was affected by the treatment applications; however, the highest protein content (14.37%) was found in control while lowest protein content (12.63%) was found in 1% of Fe foliar application.[30]

Chemical substances that are found naturally in plants are known as phytochemicals. There are many different parts of the plant that contain essential phytochemicals, such as terpenoids, flavonoids, phenolics, tannins, saponins, and steroids. In addition to providing the plant with color, scent, and flavor, they shield it from illnesses, pollution, stress, and UV radiation.[24] When considering the phytochemical analysis of GT and MK in different concentration of Fe, both showed positive results for saponin, tannins, terpenoids, steroids, and polyphenols, but they showed negative for anthraquinones.

Flavonoids are plant secondary metabolites with a polyphenolic structure.[31] In this study, TFC was performed using an $AlCl_3$ spectrophotometric assay. $AlCl_3$ forms acid-stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl groups of flavones and flavanols.[32] When looking at the TFC of GT and MK in different concentrations of Fe, GT grown at 240 ppm had the highest TFC, and 200 ppm had the lowest TFC. But the MK showed the highest TFC in 200 ppm and the lowest at 240 ppm. These results are in line with the previous study of Giordano *et al*. (2019), where a subgroup of flavonoids was

significantly increased in 2.0 mM Fe compared to 1.0 mM and 0.015 mM Fe.[33]

Phenolics are thought to have the highest ability to neutralize free radicals.[34] In this study, the Folin-Ciocalteu method was used to determine the TPC. The assay involves reducing the FC reagent with phenolic compounds in an alkaline medium. The reaction produces a blue chromophore composed of a phosphotungstic-phosphomolybdenum complex with the maximum absorption of the chromophores depending on the alkaline solution and the concentration of phenolic compounds.[35] When looking at the TPC of GT and MK in different concentrations of Fe, GT grown at 200 ppm had the highest TPC and the lowest at 240 ppm, but the MK showed the highest TPC in control and the lowest at 240 ppm. The result of GT is in line with a previous study by Buturi *et al*.,2022 and MK is not in agreement with these findings where 1 mM and 2mM of Fe application has increased the production of phenol whose main role is to control ROS production.[14]

Antioxidants play a crucial role in minimizing oxidative stress in biological processes by neutralizing free radicals, which can cause damage when combined with essential cellular elements like DNA and proteins.[36] Using the DPPH test, the antioxidant activity was assessed. When antioxidants interact with DPPH, they convert it to DPPH-H, which decreases absorbanc.[37] A higher inhibition percentage indicates the greater antioxidant activity of the sample compound. In this study, GT grown at 200 ppm had the highest inhibition percentage and control had the lowest while MK grown at 240 ppm had the highest and 200 ppm had the lowest. The results agree with the previous study of Arreola *et al.*, 2015, where an antioxidant activity of common beans was significantly increased in 50 µmol Fe compared to 25 µmol of Fe.[38] When comparing the Fe content of GT and MK in different concentrations of Fe, both plants showed the highest content at 200 ppm and the lowest content in control. Fe content increases until the plant reaches its optimum level; when it crosses the optimum level, Fe content decreases.

In interpreting the findings of this study, it is important to acknowledge several limitations. Controlled environmental conditions are essential for ensuring reliable results in future studies. Variations in factors like temperature and humidity can significantly impact outcomes, highlighting the necessity for precise control over these variables. The nutrient solution concentration was adjusted based on observed symptoms, but more precise initial trials with varied concentrations could help determine optimal levels more accurately. The study duration may not have been sufficient to observe long-term effects, suggesting that extending the duration would provide insights into the sustained impacts on plant health and nutritional content. Additionally, while qualitative tests identified the presence of phytochemicals, a quantitative analysis would offer a deeper understanding of their concentration and impact. By addressing these limitations and incorporating the recommended improvements, future studies can achieve more robust and reliable results, enhancing the overall quality and impact of the research.

5. Conclusion

Both 200 ppm and 240 ppm Fe concentrations were effective in fortifying Fe in both Mukunuwenna and Green Thampala, as evidenced by higher Fe content compared to the control group. Fe-fortified Mukunuwenna and Green Thampala also demonstrated varying levels of antioxidant activity, total flavonoid content, total phenolic content, and fresh yield. Based on the findings of the study, it can be recommended that 200 ppm of Fe can be utilized for increasing Fe content along with plant development, while 240 ppm of Fe may be preferred for enhancing Fe content along with nutrient enrichment.

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