Vitamin B_{12} and iron fortification in lettuces grown in hydroponic/aeroponic cultivation using nanoparticle technologies

Soojin Oh, MSc

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Abstract

Micronutrient deficiencies also known as hidden hunger, threaten global nutrition security and cause critical health problems. While interventions have been widely implemented for iron, iodine, and vitamin A, vitamin B_{12} (B_{12}) deficiency has received limited attention in crop fortification research. Although iron is abundant in the environment, its bioavailability for plant uptake is limited due to its chemical forms and soil interactions. Both B_{12} and iron deficiencies are leading contributors to anaemia, a major public health issue affecting young children and women of reproductive age globally. This study investigates B_{12} and iron fortification in lettuce (*Lactuca sativa*), using cyanocobalamin (B_{12}) and iron oxide nanoparticles (Fe_3O_4 NPs) under hydroponic and aeroponic conditions. B_{12} was applied via root and leaf treatments, and uptake, translocation and accumulation were assessed using elemental analysis.

The study found that both root and leaf application of B_{12} successfully enriched lettuce to levels meeting the recommended daily intake for adults (2.4 µg), without causing significant changes in plant morphology, physiology, or bioactive compound profiles. Notably, B_{12} applied at 10 mg / L via leaf application and 7.5 mg / L through root application resulted in B_{12} concentrations sufficient to exceed the adult daily requirement with 4.8 g and 1.7 g of fresh lettuce consumption respectively. Although root application showed effective B_{12} uptake and accumulation, from a cost-effective perspective, leaf application is more practical for agricultural use, as root application required approximately 100 times more B_{12} to achieve similar accumulation levels.

Additionally, the study found that Fe_3O_4 NPs capped with L-lysine enhanced lettuce growth and development, while improving iron bioavailability. Particularly, the Fe_3O_4 NPs 2.5 mg / L treatment resulted in improved physiological parameters, greater final harvest weight, and higher iron accumulation in leaf tissues compared to the Fe-EDTA treatment. These findings suggest that Fe_3O_4 NPs are a promising alternative to conventional iron fertilisers (e.g., Fe-EDTA) for hydroponic systems, with potential to reduce input costs and increase nutritional value.

Finally, the study assessed the combined fortification of B_{12} via leaf application and Fe_3O_4 NPs through root application. The Fe_3O_4 NPs 5 mg / L treatment significantly enhanced lettuce growth and development compared to the 2.5 mg / L treatment. Lettuces plants treated with high concentrations of B_{12} (100 and 1000 mg / L)

exhibited growth inhibition, whereas intermediate concentrations (10 and 50 mg / L) led to increased final harvest weight and higher accumulation of multiple elements, including Fe, K, Mg, and P. These results indicate that the effects of Fe_3O_4 NPs on plant development were generally greater than those of B_{12}, except at the highest B_{12} concentration (1000 mg / L), where inhibitory effects prevailed. This study suggests that effective and practical B_{12} fortification can be achieved using low concentrations (e.g., 5 mg / L to10 mg / L). Additionally, the Fe_3O_4 NPs 5 mg / L treatment is recommended for hydroponically cultivated lettuce to promote enhanced growth, superior harvest quality, and higher iron accumulation due to improved bioavailability.

Publications

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List of Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline -6-sulfonic acid) diammonium salt
Ado-Cbl	Adenosyl Cobalamin
B_{12}	Vitamin B_{12} (Cyanocobalamin)
Cbl	Cobalamin
CN-Cbl	Cyanocobalamin
DDT	1-dodecanethiol
DLS	Dynamic Light Scattering
EDTA	Ethylenediaminetetraacetic acid
ENMs	Engineered nanomaterials
FAO	Food and Agriculture Organisation of the United Nations
Fe-EDTA	Ferric-Ethylenediaminetetraacetic acid
FeO	Wustite
Fe_2O_3	Hematite
γ-Fe_2O_3	Maghemite
Fe_3O_4 NPs	Iron oxide nanoparticles / magnetite nanoparticles
FRO2	Ferric Reduction Oxidase 2
FT-IR	Fourier Transform Infrared
GA_3	Gibberellic acid
HCY	Homocysteine pathway
HoloTC	Holo-transcobalamin
HPLC	High Performance Liquid Chromatography
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively Coupled Plasma Mass Spectrometry

ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IF	Intrinsic factor
IRT	Iron-regulated transporter
iWUE	Intrinsic Water Use Efficiency
Ls	Stomatal Limitation
Me-Cbl	Methyl cobalamin
NA	Fe^{2}-nicotianamine
NDVI	Normalised Differential Vegetation Index
NPs	Nanoparticles
NPCI	Normalised Pigment Chlorophyll Ratio Index
NPK	Nitrogen-phosphorus-potassium
OTA	Octylamine
PAA	Polyacrylic acid
PERMDISP	Permutational Multivariate Analysis of Dispersions
PERMANOVA	Permutational Multivariate Analysis of Variance
PS	Phytosiderophores
PSRI	Plant Senescence Reflectance Index
PVP	Polyvinyl pyrrolidone
P-XRD	Power- X-ray Diffraction
RID	Radioisotope dilution
ROS	Reactive Oxygen Species
SDR	Spinning Disc Reactor
SEM/EDS	Scanning Electron Microscopy / Energy Dispersive X-ray Spectroscopy
TAA	Total Antioxidant Activity

TCII	Transcobalamin
TFC	Total Flavonoid Content
TEM	Transmission Electron Microscopy
TOP	Tri-n-octylphnosphine
TPC	Total Phenolic Content
WHO	World Health Organisation
WUE	Water Use Efficiency
XRD	X-ray Diffraction
ZnO NPs	Zinc oxide nanoparticles

Thesis outline

Chapter 1. Literature Review. This chapter focuses on micronutrient fortification in food crops, with a particular emphasis on vitamin B_{12} and iron. Nanoparticle technology is highlighted as a promising approach for micronutrient fortification in plants. Overall, this chapter provides foundational knowledge on the fortification of vitamin B_{12} and iron. Additionally, nanoparticle technology is presented as an innovative method for micronutrient fortification.

Chapter 2. General Materials and Methods. This chapter describes the experimental materials and methods utilised in this study, including study plant material (lettuce) and the chemicals employed. The methods encompass nanoparticle synthesis and characterisation, as well as assessment of plant physiology, morphology and harvest quality, biochemistry, and elemental analysis.

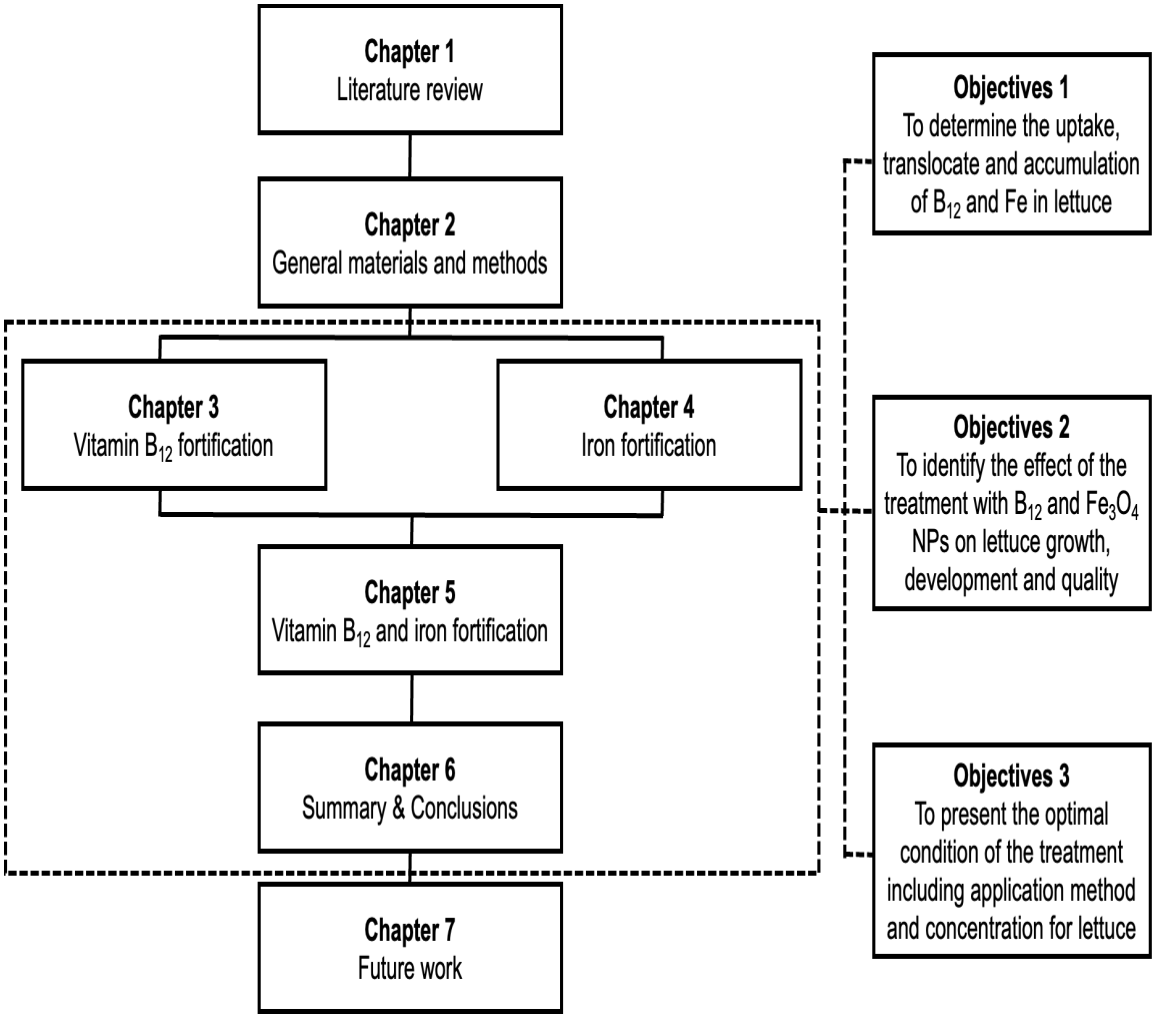
Chapter 3. Vitamin B_{12} Fortification. This chapter discusses vitamin B_{12} fortification using leaf and root application method at different concentrations. The results demonstrate the pathway of B_{12} within the lettuce tissues alongside their effect on lettuce growth, development, and nutritional quality.

Chapter 4. Iron Fortification Using Fe_3O_4 NPs. This chapter investigates iron fortification using iron oxide nanoparticles (Fe_3O_4 NPs) at various concentrations. It includes a comprehensive analysis between Fe_3O_4 NPs and conventional iron sources, such as Fe EDTA. Additionally, this chapter highlights the effect of Fe_3O_4 NPs treatment on lettuce growth, development, and nutritional quality.

Chapter 5. Vitamin B_{12} and Iron Fortification. This chapter investigates fortification of B_{12} and iron. Vitamin B_{12} was applied via leaf application, while Fe_3O_4 NPs were provided via root application. Overall, the chapter describes the effect of various combinations and concentrations of these treatments on lettuce growth, development, harvest quality and nutritional value.

Chapter 6. Summary and conclusion. This chapter presents conclusion of the thesis which provides an overall discussion and summaries the key findings from Chapter 3, 4, and 5.

Chapter 7. Future Work. This chapter has provided further investigation which can be further beneficial for B_{12} and Fe fortification using Fe_3O_4 NPs.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Literature review
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Chapter 3
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Summary & Conclusions
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Future work
Objectives 1
To determine the uptake, translocate and accumulation of B_{12} and Fe in lettuce
Objectives 2
To identify the effect of the treatment with B_{12} and Fe_3O_4 NPs on lettuce growth, development and quality
Objectives 3
To present the optimal condition of the treatment including application method and concentration for lettuce

Chapter 1. Literature Review

1.1 Micronutrient fortification in food crops

1.1.1 Introduction

A healthy human diet contains adequate amounts of macronutrients, including proteins, fats, and carbohydrates, and essential micronutrients, such as vitamins and minerals. Healthy diets play a crucial role in supplying essential nutrients, such as vitamins, minerals, dietary fibre and other bioactive compounds that are vital for optimal human growth, development, and overall health. To support optimal human health and development, daily recommended dietary intakes (RDI) have been established for key vitamins and minerals, providing guidelines to ensure adequate consumptions of these essential nutrients (See Table 1.1) [1]. However, despite these nutritional recommendations, a significant proportion of global population is unable to meet these requirements due to persistent food insecurity. Food insecurity restricts the availability of sufficient and balanced nutrients and energy intakes for human health, causing malnutrition symptoms such as undernutrition, micronutrient deficiencies, and obesity. The current global food system does not meet the universal requirement of adequate nutrition in food production, which directly influences human health. According to the Food and Agriculture Organization (FAO), over 690 million people worldwide are suffering hunger and nearly 750 million people have been exposed to food insecurity in 2019 [2].

According to Food and Agriculture Organisation (FAO), over two billion people worldwide are at risk of micronutrient deficiency, which is also known as 'hidden hunger' due to its often unnoticed yet significant effects on immune system function, growth and development in human health [3]. Micronutrient deficiency is strongly associated with inadequate food access, a prevalent challenge in low and lower-middle-income regions such as Sub-Saharan Africa, South-Central Asia and South-East Asia [4]. The rationale underlying this is that these regions heavily rely on starchy staple foods such as cereals, tubers, and roots, and the overall availability of animal-based food is lower than that in high-income regions [2]. The population groups at high risk for micronutrient deficiency include children under five and pregnant women, who have a considerable need for micronutrient supply for healthy development and growth. Stunted growth in children is one of the major adverse symptoms due to malnutrition, occurring in 21.3 % or 144 million children

in 2019 worldwide. However, micronutrient deficiencies are not confined to specific demographic or geographic regions; they can also affect the general populations in high-income countries, especially given the increasing trend toward plant-based diets. Plant-based diets, while beneficial for health and sustainability, may increase the risk of deficiencies in certain micronutrients such as vitamin B_{12}, vitamin D, and calcium due to their limited accesses or lower bioavailability in plant-based food compared to animal sources [5].

Integrated mitigation programmes have been developed to address macro and micro-nutrient deficiencies, including biofortification for food crops, food fortification and supplementation, sustainable agricultural practices, and improvement in post-harvest handling and storage. Globally, large-scale food fortification efforts have focused on key micronutrients that pose significant public health risks when deficient. These risks are especially critical during periods such as pregnancy, lactation, and infancy, making women and infants particularly vulnerable to deficiencies. Iodine deficiency has significantly declined worldwide due to the implementation of universal salt iodisation, a cost-effective strategy that ensure sufficient iodine intake, especially among vulnerable groups such as pregnant women and children [6]. Vitamin A fortification programmes have also been designed and implemented through the addition of vitamin to staple food vehicles such as edible oil and processed foods like sugar, particularly in low- and lower-middle income countries [7]. Mandatory folic acid fortification of wheat flour has been implemented in over fifty countries worldwide, alongside recommended supplementation programmes for pregnant women. Similarly, wheat flour has been fortified with iron in many countries, though its effectiveness in mitigating anaemia has shown limited or mixed success [8]. Vitamin D fortification of milk became a widespread public health intervention during 1930s and 1940s in several European countries, including United Kingdom, Finland, and Germany [9].

Despite the widespread implementation of fortification programmes targeting key micronutrients, such as iron, iodine, vitamin A, folic acid, and vitamin D, relatively limited attention has been given to the fortification of vitamin B_{12}. This study addresses this gap by investigating the co-fortification of vitamin B_{12} and iron in hydroponically grown lettuce using advanced delivery systems such as nanoparticle-based carriers. Lettuce (Lactuca sativa) was selected as the model crop for this research based on several practical and scientific considerations. Firstly, lettuce is widely cultivated and readily accessible across the globe, making it a suitable representative crop for translational research with potential for global application. Secondly, it has relatively short growth cycle, which allows for rapid

experimental turnover and multiple repetitions within a limited timeframe which is an important factor in controlled environmental studies. Thirdly, lettuce is known for its nutritional value, containing health-promoting compounds such as dietary fibre, vitamins and antioxidants [10]. This highlights its potential for studies targeting nutritional enhancement through biofortification. Lastly, lettuce is well-adapted to soilless cultivation systems, particularly hydroponics, which aligns with the research aim of controlled nutrient delivery and precise environmental monitoring. Its compatibility with hydroponic systems also supports sustainability goals of this research, as these soilless cultivation methods enable efficient water and nutrient use, reduce the risk of soil-borne diseases, allow for precise environmental control, and are highly suitable for controlled experimental designs and urban agriculture [11].

Table 1.1 Recommended dietary intakes (RDI) of major vitamins and minerals for human health [1].

	Vitamin A (µg RE /day)		Vitamin D (µg /day)		Vitamin K (K_1) (µg / day)	
Age	**Male**	**Male**	**Male**	**Female**	**Male**	**Female**
Infant (0 to 12 months)	180-190	180-190	5	5	10	10
1 to 3 years	200	200	5	5	15	15
4 to 8 years	200	200	5	5	20-30	20-30
9 to 13 years	250-330	250-330	5	5	30	30
14 to 19 years	330-400	330-400	5	5	45-65	45-55
19-65 years	270	270	10	10	65-85	65
65 + years	300	300	15	15	75-80	65-80
Female - pregnancy		370		5		
- Lactation		450		5		
	Vitamin C (mg /day)		**Vitamin B_9 (Folate / folic acid) (µg /day)**		**Magnesium (mg / day)**	
Age	**Male**	**Male**	**Male**	**Female**	**Male**	**Female**
Infant (0 to 12 months)	25-30	25-30	80	80	26-54	26-54
1 to 3 years	30	30	150	150	60-76	60-76
4 to 8 years	30-35	30-35	200-300	200-300	76-100	76-100
9 to 13 years	35-40	35-40	300-400	300-400	100-230	100-220
14 to 19 years	40	40	400	400	230	220
19-65 years	45	45	400	400	260	220
65 + years	45	45	400	400	224	190
Female - pregnancy		55		600		
- Lactation		70		500		
	Iron		**Vitamin D (µg /day)**		**Vitamin K (K_1) (µg / day)**	
Age	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Infant (0 to 12 months)	9	9	5	5	10	10
1 to 3 years	13	13	5	5	15	15
4 to 8 years	19-28	19-28	5	5	20-30	20-30
9 to 13 years	28-45	28-46	5	5	30	30
14 to 19 years	45-64	46-56	5	5	45-65	45-55
19-65 years	75	62	10	10	65-85	65
65 + years		62	15	15	75-80	65-80
Female - pregnancy		62		5		
- Lactation		62		5		

1.1.2 Current strategies for micronutrient fortification in food crops

1.1.2.1 Biofortification

Biofortification is acknowledged as a multidisciplinary and multi-institutional effort that integrates expertise in the development of new varieties of staple food crops. It focuses on enhancing the bioavailability of vitamins and minerals through agronomic practices, breeding, or biotechnology-based approaches[12,13]. Biofortification provides a cost-effective strategy for long-term application with feasibility for underserved rural populations [14]. Biofortified crops have already been widely adopted in low- and middle-income countries, where staple crops, such as cereals, tuberous root, and legume crops, form the dietary foundation. Recognising the scientific and developmental complexity of biofortification, the HarvestPlus programme was launched in 2003 as a 'Challenge Programme' under the Consultative Group on International Agricultural Research (CGIAR), demonstrating the global prioritisation of biofortification as a key strategy for improving public health and food security [15].

This programme mainly targeted increased bio-availability of major micronutrient such as vitamin A, iron, and zinc. For instance, vitamin A has been successfully biofortified in crops such as sweet potato (e.g., orange-fleshed sweet potato), maize, cassava, banana, and plantain. In addition to improved micronutrient content, biofortification efforts have also enhanced agronomic traits such as increased yield and stress resistance.

Iron has been fortified with legume crops (i.e., beans) and pearl millet, which also found improvements in yield and disease resistance. Similarly, zinc fortification has been applied to staple crops such as wheat, rice and maize [16]. According to CGIAR findings, vitamin A, iron, and zinc biofortified crops have successfully delivered sufficient amounts of essential micronutrients for human consumption and has significantly improved their bioavailability in major staple foods [15].

Biofortification presents two main advantages: long term cost-effectiveness and the ability to reach rural populations which are underserved [13]. Through various stages of biofortification, such as the exploration development and delivery stages, crop improvement activities are conducted with consideration of applicability and cost-effectiveness worldwide, especially for low- and middle-income countries [12,13]. The main challenges for biofortification development consist of three critical

elements: supply, policy, and demand. Agronomic traits should meet the micronutrient requirements and biofortified crops should be supplied to both rural and urban consumers through supportive policy [13]. Supportive policy is necessary for further intervention programmes with sustainability. Consumer and growers' participation can also limit the biofortified food application which presents as an acceptance of biofortified crop produce. Additionally, conventional plant breeding or genetic engineering technology requires long-term development and adequate expenses. Utilising the agronomic method has also been highlighted for the biofortification method which mainly focuses on fertilisers with micronutrients using nanotechnology.

Previous study suggested that the micronutrient-based nanoparticle application would be a great alternative for biofortification without further breeding or genetic variation by enhancing micronutrient content and crop quality. The study found that the micronutrient contents including iron, zinc and calcium was enhanced in potato, tomato and chilli pepper grown in hydroponic systems utilising metal oxide nanoparticles synthesised with amino acids, which enhances the stability of nanoparticles [17].

1.1.2.2 Food fortification

Food fortification refers to an approach to enhance essential micronutrient content by adding vitamins or minerals into food crops so that it contributes to fortifying the contents of nutrients/minerals and providing health benefits to the public [12]. Food fortification technology positively regulates micronutrient deficiency and prevents malnutrition problems, especially in staple crops. Unlike supplementations, which typically requires large dose of micronutrients in the form of capsules, tablets, and

syrups [18,19], food fortification involves incorporating relatively small amounts of micronutrients directly into food products such as wheat flour, edible oil, rice, and salt. In comparison to supplementation which can easily cause overdose [18], food fortification provides adequate amounts of micronutrients to consumers from a mass scale to a specific targeted scale [19].

Globally, the iodised salt programme is considered the most successful large-scale food fortification initiative, achieving a 74 % to 90 % reduction in iodine deficiency symptoms, including goiter incidence between 1924 and 1935. During this period, vitamin D was also fortified in milk in Europe and North America for children's health. Iron, vitamin B and other micronutrient were also fortified in cereals and white flours in the USA [20]. From the 1970s, several Latin American countries have fortified sugar with vitamin A, resulting in a reduction of a vitamin A deficiency prevalence from 22 % to 5 %. Currently, approximately 140 countries worldwide have implemented regulatory guidelines for food fortification programmes [20].

In the UK, the government has implemented mandatory food fortification practices by requiring the addition of iron, niacin, thiamine, and calcium carbonate to non-whole wheat flour during milling This is regulated under The Bread and Flour Regulations 1998, which mandate the fortification of all non-wholemeal wheat flour with these nutrients [21]. Similarly, The Bread and Flour (Amendment) (England) Regulation 2024 introduced the mandatory addition of folic acid to non-whole meal wheat flour, aiming to further support daily recommended nutrient intakes [21]. Additionally, legislation such as The Food for Specific Groups (Information and Compositional Requirements) (England) Regulations 2016, enforces compositional standard for infant formula in line with Commission Delegated Regulation (EU) 2016/127, specifying the inclusion of essential nutrients such as iron, phosphorus, zinc, vitamin A, D, C, K, E, thiamine, Niacin, vitamin B_6, and vitamin B_{12} to meet the nutritional requirements of infants [22]. Unlike mandatory food fortification, voluntary fortification has also been widely implemented in commonly consumed food such as dairy products (e.g., milk and yoghurt), breakfast cereal, biscuits, bread, beverages, snack foods, and plant-based milk alternatives [23].

There are several opportunities and challenges in food fortification. First, fortified foods would supply a better-quality diet with micronutrients which are necessary for women and children by reducing the requirements of supplementation. Second, fortified food for staple crops could contain micronutrients at a natural level. In addition, the application of fortified food could cost-effectively improve the nutritional status in a large population or targeted population worldwide [8]. Although

food fortification has numerous impacts on the public diet, there are several challenges to be considered. First, although fortified food applies to the public or targeted group, a specific fortified food product might be consumed only by some part of the targeted group. It is required that adequate programmes or advertisements regarding fortified food are given for targeted group [19]. Second, due to accessibility, there is a high chance to access fortified food in urban areas compared to rural areas [18]. Therefore, research should focus not only on the fortification process but also on the distribution of fortified products. Third, fortified food generally targets young children and women due to their extra requirement of micronutrients. However, the amount of uptake of fortified food is relatively small in infants or young children. Therefore, they are less likely to consume recommended intakes compared to their requirement of micronutrients. In addition, further knowledge is required about the effect of interaction among nutrients when micronutrients are added. To achieve sustainable food fortification and its implementation, it is necessary to consider transdisciplinary aspects based on understanding the effects of agriculture, environment, socioeconomic and politics [19]. Also, further supportive national-scale involvement is significantly vital for sustainable food fortification with increased bioavailability in the long term.

1.2 Vitamin B_{12} (Cobalamin) fortification

1.2.1 The role of vitamin B_{12} in human health

Vitamin B_{12}, known as cobalamin (Cbl), is a water-soluble vitamin and it is synthesised by bacteria such as heterotrophic bacteria and most of the aerobic photosynthetic cyanobacteria [24]. It is originated by bacteria mainly found in the gastrointestinal tract of animals and it should be synthesised via the fermentation process of the lactic acid [25]. There are two different B_{12}-biosynthetic routes for prokaryotes in nature based on their oxygen dependency: aerobic and anaerobic pathways. For instance, *Paracoccus denitrificans* takes an aerobic (oxygen-dependent) pathway, whilst *Bacilus megaterium, P. shermanii* and Salmonella typhimurium take an anaerobic (oxygen-independent) pathway [26]. Enteric bacteria are the primary source of B_{12}, responsible for most B_{12} biosynthesis in animals. However, eukaryotes are not capable of synthesising B_{12} and instead rely on B_{12}-producing bacteria [27]; organisms such as nematodes and animals acquire B_{12} through their diet.

Vitamin B_{12} is a nutritional requirement for animals, whereas plants neither require nor synthesise it. This is mainly because higher plants contain cobalamin-independent methionine synthase (MetE) which catalyse the final stage of methionine biosynthesis [28]. Unlike plants, animals contain a B_{12}-dependent enzyme called methionine synthase (METH), whilst plants contain B_{12}-independent methionine synthase (METE) enzyme [24]. Only traceable amounts of B_{12} are found in plants due to a nitrogen-fixing actinobacteria, *Frankia alni*, which associates symbiotically with actinorhizal plants and contributes B_{12} [29]. Thus, it is mainly found in animal-derived sources, including meat (e.g., ruminant or omnivorous animals), dairy, fish, and shellfish [30].

As illustrated in Figure 1.1, Vitamin B_{12} (Cobalamin) consists of central corrin rings containing four pyrrole rings around a central cobalt atom, a lower ligand (α-ligand) donated by 5,6-dimethylbenzimidazole (DMBI), and an upper ligand (β-ligand) synthesised from an adenosyl or methyl group [31,32]. Dependent on the different ligands on the upper surface of cobalt atoms, B_{12} is classified as four different chemical forms: (1) Hydroxocobalamin(H-Cbl), (2) Methylcobalamin (Me-Cbl), (3) Cyanocobalamin (CN-Cbl), and (4) adenosylcobalami (Ado-Cbl). As coenzymes in the cell, Me-Cbl and Ado-Cbl are the active forms of B_{12}. CN-Cbl and H-Cbl are

provitamin forms requiring Me-Cbl or Ado-Cbl to be utilised by the cells [32]. By participating in the metabolic homocysteine pathway (HCY), Me-Cbl acts a cofactor of methionine synthases. HCY pathway impacts on DNA synthesis and is involved in regenerating methyl donor S-adenosylmethionine (SAM).

Vitamin B_{12} functions as a coenzyme in two essential biochemical reactions: methionine synthase activity and methylmalonyl-CoA mutase activity. Methylcobalamin (Me-Cbl), the coenzyme form involved in methionine synthase, facilitates the remethylation of homocysteine to methionine which is a critical reaction for DNA synthesis and methylation, and for supporting gene expression, red blood cell formation, and neurological function [33,34]**.** Methionine is not only a precursor to S-adenosylmethionine (SAM), a universal methyl donor, but is also vital for the metabolism of nucleic acids, proteins, and lipids [35]. Adenosylcobalami (Ado-Cbl), another active form of B_{12}, serves as a cofactor for methylmalonyl-CoA mutase, which catalyses the isomerisation of methylmalonyl-CoA to succinyl-CoA. This reaction is essential for the catabolism of certain amino acids and odd-chain fatty acids, contributing to cellular energy production [34]. Among the various forms of B_{12} (cobalamin), cyanocobalamin (CN-Cbl), is a synthetic chemically stable form produced by the reaction of natural cobalamin with cyanide. Due to its stability and cost-effectiveness, CN-Cbl is the most widely used form in food fortification, nutritional supplements, and pharmaceutical application [31,32].

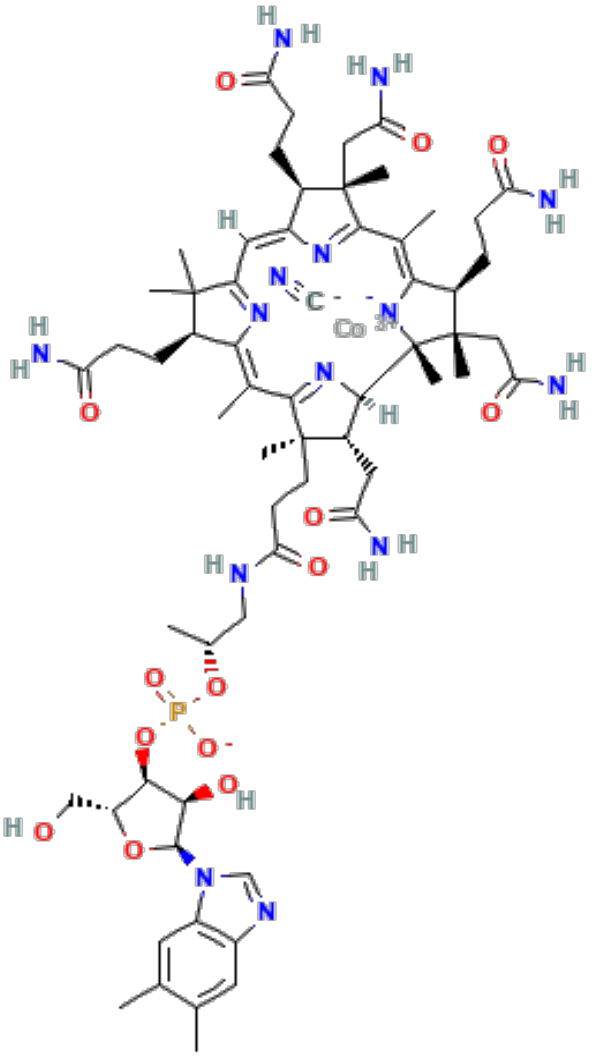


Figure 1.1 Chemical structure of Vitamin B12 (cyanocobalamin). Adopted from PubChem (CID:5311498).

1.2.2 Vitamin B_{12} absorption, digestion, and circulation in human health

Vitamin B_{12} is predominately found in animal-based food. Following the ingestion of food-bound cobalamin, B_{12} is initially attached to animal-derived proteins. In the stomach, these dietary proteins are released by the acidic environment through proteolysis, facilitated by pepsin and hydrochloric acid (HCl). Vitamin B_{12} is then bound to haptocorrin (also known as R-protein), which is secreted by the salivary glands and serves to protect B_{12} from acid degradation [36]. Intrinsic factor (IF), secreted by the gastric parietal cells, is also present in the stomach but initially binds weakly to B_{12} due to the higher affinity of haptocorrin. Upon entering the duodenum, the increased pH and the action of pancreatic enzymes lead to the degradation of the B_{12}-haptocorrin complex, releasing free B_{12}. This free B_{12} then binds to intrinsic factor to form the B_{12} Intrinsic Factor complex, which is transported through the small intestine towards ileum [36] (Figure 1.2).

In the ileum, the B_{12}-IF complex binds to the CUBAM receptor complex, which contains cubilin, and is internalised via receptor-mediated endocytosis [37–39]. Once inside the enterocyte, B_{12} dissociates from intrinsic factor and binds to one of several transport proteins: transcobalamin I, II, and III. Among these, transcobalamin II (TCII) is primarily responsible for delivering B_{12} to cells throughout the body. The TCII-B_{12} complex enters cells via endocytosis, after which free B_{12} is enzymatically converted into its two active coenzyme forms: methyl-cobalamin (Me-Cbl) and adenosyl-cobalamin (Ado-Cbl). Most of absorbed B_{12} is stored in the liver, while a portion is secreted into the bile and reabsorbed via enterohepatic circulation [36,37,40].

Previous studies have identified several endogenous and exogenous factors that affect the absorption of B_{12} in gastrointestinal tract and during enterohepatic circulation [36]. Firstly, achlorhydria – a condition characterised by reduced or absent hydrochloric acid in gastric secretions – can impair the release of B_{12} from food proteins, leading to cobalamin malabsorption. A deficiency in intrinsic factor (IF), often associated with chronic gastritis, can also result in poor B_{12} absorption, potentially causing megaloblastic anaemia and neurological disorders [36]. Secondly, exocrine pancreatic insufficiency can contribute to B_{12} malabsorption due to the reduced degradation of haptocorrin and persistently low pH in the small intestine, which interferes with the proper release of B_{12} from its binding proteins [37]. Thirdly, bacterial overgrowth in the small intestine – particularly involving

species such as *Pseudomonas* spp. and *Klebsiella* spp. – can compete for B_{12} and impair its absorption. Such overgrowth may result from gastrectomy, ileocolic intestinal resection, or reduced gastric acid secretion [36,37]. Lastly, genetic disorders associated with B_{12} deficiency can interfere with its plasma transport and conversion into active coenzyme forms [36] (Figure 1.2).

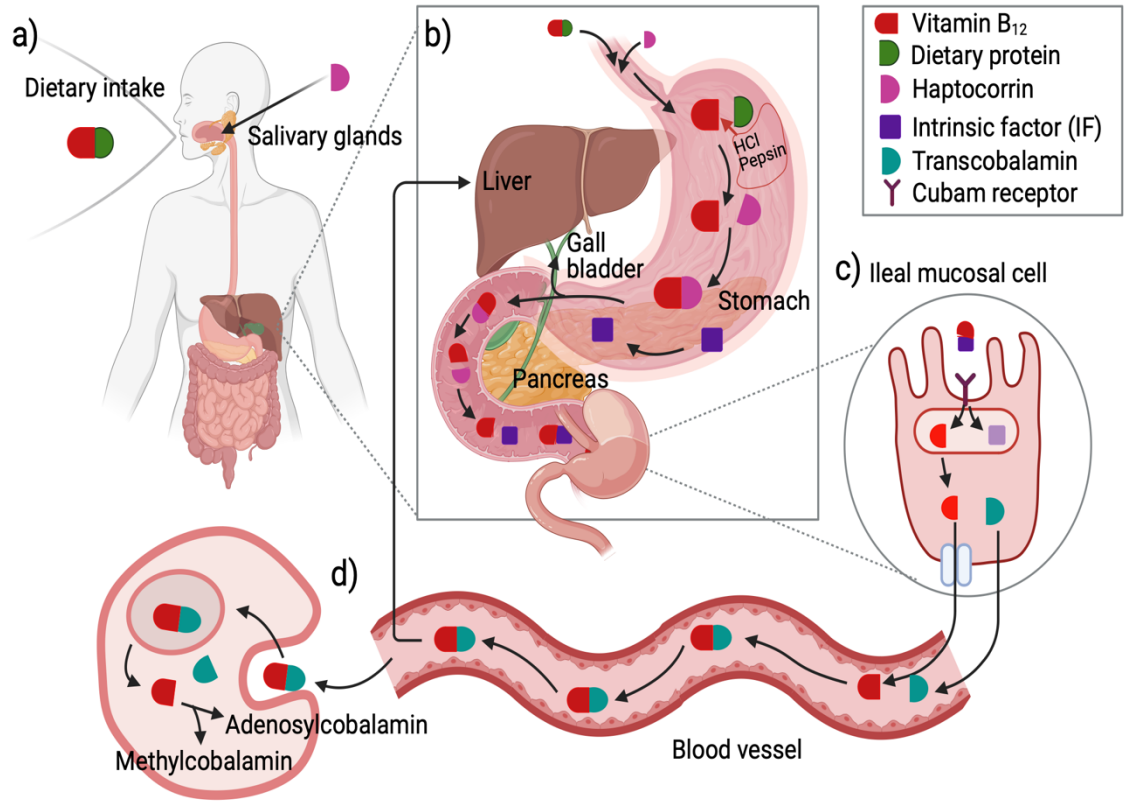


Figure 1.2 Gastrointestinal metabolisms and enterohepatic circulation of vitamin B12 (Cobalamin)

A. Dietary vitamin B_{12} is bound to dietary animal-derived protein in food. **B.** Upon ingestion, gastric acid and pepsin facilitate the proteolysis of dietary proteins in the stomach, releasing B_{12}. The free B_{12} then binds to haptocorrin (R-protein), which is secreted by the salivary glands and protects B_{12} from acidic degradation. Intrinsic factor (IF) is also secreted in the stomach; however, it does not bind strongly to B_{12} while haptocorrin is present. In the duodenum, the increased pH and the action of pancreatic enzymes lead to the degradation of the B_{12}-haptocorrin complex, releasing free B_{12}. This free B_{12} then binds to IF, forming the B_{12}-IF complex, which is transported to the ileum. **C**. In the ileum, the B_{12} -IF complex binds to the CUBAM receptor complex, which includes cubilin, facilitating receptor-mediated endocytosis. Once internalised, B_{12} is released from IF and binds to one of several transcobalamin (TCI, TCII, or TCIII). Among these, transcobalamin II (TCII) is primarily responsible for transporting B_{12} to cells throughout the body via the portal circulation. **D.** The TCII- B_{12} complex is taken up by cells via endocytosis, and intracellular enzymes convert B_{12} into its two activity coenzyme forms: methyl-cobalamin (Me-Cbl) and adenosyl-cobalamin (Ado-Cbl). The majority of absorbed B_{12} is stored in the liver, while a portion is secreted into the bile and reabsorbed through enterohepatic circulation [41–44]

1.2.3 Vitamin B_{12} deficiency

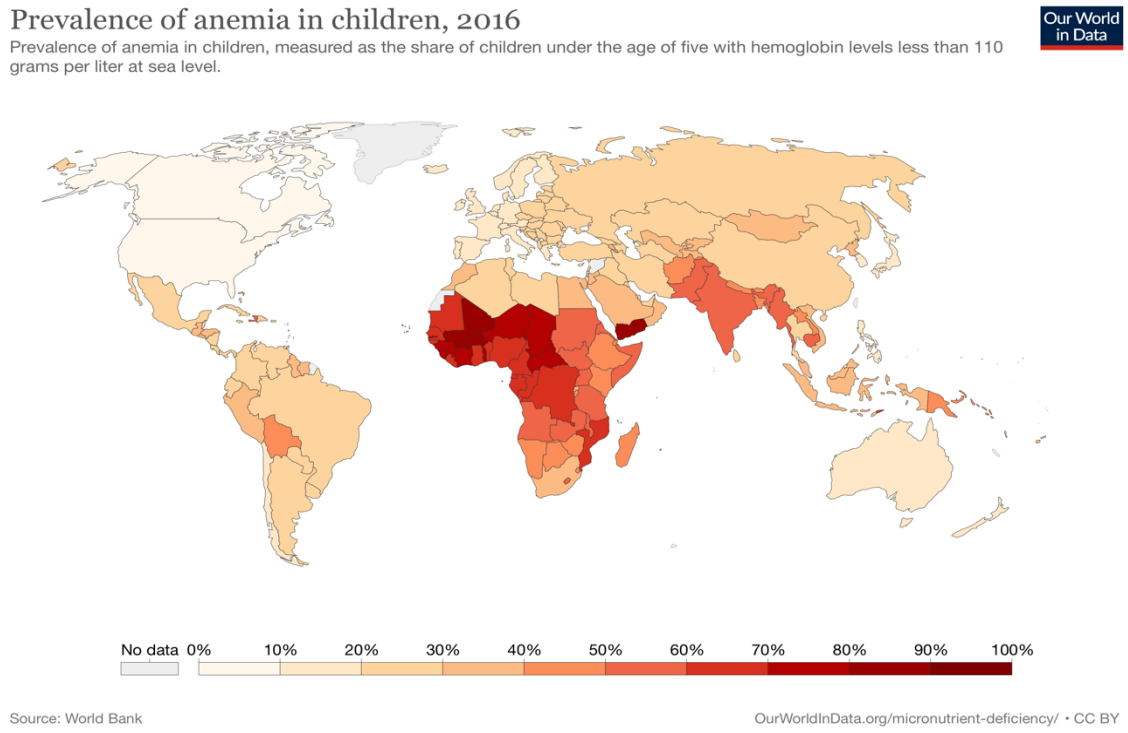
According to the World Health Organisation and the Food and Agricultural Organisation of the United Nations, the recommended daily intake amount of B_{12} is 0.4 µg (microgram) to 0.5 µg for infants, 0.9 to 2.4 µg for children to adolescents, and 2.4 µg for adults (2.6 µg and 2.8 µg for pregnancy and lactation respectively) (See Table 1.2) [1].

Table 1.2 Recommended dietary intake (RDI) of vitamin B_{12} for human (micrograms per day) [1].

Age	Male	Female	Pregnancy	Lactation
Infant (0 to 12 months)	0.4 – 0.5 µg	0.4 – 0.5 µg		
1 to 3 years	0.9 µg	0.9 µg		
4 to 8 years	1.2 µg	1.2 µg		
9 to 13 years	1.8 µg	1.8 µg		
14 to 19 + years	2.4 µg	2.4 µg	2.6 µg	2.8 µg

When B_{12} intake falls below recommended levels, either due to insufficient dietary consumption or impaired absorption, it can lead to a range of deficiency symptoms. Vitamin B_{12} deficiency is characterised by low level of circulating serum B_{12} and holo-transcobalamin (holoTC) along with the elevated level of total homocysteine in plasma and methylmalonic acid in serum or urine [46]. Homocysteine, as an amino acid, is generated by the demethylation of methionine and is accumulated in the blood when folate, B_{12} and B_6 are insufficient [47]. Vitamin B_{12} deficiency is prevalent not only in low and lower-middle-income countries but also in upper-middle-income countries.

Geographically, B_{12} deficiency is more prevalent in low- and middle-income countries, primarily due to dietary patterns dominated by staple crops and plant-based foods with limited intake of animal-source product[48]. In contrast, in high-income countries, the predominant cause of B_{12} deficiency is pernicious anaemia, an autoimmune disorder that impairs intrinsic factor production and thus inhibits B_{12} absorption [42]. Pernicious anaemia mainly threatens pregnant women and children under five particularly in South Asia and Sub-Saharan African countries [49]. Vitamin B_{12} deficiency is also prevalent in the elderly, affecting up to 20 % of people over 60 and approximately 6 % of adults (younger than 60 years) in the United States and the United Kingdom (Figure 1.3) [35].



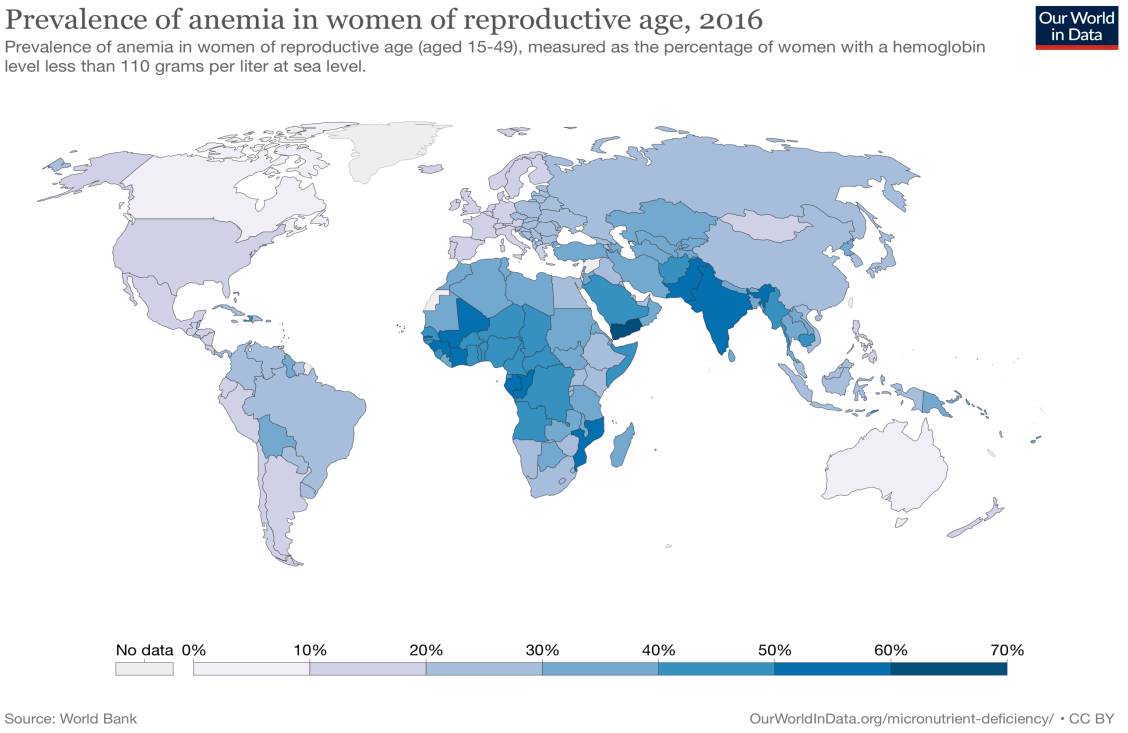


Figure 1.3 Vitamin B_{12} and iron deficiency driven anaemia prevalence in children and women of reproductive age worldwide.

Prevalence of anaemia in children under five predominantly occurs in Sub-Saharan Africa regions with average 60 % and Southern Asia 55 % in 2016. Prevalence of anaemia in women of reproductive age (aged between 15 and 49) mainly occurs in South Asia (24.2 – 69.6 %) and Sub-Saharan Africa (23.2 – 59.1 %), and Middle East and North Africa regions (23.4 – 49.5 %). (The image source: www.WorldBank.org)

Populations at high risk for B_{12} deficiency include the elderly, children, pregnant women of reproductive age, patients (e.g., autoimmune diseases including pernicious anaemia and atrophic gastritis), and those with malabsorption of food-cobalamin including those on strict vegetarian or vegan diets [50]. Within the elderly population, B_{12} malabsorption is mainly due to age-related gastric atrophy, which causes a reduction in acid and intrinsic factor (IF) [51].

Also, the populations with atrophic gastritis with low levels of stomach acid secretion generally have B_{12} malabsorption with certain gastric dysfunctions from food bound B_{12} sources[52]. Vitamin B_{12} deficiency is generally measured by serum methylmalonic acid (MMA) or the level of total plasma homocysteine which is normally lower than 200 or 250 picogram / mL (148 to 184 picomoles / L) [35]. Maternal B_{12} deficiency during the period and/or lactation increases the possibility of birth defects or growth retardation in infants [46]. In addition, low levels of B_{12} in breastfeeding and insufficient maternal intake of animal-based food causes brain development defects and overall developmental regressions, particularly in 4-6-month-old infants [38].

Pathophysiology of vitamin B_{12} deficiency includes subclinical symptoms because B_{12} is stored 1 - 5 mg in the human body which makes it difficult to diagnose deficiency [35]. Vitamin B_{12} deficiency is often accompanied by other micronutrient deficiencies such as folate (as known as B_9), iron, zinc, and protein deficiencies [50]. In particular, various studies have highlighted the correlation between B_{12} and folate [53,54]. Folic acid fortification neither prevents nor treats B_{12} deficiency, but it also does not impact adversely B_{12} deficiency [53]. Severe B_{12} deficiency induces megaloblastic anaemia (abnormal nucleated red blood cells) and abnormal neurologic diseases [35]. This deficiency also causes haematological and psychiatric adverse symptoms by affecting the formation of red blood cells and the normal functions of the nervous system [31,32,55].

Previous research has highlighted several endogenous and exogenous factors that impair B_{12} absorption: (1) Food-cobalamin malabsorption, often resulting from inadequate dietary intake or low bioavailability of B_{12} in certain food matrices; and (2) Impaired absorption due to chronic condition or autoimmune disorders including genetic disorders affecting B_{12} transport, pernicious anaemia, atrophic gastritis, *Helicobacter pylori* infection, or chronic alcohol abuse which disrupt intrinsic factor production or damage the gastric and intestinal mucosa [38,46,51]. Currently, patients suffering from autoimmune diseases with B_{12} deficiency are recommended to be injected with 1000 µg of B_{12} several times weekly. High-dose oral treatment is also

recommended for patients providing effective treatments. Vitamin B_{12} replacement therapy via intramuscular administration was also conducted for treating B_{12} deficiency [38,51]. However, it has been reported that deficiency symptoms are alleviated but not fully improved. Extensive medical and pharmacological research has focused on B_{12} supplementation. A more comprehensive study would be conducted on B_{12} enrichment in living plants.

1.2.4 Vitamin B_{12} sources: supplementations and natural food sources

It is common to treat vitamin B_{12} deficiency with high-dose injection, oral treatments, and nasal gel spray with cyanocobalamin. Although the supplementations have a high level of B_{12} doses, it is generally believed to be safe since only limited amounts of B_{12} are stored in the human body (1.4 to 5.1 μg) [35,56]. For instance, B_{12} is absorbed 50 % from 1μg oral dose and 20 % from 5 μg dose and the absorption is decreased by saturation from B_{12}-IF complex in the ileum from 1 to 2 % of an oral dose [46]. Daily losses of B_{12} presents 3.8 to 20.7 μg in healthy populations (over 25 years old), which is 1.4 - 8.6 times higher than the required amount of B_{12} preventing deficiency [56]. Vitamin B_{12} is generally concentrated in animal-based sources such as meat, dairy products, eggs, fish, and shellfish [32]. Extensive research regarding B_{12} fortification has focused on supplementation for medical or pharmacological purposes. Supplements of B_{12} from multivitamins or minerals are generally 500 to 1,000 μg of B_{12} with 2 % and 1.3 % of absorption respectively [35]. Due to the IF-mediated gastrointestinal absorption and circulation system, B_{12} bioavailability decreased ranging from 1.5 to 2.0 μg per meal under physiologic conditions or food processing [52]. In healthy populations, B_{12} bioavailability presents 42 % from fish or meat (beef, lamb, and chicken), ranging from 56 to 89 %, and although traceable amounts of B_{12} content are found in the yolk, eggs have poor bioavailability compared to other B_{12}-contained food sources. Vitamin B_{12} bioavailability can be significantly degraded by dysfunctions such as atrophic gastritis with low levels of stomach acid secretion. According to Dietary Reference Intake, it is assumed that a healthy population with normal gastrointestinal function can absorb 40 % to 50 % of B_{12} from animal-based food sources [57].

Wantanabe and Bito (2018) highlighted the meat and livers of ruminant animals contain higher amounts of vitamin B_{12} (cooked beef liver contains 83 μg / 100 g) than those found in omnivorous animals (cooked chicken meat contains 0.4 - 0.6 μg / 100 g). Trace amounts of B_{12} are present in various animal-derived foods,

including milk (0.3 - 0.4 µg / 100 g), egg (0.9 - 1.4 µg / 100 g), shellfish (104 µg / 100 g) and fish including salmon, trout, and tuna (3.0 - 8.9 µg / 100 g) [30]. Compared to animal-based food, plant-based food contains very low or negligible amounts of B_{12} [58]. For instance, only negligible amounts of B_{12} were found (< 0.1 µg / 100 g) in vegetables, including broccoli, asparagus, and mung bean sprouts [30]. Several studies have focused on plants, fungi, and algae with traceable amounts of B_{12} within these symbioses including edible mushrooms, edible algae, fermented soybeans (0.1 - 1.5 µg / 100 g) and vegetables, and processed food such as cereal, bread, and beverages [30,59]. The B_{12} content in edible plants are generally very low with different degrees of stability and bioavailability which contribute complex analysis of B_{12} [29]. Previous studies have emphasised that the biological activity of B_{12} is uncertain in most cases due to the limited availability of natural sources of B_{12} [29]. Therefore, further studies are required to focus on the fortification of B_{12} in food crops and evaluate their applicability in the dietary system [59] .

1.2.5 Status of vitamin B_{12} fortification

One of the effective strategies to enhance the vitamin B_{12} content is flour fortification on a national scale with a uniform dose. Various processed food products have been fortified with B_{12} such as cereal grain products [47,60], dried soup powder, and powdered milk drink [61]. Vitamin B_{12} can also be supplemented in fresh-cut fruits and vegetables for alleviating its deficiency in the population groups who are at high risk. A combination of B_{12} and chitosan is applied in fresh-cut salad mixes (melon, pineapple, and carrot) which are for 'ready-to-blend' beverages. There was an attempt made to develop B_{12} fortified beverage contains up to 8.6 µg / 1 kg of B_{12} along with an enhanced shelf-life [62]. A recent study investigating the nutrient composition of functional beverages, known as energy drinks, reported that B_{12} was present in approximately 67 % of the tested products, including 20 energy shots and 55 energy drinks. Notably, the actual B_{12} content in these beverages were often far above the recommended daily intake. In several products, the B_{12} levels exceeded 5,000 % of the daily value. This indicates that while B_{12} fortification is widespread in functional beverages, the concentrations are frequently above recommended daily intake, raising potential question about the regulation and nutritional necessity of such high dose in consumer products [63].

Further efforts were made to quantify the bioavailability of fortified vitamin B_{12} in bread in five healthy elderly people aged over 60 [62]. The study shows that B_{12}-fortified flour retains approximately 50 % of its bioavailability after the processes of

fermentation and baking containing 2 µg / 100 g. The study shows the healthy elderly individuals can absorb sufficient B_{12} from the fortified bread, suggesting that the B_{12} fortified wheat (flour) may serve as a promising plant-based alternative to animal-derived sources, particularly when fortified with high-purity crystalline ^{14}C-labeled B_{12} [62]. Further study would be required to investigate the absorption of fortified flour for different targeted subjects who have a high risk of B_{12} deficiency such as children or pregnant women or larger populations of the elderly. Accordingly, vitamin B complex fortified breakfast cereals contribute to the enhancement of their contents including vitamin B_6, B_9 (folate), and B_{12} (cobalamin). Fortified cereal significantly improved B_{12} concentrations and reduced the prevalence of high levels of plasma homocysteine from 13 to 3 %. This study highlights that B_{12} fortified cereal would be great B_{12} sources for general populations who frequently have poor vitamin status [47] . It was highlighted that free B_{12} - fortified breakfast cereals present better absorption compared to B_{12} -bound proteins in food which may be due to the Intrinsic Factor-mediated gastrointestinal metabolism effects on B_{12} absorption. Vitamin B_{12} fortification is also targeted to adults aged over 50 years by using the microencapsulation approach [47]. Additionally, the study found that the application of the encapsulated B_{12} powder in yoghurt (50 µg of B_{12} was added into 175 g of yoghurt) successfully fortified B_{12} [60].

Several in situ fortification methods have been developed to enhance vitamin B_{12} levels in fermented plant-based food. In situ fortification using bacteria (*Propionibacterium freudenreichii* DSM 20271 and *Levilactobacillus brevis*) has been shown to successfully increase B_{12} content in fermented cereals, pseudo-cereal (e.g., rice bran and buckwheat bran), and legume-based products [64]. Fermented legume materials have been reported to contain 30 to 40 µg / 100 g (dry weight) of B_{12}, as determined by ultra-high-performance liquid chromatography (UHPLC). These findings suggest that fermented plant-based food hold great potential as alternative source of B_{12} for plant-based diets. Furthermore, this study found that optimal pH conditions for P. *freudenreichii* significantly increase the B_{12} content in fermented grain materials. Further research is required to examine the contents of other micronutrients and minerals in the fermented crop materials using in situ fortification which is necessary to be considered for B_{12} amelioration along with other micronutrient deficiencies [64]. Vitamin B_{12} also successfully fortified up to 0.97 µg / 100 g in tempeh by in situ approach using P. *freudenreichii* (Log 7 CFU/g) and Rhizopus oryzae spores (Log 4 CFU/g) [65]. This study also evaluated the effect of in situ fortification on tempeh

quality by analysing the consumer acceptance traits including microbial composition, texture (firmness), and volatile organic compounds to measure aroma quality. The results indicated that the fortification with both food-grade micro-organisms did not negatively impact these quality traits. However, it is recommended that future studies explore the contents of other micronutrient and/or minerals, especially those relevant to address B_{12} deficiency and fortification strategies.

Food crops including kaiware daikon (Japanese radish) [66], common bean [67], lettuce [68], tomato [69], pea [70], and spinach [71] have been a focus of B_{12} fortification. Seed priming techniques [66,72] , and a hydroponic system with B_{12}-enriched solution[68] [69] have been utilised for B_{12} fortification in food crops. According to Keshavarz and Moghadam (2017), common bean was processed via seed priming with high concentrations of B_{12} exhibit more favourable growth performance and an increased B_{12} content in the crops [73]. The study found that B_{12} increases the resistance capacity against abiotic stress and reduces oxidative stress by providing an effective antioxidant and regulating osmotic balance. Common bean seeds soaked with 11 and 22 µM of B_{12} concentrations show enhanced chlorophyll content, catalase, and peroxidase activity in the leaves in the condition of salt stress compared with the control treatment. Also, the application of seed priming with 22 µM of B_{12} under saline conditions increased the level of proteins in the bean plants, whilst there was no effect of protein improvement under non-saline conditions. The outcome of this study highlights that the application of B_{12} enhances not only salinity tolerance but also effective photosynthetic biosynthesis by alleviating the adverse effect on photosynthesis pigments in salinity stress [67,74]. Although plants take B_{12} -independent enzymatic pathway, exogenous application B_{12} has been shown to alleviate abiotic stress in common bean. These effects are likely mediated through antioxidant activity or stress response modulation rather than direct metabolic incorporation of B_{12}.

Seed priming with B_{12}-enriched solution also increases the B_{12} content in kaiware daikon sprouts up to 1.5 µM / g in any concentration of B_{12} solutions ranging from 0 to 200 µg / mL [75]. This study addresses the possibility of reduction of B_{12} by cooking processes, such as boiling, and highlights that prolonged boiling (3 to 10 minutes) will decrease the B_{12} content in kaiware daikon. Based on this finding, further studies can be conducted to identify the effect of B_{12} enriched coated with micronutrients/minerals in food crops and how much B_{12} content will be retained after cooking processes [75].

High concentrated B_{12} solutions were applied via hydroponic cultivation for lettuce [68], tomato [69] and spinach [71]. Bito et al (2013) show that the majority of B_{12} (Cyanocobalamin) accumulated in leaves (86 %) which may be a promising source of free B_{12} in food crops. Approximately 164.6 µg / g fresh weight of lettuce would provide the recommended daily allowance for vitamin B_{12} (2.4 µg / day) [68]. This study addressed the expected costs for B_{12}-nutrition solution for lettuce fortification which was calculated to be approximately U.S $ 0.06 [68]. Similarly, the outcomes of B_{12} fortification in tomato indicated that B_{12} was successfully fortified in ripened tomato fruit up to 0.4 µg / 100 g of dry weight [69]. Additionally, the study highlighted that the B_{12} treated tomato showed significant increases in glucose and lycopene content [69]. Additionally, Zheng et al. (2021) showed that B_{12} fortification was successfully conducted for spinach grown in hydroponic cultivation. The results indicated that the spinach contained remarkable amount of B_{12} after boiling process (0.94 ± 0.11 µg / g) highlighting the stability of B_{12} absorbed by spinach [71] (See Table 1.3). This result indicated a cost efficient B_{12}-enriched solution for hydroponic cultivation which could meet the daily requirement of B_{12} for adults [68]. This study highlighted that B_{12} fortification not only enhanced plant growth and development by increasing glucose and lycopene levels, compounds associated with improved stress tolerance, but also offered potential benefits for human health, including cancer and cardiovascular disease prevention [69,76], as well as improved gut microbiota and increased production of short-chain fatty acids [71].

Previous studies investigated the effects of foliar aplication of B_{12} alone and in combination with vitamin C [77] and Gibberellic acid (GA_3) [78] on overal plant growth, biomass, harvest yield and nutritional quality. El-Baz et al. (2016), demonstrated that foliar application of B_{12} alone significantly enhanced total sugar content, total soluble solids to titratable acid ratio, and vitamin C level in banana plants compared to untreated controls [77]. Simliarly, pear trees treated with a combined application of B_{12} (4 mg / L) and gibberellins (30 mg / L) exhibited notable improvements in growth, harvest yield, nutritional quality, and elevated chlorophyll content [78]. These improvements are likely associated with enhanced carbohydrate metabolisms. According to El-Baz et al. (2017), B_{12} activates the citric acid cycle and pentose phosphate pathway, thereby increasing carbohydrate accumulation and promoting shoot elongation. This carbohydrates accumulation was found to support further vegetative development, such as increased shoot diameter and expand leaf area, suggesting a progressive enhancement in photosynthetic capacity and biomass production during plant growth [78].

It was highlighted that a high intake of B_{12} did not show adverse effects on human bodies. However, there are limited studies on the effect of fortified B_{12} in food crops and its specific health effects on human health [84]. Further research would be required to identify sustainable fortification methods for B_{12} and its stability and effects on human health when it is digested or accumulated in the long term. Additionally, it remains challenging to quantify and determine the exact B_{12} content in food crops because B_{12} mainly exists as a bound form in food crops with different degrees of stability [74]. Nakos et al. (2017) pointed out that immunoaffinity chromatography and high-performance liquid chromatography (HPLC) analysis can provide quantitative chromatographic isolation of B_{12} in food crops. Further studies should distinguish between active or inactive analogues of B_{12} forms in food crops [74]. It is difficult to evaluate vitamin B_{12} bioavailability by quantifying active B_{12} or inactive corrinoids in certain foods, such as B_{12}-fortified plant-based food. Therefore, for B_{12} fortification strategies, it is necessary to enhance the B_{12} content in food sources along with the high bioavailability which is the actual amount of available uptake [57].

Little is known about vitamin B_{12} biofortification based on crops. However, a relatively limited number of studies have been conducted to investigate B_{12} fortification and understand their mechanisms of uptake/distribution in living plants. According to previous studies, several plants, fungi, and algae (e.g., Kaiware daikon, mushrooms, dry seaweed, and garden cress) absorb and translocate B_{12} if they are grown in nutrient-sufficient conditions with organic fertiliser or vitamin B_{12}-enriched growing media [75,85,86]. Additionally, more various concentrations of B_{12} should be explored to investigate their effcts on plant growth and development. Previous studies do not account for the effect of B_{12} treatment on plant growth or development throughout the growing period as the treatment was applied 24 hours before final harvest [68,69,71].

Table 1.3 Vitamin B_{12} fortification in food crops [1].

B_{12} application		B_{12} concentration	Impacts on plant
B_{12} fortified food and supplementation			
B_{12} fortified food	Flour	2 µg / 100 g	- B_{12} fortified flour retains its bioavailability 50 % after the process of fermentation and baking [62]
	Yoghurts	50 µg / 175 g of yogurt	- B_{12} microencapsulated fortified yogurt showed stable fortification throughout shelf-life [87]
In situ fortification in fermented food	Cereal, pseudo-cereal, and legume	In situ with *Propionibacterium freudenreichii* and *Levilactobacillus brevis*	- Effectively fortified B_{12} during fermentation process in study materials - Fermented raw materials impact on the fortification [64].
	Lupin tempeh	*Propionibacterium freudenreichii*	- A significant increase of B_{12} (up to 0.97 µg / 100 g) via fermenting lupin [65]
B_{12} fortification in plants			
Seed priming	Kaiware daikon	0 - 200 µg / mL	- Increased B_{12} content in plant up to 1.5 µM / g in any concentration [66]
	Common bean	11 and 22 µM	- Increased capacity against abiotic stress - Enhanced chlorophyll content, catalase, and peroxidase activity [74]
B_{12} enriched - hydroponic solutions	Lettuce	1,5, 10 µmol / L	- B_{12} accumulated in lettuce leaves with cost-effective method [68]
	Tomato	5 µM	- No significant differences in fresh weight - Identify B_{12} move from root to fruit - B_{12} treated tomato showed increases in glucose and lycopene content [69]
	Spinach	0.1, 6.78, and 13.35 mg / L	- B_{12} was accumulated in lettuce leaves after boiling process (0.94 ± 0.11 µg / g) with beneficial effects on gut microbiota [71]
Foliar spraying	Pear tree	2 & 4 mg / L B_{12} and GA_3 + B_{12}	- Pear trees treated with B_{12} alone and in combination with GA_3 (30 mg / L) + B_{12} (4 mg / L) showed increases in shoot length, leaf area, high content of carbohydrate and nitrogen %, and harvest yield (e.g., fruit weight and volume) compared to control group [78]
	Banana plant	50, 100 mg / L and combination of cobalt & vitamin C	- Banana plant treated with B_{12} alone showed significance increases of bunch weight, harvest yield, biomass, and finger weight compared to the control group. - Total sugar, total soluble solids to titratable acid, and vitamin C also significantly increased by B_{12} treatment compared to control [77]

1.3 Iron fortification using nanoparticles

1.3.1 The role of iron in human health and plant growth

As a main component of cytochromes and haemoglobin, iron is an essential for human health. The daily required amount of iron is variable depending on ages, gender, and dietary condition such as plant-based diets. According to the National Institute of Health, the recommended daily amount of iron is 0.27 mg to 11 mg for infants (0 to 6 months and 7 to 12 months infants), 7 to 15 mg for children to adolescents and 18 to 27 mg for pregnancy [88]. The food sources contain abundant iron includes red meat, liver, beans, soybean flour and fortified products such as breakfast cereals [89]. As a predominant cause of anaemia, iron deficiency causes on public health concerns particularly overall 40 % of young children (6 to 59 months of age), and 37 % of women with pregnancy and 30 % of women 15 to 49 years of age are estimated anaemia, globally [90]. Iron deficiency anaemia has been highlighted via extensive mitigation programmes such as food fortification, biofortification and supplements for dietary. However, iron fortification in food remains a global challenge due to several issues, including undesirable changes in food quality (texture, colour, and taste), degradation of vitamins, and the low bioavailability of iron caused by the presence of absorption inhibitors [91,92].

Iron plays an important role in plant's metabolic processes by involving the following mechanisms: (1) Photosynthesis activity with the presence of iron atom in photosynthetic apparatus; (2) Chlorophyll synthesis as a major element of chloroplasts within a porphyrin structure of chlorophyll; (3) Respiration process associated to the electron transport chains; (4) Nitrogen fixation in plants as a cofactor of various enzymes, including lipoxygenase and 1-aminocyclopropane acid-1-carboxlylic acid oxidase; (5) DNA synthesis via the action of the ribonucleotide reductase [93,94]. Although, iron is one of the abundant elements in ecosystem, it often exists in soil as an insoluble ferric hydroxide form. In other worlds, iron bioavailability for plant is limited which impacted by their chemical forms (ferrous Fe^{2+} or ferric iron Fe^{3+}) and soil condition including anaerobic or aerobic condition along with pH level [93]. Iron deficiency negatively affects plant growth and development by increasing chlorosis and wilting leaves, reducing biomass of the leaf and root which eventually leads loss of harvest yield and crop failure [93]. Additionally, excessive amount of iron is potentially toxic to plants which causes formation of the reactive oxygen-based radicals. This may cause vital

damage of plant membranes by lipid peroxidation and further bronzing, acidity and blackening the root in the above-optimal condition of iron [93]. For the optimum growth and development, plants required the range of 10^{-9} to 10^{-4} M of iron. As mentioned above, the bioavailability of iron is limited for plants to uptake because it predominantly exists in the Fe^{3+} form. Fe^{3+} primarily forms oxyhydroxide polymers with extremely low solubility, making it unavailable for direct absorption by plants [93]. The plants grown in iron deficiency condition; morphological structure of the root presents lateral roots increases to facilitate iron uptake by increasing the root surface [94]. Furthermore, under the iron deficiency environment, plants have developed sophisticated mechanisms so called Strategy I and II to facilitate iron uptake in a limited availability of the soluble iron. Strategy I accounts for the dicots and non-gramineous monocots to adapt to iron deficiency, while Strategy II has developed by gramineous monocots [94].

Strategy I pathway is most exhibited in plants except grasses presenting the following stages under iron deficiency condition: acidification of the rhizosphere induced by the plasma membrane called H+-ATPase (P type-ATPase); Ferric Reduction Oxidase 2 (FRO2) involves the reduction of Fe^{3+} to Fe^{2+} that transported to the plant cell by Iron-Regulated Transporter (IRT1 transporter)[94–96] . While Strategy I is reduction-based strategy, Strategy II is based on chelation of Fe^{3+} by phytosiderophores (PS). PS chelated Fe^{3+} to PS-FE (III) complex which transport to the plant cell [96]. Once iron is entered to the rhizodermal cells of the plants, iron is moved from the endodermis to the vasculature of the plant including xylem and phloem as a Fe^{2}-nicotianamine (NA) form via symplastic pathway [95]. Via the xylem parenchyma cells, iron is mobilised to symplast to apoplast pathway within the xylem vessels as a form of Fe^{3}-citrate. Iron located in the parenchyma cells may transport to the leaf mesophyll cells via Fe^{2}-NA meditation. Approximately 90 % of iron is stored in the chloroplasts and mainly found in stroma bound to a protein called phytoferritin. Further studies are required to clarify the pathway of iron to chloroplast [95] (Figure 1.4).

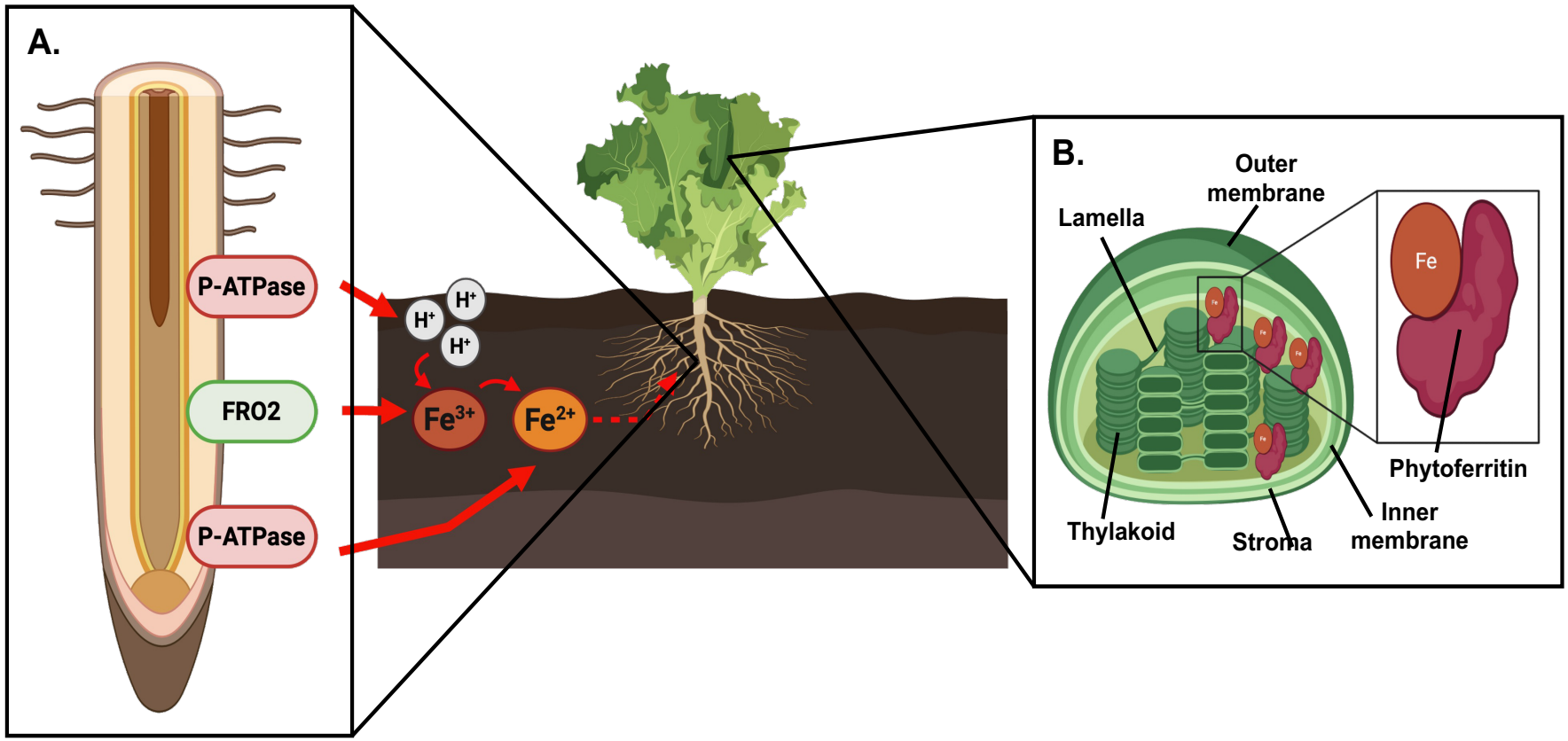


Figure 1.4 Iron uptake and translocate in lettuce plant.

Modified and referred the previous studies [82,83,86,87]. **A**. Strategy I pathway for iron uptake and circulation in iron deficiency environment based in reduction. **B**. Iron stored in chloroplasts in the leaf tissues which bound with phytoferritin. **C**. Translocation of iron in root tissues via a) apoplast and b) symplast pathway (created with BioRender.com).

1.3.2 Nanoparticle technology for micronutrient fortification

Engineered nanomaterials (ENMs), called nanoparticles, feature at least one dimension ranging from 1 to 100 nm and consist of organic, inorganic, or hybrid materials [97]. There are two major approaches for nanoparticle formation including top-down and bottom-up. The top-down approach refers the mechanical reduction of the size and structure towards to the nanoscale, while bottom-up approach is involving physical and chemical process for formation of the nanoparticles from atoms and molecule level to large nanostructure [98]. The bottom-up approach is most widely taken for nanoparticle synthesis as it allows to control the size. Various synthetic approaches have been developed including physical methods such as mechanical grinding and chemical methods such as co-precipitation [99], sol-gel synthesis [100], microemulsion [101], thermal decomposition [100], electrochemical synthesis, and hydrothermal methods [102,103] (See **Table 1.4**).

Table 1.4 Synthesis method for Fe_3O_4 NPs: advantages and disadvantages

Method	Advantages	Disadvantages
Co-precipitation	(a) Rapid process with high yield, (b) Cost efficiency, (c) Relatively safe process [103,104]	(a) Presence of agglomeration and variation in size and stability [104]
Sol-gel	(a) Control the size and internal structure, (b) Uniform size and simple procedure [105]	(a) High permeability and relatively weak bonding[105]; (b) Long process [106]
Microemulsion	(a) Relatively safe method, (b) Easy regulation of the size, shape, and composition [101]	(a) Effect of factors: temperature, pH, and washing method [101]
Thermal decomposition	(a) Enable to control size, polydispersity, and shape [100]	(a) High labour and cost requirements; (b) Safety concerns related to high temperature[100]; (c) Time consuming post-processing, for aqueous solutions [104]
Electrochemical synthesis	(a) Obtain uniform polarisation, (b) access for altering the cell [107], (c) Controllable size of particles[105]	(a) Limited reproductivity [105]
Hydrothermal	(a) Easy to control size and shape of particles [105]	(a) High expenses of equipment [106]; (b) High temperature and pressure [105]

Owing to their small particle size, nanoparticles have a large surface area and exhibit high solubility and mobility that is widely exploited for smart delivery for pharmaceutical, medical, and agricultural purposes [97]. The size of engineered nanomaterials (ENMs) is critical in determining their ability to cross biological barriers, such as the plant cell wall and membrane, following root or foliar application. This property enables the development of smart delivery systems for nutrients and pesticides[108,109]. The characteristics of nanoparticles, such as structure or surface chemistry traits, should be selected properly for different functions or nanotechnological strategies [108]. Through interactions between nanoparticles and plants, including nutrient interactions, it is possible to enhance the nutritional quality of food crops by accumulating specific macro- and micronutrients.

The agronomical biofortification of food crops with micronutrients/minerals is a promising technique with a fast and easy way to mitigate inadequate essential nutrients/minerals in plants [18]. Nano-agrochemicals can be classified by their types or delivered nutrients, such as macronutrient and micronutrient fertilisers and macronutrient carriers [110]. Macronutrients, particularly NPK fertilisers, can be applied with nanoparticles in nano-capsule form, or particles can be coated with nutrients/minerals (such as in urea-coated zeolite chips) with slow-release application to enhance the uptake and efficiency of fertilizer [110]. Nano-NPK fertilisers have been shown to enhance crop yield and growth quality, including improvement in the size and weight of edible vegetative parts, as well as the concentration of physiochemical compounds. These effects have been observed in several crops, such as wheat [111,112], potato [113], maize, kale, and capsicum crop [113]. Among the various techniques involving nano-NPK fertilisers, such as the slow-release formulations or coatings with nutrient ions, nano-NPK has been shown to shorten crop life cycles compared to conventional fertilisers, a factor that contributes significantly to increased crop yield [114]. Micronutrient-loaded nanoparticles may provide a more favourable uptake or distribution of micronutrients in plants by providing a slow release of the nutrient by plants or soils and reducing environmental pollution (e.g., leaching) or agroecology degradation [18]. Compared to conventional fertilisers, nano-fertilisers can triple the nutrient effectiveness, reduce the requirement or usage of fertiliser applications, develop crops with stress resistance, and cause less adverse environmental impacts (e.g., leaching) [18].

Micronutrient fertilisers are being developed with various micronutrient ions, such as Fe [115]; Mn, Zn [114]; and Cu [116]. The translocation and accumulation of

micronutrient-based nanoparticles in plant tissues such as leaves, shoots, and grains, vary depending on the application method, leading to distinct effects on plant growth and development. For instance, hydroponic cultivation enables more efficient and effective root application of nano-fertiliser [117]. Nano-fertilisers are also widely applied for foliar applications using spraying methods. Furthermore, nano-fertiliser treatments can be conducted together, such as the combination of seed priming and foliar application of zinc oxide nanoparticles (ZnO NPs), which showed enhancement of seedling growth and increased biomass, chlorophyll content, and yield in rice [115].

1.3.3 Iron oxide nanoparticles (Fe_3O_4 NPs)

Iron oxide nanoparticles (Fe_3O_4 NPs) are widely applied for medical, pharmaceutical, and agricultural purposes as they have several attractive attributes: (1) Cost effective synthesis process; (2) Physiologically safe for the application of human body; (3) Biocompatibility and biodegradability; (4) excellent dispersibility as solution form; (5) Fulfil smart delivery purpose as it can be detected by magnetic resonance imaging technique [118,119]. Iron oxide presents different characteristics within the different chemical compounds including magnetite (Fe_3O_4), hematite (Fe_2O_3), maghemite (γ-Fe_2O_3), and FeO (wustite)[119] . Among different phase of iron oxide, Fe_3O_4 and γ-Fe_2O_3 are widely applied for biomedical and biotechnological application owing to multiple polymorphs within favourable magnetic properties [118].

The magnetitic properties of Fe_3O_4 NPs are size-dependent, with smaller particles exhibiting superparamagnetic behaviour due to a decrease in relative oxygen concentration. Notably, Fe_3O_4 NPs below 20 nm demonstrate enhanced stability and superparamagnetism, attributed to surface and structural changes associated with reduced oxygen content [119]. To achieve high performed Fe_3O_4 NPs, the nanoparticle synthesis process should effectively control the environmental condition such as pH, temperature, solvent type, solutions, and precursor concentrations which affect nanoparticle attributes including purity, crystallinity, oxide phase, surface chemistry, morphology, size, and uniformity of size [103,104,120]. One of the key features of nanoparticles is the size of the particles influencing physical properties such as responsive reaction to the applied magnetic field and biological outcomes for the application subject such as their bioavailability for human body or living plants [120]. The size of nanoparticles can be managed by

adjusting three factors including temperature, concentration of the base solutions and the surfactant presence [120].

Along with the synthesis method, coating agents also impact on nanoparticle properties by modifying structural or conductivity properties [121]. This is due to that fact that the magnetic Fe_3O_4 NPs have large hydrophobic surface area which tends to agglomerate. Particularly, Fe_3O_4 NPs and γ-Fe_2O_3 have relatively high chemical activity which can easily oxidise in air by losing their magnetic attributes [120]. Further efforts were made for increasing stability and applicability of Fe_3O_4 NPs by coating with different type of capping agents including organic macromolecules, polymers, proteins, or inorganic molecules to prevent agglomeration of nanoparticles [102]. Various capping agents have been employed to improve the stability of Fe_3O_4 NPs, including polyacrylic acid (PAA) [122], sodium citrate, polyvinyl pyrrolidone (PVP), starch [123]; octylamine (OTA), 1-dodecanethiol (DDT) and tri-n-octylphosphine (TOP) [124].

1.3.4 Fe_3O_4 NPs uptake and translocation in plant

It is necessary to understand how the plant absorbs, transports, and accumulates the nanoparticle in order to enhance nutrient/mineral contents in food crops in targeted locations such as edible parts of the food crops. Nanoparticle application enables effective smart-delivery functions owing to their high solubility and mobility [125]. Several studies have been conducted to investigate the mechanisms of uptake, transport, and accumulation of micronutrient/mineral nanoparticles in living plants by applying integrated analytic methods such as microscopy and mass spectrometry. However, due to the complexity and interaction between nanoparticles and plants, exact mechanisms should be investigated. Additionally, various factors significantly impact the absorption and uptake of nanoparticles in living plants. First, the size of nanoparticles is a key factor limiting their penetration through plant cell wall pores, which typically range from 5 to 20 nm in diameter [126]. Second, the type and chemical composition of nanoparticles have a great influence on the absorption or uptake of nanoparticles [126,127]. Coating materials for nanoparticles and the chemical composition of their surfaces can also alter their absorption or accumulation in living plants [126,128]. Third, plant species impact the speed of absorption and distribution of nanoparticles due to different plant physiological traits, such as the thickness or architecture of the barriers, including the size of the cell wall pore, Casparian strip, and xylem thickness [129]. For instance, paramagnetic-coated nanoparticles were applied to four different crops, wheat,

pea, sunflower, and tomato, with results indicating that the nanoparticles accumulate in different plant locations, such as vascular tissues or trichomes [128]. The effects of ENMS on plants can be highlighted as dependent on crop life stages, including: (1) germination and early seedling growth; (2) post-transplant and further growth of seedlings; (3) mature/harvest stages [130]. Finally, the method of application plays an important role in nanoparticle pathways in plants. Nanoparticles in terrestrial plants generally accumulate and aggregate at the root zone, which is greatly influenced by microenvironmental conditions, including symbiosis with bacteria or fungi [128]. According to Schewab et al. (2016), the application of foliar ENMs in soil via roots is particularly challenging due to the interaction between microorganisms in soils and additional complex soil conditions. Delivering ENMs via foliar application or seed coating may improve the uptake or distribution of ENMs in living plants. Further study is required to investigate the optimum application method of ENMs based on understanding the transdisciplinary factors of growing plants [128].

There are two primary paths for uptake, translocation and accumulaton of nanoparticles: foliar and root (Figure 1.5). In foliar application, the cuticle acts as a major barrier to nanoparticle penetration into plant tisuses due to its waxy, water-resistant components. As a result, the primary route for nutrient uptake is through the cuticular pathways, including lipophilic pathways. The lipophilic pathway involves diffusion via cuticular wax layers, whilst the hydrophilic pathway occurs via polar aqueous pores located in the cuticle or stomata in leaves: the “stomatal pathway.” Uptake via the hydrophilic pathway is influenced by stomatal openings, such as the morphological traits of stomatal apertures in various plant species. After the stomatal pathway, nanoparticles are transported via the vascular pathway, consisting of the xylem and phloem systems. The direction of flow in the xylem system is from root to shoot, whilst the flow direction in the phloem system is from shoot to root. Thus, in foliar applications, nanoparticles are translocated only via the phloem system from leaves to roots. Accordingly, previous studies have found that nanoparticles can be transported via xylem and phloem systems. However, the mechanisms underlying nanoparticle translocation in the xylem and phloem still require further investigation [131]. Due to the size of the cuticular pore, which is approximately 2 nm in diameter, the stomatal pathway is the most likely route for nanoparticle translocation. Therefore, from foliar spraying application, nanoparticles can be found in leaf stomata and deeper plant tissues, such as phloem, with translocation [131].

In root application (Figure 1.5), nanoparticles passively cross the permeable regions of the cuticle and the pores of the cell wall. Several factors influence the uptake of nanoparticles in plants via root application, such as nanoparticle size, fictionalisation of surface (e.g., positive, and negative charge), morphological traits, conditions of exposure, plant species, and growth stage, root integrity, and rhizospheric processes [131]. When applied in the root zone, there are several barriers that nanoparticles cross, including physiological barriers (e.g., root hair) or environmental conditions of the root (e.g., bacteria and mycorrhizae), mucilage/exudates, and cuticle of the root periderm. During interactions with barriers, nanoparticles penetrate the cell membrane by fluid-phase endocytosis, passive diffusion, or mechanical piercing and then accumulate in the mucilage, cuticle, and cell wall. Afterwards, microorganisms, through processes such as mucilage exudation or biomineralization, assist nanoparticles in transport 128.

Once nanoparticles enter the root tissues, they may move through two main pathways: (1) The apoplastic route, which involves movement through the cell walls and intercellular spaces without crossing the plasma membrane; (2) The symplastic route, which involves movement through the cytoplasm from cell to cell via plasmodesmata (Figure 1.5C). Upon root penetration, nanoparticles move from the root surface through intercellular structures, including epidermis, cortex, endodermis, and Casparian strip, and are eventually transported to the shoot via xylem [131]. When nanoparticles approach the root epidermis, two major pathways have been previously investigated, namely the apoplast and symplast, connecting cell wall and intercellular spaces, and protoplasmic connections through ion channels [126].The apoplastic pathway plays an important role in radial movement within plant tissues, and it allows nanoparticles to translocate upwards to the aerial part. In addition, once nanoparticles enter the central cylinder, they can move towards the aerial part via the transpiration stream through the xylem.

When nanoparticles translocate through the apoplastic pathway, they encounter a barrier known as the Casparian strip, which blocks further apoplastic movement and requires nanoparticles to switch to the symplastic pathway to reach the xylem. The Casparian strip is a band of cell wall material enriched with lipophilic hydrocarbons, such as lignin, located in the endodermis adjacent to the vascular system [132]. It acts as a selective barrier that can hinder the translocation of nanoparticles from roots to shoots, particularly when their size exceeds 20 nm [131,133]. The symplastic pathway, which facilitate the movement of water and nutrients, utilises sieve tube elements in the phloem to enable the translocation of nanoparticles towards non-photosynthetic tissues and organs. In this pathway,

nanoparticles may enter the cytoplasm and move between adjacent cells via plasmodesmata, microscopic channels that enable direct cytoplasmic continuity. Several hypotheses have been proposed regarding nanoparticle movement through the symplast, involving aquaporins, ion channels, endocytosis, and disruption of membrane integrity. Endocytosis is considered one of the most feasible transmembrane pathways, playing a role in nutrient uptake and microbial interactions [131]. In root application, the nanoparticles are primarily translocated via the xylem, moving from the roots to the shoots and leaves, but not through the phloem [126].

Nanoparticles are translocated and accumulate at different speeds and locations in plants based on their component materials [126]. Generally, nanoparticles accumulate in fruits, grains, flowers, or young leaves by travelling to the vascular system [134,135]. Owing to advanced spectrometry or mass spectrometry techniques, it is possible to determine the overall uptake, translocation, and accumulation of nanoparticles in living plants. For instance, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and X-ray spectroscopy analysis allow us to determine the sizes, shapes, and locations of nanoparticles in plant cell [17].

Inductively coupled plasma optical emission spectroscopy (ICP-OES) and ICP-mass spectrometry (ICP-MS) precisely analyse nanoparticle compounds [126]. However, further studies should be conducted to identify the interaction between nanoparticles that are absorbed by plants and animals or humans that consume plants treated with nanoparticles [126]. Additionally, the uptake, distribution, and accumulation mechanisms vary and are greatly influenced by several components. Mozafar and Oertli (1992) found the uptake of vitamin B_{12} content in soybean plants using the radioisotope dilution (RID) technique. By identifying vitamin B_{12} and vitamin B_{12}-binding sites, the study showed that vitamin B_{12} transport from vitamin B_{12}-enriched nutrients to soybean plants that were found in unifoliate, trifoliate leaves, and stems. Vitamin B_{12} is rapidly transported from roots to shoots, with greater accumulation in the leaves of soybean plants when the rooting medium is aerated with nitrogen gas, as demonstrated by Mozafar and Oertli (1992) [136].

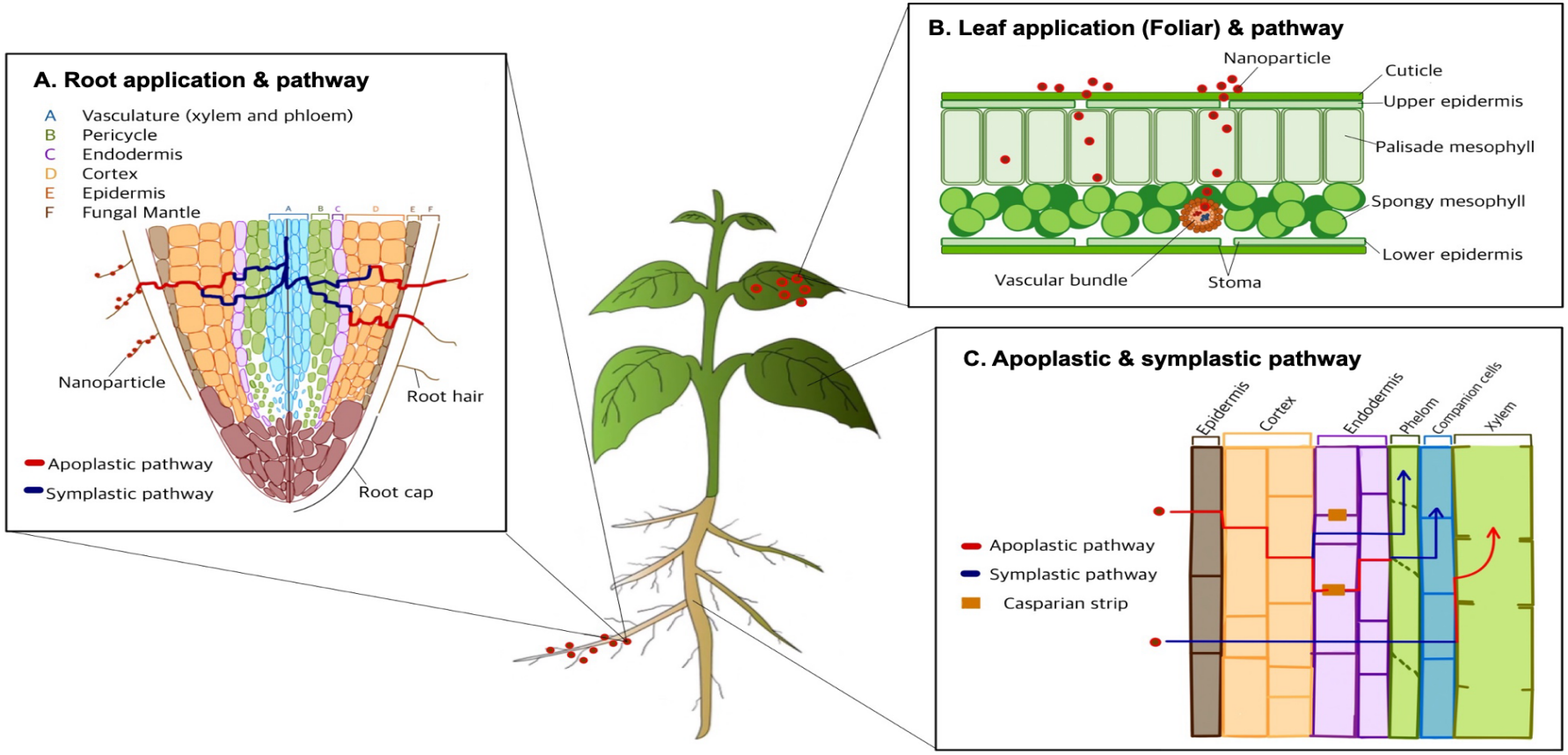


Figure 1.5 Nanoparticle uptake and translocation in living plant: pathway of foliar spray and root application.

A. Root application: uptake and translocate. There are two pathways for uptake and translocation of nanoparticles (NPs) once they traverse the root hair: apoplastic pathway and symplastic pathway. **B.** Leaf application: uptake and translocation. NPs can be applied via foliar spraying method allowing to uptake and accumulate the NPs into leaves efficiently. **C.** Apoplastic and symplastic pathway. When NPs approach the root epidermis, two major pathways have been previously investigated: apoplast and symplast pathway. These allow the translocation of NPs towards non-photosynthetic tissues and organs.

1.3.5 Iron fortification in food crops using Fe_3O_4 NPs

As a co-factor in photosynthetic, iron is an essential nutrient for photosynthetic organisms involving various metabolic mechanisms such as an electron transport chain [137]. Iron deficiency frequently coexists with vitamin B_{12} deficiency, and the resulting microcytosis may mask the macrocytosis characteristic of B_{12} deficiency. In cases of B_{12} or folate deficiency, impaired DNA synthesis leads to ineffective erythropoiesis and reduced iron utilisation, which may elevate serum iron levels. If haemolytic anaemia persists, iron stores may become depleted over time, potentially leading to iron deficiency anaemia [138].

There were various attempted made for iron fortification using iron nanoparticles with various chemical compound including magnetite (Fe_3O_4), hematite (Fe_2O_3), and maghemite (γ-Fe_2O_3) in food crops including potato [17], bean [139], tomato [140], strawberry [141], barley [142], rice [143], muskmelon [144], wheat [145]. Iron NPs have been applied via various application method including seed priming [139,145,146], via root application in hydroponic system [17,141–143,146,147], via leaf application using foliar spray method [140], and via irrigation practice [144]. The Fe NPs treatment via root application enhanced the growth of root and stem, and biomass in potato [17], barley [142], and spinach [147] grown in hydroponic system (see Table 1.5).

Specifically, Fe_3O_4 NPs capped with histidine at a concentration of 8 mg / L promoted optimal tubers growth in hydroponically grown potatoes, resulting in better growth and higher harvest yield (e.g., plant height, dry weight, number of tubers) compared to treatments with 12 mg / L and 16 mg / L [17]. Similar results were also observed with the foliar application of Fe_3O_4 NPs in greenhouse- and polytunnel-grown potatoes. The highest Fe content was quantified in hydroponically grown potatoes treated with Fe_3O_4 NPs capped with histidine at a concentration of 12 mg / L. In contrasts, potatoes treated with 16 mg / L via foliar spray showed significantly higher iron content in tubers. Fe_3O_4 NPs is also treated in tomato and chili pepper with different concentrations. All concentrations (6, 12, and 24 mg / L) of Fe_3O_4 NPs significantly increased Fe content in tomato with the greatest increase obtained by 6 mg / L. Furthermore, tomatoes treated with Fe_3O_4 NPs (particularly with 12 mg / L) showed increased weight in the ripened fruits and produced 146.38% more than the control. Fe_3O_4 NPs with 6 mg / L contributes significantly to increasing the fresh weight of tomato from 287.21 to 501.08 g. Fe-fortified tomato displayed no phytotoxicity effects on excessive amounts of Fe treatment. In chilli pepper trials, Fe_3O_4 NPs 6 mg / L treatment increases plant

height (70 nm) and fresh weight. This study highlights that hydroponic propagation contributes to fortifying micronutrient levels with advantageous conditions by providing adequate soil and compost substrates [17] .

Azizkhani et al. (2023) determined the effect of Fe_3O_4 NPs on strawberry compared with the conventional iron fertiliser. The results showed that the Fe_3O_4 NPs treatment (0.06 and 6 mg / L) showed increases in Fe content in leaf and fruit, chlorophyll pigments, and phytochemical content including total soluble carbohydrate and total soluble protein in comparison with the control group with Fe sulphate 6 mg / L treatment [141]. Additionally, this study determined that strawberry treated with Fe_3O_4 NPs increases in total phenolic, flavonoids, and antioxidant content compared to the control group. Tombuloglu et al. (2019) found that the Fe_3O_4 NPs treatment up to 500 mg / L (125, 250, and 500 mg / L) enhanced barley's growth including significant increases in leaf morphology and fresh and dry weights compared to the control group without Fe_3O_4 NPs application. Additionally, relatively high level of hydrogen peroxide (H_2O_2) which have destructive effect on plant cells and catalase alleviate this adverse effect by converting H_2O_2 to water and oxygen [142]. As the given Fe_3O_4 NPs concentration have increased up to 500 mg / L treatment, catalase activity and H_2O_2 in root zone significantly increased compared to the control group indicating that the Fe_3O_4 NPs triggered catalase enzyme activity to protect the cell against H_2O_2 accumulation. Barley treated with Fe_3O_4 NPs showed significant increases pigment content, chlorophyll a, chlorophyll b and carotenoids, compared to the control group. There was no phytotoxic effect in barley treated with Fe_3O_4 NPs 1000 mg / L [142]. The study conducted by Jeyasubramanian et al. (2016) highlighted that the spinach treated with Fe_2O_3 NPs (100, 150, and 200 mg) showed root and stem elongation with increases of 1.45, 1.91, and 2.27 folds, compared to the control group without Fe_2O_3 NPs treatment. An increase in Fe_2O_3 NPs treatment resulted in enhanced biomass and corresponding increases in Fe quantification within the spinach [147].

Table 1.5 Iron fortification using Fe_3O_4 NPs in food crops

Plant materials	NPs	Concentration	Application method	Effects on plant growth, yield, and nutritional quality
Bean	Fe_3O_4 & Fe_3O_4 + PEG	1-1000 mg / L	Seed priming	- Improved growth (increased radicle elongation) root vigour and final yield - The phytotoxicity was found in high concentration (100 and 1000 mg / L) - Enzymatic assays revealed that Fe_3O_4+PEG affect less negatively on bean seed [139]
Wheat	Fe_3O_4	0, 40, 200 and 500 mg / L	Seed priming	- Wheat treated with 200 and 500 mg / L showed better plant growth, photosynthesis, and respiration activity along with increased photosynthetic pigments in leaves - The activity of ascorbate peroxidase was increased while malondialdehyde content were decreased in wheat [145]
Tomato	Fe_2O_3	50-800 mg / L	Seed priming	- Improved germination rate, seedling growth (50-200 mg / L), root vigour and Fe content quantified in root hair and root tip [146]
Potato and tomato	Fe_3O_4	8-32 mg / L	Hydroponic	- Increased tuber size and seedling; improved root vigour and final yield - Increased Fe in skin and tuber of potato [17]
Spinach	Fe2O3	100, 150, and 200 mg	Hydroponics	- Spinach grown in hydroponically treated with Fe_2O_3 showed increases in growth of roots and stems, biomass and elemental analysis for iron content compared to the untreated plant [117].
Strawberry	Cysteine-coated Fe_3O_4	0.06 and 6 mg / L	Hydroponic	- Increased biomass, yield, chlorophyll pigments in strawberry leaves, - Increased total soluble carbohydrate and total soluble protein compared to conventional fertiliser treated group - Increase secondary metabolites (e.g., anthocyanin, flavonoid, phenol, and antioxidant capacity) [141]
Barley	Fe_3O_4	125, 250, 500 and 1000 mg / L	Hydroponic	- Up to 500 mg / L of treatment showed increases in fresh weight (leaf and root) and no phytotoxic effect was observed at high doses - Fe_3O_4 treated barely showed significant alterations of the photosystem genes in leaves [142]
Rice	Fe_3O_4 & Fe_2O_3	50, 250, and 500 mg / L	Hydroponic	- Under iron deficiency condition, rice treated with 50 mg / L increased root length and leaf elongation [143]
Tomato	Fe_3O_4	500 mg / L	Foliar spray	- Improved physiological and photosynthetic development - Frequent Fe_3O_4 spray might cause toxic effect showing growth inhibition and suggested low-repat application via foliar spray [140]
Muskmelon	γ- Fe_2O_3 & Fe_3O_4	0, 100, 200 and 400 mg / L	Irrigation - soil	- Muskmelon treated with γ- Fe_2O_3 & Fe_3O_4 showed increased growth and chlorophyll content, vitamin C, and fruit weight [144]

1.3.6 Opportunities and challenges of nanoparticles for food crop

As a promising technology for sustainable agriculture, nanotechnology poses numerous opportunities. First, engineered nanomaterials (ENMs) enhance the overall efficiency of water, light, and agrochemical use [117]. In nano-fertiliser application, increased water use efficiency is achieved by enhancing water retention, reducing losses, and improving the uptake and utilisation of water and nutrients [148]. ENMs show the potential to increase light use efficiency in plants by augmenting chloroplast photosynthetic activities and enhancing chloroplast reactive oxygen species (ROS) [148]. Agrochemical application shows significant enhancement of performance by decreasing adverse environmental effects, including leaching. Nano-fertilisers constitute an alternative technique to the use of conventional fertilisers because they boost crop yield, quality, and tolerance to abiotic stress owing to their high efficiency [149]. Compared to conventional fertilizer, nano-fertiliser can increase crop production by approximately 20 to 30 % and achieve similar levels of plant protection and nutrient enhancement with lower fertilizer usage [150]. Owing to their biochemical and physical characteristics, ENMs can be delivered precisely to the plant interior to enhance the uptake of nutrients and fertiliser [108]. Second, nanotechnology promotes soil sustainability by lowering additional fertilizer inputs via increased nutrient efficiency. Additionally, ENMs have the potential to increase fungi, which is beneficial for plant growth, or to encourage plant root vitality via nitrogen-fixing bacteria. Furthermore, nanotechnology enables the control of biotic stress such as pathogens or weed competitors and abiotic stress, including extreme temperature and water stress [108].

There are several challenges for further deployment of nanotechnology in agri-food. One of the most significant challenges in nanotechnology is the potential risks and risk perceptions regarding nanoparticles [108]. Consumer perception and/or acceptance are negative towards ENMs in food crops. According to Giles et al. (2015), nanotechnology integrated into food product is less likely to be accepted by consumers than its use in food packaging [151]. However, of most significant concern regarding ENM applications in agricultural food is that sectors have only focused on the input of nanoparticles as ingredients or additives to food products directly, whilst comparatively little concern has been aroused regarding nano-agrochemical applications, including nano-fertilisers or nano-pesticides. Furthermore, these observations indicate that the consumer accepts nanotechnology applications in agricultural food production when perceived

benefits outweigh the perceived risks. For the future application of nanotechnology in food crops, it is necessary to encourage marketing and commercialization for nanotechnology-processed food crops by increasing acceptance along with expert opinions regarding safety or acceptability [151]. Further efforts should address and secure the availability of nano-agrochemicals within effective legislation and long-term risk assessment for the entire life cycle of nanoparticle application to food crops and further human/animal intake [152].

Additionally, although there have been several attempts, it is still necessary to investigate the interaction between ENMs and plants and to understand the mechanisms of uptake and translocation of nanoparticles in living plants [108,109,126]. Precisely delivering ENMs remains a challenge considering precise target location, exact time of application, and appropriate dose of ENMs. Additionally, in nanoparticle applications, particle size plays an important role, influencing the translocation of nanoparticles in living plants. Nanoparticle size can be increased beyond the nano range and attain different shapes due to continuous aggregation which also may result in Ostwald ripening. Thus, synthesis process of nanoparticles should be developed to control nano-size and composition of nanoparticles, particularly when scaling up for commercial usages [137,153]. Davies (2018) presents a successful synthesis approach for producing stable sizes of nanoparticles ranging from 3.62 to 20.18 nm for calcium oxide, 3 to 7.6 nm for iron oxide, and 7.03 to15.41 nm for zinc oxide. The study exploited a spinning disk reactor (SDR) to synthesise nanoparticles, which provided a relatively rapid, efficient, and cost-effective synthesis process. SDR method allows the production of large amounts of nanoparticles with uniform size, and the electrostatic coating method with histidine (amino acid) also enhances the mobility and solubility of metal oxide nanoparticles by enhancing retention capabilities and decreasing leaching issue [137]. Further guidance documents and validated methods for nanoparticle size measurement should be established [154].

1.4 Soilless cultivation for micronutrient fortification

Hydroponic and aeroponic systems support the effective fortification of micronutrients with high productivity and efficiency by providing optimised year-round production [155]. Hydroponic cultivation refers to the growing method based on the recirculated inorganic/organic nutrient solution instead of soil cultivation. In hydroponic systems, the plant roots are immersed partially or completely in a nutrient solution. On the other hand, the aeroponic system exposes the plant roots

to aerosol droplets containing micro-/macro-nutrient [156]. In aeroponic systems, droplet size is one of the major parameters for determining the absorption effectiveness directly influencing plant growth and it can be classified into spray (over 100 μm), fog (1 to 100 μm), and mist (1 to 35 μm) [157]. Both hydroponic and aeroponic cultivations provide favourable environments for plant growth. Nanoparticles can be applied to nutrient solutions for hydroponic cultivation. Better absorption and translocation of nanoparticles was observed in plants grown in hydroponic systems compared to soil cultivation due to more conductive aggregation and dissolution of nanoparticles in the root zone in hydroponic system [150]. Some minerals/micronutrients present low mobility or are even unavailable to plants in the soil depending on the physicochemical characteristics including pH, composition, and electrical conductivity [158]. Therefore, hydroponic cultivation systems might be an efficient strategy for vitamin B_{12} fortification by applying vitamin B_{12}-enriched nutrient solution precisely with nanoparticle coating techniques.

Chapter 2. General Materials and Methods

2.1 Iron oxide nanoparticles (Fe_3O_4 NPs)

2.1.1 Materials

The following chemicals/reagents were purchased from Sigma-Aldrich® (Merck Life Science UK Ltd. Gillingham, UK); iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥ 97 %), iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$, 98 %), ammonia (NH_3, anhydrous, ≥ 99.98 %), and nitric acid (HNO_3, ACS reagent, 70 %). Additionally, vitamin B_{12} (cyanocobalamin, $C_{63}H_{88}CoN_{14}O_{14}P$, ≥ 98 %), histidine ($C_6H_9N_3O_2$), starch from potato ($C_6H_{10}O_5$) and L-lysine ($H_2N(CH_2)_4CH(NH_2)CO_2H$, ≥ 98 %) were purchased from Sigma-Aldrich®. Iron oxide nanoparticles capped with L-lysine were obtained by Pharm2Farm in Nottingham, UK. Multielement standard solutions were purchased from Sigma-Aldrich® (Merck Life Science UK Ltd. Gillingham, UK); 1 mg / L of calcium (Ca), cobalt (Co), iron (Fe), potassium (K), magnesium (Mg) and phosphorus (P), and zinc (Zn) in nitric acid, respectively. Ultrapure type I water was used in all studies.

2.1.2 Synthetic methods

2.1.2.1 Co-precipitation

Magnetite nanoparticles (Fe_3O_4 NPs) were synthesised via a slightly modified co-precipitation method adapted from Kinana (2023) [159]. The following equation was applied.

$$FeCl_2 \cdot 4H_2O + 2FeCI_3 \cdot 6H_2O + 8\,NH_4OH = Fe_3O_4 + 8NH_4Cl + 10H_2O$$

Fe^{2+} (ferrous cation) and Fe^{3+} (ferric cation) salts were dissolved in a 1 : 2 ratios in 1000 mL of type I water at 45 °C under vigorously stirring with 1500 RPM of a hotplate stirrer (Asynt Ltd., Cambridge, UK). Then the capping agents such as histidine, potato starch or L-lysine was added in the iron solutions previously dissolved with ultrapure water to stabilise the nanoparticle [160]. When the precursor solutions had no sediment, the reaction was conducted in airtight condition

supplied with nitrogen gas to stabilise the synthesis process by controlling the oxidation which shown in Figure 2.1.

Ammonia solution (NH_4OH) was slowly loaded via syringe into the precursor solutions which was used as a precipitating agent to facilitate the homogenous synthesis of magnetite (Fe_3O_4) [161]. After completing ammonia injection, the black precipitation of Fe_3O_4 was formed by the co-precipitation reaction. The solution was stirred vigorously at room temperature for an hour with 1000 to 1500 RPM of a hotplate stirrer. Once the synthesis reaction was completed, approximate 300 to 500 mL of deionised was added in to the final product of the synthesis and shaken to remove the residue of the reaction. It was transferred into a beaker and placed on top of a neodymium magnet (ultra-high performance N52, first4magnets®) to separate the reaction residue from magnetite. Then the supernatant was carefully removed using a pipette. This process was repeated multiple time to remove the residues of the reaction.

After rinsing procedure, the collected final solution with type I water, several drying methods were applied to achieve pure Fe_3O_4 NPs. The method includes vacuum dry and air dry using various equipment. The evaporation dry method was conducted by Buchi rotary evaporator® (Vacuum controller V-850 and Rotavapor R-210, Buchi Ltd., Suffolk, UK). Vacuum dry was also conducted using a mini vacuum pump (Laboport®, KNF Neuberger Inc., UK) with glass filter used to collect the final product. The air-dry method used a funnel and filer paper to collect the Fe_3O_4 NPs and dried at the room temperature for overnights inside a fume hood.

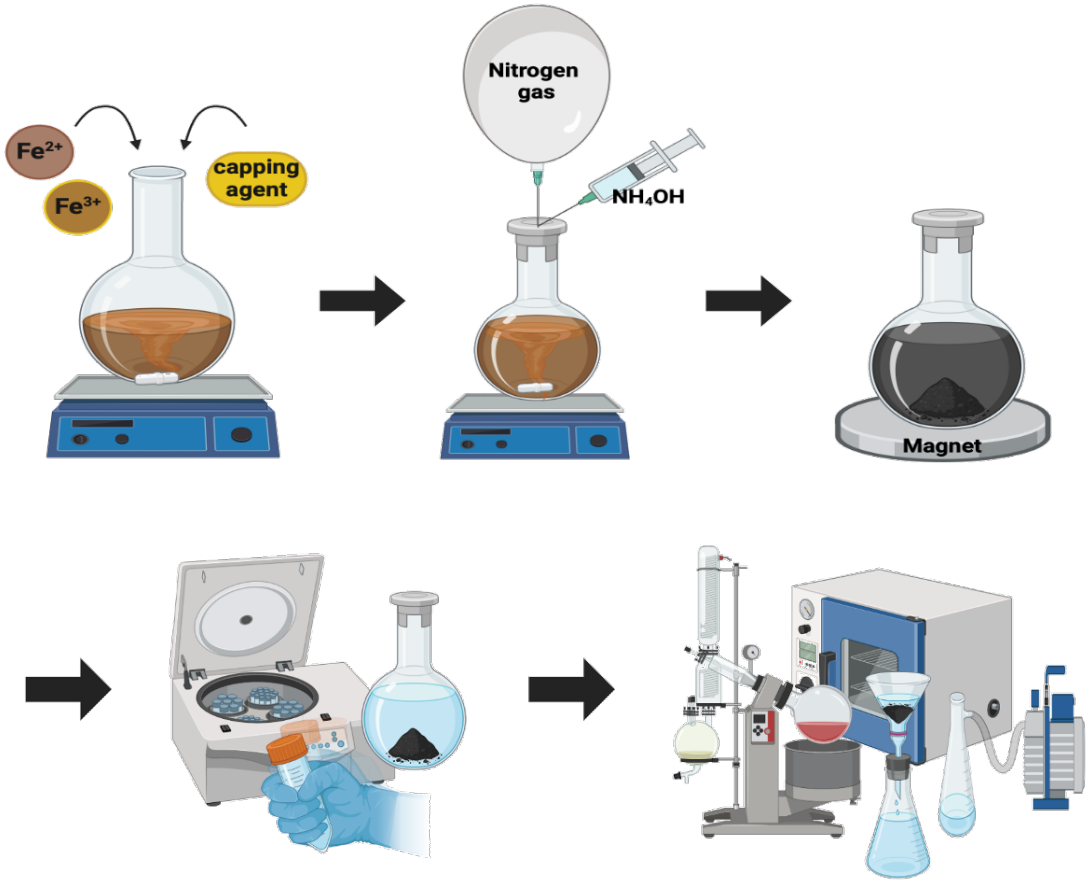


Figure 2.1 Fe_3O_4 NPs synthesis process

1. Dissolve chemical materials. **2.** Fe_3O_4 NPs synthesis.**3.** Completion of synthesis reaction. **4.** Rinse process via centrifuge. **5.** Drying methods including air dry, oven dry, vacuum dry (created with BioRender.com).

2.1.2.2 Spinning disc reactor (SDR)

Fe_3O_4 NPs were also synthesised using a novel synthetic method that combines co-precipitation and sol-gel process patent by Dr. Gareth Cave (EP2825515A2) 162. The method using spinning disc reactor (SDR) was partially modified from the previous study conducted by Davies (2018). The SDR enables to produce large amounts of Fe_3O_4 NPs as it works with a continuous flow system and a high centrifugal force 17. Fe^{2+} (ferrous cation) and Fe^{3+} (ferric cation) salts were dissolved in a 1 : 2 ratios in 1000 mL of type I water at 45 °C under vigorously stirring with 1500 RPM of a hotplate stirrer (Asynt Ltd., Cambridge, UK).

An ammonia solution was prepared as the primary precursor for the magnetite synthesis. Subsequently, a spinning disc reactor was operated by connecting separate peristaltic pump (a roller pump) into each precursor solutions, iron salt solution and ammonia solution. The peristaltic pump was running at 60 mL / s flow rate with 2000 – 3000 RPM of centrifugal force which could be adjusted by control panel. The final product of the synthesis was collected from the collection vessel, then processed for rinse and coating process which described the above (Figure 2.2).

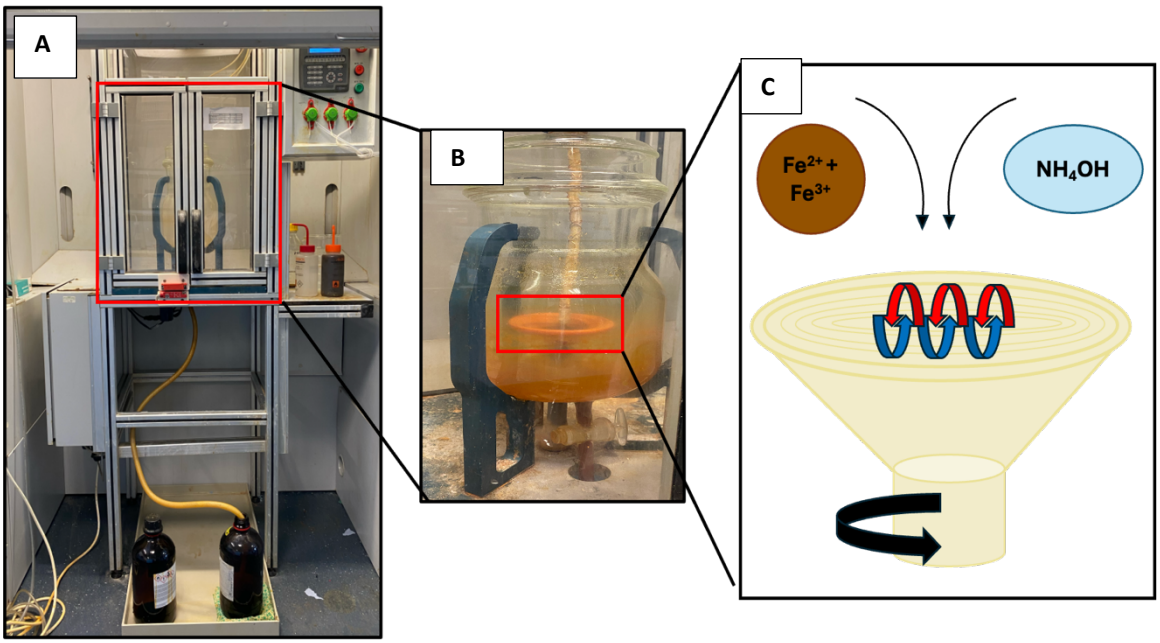


Figure 2.2 Spinning disc reactor (SDR).

A. SDR within a control panel, which allows adjustment of pump speed and disc rotation, with collections vessels for Fe_3O_4 NPs located beneath the SDR. **B**. SDR consists of a reaction vessel, a spinning disc, and a collection system. **C**. SDR disc for the Fe_3O_4 NPs synthesis: 1) Precursor solutions are introduced via pumps and mixed on the spinning disc (rotating at 500 – 3000 RPM) with the aid of ridges on the disc; 2) The synthesised Fe_3O_4 NPs are propelled to the wall of the reaction vessel and eventually collected in the vessel located beneath the SDR.

2.1.3 Coating with capping agents

To enhance the stability of the Fe_3O_4 NPs, this study tested different coating methods using various capping agents (e.g., histidine, potato starch and L-lysine). The capping agents were applied at a 1 : 1 ratio (w/w) with Fe_3O_4 NPs to increase coating rate. In the first method, the capping agent was added during nanoparticle synthesis by co-precipiation at 70 °C.

For the starch-coated Fe_3O_4 NPs, vitamin B_{12} was subsequently coated at a 1 : 66 ratio (v/v) by vigorous strring at 1500 RPM using a hotplate stirrer (Asynt Ltd., Cambridge, UK). The reaction mixture was continuously stirred over two hours to complete the coating. Afterwards, all the nanoparticles were washed multiple times with ultrapure Type I water to remove unbound residues and by-products.

The second coating method was based on a method patented by Cave [162]. The method consists in coating the Fe_3O_4 NPs after being synthesised and coated with organic compound using dry mixing process. The procedure of the electrostatistic coating was conducted after drying the Fe_3O_4 NPs. Using the pestle and mortar, Fe_3O_4 NPs and the capping agents were ground together for 15 to 20 minutes until the mixture gained a pasty clay texture. After the coating, the final product was rinsed twiced with type I water to remove uncoated residues.

2.1.4 Characterisation of Fe_3O_4 NPs

2.1.4.1 Transmission electron microscopy (TEM)

Transmission electron microscopy was employed to determine the size (diameter of the core of Fe_3O_4 NPs), size distribution, and shape of the Fe_3O_4 NPs (Jeol JEM-2100 Plus TEM, JEOL Ltd, Japan). The operation of TEM was supported by Imaging Suite technicians (Medical Technologies Innovation Facility, Nottingham Trent University). Fe_3O_4 NPs was diluted with type I water and sonicated for 15 minutes. Then using a micropipette, 7 µL of dispersion was carefully loaded into a carbon filmed copper grid (Agar Scientific Ltd., UK) and the excess removed using filter paper. The sample loaded grid was left for drying 30 to 45 minutes and then loaded into the double tilt sample holder using tweezer. The sample holder was injected into TEM (voltage of 200 kV). The images of the Fe_3O_4 NPs were captured for posterior analysis of the size using Image J software (software (NIH, USA).

2.1.4.2 Scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDS) (SEM/EDS)

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDS) was used to determine the size, size distribution, shape, and the surface topography of Fe_3O_4 NPs (JSM-7100F LV FEG-SEM, JEOL Ltd., Tokyo, Japan. The operation of the SEM was partially supported by Imaging Suite technicians at the Medical Technologies Innovation Facility, Nottingham Trent University). Fe_3O_4 NPs was diluted with type I water and sonicated for 15 minutes. Then 15 µL of diluted Fe_3O_4 NPs solution was loaded into the double-sided conducted carbon tapes which was left for dry overnight. Then it was injected into the SEM (2.0 – 10 kV voltage). The size and surface topography were analysed using ImageJ software (National Institute of Health, USA). The EDS image was obtained by AZtecLive programme installed with SEM/EDS (Oxford Instrument, UK).

2.1.4.3 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) analysis enables to determine the average of hydrodynamic size and zeta-potentials of Fe_3O_4 NPs and capping agents [1]. The Fe_3O_4 NPs sample dispersion was diluted using deionised water and sonicated for 15 minutes before running the analysis. The diluted Fe_3O_4 NPs dispersion was loaded into disposable folded capillary zeta cells (Malvern Panalytical Ltd. UK) and analysed in a Zetasizer Nano ZS (Malvern Instrument, UK). The hydrodynamic size was determined by averaging measurements from 30 runs, while zeta-potentials calculations were based on 50 runs. The data obtained from DLS were analysed using Microsoft Excel.

2.1.4.4 Fourier transform infrared (FT-IR) spectroscopy

Fourier transformation infrared (FT-IR) spectroscopy was operated to identify the chemical bonds at the surface level of the Fe_3O_4 NPs using an FT-IR spectrometer (Cary 630 FT-IR spectrometry, Agilent Technologies®, California, USA). Before analysis, the Fe_3O_4 NPs sample was prepared by grinding to achieve fine powder form and dried overnight at 60 °C in the oven (MINO Series Class oven, GenLab Ltd., Sulfolk, UK) to avoid the detection of water peaks. Approximately 1 mg of the sample was loaded into the sample compartment. Each sample comparts and the

probe was cleaned with ethanol to avoid any contamination. FT-IR was running for 32 scans and the scan wavenumber ranged between 500 and 4000 cm^{-1}, with a resolution of 4 cm^{-1}. Compliance software called MicroLab Pharma Software was used for the analysis (Agilent Technologies®, California, USA).

2.1.4.5 Powder-X-ray diffraction (P-XRD)

Powder-X-ray diffraction (P-XRD) was operated to determine the crystalline structure of Fe_3O_4 NPs using RigaKu Smart Lab® (Rigaku Ltd., Tokyo, Japan). The samples were prepared the same way for FT-IR analysis. The sample was loaded in the powder specimen holder and sample stage with a Kß filter for cupper (λ = 0.1392 nm). The samples were analysed using a θ/2θ scan axis. The scan range varied between 20 ° and 80 °, and the scan mode and speed were 1 D and 5 °/min speed, respectively[160].

2.1.4.6 Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) enables to measurement of individual isotopes of an element from the fluid samples (NexION 1000/2000 ICP-Mass spectrometer, PerkinElmer®, USA). Via ICP-MS analysis, the concentration of Fe_3O_4 NPs was quantified after the synthesis to determine the efficiency of the coating method including yield of the synthesis. Also, the samples of Fe_3O_4 NPs coated with vitamin B_{12} were analysed to quantify the concentration of each element for further application to the hydroponic system. Cobalt (Co) content determined by ICP-MS was used to calculate the mass of vitamin B_{12} using the following equation:

$$Vitamin\ B_{12}\ (Cyanocobalamin)\ molecular\ weight\ (g/mol) = Cobalt\ (Co)\ molecular\ weight * 22.999576\ (g/mol)$$

The fine powder samples of Fe_3O_4 NPs were weighed up to 50 mg then digested in nitric acid at 70 % (HNO_3). To facilitate the digestion process, the samples were placed into an ultrasonic bath and heated up to 60 °C overnight (Bennett Scientific Ltd., UK). Upon completion of the digestion process, the resulting solutions exhibited a clear yellow colour. To avoid the blockage of the injector (2.0 mm) and nebuliser of the ICP-MS, the digested solutions were filtered using glass Pasteur

pipettes filled with cotton wool. After filtration, 1 mL of the digested sample was diluted into 4 mL of 2 % HNO_3 (DF = 5). The samples were further diluted using the same method.

For the ICP-MS (Syngistix, PerkinElmer®, UK) calibration curve of the standard, the serial dilutions were prepared for Fe and Co. Indium (In) was selected as an internal standard. The quantitative method was chosen for sample analysis. Isotopes of each element were selected as analytes (e.g., Fe isotope 56 and Co isotope 59). The probe sweeps/reading were conducted 10 times with three replicates each. The ICP-MS profile was set as Helium KED with a flow rate of 5. Once the reading was completed, the concentration of the analyte in the sample was determined by interpolation in the calibration curve of the standard.

2.2 Experiment for micronutrient fortification

2.2.1 Materials

Lettuce (*Lactuca sativa L*. var. *Longifolia*) seeds were purchased from Moles seeds Ltd. (Essex, UK) with varieties Paris Island Cos. The seeds were sowed in the seedling module trays with the composts purchased from Sinclair All-Purpose growing medium (BHGS Ltd, UK). The hydroponic/aeroponic propagators called X-stream Aeroponic Propagators, were purchased from Nutriculture Ltd. UK. The size of the propagators was 45 cm (L) x 38 cm (W) x 39 cm (H) with 20 seedlings capacity. Total volume of the tank is 9 litres, and the pump was located the middle of the tank, and it continuously mist the nutrient solutions to the root zone of the lettuces. The nutrient fertiliser solution was purchased from Canna COCO which consists of COCO A and B solution (Canna COCO Professional Nutrients, Growell Ltd. UK).

The nutrient fertiliser was mixed with the tap water pH 7.0 and the pH was controlled using 2 % HNO_3 to maintain pH 6 to 6.2. For pH and EC calibration, the calibration and storage package were purchased from Growell® including pH buffer 4 and 7, pH probe refill & storage, and conductivity standard solutions (Growell Ltd. UK). Consumable materials were purchased respectively: 15 mL and 50 mL of conical centrifuge tubes (Falcon™, Thermo Fisher Scientific Inc. Loughborough, UK); glass Pasteur pipettes (Fisherbrand™, Thermo Fisher Scientific Inc. Loughborough, UK); and Fine mist spray bottles (Cole-Parmer® LLC, Cambridge, UK). Canna COCO nutrient base has been prepared and applied based on the

manufacturer's protocol (40 mL of each Canna COCO solution A and B for 10 L of tap water pH 7.0) (Canna COCO Professional Nutrients, Growell Ltd. UK). For Hoagland nutrient fertiliser, the following chemicals were purchased from Sigma-Aldrich® (Merck Life Science UK Ltd. Gillingham, UK); ammonium nitrate (NH_4NO_3, ≥ 98 %), sodium nitrate ($NNaO_3$, 99.99 %), calcium nitrate tetrahydrate ($CaNO_6.4H_2O$, ≥ 99 %), potassium hydroxide (KOH, ≥ 85 %, pellets), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4, ≥ 99 %), calcium phosphate monobasic monohydrate ($Ca(H_2PO_4)2{\cdot}H_2O$, ≥ 80 %), calcium phosphate ($Ca_3(PO_4)_2$, ≥ 96 %), potassium sulphate (K_2SO_4, ≥ 99 %), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, ≥ 99 %), magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$, ≥ 98 %), iron sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$ & EDTA $2Na{\cdot}2H_2O$) ($FeSO_4{\cdot}7H_2O$, ≥ 99 % & EDTA (Disodium salt, molecular biology grade)), copper sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$, ≥ 98 %), zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$, ≥ 99 %), manganese sulphate ($MnSO_4{\cdot}H_2O$), boric acid (H_3BO_3 ≥ 99.5 %), ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$, 99.98 %), and sodium molybdate dihydrate ($Na_2MoO_4{\cdot}2H_2O$, ≥ 99.5 %) (See Table 2.1).

2.2.2 Growing condition

Once the third true leaf of each seedling was fully expanded with the root development (5 - 10 cm), the seedlings were transplanted into the hydroponic /aeroponic cultivations. Before transferring the seedlings, the root zone was washed by running water to remove the compost residue. The seedlings were held by neoprene rooting discs within the rooting baskets. The uniform seedlings were selected for the experiments 24 hours after the transplanting, having been grown in hydroponic/aeroponic propagators filled with water and nutrient fertilisers. The experiment was conducted in a growth room providing a controlled environment with the temperature 23 °C / 18 °C (day/night) controlled by AC. Tinytag (Gemini Data Loggers Ltd., Chichester, UK) monitored the microenvironmental parameters for a growing period, including temperature (average 21°C), dew point 14.6°C, and relative humidity 66.8 %. The photoperiod was 16 hours for the day and 8 hours dark with 200 µmol m-2s-1 light intensity measured by UV spectrometers (Avantes®, Appeldoorn, Netherlands) (Figure 2.3).

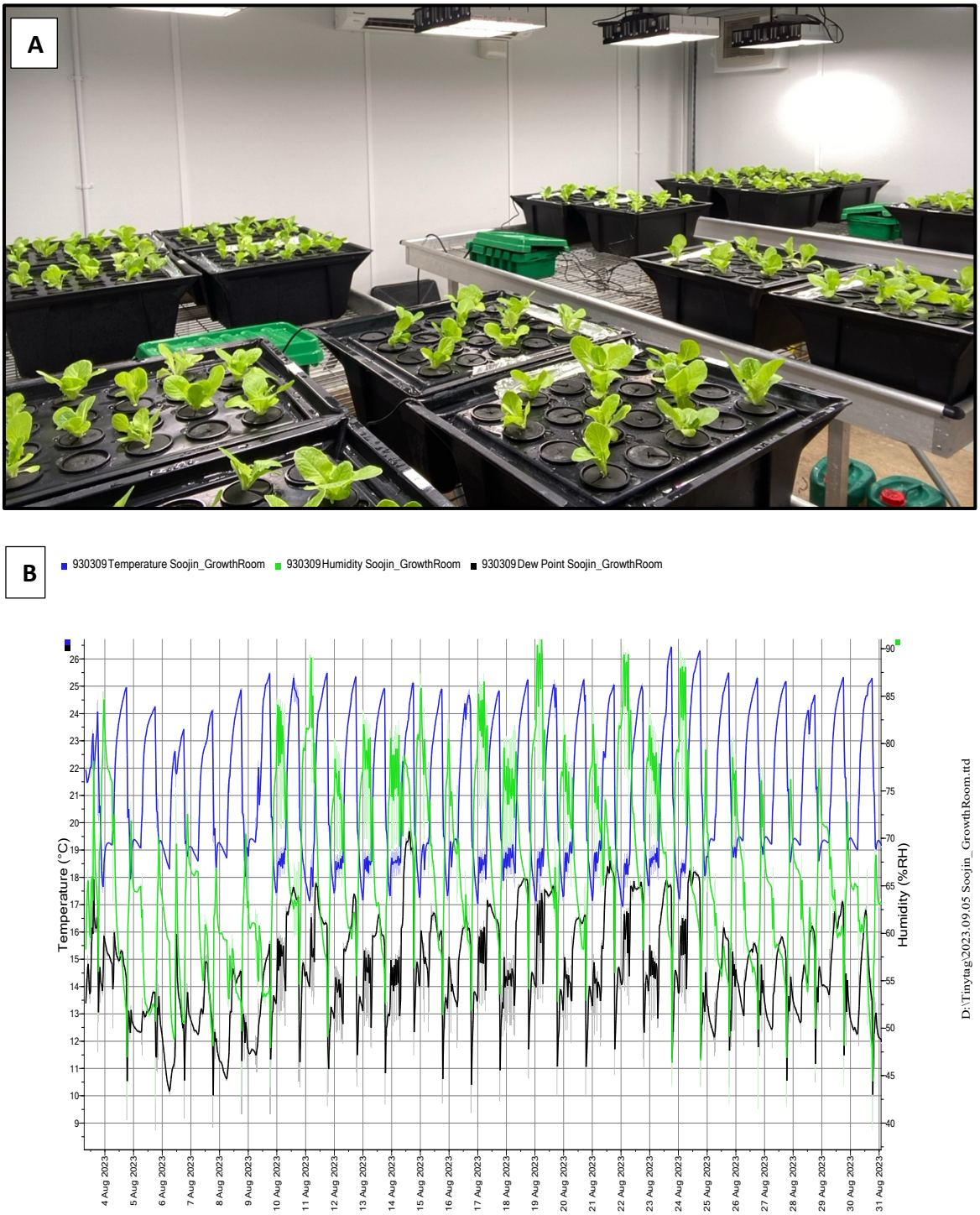


Figure 2.3 Growth room: Hydroponic/aeroponic cultivations within the LED lights.

A. The hydroponic/aeroponic systems were placed in benches at the growth room. The growth room had controlled microenvironmental condition (temperature, light, and humidity). **B.** Microenvironmental parameters measured by Tinytag including temperature, relative humidity, and dew point. These conditions were maintained throughout the entire experiment. Blue solid line represents temperature, the green solid line represents humidity, and black solid line represents dew point in the growth room.

2.2.3 Nutrient solutions and treatment

Two different nutrient fertilisers were used for this study. Firstly, Canna COCO nutrient base has been prepared and applied based on the manufacturer's protocol (40 ml of each Canna COCO solution A and B for 10 L of tap water pH 7.0). The Canna COCO nutrients were used for vitamin B_{12} fortification experiments in **Chapter 3**. Secondly, Hoagland nutrient fertiliser was prepared and used for iron fortification and iron and vitamin B_{12} fortification in **Chapter 4** and **Chapter 5**. As shown in Table 1.3, the slightly modified Hoagland nutrient recipe was prepared and applied into the hydroponic / aeroponic cultivation[163]. The stock solutions for each compound were prepared and applied individually after diluted with tap water to the hydroponic nutrient thanks.

The propagators were continuously aerated and sprayed nutrient solution by the pump. The hydroponic tanks, so called hydroponic/aeroponic propagators, were emptied, and washed including the pumps weekly to avoid the blockage of the aeroponic nozzles. Subsequently, the conditions of the cultivation systems were monitored via measuring the pH, EC, and temperature of the hydroponic solutions. The solutions were monitored every day using Bluelab combo metre (Bluelab®, UK). The pH was maintained ranging from 6.0 to 6.2 using 2 % HNO_3 and EC level was sustained ranging 2.3 to 2.5.

Table 2.1 Preparation of stock to be used to make customised Hoagland nutrient solutions.

#	Compound	Compound MW (g / mol)	Desired stock (g/L)	Final volume of stock (mL)	Stock solutions (g)	Final stock (M)
1	NH_4NO_3	80.04	160.08	500	80.04	2
2	$NNaO_3$	84.99	169.98	500	84.99	2
3	$CaN_2O_6{\cdot}4H_20$	236.15	472.3	1000	472.3	2
4	KOH	56.11	56.11	1000	56.11	2
5	K_2HPO_4	174.18	174.18	500	87.09	1
6	KH_2PO_4	136.08	136.09	250	34.0225	1
7	$CaCl_22H_20$	147.01	262.52	500	131.26	1.786
8	$MgSO_4{\cdot}7H_2O$	246.48	492.96	500	246.48	2
9	**$FeSO_4{\cdot}7H_20$ & $Na_2EDTA{\cdot}2H_20$***	650.25	29	500	14.5	0.0446
	$CuSO_4{\cdot}5H_2O$	249.68	0.08		0.04	0.00032
	$ZnSO_4{\cdot}7H_2O$	287.56	0.22		0.11	0.00077
10	*$MnSO_4{\cdot}4H_2O$*	173.01	1.57	500	0.785	0.00907
	$(NH_4)6Mo7O_{24}$	1163.9	0.1639		0.08195	0.00014
	H_3BO_3	61.83	2.86		1.43	0.04625
11	**Fe_3O_4 Nanoparticles***	231.53	10	500	1.72	0.015

The Fe-EDTA treatment was prepared based on the molecular weight of the $FeSO_4{\cdot}7H_2O$ & $NA_2EDTA{\cdot}2H_2O$ complex (650.25 g / mol), with the compound mass adjusted to deliver the desired concentration of elemental iron (mg Fe / L), accounting for the iron fraction in the molecule. Similarly, the Fe_3O_4 NPs treatment was prepared considering the molecular weight of Fe_3O_4 NPs (231.53 g / mol) and its elemental iron content, containing three iron atoms per molecule. The iron fraction in Fe_3O_4 NPs was reflected in the calculation of the compound mass to achieve equivalent elemental iron concentrations across treatments. Concentrations are expressed as elemental iron (Fe) content (mg / L) unless otherwise indicated.

2.3 Method: Data collection

2.3.1 Elemental analysis using ICP-MS

Lettuce tissues, containing of leaves and roots, were dried in an oven at 65 °C for 72 hours, then the samples were homogenised in powder, and approximately 50 mg of lettuce tissues were digested in 5 mL of 70 % HNO_3. Sample solutions were sonicated at 60 °C for overnight to fasten the digestion process. The digested sample solutions were filtered using glass Pasteur pipettes with cotton wool and then diluted 1mL of the sample solution in 4 mL of 2 % HNO_3 (dilution factor = 5) (Figure 2.4). The analysis method was the same as Fe_3O_4 NPs as below, but analytes were included: calcium (Ca), cobalt (Co), iron (Fe), potassium (K), magnesium (Mg) and phosphorus (P), and zinc (Zn). To avoid the contamination from previous readings, the probe was washed thoroughly before each use, and a blank run was conducted triple times after each treatment batch. Compliance software called Syngistix software version 4.0 was used for the NexION® ICP-MS analysis (Syngistix™, PerkinElmer Inc, UK).

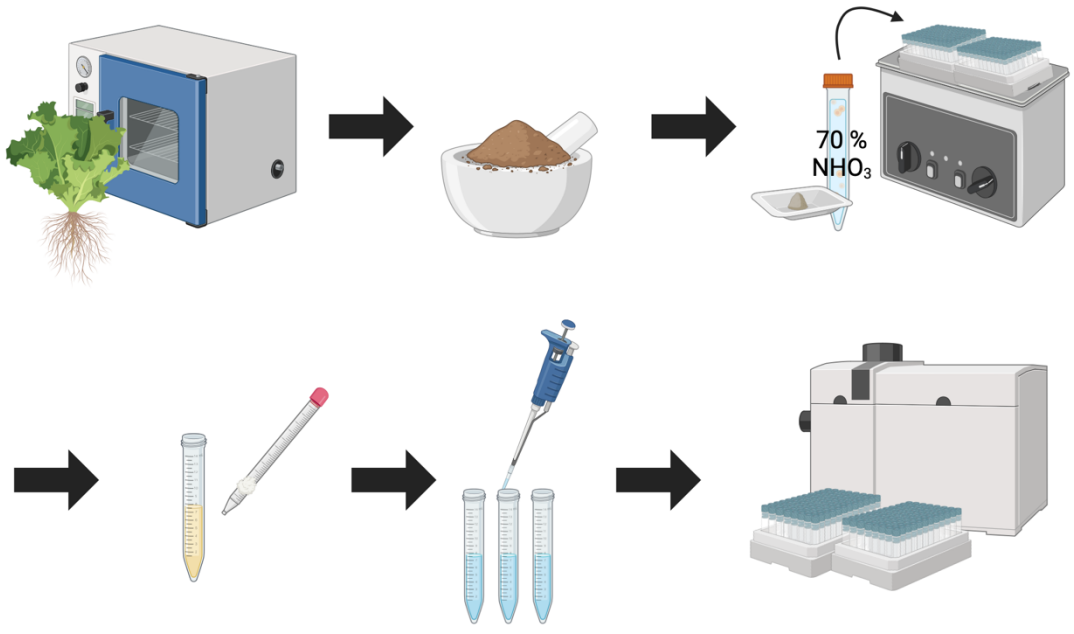


Figure 2.4 Schematic representation of ICP-MS analysis

1. Drying lettuce samples. **2.** Sample homogenisation. **3.** Digestion of the sample using heated ultrasonic bath. **4.** Filtering. **5.** Preparing serial dilutions. **6.** ICP-MS analysis (created with BioRender.com).

2.3.2 Morphological measurements: PlantEye

The PlantEye F600, a 3D multispectral scanner (Phenospex, Netherlands), was employed for plant phenotyping (Figure 2.5). Compliance software called HortControl 3.8 was used for data collection and analysis. The measurement was taken every week in the glasshouse, on Brackenhurst campus at Nottingham Trent University. Each treatment was designated a barcode with eight lettuce samples and seedlings were transferred into a bucket filled with tap water.

PlantEye was conducted to measure plant growth (e.g., digital biomass, height, and leaf area index), spectral parameters (e.g., hue and greenness) and spectral vegetation indices (e.g., Normalised Differential Vegetation Index (NDVI), Normalised Pigment Chlorophyll Ratio Index (NPCI) and Plant Senescence Reflectance Index (PSRI)). Digital biomass (cm^3) is measured as a combination of height and leaf area presenting the morphological growth and/or development of lettuce.

The parameter of hue value ranges from 0 to 360 ° expressed by the colour spaces which is measured independently from its saturation (pale or intense) or value. Each colour spectrum is covered by the degrees such as yellow for 60 °, green for 120 °, and cyan for 180 °. Likewise, the greenness index also referred to as the green leaf index, ranges from -1 to 1. The parameters of NDVI, NPCI and PSRI were estimated for the physiological health of the lettuces. As an index of plant vigour and biomass, PlantEye measured NDVI which calculated the ratio of NIR and the red channel by PlantEye. NPCI presents the chlorophyll content as a measure of red and blue channels from PlantEye. PSRI estimates plant aging.

Figure 2.5 PlantEye (Phenospex)

A. Operation of scanning procedure by PlantEyeF600 installed in the greenhouse, Nottingham Trent University. **B.** A multispectral 3D scanner as a non-destructive phenotypic measurement. **C.** An example of the collected image within a real-time scanning.

2.3.3 Physiological measurements: LI-COR

Indirect photosynthetic activity and gas exchange were measured by an LI-6800 Portable Photosynthesis System (LI-COR Bioscience™, Lincoln, USA) and the programme measures transpiration rate (*E*), Net assimilation rate (*A*), intercellular CO_2 (*Ci*), ambient (to leaf) CO_2 (*Ca*), and stomatal conductance to H_2O (*gsw*). Transpiration rate (*E*) reflects water loss in plants and is closely linked to stomatal opening. Net assimilation rate (*A*) refers to the net photosynthetic CO_2 uptake by the leaf, indicating photosynthetic efficiency. Intercellular CO_2 (*Ci*) represents the level of CO_2 concentrations within the leaf air spaces, while ambient to leaf CO_2 (*Ca*) refers to the CO_2 concentration in the air surrounding the leaf surface. Stomatal conductance to H_2O (*gsw*) represents the rate at which water vapour exits the leaf through the stomata, indicating the efficiency of gas exchange.

The relation to each parameter estimated the following traits of the efficiency of water management and gas exchange for lettuce: Water Use Efficiency (*WUE*),

Intrinsic Water Use Efficiency (*iWUE*), and Stomatal Limitation (L_s). Each calculation was based on the below equation as follows:

$$Water\ Use\ Efficiency\ (WUE) = \frac{Net\ assimilation\ rate\ (A)}{Transpiration\ rate\ (E)}$$

$$Intrinsic\ Water\ Use\ Efficiency\ (iWUE) = \frac{Net\ assimilation\ rate\ (A)}{Stomatal\ conductance\ to\ H_2O\ (gsw)}$$

$$Stomatal\ Limitation\ (L_s) = 1 - (\frac{Intercellular\ CO_2\ (Ci)}{Ambient\ (to\ leaf)\ CO_2\ (Ca)}$$

The environmental condition of the leaf chamber was as follows: flow rate 500 µmol s^{-1}, fan speed 3000 RPM, temperature 25.0 °C and relative humidity at 60 %. The second fully expanded eight plants across ten treatments were measured on 3 weeks and 4 weeks after transplanting. The measurement was collected by an auto programme named light response which was set up from 200 to 1800 µmol m^2s^{-1} till the data readings had stabilised. Microsoft Excel (Microsoft, USA) was used for data analysis.

2.3.4 Relative chlorophyll measurements: SPAD

Relative chlorophyll fluorescence absorbance was measured by a portable chlorophyll metre called SPAD-502Plus (Konica Minolta Ltd., Osaka, Japan) which is a non-destructive hand-held device. SPAD-502 measured two wavelength regions, red (650 nm) and near-infrared-red (650 - 940 nm), within the average of four readings. The measurements were taken weekly from the fully expanded leaf of each plant across four replicates for each of the ten treatments. The amount of chlorophyll fluorescence was calculated by the average SPAD values for each replicate.

2.3.5 Harvest quality: fresh and dry weights

This study measured fresh and dry weights of lettuce as a harvest quality. The lettuce seedlings were harvested two times: The first harvest was undertaken 2 weeks after transplanting and the second harvest was implemented 4 weeks after

transplanting. The week 4 harvest was recorded as a final harvest data (Figure 2.6). Three to eight lettuces were weighed for each harvest date. 24 hours before harvest date, the whole lettuces were thoroughly rinsed by tap water for twice to minimise the residue of the treatment. The hydroponic nutrient tank was empty and filled with fresh water. On the harvest date, the fresh weight was measured using a four-digit analytical balance (Adam Equipment Co Ltd., UK). The leaf and root tissues were sectioned by a secateur. For the fresh weight measurement, the whole lettuces were gently patted using paper roll and it was measured leaf and root, respectively. For dry weight measurement, the freshly harvested lettuce samples were packed in a brown paper bag (Amazon, UK) then transferred to an oven. The samples were dried at 65 °C for 72 to 96 hours until the lettuce tissues were fully dehydrated. The dry weight was measured using four-digit analytical balance.

Figure 2.6 Growth and development of lettuce.

A. Week 1 - Seedling stage 7 days after transplanting. **B.** Week 2 - Vegetative stage 14 days after transplanting **C.** Week 3 - Heading stage 21 days after transplanting; **D.** Week 4 - Maturation stage 28 days after transplanting. A portion of lettuces were harvested on week 2 and week 4 to quantify in leaf and root lettuce tissues.

2.4 Secondary metabolites analysis

2.4.1 Materials

The following chemicals/reagents were purchased from Sigma-Aldrich® (Merck Life Science UK Ltd. Gillingham, UK); methanol (CH_3OH, ≥ 99.6 %,), hydrochloric acid (HCl, 37 %), Folin-Ciocalteu's phenol reagent, Sodium carbonate (Na_2CO_3), Gallic acid ($C_7H_6O_5$, > 97.5 %,), aluminium trichloride solution ($AlCl_3$, 99.9 %,), ethanol (CH_3CH_2OH, ≥ 99.5 %, anhydrous), sodium nitrite ($NaNO_2$, ≥ 99 %,), sodium hydroxide (NAOH, ≥ 98 %, anhydrous,), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Trolox® ($C_{14}H_{18}O_4$, ≥ 98 %), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid ($C_{14}H_{18}O_4$, ≥ 98 % titration), potassium persulfate ($K_2S_2O_8$, ≥ 99.0 %), sodium chloride (NaCl, ≥ 99 %), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, ≥ 98 %), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$). The following reagents/chemicals were purchased from Thermo Fisher Scientific (Loughborough, UK); Rutin (≥ 97 %), and 2,2'-Azino-bis (3-ethylbenzothiazoline -6-sulfonic acid) diammonium salt (ABTS, 98 %).

2.4.2 Extraction

The lettuce leaves tissues were kept in the -80 ℃ freezer (Pro ULT Freezers -86 °C, PHC Co, Tokyo, Japan) until conducting the homogenisation process. Three to four randomly harvested leaves were homogenised for 1 minute by using a mortar and pestle while supplying liquid nitrogen. The homogenised tissues were measured approximately up to 1 g and mixed with 10 mL of 2 % acidified methanol solution (MeOH : HCl = 98 : 2 v/v) for the extraction. To facilitate the extraction process, the sample solutions were covered with aluminium foil and were placed in the VWR® Orbital Shaker (Avantor®, Leicester, UK) overnight at 150 min^{-1} speed with a room temperature. After the extraction process, the sample solutions were centrifuged (VMR® Mega Star Benchtop Centrifuges, Avantor®, Leicester, UK) at 4700 RPM for 30 minutes and took only clear supernatant using a pipette.

2.4.3 Total Phenolic Content (TPC)

Folin-Ciocâlteu reagent was used in this study to determine the Total Phenolic Content (TPC) in lettuce leaves using the gallic acid equivalence method (GAE) [164]. Folin-Ciocâlteu phenol reagent was diluted with deionised water in 10:1 ratio (v/v) and kept at 4 ℃ fridge until use (Sparkfree Fridge, Labcold™, UK). Sodium carbonate was prepared as a buffer solution with 7.5 % kept at room temperature until being used. Gallic acid was used as a standard solution for preparing serial dilutions 100, 50, 25, 12.5, 6.25, 3.13, 1.57, and 0.78 µg / mL diluted with deionised water. For spectroscopy measurement, 20 µL of each reagent, including supernatant of digested plant solution, blank (2 % acidified MeOH), and serial dilutions of GA standard solution, were transferred into cuvettes (pathlength 10mm, PMMA). Then 100 µL of Folin-Ciocâlteu was added into the cuvettes and diluted with 1.58 mL of deionised water. After 3 minutes of incubation at room temperature, 300 µL of 7.5 % sodium carbonate solution was added. At last, the cuvettes were covered with a lid and kept it at room temperature for 2 hours for final incubation. The absorbances were read at 765 nm wavelengths by Ultraviolet-visible spectroscopy (UV mini-1240, Shimadz, Kyoto, Japan) for duplicated blanks, standards, and triplicated sample solutions. To improve the accuracy of absorbance readings, every sample was measured twice on separate dates (Figure 2.7).

2.4.4 Total flavonoid content (TFC)

Total Flavonoid Content (TFC) was determined using an aluminium chloride assay[164]. Stock solutions were prepared including 100 µg / mL of Rutin diluted with methanol, 2 % $AlCl_3$, 5 % $NaNO_2$ and 1 M NaOH. As a standard solution, Rutin stock solution was diluted with methanol for preparing serial dilutions 100, 50, 25, 12.5, 6.25, 3.13, 1.57 and 0.78 µg / mL. To Prepare working solutions for the analysis, 1 mL of each reagent was transferred into 5 mL centrifuge tubes, including the supernatant of digested plant solution, a blank solution consisting of 99.6 % of methanol and deionised water, and serial dilutions of Rutin standard solutions, so called as Rutin equivalents (RE). Then 0.3 mL of 5 % $NaNO_2$ was added into the tubes and incubated for 5 minutes to allow the reaction to occur. This was followed by adding 0.3 mL of 2 % $AlCl_3$, 2 mL of 1 M NaOH, and 2.4 mL of deionised water immediately. The solution was then shaken until it turned to pink in colour. For the spectroscopy measurement, take 2 mL of each working solution

into cuvettes (path length 10mm, PMMA). The absorbances were read at 510 nm wavelengths by UV-Vis (UV mini-1240, Shimadz, Kyoto, Japan) for duplicated blanks, standards, and triplicated sample solutions. To improve the accuracy of absorbance readings, every sample was measured twice on different dates (Figure 2.7).

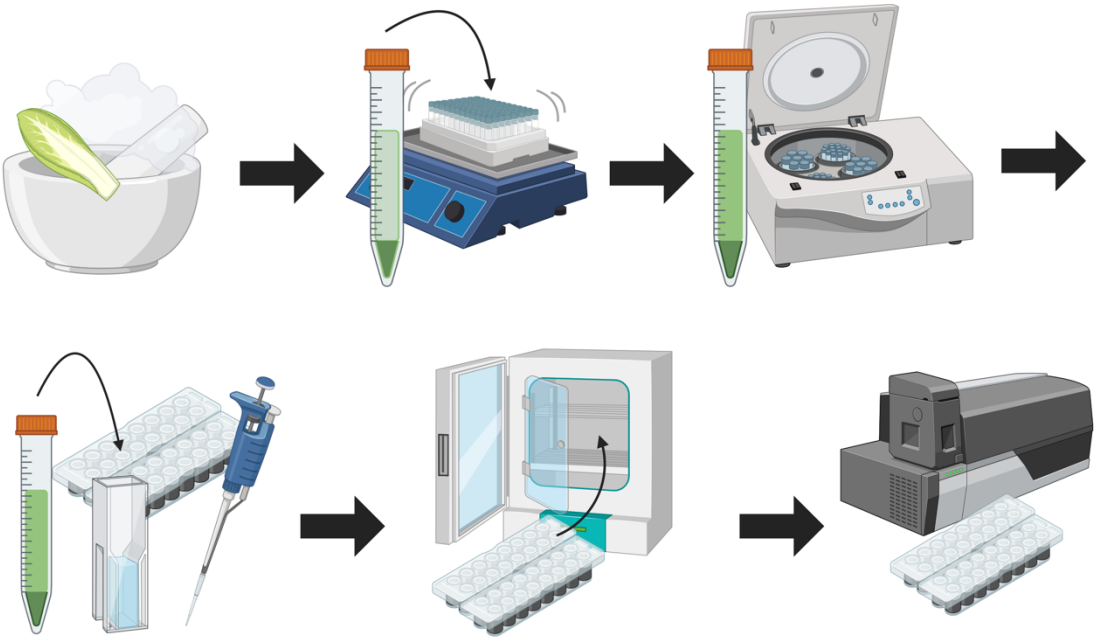


Figure 2.7 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) analysis.

1. Sample homogenisation**. 2.** Reaction for overnight. **3.** Collection of the reaction via centrifuge. **4.** Preparation for samples. **5.** Sample incubation **6.** Reading the samples using UV-VIS spectrophotometer.

2.4.5 Total antioxidant activity (TAA)

Total Antioxidant Activity (TAA) was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay with a slight modification [164]. Trolox® equivalent antioxidant capacity (TEAC) was used as a standard reference to calculate both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging methods. Specifically, TEAC standard solution was prepared by dissolving 6.3 mg of Trolox in 50 mL of 50 % methanol solution in deionised water (1:1 ratio v/v). Then make serial dilution using 50 % methanol solutions including 250, 125, 62.5, 31.25, and 15.625 µg / mL (mg / L) to get a standard calibration curve.

For the DPPH assay, the DPPH stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol which was incubated in the fridge (4 °C) overnight for the reaction. The DPPH working solution was prepared by adding 80 mL of methanol into 30 mL of DPPH stock solution. A stable free radical working solution of DPPH shows dark purple in colour. The ABTS stock solution was prepared by dissolving 384.90 mg of ABTS in 100 mL of deionised water. For forming $ABTS^{\bullet+}$, potassium persulfate ($K_2S_2O_8$) was used as an oxidising agent and prepared by dissolving 66.2 mg of $K_2S_2O_8$ in 100 mL of deionised water. The working solution for ABTS was prepared by mixing ABTS stock solution and $K_2S_2O_8$ solution (1 : 9 ratio, v/v), then incubated in dark conditions at room temperature overnight to complete the reaction. Once the reaction was completed, the ABTS working solution showed a dark blue-green colour.

The antioxidant activity of lettuce was measured by a microplate reader Tecan Spark® Tecan Group Ltd., Männedorf, Switzerland). Both DPPH and ABTS working solutions were prepared on each plate. For the DPPH assay, 15 µL of each solution was loaded on the plates including blank (deionised water and methanol), TEAC standard solutions, and the supernatant of the homogenised lettuce solution. Added 285 µL of DPPH working solution then incubated 30 minutes at 30 °C in a dark environment. After the incubation, the absorbance of the DPPH assay was measured at 517 nm. For the ABTS assay, the pH of the ABTS working solution was adjusted priorly. Phosphate buffer solution was prepared for 5 mM of pH 7.4 and used for adjusting the pH of the ABTS working solution to 0.7 by reading absorbance at 734 nm. Then the plates loaded with 10 µL of each solution which is the same as DPPH assay. Then 290 µL of ABTS

working solution was added to the plate and incubated it 30 minutes at room temperature in a dark environment. Once the incubation was over, the plate was read at 734 nm wavelength (Figure 2.8).

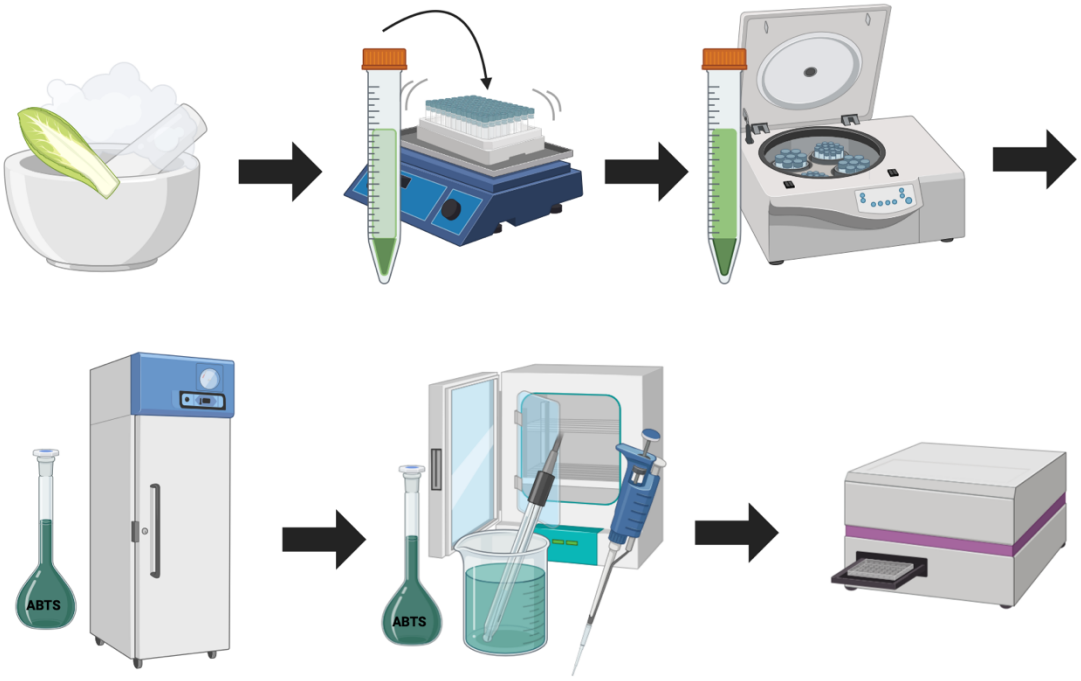


Figure 2.8 Total Antioxidant Activity (TAA) analysis.

1. Sample homogenisation. **2**. Reaction for overnight. **3**. Collection of the reaction via centrifuge. **4**. Incubate the sample for the reaction. **5.** Sample preparation in a plate and incubated. **6.** Reading the samples using a plate reader.

2.5 Statistical analysis

Overall data was presented as descriptive statistic including mean, standard deviation (SD), minimum and maximum value from each measurement. Considered the data distribution for this study, statistical analysis made using Permutational Multivariate Analysis of Variance (PERMANOVA) using R studio (Posit® Software, Boston, USA). PERMANOVA method is ideal for multivariate data with more than two intendent variables, also known as explanatory or predictor variables. The R scripts were created based on the book written by Bakker and referred the GitHub (Github.com, GitHub Inc, California, USA)[165,166]. Via PERMANOVA test, this study was able to determine the statistically significant difference between the groups. The pair-wise test was also conducted to identify the difference between specific groups via multiple permutational comparison test. Subsequently, Permutational Multivariate Analysis of Dispersion (PERMDISP) test was conducted after PERMANOVA test to determine the dispersion of the samples which validate the outcomes of PERMANOVA test [165,166].

Chapter 3. Vitamin B_{12} Fortification

Investigations into vitamin B_{12} fortification has largely been limited to studies on the living food crops. Only a limited number of studies have focused on B_{12} fortification, and these are restricted to specific application conditions, such as utilising a using a designated application method (leaf or root application) or an intermittent B_{12} treatment. Recent study investigated B_{12} fortification in lettuce grown in hydroponic cultivation, this study was however limited to root application of B_{12} within a single timeframe of treatment 24 hours before the harvest [68]. Another recent study conducted by Yamamoto et al. (2023) examined the B_{12} fortification in tomato grown hydroponically which included nutritional analysis including analysis of sugar, ascorbate, and lycopene levels[69]. This study was however limited to using a single concentration of B_{12} (5 μM) within a root application. The present study aims to fortify B_{12} using root and leaf application methods within several concentrations of B_{12} (0, 2.5, 5, 7.5 and 10 mg / L).

This chapter investigates the optimal method of vitamin B_{12} fortification in hydroponically grown lettuce using two different application approaches: leaf (foliar spray) and root applications via the hydroponic nutrition solution. Building upon the findings from the established literature on vitamin B_{12} fortification, this study aims to determine the effect of B_{12} treatment on lettuce and to explore the mechanisms of uptake, translocation and accumulation of B_{12} within lettuce tissues. It is hypothesised that vitamin B_{12} treatment may not significantly affect the morphological parameters of hydroponically grown lettuce, considering the species' inherent growth characteristics and the concentrations applied in this study. However, it is expected that exogenous B_{12} will be absorbed, translocated, and accumulated within the lettuce tissues, and that B_{12} treatment may produce measurable effects on the physiological responses and nutritional composition of the plants. Additionally, it is hypothesised that the efficiency of B_{12} uptake may vary between application methods, with potential differences expected between foliar spray and root application via the hydroponic nutrition solution.

The aim of this chapter is to determine the optimal method of B_{12} fortification and evaluate the effects of different B_{12} concentrations on lettuce growth, physiology, and nutritional quality. The objectives are: (1) to compare the efficiency of foliar spray versus root application methods for B_{12} uptake, translocation, and accumulation in lettuce; (2) to assess the impact of varying B_{12} concentrations on the morphological, physiological, and nutritional parameters of hydroponically

grown lettuce; and (3) to elucidate possible mechanisms of B_{12} uptake and translocation within lettuce tissues under different treatment conditions.

3.1 Vitamin B_{12} fortification - Leaf application

3.1.1 Material and method

The materials and growing conditions are described in **Chapter 2.2.** The present experiment aimed to determine the effect of the leaf application along with different concentrations of vitamin B_{12} 0 (control with H_2O application), 2.5, 5, 7.5, and 10 mg / L. Vitamin B_{12} (Cyanocobalamin, $C_{63}H_{88}CoN_{14}O_{14}P$, ≥ 98 %) was weighed and diluted with type I water for serial dilutions using 50 mL conical centrifuge tubes. Subsequently, the B_{12} solutions were loaded into the fine mist spray bottles which were covered with foil to avoid light response for further application. The total volume of B_{12} solutions of 50 mL were prepared for the application during the vegetative stage of the lettuce growth (1 week and 2 weeks after transplanting). During the maturation period of growth (3 weeks and 4 weeks after transplanting), the total volume of B_{12} solutions were increased from 100 to 200 mL to account for the increase of lettuce biomass. The treatments were applied twice a day at 9 am and 5 pm weekly until harvest. Vitamin B_{12} solutions were sprayed on the entire leaves of lettuce for harvest sample collection, the entire lettuces were washed multiple times with running water to minimise the residues from the treatment application.

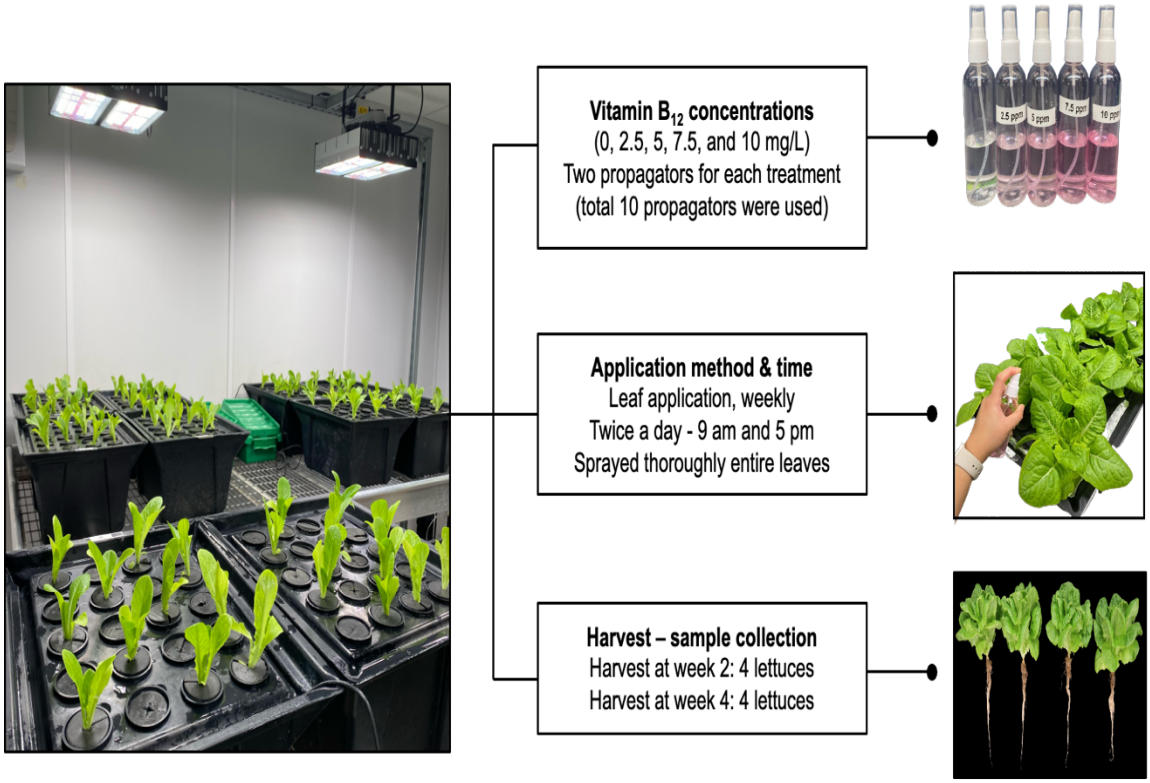


Figure 3.1 Schematic representation of growing condition for B_{12} fortification via leaf application.

Vitamin B_{12} concentrations (0, 2.5, 7.5, and 10 mg / L); Leaf application method (using foliar spray method), and harvest details.

3.1.2 Data collection and statistical analysis

This experiment has eight biological replicates per different concentration vitamin B_{12} treatment. Five different treatments of B_{12} (0, 2.5, 5, 7.5, and 10 mg / L) were applied for two propagators each and total 10 propagators were used for this experiment which shown in **(**Figure 3.1**).** A portion of lettuces were harvested 14 days and 28 days after transplanting to quantify B_{12} uptake and translocation over time in lettuce tissues via elemental analysis.

The growth and morphological traits were obtained weekly via PlantEye F600 using a 3D scanner (Phenospex, Netherlands). The growth and morphological data were collected weekly for parameters such as height (cm), leaf area (cm^2), and digital biomass (cm^3). The spectral reflectance of the lettuce was measured to quantify the hue (º) and greenness parameters. Then spectral parameters were analysed for NDVI, NPCI, and PSRI related to lettuce growth. As physiological

measurement, water management and gas exchange traits were measured by LI-6800 (LI-COR Bioscience, Lincoln, UK) weekly for the following parameters: transpiration rate (*E*), net assimilation rate (*A*), ambient to leaf CO_2 (*Ca*), intercellular CO_2 (*Ci*) and stomatal conductance to water vapour (*gsw*). The measurements of the LI-COR parameters were interpreted as Water Use Efficiency (*WUE*), Intrinsic Water Use Efficiency (*iWUE*) and Stomatal Limitation (L_s). The Harvest quality was obtained by measuring the fresh weight of lettuces for the final harvest day. Furthermore, the elemental analysis was conducted by ICP-MS analysis from lettuces root and leaf tissues which were harvested week 2 and week 4 after transplanting to hydroponic system. The detailed processes of data collection are described in **Chapter 2.**

The data was statistically analysed using Permutational Multivariate Analysis of Variance (PERMANOVA) and Permutational Analysis of Multivariate Dispersions (PERMDISP) in R studio (R version 4.4.0). PERMANOVA was applied to assess the significant difference between B_{12} treatment groups, followed by multiple pairwise comparison to identify which specific groups differed significantly (number of permutations = 9,999).

3.1.3 Results

This section investigates the effect of B_{12} fortification via leaf application, also known as foliar spray, on hydroponically grown lettuce. Given that leaf application delivers B_{12} directly to the edible parts of leafy vegetables, this method may facilitate more rapid and efficient B_{12} uptake and accumulation compared to root-based applications. The aim of this chapter is to evaluate how varying B_{12} concentrations affect lettuce growth, physiology, and nutritional quality. The objectives are: (1) to quantify the uptake, translation, and accumulation of leaf applied B_{12} in lettuce leaves and root tissues using ICP-MS; and (2) to assess the effects of varying B_{12} concentrations (0, 2.5, 5,7.5, 10 mg / L) on morphological traits such as leaf area, biomass, physiological parameters (photosynthetic gas exchange, water management, and relative chlorophyll content, and nutritional quality including B_{12} content, secondary metabolites.

3.1.3.1 Growth and development measurements

Lettuce growth and morphological parameters, presented as mean ± standard deviation, were measured using the PlantEye F600 system. Further details of the methods and parameters are described in **Chapter 2.3.2**. Statistical analysis was conducted to determine the significance between the groups of different B_{12} treatments by implementing PERMANOVA and multiple permutational comparison test (pairwise) with 9,999 times of permutation.

Figure 3.2 presents the weekly measurements of plant height (cm) and leaf area (cm^2), illustrating the morphological development of lettuce over the growth period. Both parameters showed an overall increase throughout the weeks, with comparable variation observed between week 3 and week 4. However, no statistically significant differences were observed among the different B12 treatment concentrations based on permutational pairwise tests using 9,999 permutations.

At the final harvest (week 4), the control group (no B_{12} treatment) exhibited the highest mean plant height (15.26 ± 1.71 cm), closely followed by the B_{12} 2.5 mg / L treatment group (15.11 ± 0.70 cm). The B_{12} 5 mg / L treatment group showed the lowest mean plant height (13.13 ± 2.69 cm). Regarding leaf area (cm^2), lettuces treated with B_{12} 5 mg / L recorded the largest leaf area (771 ± 90 cm^2), closely followed by the control group (768 ± 95 cm^2). The B12 10 mg / L treatment group resulted in the lowest mean leaf area (577 ± 141 cm^2).

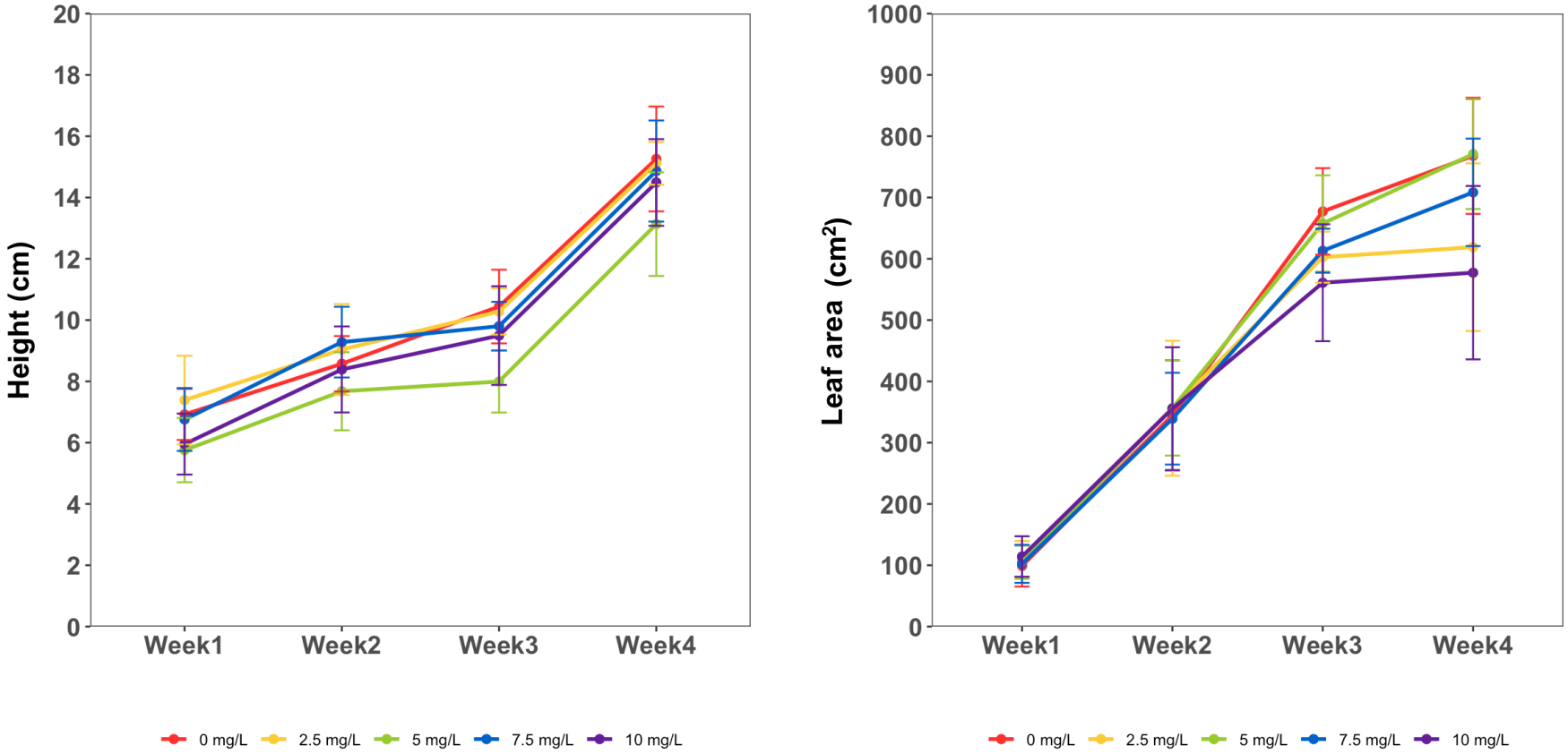


Figure 3.2 Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Figure 3.3 presents the digital biomass (cm3) and hue measurements. As a volumetric estimate for mature lettuce, digital biomass reflects plant height and projected leaf area as measured by the PlantEye scanner. Increased variation between the B_{12} treatments was observed between week 2 and week 4. At week 4, the highest digital biomass value was recorded in the control group (11,637 ± 1,353 cm^3), followed by B_{12} 7.5 mg / L treatment (10,584 ± 2,060 cm^3). The lowest value was observed in B_{12} 10 mg / L (8,312 ± 2,027 cm^3). Although some numerical variations were observed, these differences were not statistically significant ($p > 0.05$). Overall, no statistically significant differences were observed in plant morphological parameters including plant height, leaf area, and digital biomass among the B_{12} treatment groups ($p > 0.05$).

Hue measurements, which range from 0 to 360 º, indicate the colour and can reflect the morphological health. Hue values between 90 º to 140 º typically correspond to green colouring, suggesting healthy plants. Hue measurements across all treatment groups remained within the healthy green range (90 º to 100 º), with only minor variation between B_{12} treatments. At week 4, the B_{12} 5 mg treatment group exhibited the highest mean hue value (103.16 ± 2.44 º).

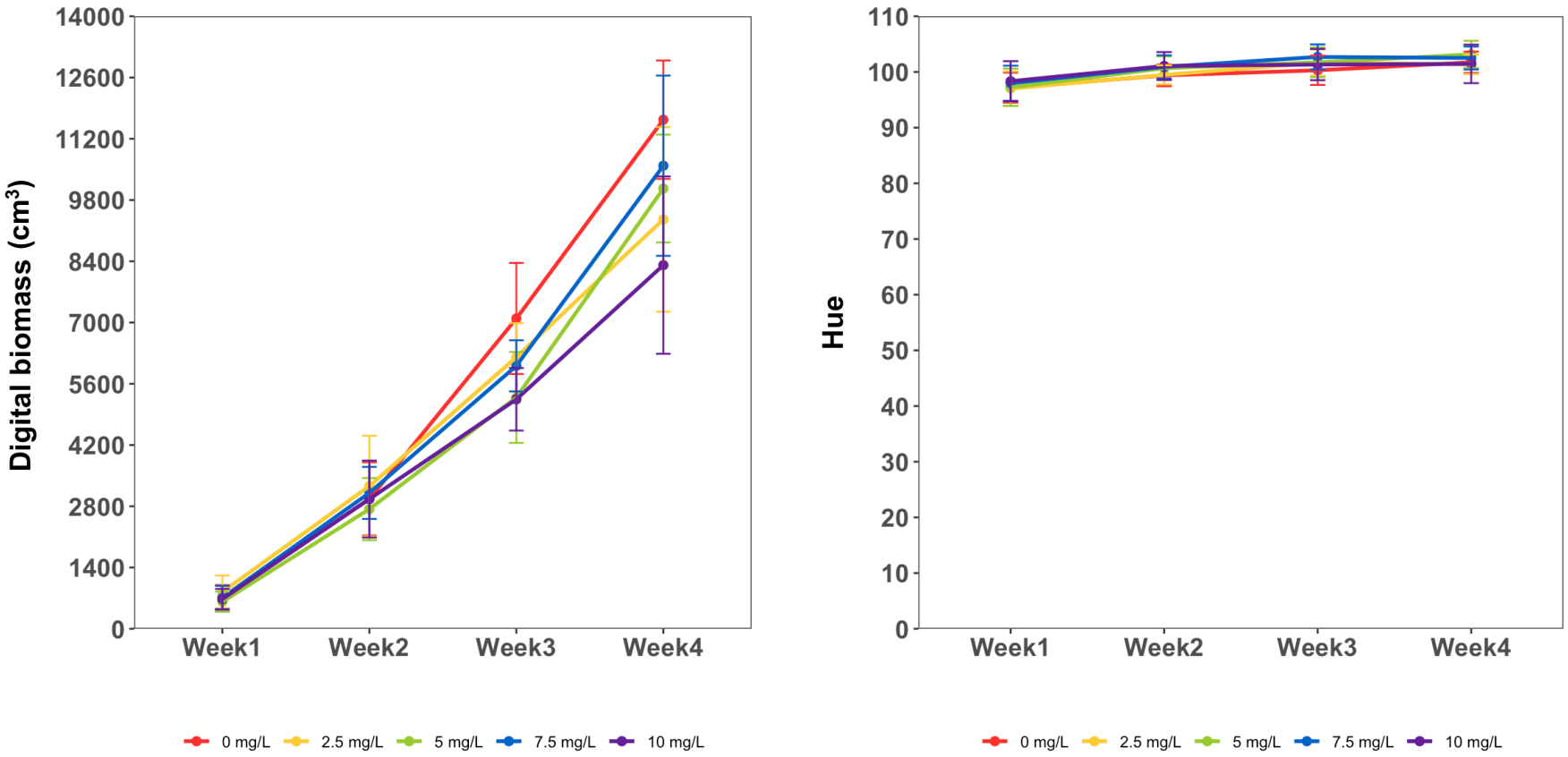


Figure 3.3 Digital biomass (cm^3) and Hue (°) of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Greenness measurements, which range from -1 to 1, represent the green leaf index, where higher values indicate greater greenness typically associated with healthy leaves. As shown in Figure 3.4, greenness values were generally higher around week 2 during the growth period. At week 4, the control group (no B_{12} treatment) exhibited the highest mean greenness value (0.51 ± 0.01), while the B_{12} 7.5 mg / L treatment group showed the lowest mean value (0.47 ± 0.01). However, no statistically significant differences were found between the B_{12} treatments for either hue or greenness ($p > 0.05$), indicating that B_{12} application did not have a measurable impact on leaf colour or pigmentation.

Normalised Difference Vegetation Index (NDVI) is an indicator of vegetation health and relates to the interception of photosynthetically active radiation. Healthy vegetation typically presents NDVI values between 0.6 to 0.9. Overall, NDVI values across all B_{12} treatments groups remained within this healthy range from week 2 onwards. At the final measurement (week 4), lettuces treated with B_{12} 5 mg /L showed the highest mean NDVI value (0.72 ± 0.03). However, no statistically significant differences were found between the groups ($p > 0.05$).

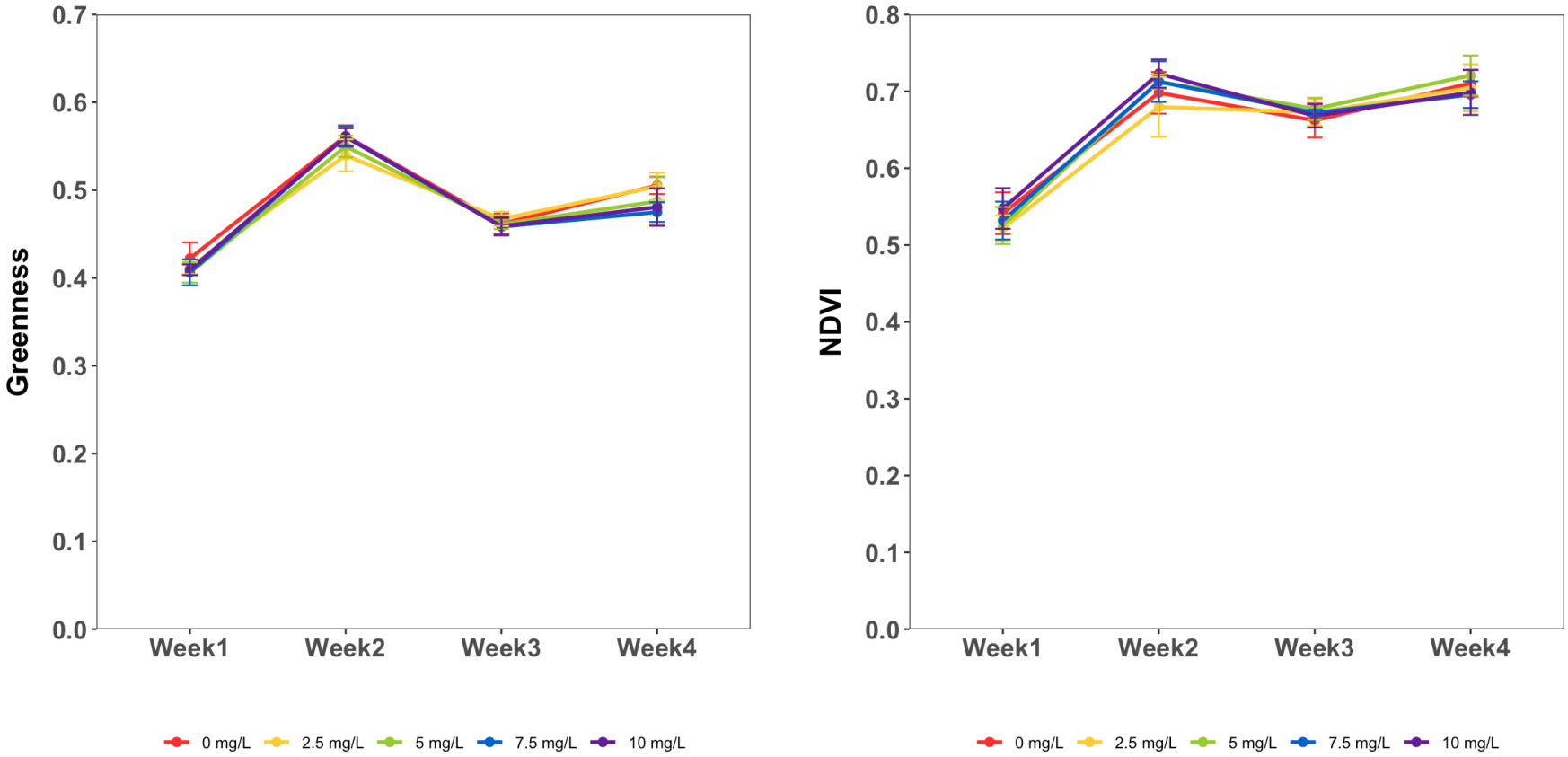


Figure 3.4 Greenness and NDVI of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Figure 3.5 presents Normalised Pigment Chlorophyll Ratio Index (NPCI) and Plant senescence reflectance index (PSRI). NPCI reflects the ratio of pigments in the leaf, particularly the balance between chlorophyll and carotenoids, and serves as an indicator of physiological status and stress. Overall, NPCI values peaked at week 2, followed by a decline between week 2 and week 3. At week 4, the highest mean NPCI value was observed in the B_{12} 2.5 mg / L treatment group (0.41 ± 0.04), closely followed by the control group (0.40 ± 0.04).

Likewise, the PSRI indicates plant senescence by assessing the ratio of carotenoid to chlorophyll, serving as an indicator of physiological aging and leaf senescence. PSRI values peaked at week 1, followed by a gradual decline over the growth period, decreasing from an average of 0.15 to 0.09. At the final harvest (week 4), the B_{12} 5 mg / L treatment group exhibited the lowest mean PSRI value (0.08 ± 0.01), suggesting relatively reduced senescence. However, no statistically significant differences were detected in PSRI values among the B_{12} treatment groups ($p > 0.05$).

In conclusion, B_{12} treatment via leaf application did not significantly affect the morphological or physiological developments of lettuces. Lettuces from the control group, which did not receive B_{12} treatment, generally exhibited greater biomass accumulation and heathier growth conditions. Although the B_{12} 5 mg / L treatment resulted in increases in leaf area, hue, and vegetation indices compared to the control, these differences were not statistically significant differences ($p > 0.05$). This is consistent with the biological expectation that most higher plants, including lettuce, do not require exogenous B_{12} for their growth and development. Plants typically lack B_{12}-dependent metabolic pathways, which likely explains the minimal physiological response observed in this study. Additionally, the concentrations tested may not have been sufficient to surpass physiological thresholds required to induce detectable growth responses. It is also possible that the endogenous B_{12} levels present in the lettuces were already adequate to support normal growth and development, further reducing the potential benefit of external B_{12} supplementation.

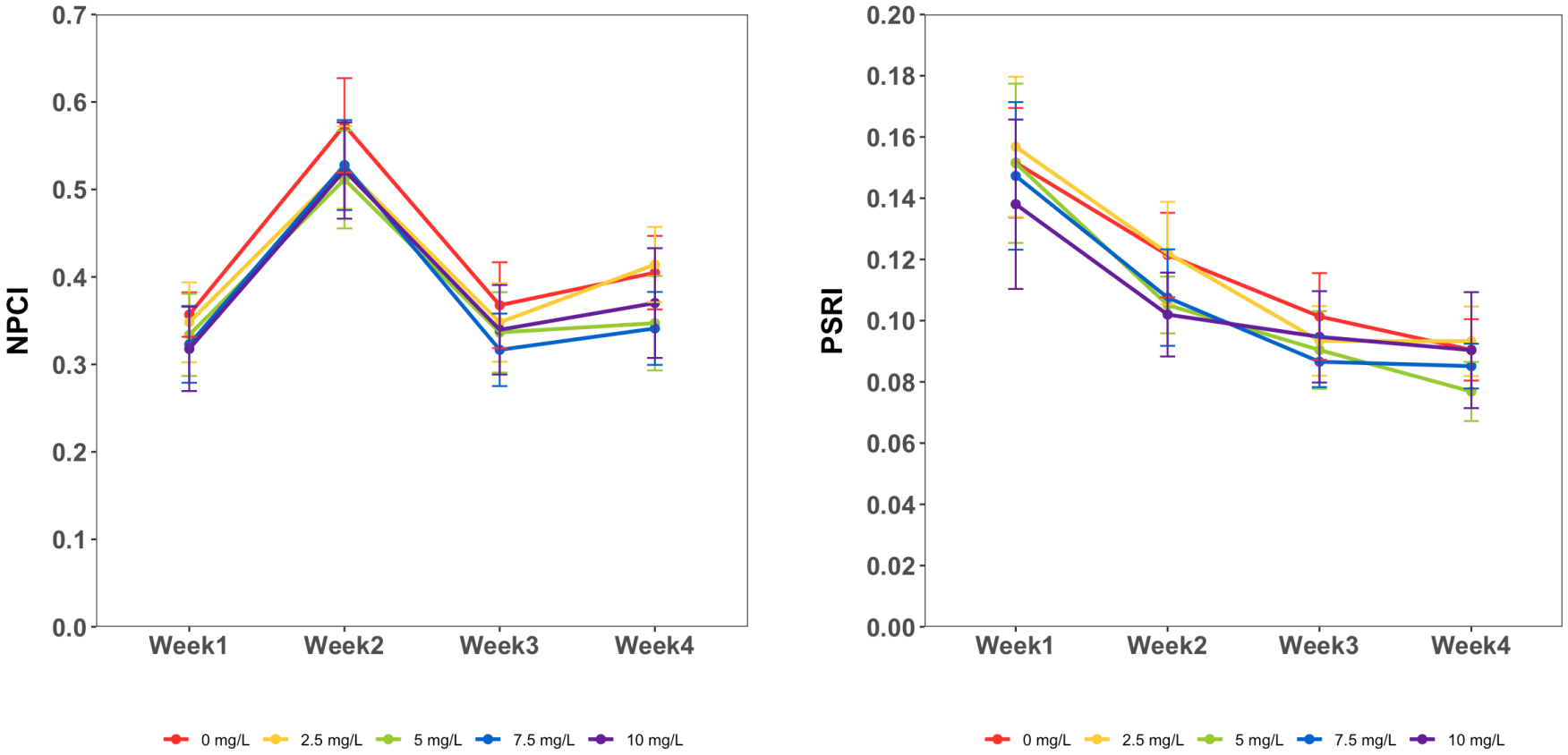


Figure 3.5 NPCI and PSRI of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

3.1.3.2 Water management and gas exchange

The efficiencies of water management and gas exchange were measured by LI-COR (LI-6800, LICOR Bioscience, Lincoln, UK). Although measurements were taken biweekly at weeks 2 and 4 of the growth stage, only the data from week 4 are presented. The parameters assessed included Water Use Efficiency (*WUE*), Intrinsic Water Use Efficiency (*iWUE*), and Stomatal Limitation (L_s). Further methodological details are provided in **Chapter 2.3.3**.

Water Use Efficiency (WUE)

Water Use Efficiency (*WUE*), which represents the ratio of carbon dioxide assimilation to water loss through transpiration, was measured at week 4 and is highlighted in Figure 3.6. Lettuces treated with B_{12} generally exhibited higher *WUE* compared to the control group, with the highest *WUE* observed in plants treated with B_{12} 5 mg / L. As light intensities increased from 200 to 1800 µmol m^2s^{-1}, *WUE* values of 3287, 5354, and 5971 µmol CO_2 / mmol H_2O were recorded, representing an overall increase of approximately 35 % compared to the control group. This improvement suggests that lettuces treated with B_{12} 5 mg / L may utilise water more efficiently by maintaining higher rates of carbon assimilation per unit of water transpired. However, it is also possible that increased *WUE* is a result of reduced stomatal conductance, which may limit water loss rather than actively enhancing photosynthetic capacity.

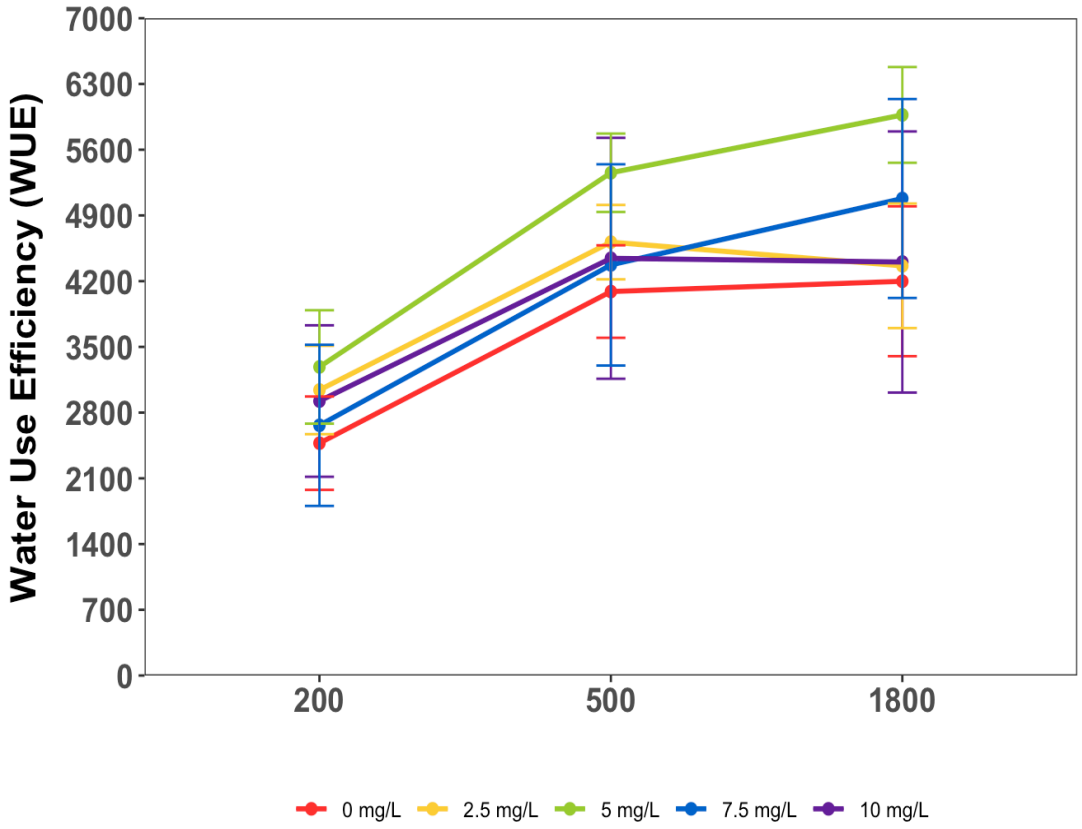


Figure 3.6 Water Use Efficiency (WUE) measured using LI-COR at Week 4.

Water use efficiency (WUE), calculated as net assimilation rate (A) divided by transpiration rate (E), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

Intrinsic Water Use Efficiency (iWUE)

As an indicator directly related to stomatal behaviour including opening and closing of stomata, the *iWUE* measurements from week 4 are presented which shown in Figure 3.7. At the lowest light intensity (200 µmol m^2s^{-1}), the control group (without B_{12} treatment) recorded the highest *iWUE* value (24.38 µmol CO_2 / mmol H_2O). However, as light intensity increased from 500 to 1800 µmol m^2s^{-1}, the B_{12} 5 mg / L treatment group exhibited the highest *iWUE* values, reaching 45.63 and 83.57 µmol CO_2 / mmol H_2O, respectively. This trend suggests that lettuces treated with B_{12} 5 mg / L may have more effective internal regulation of gas exchange under high light conditions, potentially maintain photosynthetic activity with relatively lower stomatal conductance and reduced water loss. Alternatively, the elevated *iWUE* could reflect a stomatal limitation on gas exchange, indicating that carbon assimilation was maintained despite reduced stomatal aperture.

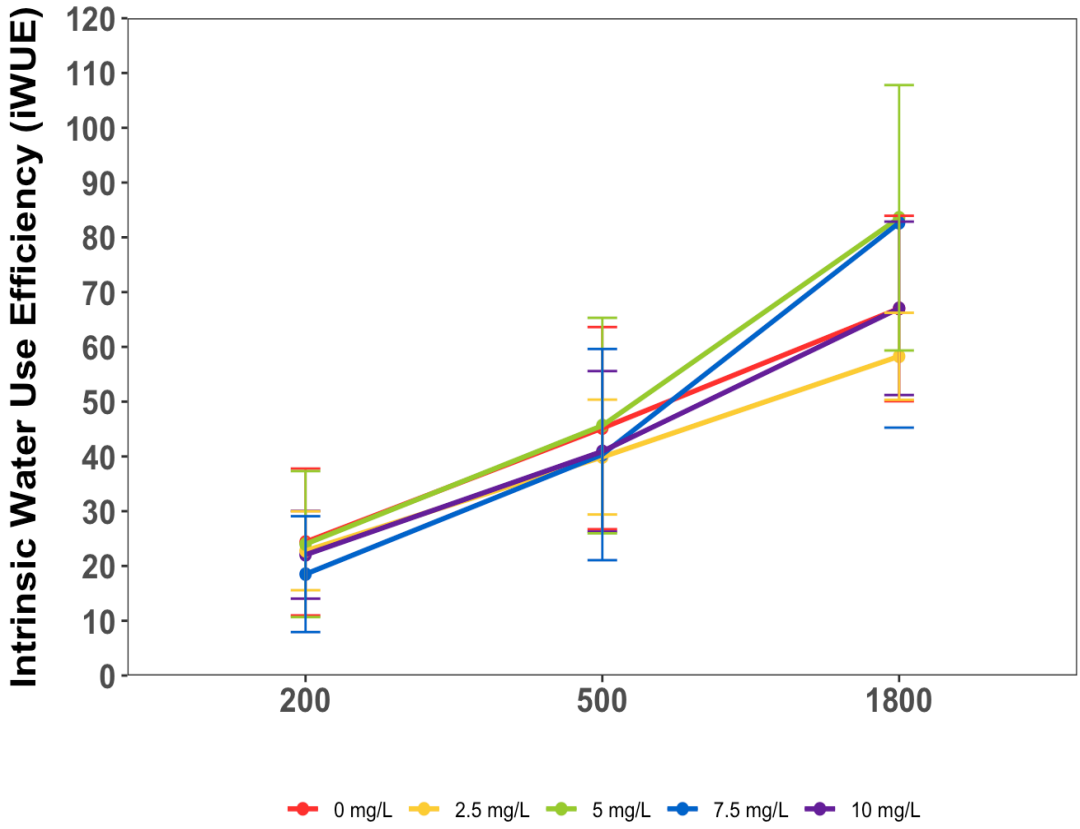


Figure 3.7 Intrinsic Water Use Efficiency (iWUE) measured using LI-COR at Week 4.

Intrinsic Water use efficiency (iWUE), calculated as net assimilation rate (A) divided by stomatal conductance to H_2O (gsw) , plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured line represents B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

Stomatal Limitation (L_s)

Stomatal limitation (L_s) measures the extent to which stomata limit CO_2 diffusion into the leaf. Low stomatal limitation indicates that the stomata are open, allowing CO_2 to enter freely. In contrast, high stomatal limitation reflects more closed stomata, restricting CO_2 intake and likely reducing photosynthesis. As shown in Figure 3.8, lettuces treated with B_{12} at 2.5 to 10 mg / L showed improved stomatal activity compared to the control group (without B_{12} treatment) at 200 µmol m^2s^{-1} light intensity. As light intensity increased from 500 and 1800 µmol m^2s^{-1}, the B_{12} 2.5 and 10 mg / L groups showed exhibited lower L_s values than the control group. Overall, B_{12} treatments demonstrated reduced stomatal limitation, suggesting more efficient stomatal function that may enhance photosynthetic performance by facilitating greater CO_2 diffusion into the leaves without restricting gas exchange.

The combined results from *WUE*, *iWUE*, and L_s measurements indicate that B_{12} treatment, particularly 5 mg / L, enhances stomatal efficiency and water use in lettuce, while B_{12} treatments at 2.5 and 10 mg / L demonstrate efficient stomatal activity. These findings suggest that B_{12} treatment may improve the balance between gas exchange and water conservation, potentially contributing enhanced photosynthetic efficiency and water use under varying light conditions, ranging from low light (200 µmol m^2s^{-1}) to near-saturating condition (1800 µmol m^2s^{-1}). However, it should be noted that statistical analyses were not performed on the LI-COR data due to the characteristics of the dataset. Therefore, these physiological trends, including water use efficiency and gas exchange related to photosynthetic activity, should be interpreted with caution as preliminary observations.

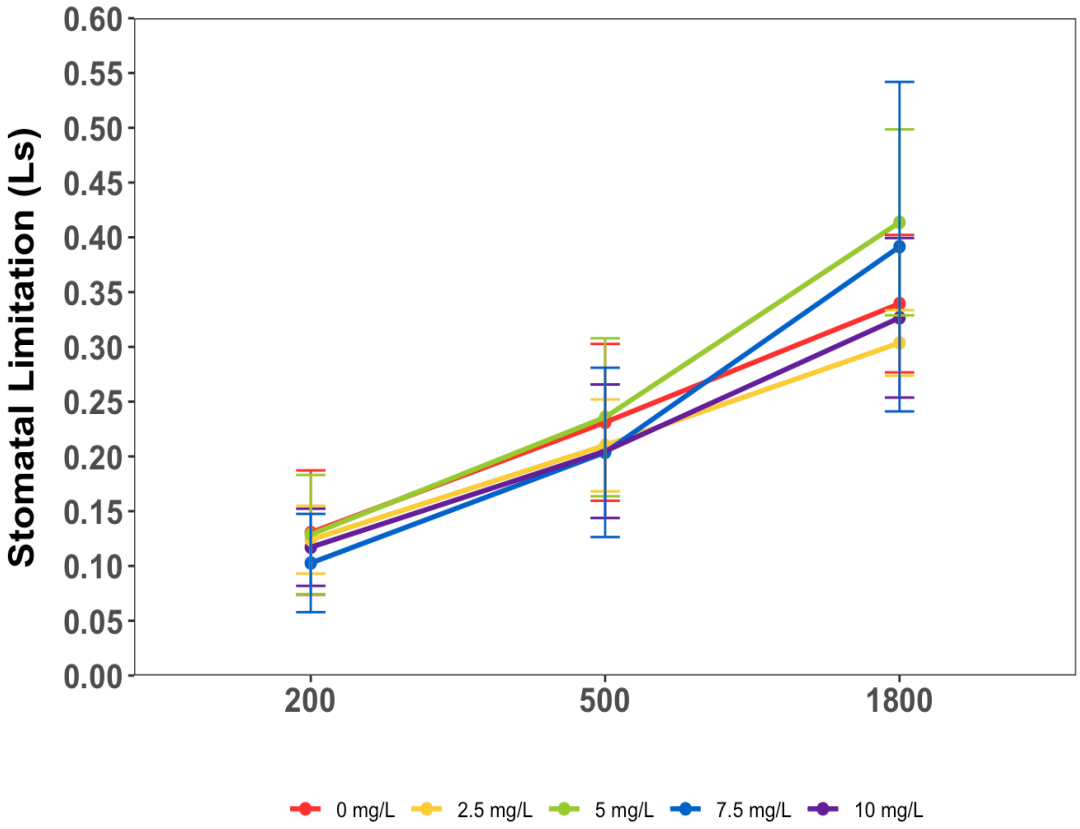


Figure 3.8 Stomatal Limitation (Ls) measured using LI-COR at Week 4.

Stomatal Limitation (L_s), calculated as 1- (intercellular CO2 concentration (Ci) divided by ambient CO_2 concentration (Ca), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured line represents B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

3.1.3.3 Relative chlorophyll measurements

Relative chlorophyll measurements were conducted using SPAD-502Plus (Konica Minolta Ltd., Osaka, Japan) on week 2 and week 4. The amount of chlorophyll fluorescence was calculated by the average SPAD values for eight individual lettuces per treatments. Further details are presented in **Chapter 2.3.4**. SPAD values correlate with chlorophyll content, which is crucial for photosynthesis. Higher SPAD values generally indicates healthier plants with more chlorophyll leading to more efficiency photosynthesis and potentially better growth and yield. Figure 3.9 shows the SPAD readings taken at week 2 of growth stage. Compared to the control group (without B_{12} treatment), lettuces treated with B_{12} 5, 7.5, and 10 mg / L displayed higher SPAD values. The highest reading value was observed in B_{12} 10 mg / L treatment (21.7 ± 2.1). However, there were no statistically significant differences in SPAD values between the B_{12} concentrations ($p > 0.05$). Although the differences were not statistically significant, the trend of higher SPAD readings in B_{12} -treated groups may suggest a potential improvement in chlorophyll content and photosynthetic capacity, though further confirmation would be required.

SPAD measurements taken at week 4 were used as an indicator of final harvest quality. The highest SPAD value (26.4 ± 2.0) was recorded in lettuces treated with B_{12} 10 mg / L, which was statistically significantly higher ($p < 0.05$) than those in the control group and 2.5 mg / L groups. Specifically, SPAD values in the B_{12} 10 mg / L treatment increased by approximately 13 % and 16 % compared to the control (without B_{12} treatment), and B_{12} 2.5 mg / L, respectively. Although lettuces treated with B12 at 5 mg / L (25.8 ± 2.0) and 7.5 mg / L (24.5 ± 1.3) showed numerically higher SPAD values compared to the control group, these differences were not statistically significant ($p > 0.05$), as indicated by overlapping statistical groupings. These results suggest that B_{12} application at concentration of 5 mg / L and above may enhance chlorophyll content in lettuce, potentially improving photosynthetic capacity. However, the relatively small variation in SPAD values between 5 and 7.5 mg / L B_{12} treatments indicates a potential threshold effect rather than a proportional increase with B_{12} concentrations.

Overall, this study found that B_{12} treatment may contribute to increased chlorophyll content in lettuce leaf tissues compared to the untreated control. Notably, the B_{12} 10 mg / L treatment significantly enhanced chlorophyll content, whereas the 2.5 mg / L treatment resulted in the lowest SPAD values among the B_{12} groups. These

findings indicated that relatively higher B_{12} concentration (5, 7.5, and 10 mg / L) may promote chlorophyll synthesis or retention in lettuces. This suggests a potential role of B_{12} in improving photosynthetic capacity and overall plant health. Since the SPAD measurement were taken at the final harvest stage (week 4) and are associated with harvest quality, the results imply that lettuces treated with B_{12}, except the 2.5 mg / L, may exhibit improved harvest quality due to their higher chlorophyll content compared to the control.

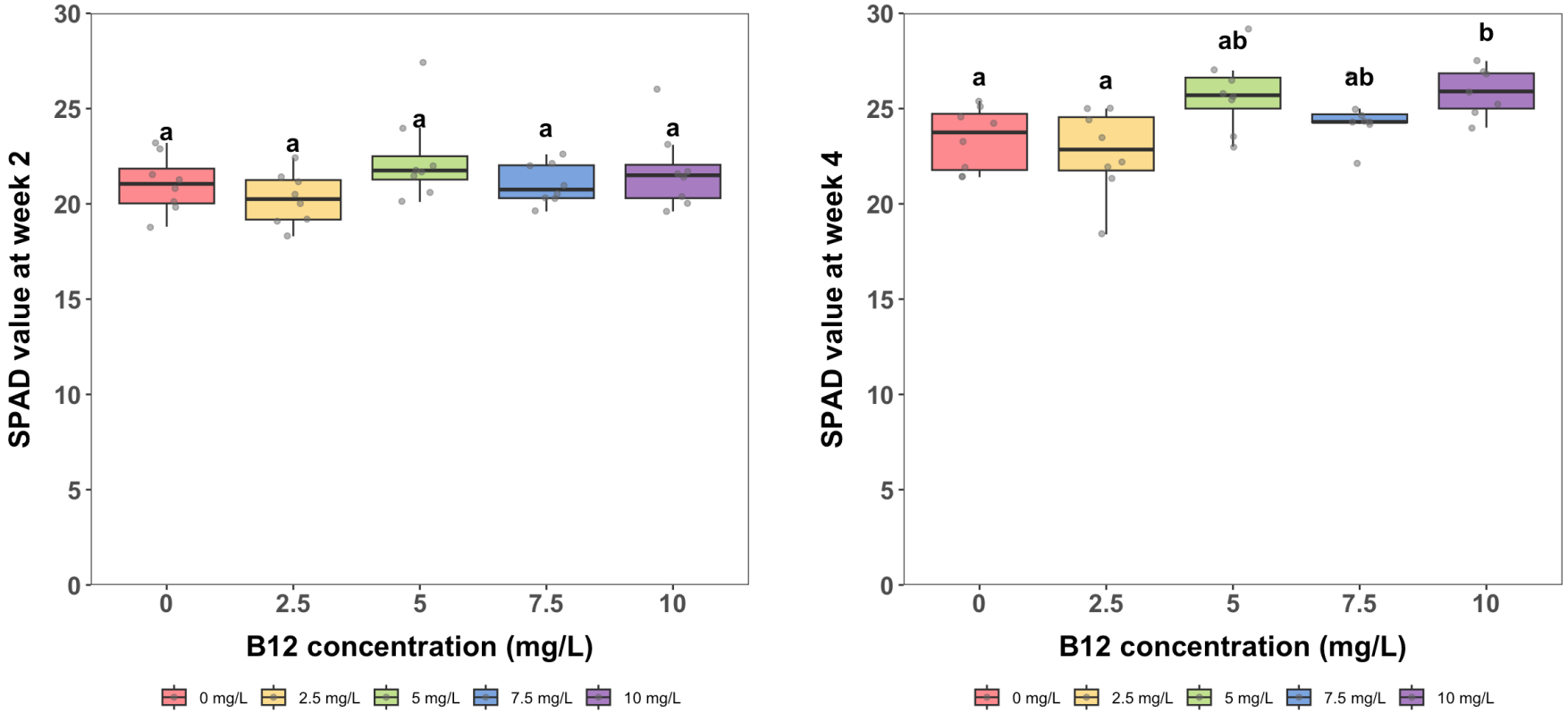


Figure 3.9 Relative chlorophyll content measured by SPAD meter at Week 2 and Week 4.

Coloured box plots indicate B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as shown in the legend. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups. (A) SPAD value at week 2, (B)SPAD value at week 4.

3.1.3.4 Harvest quality: fresh weight

Fresh weight (g) was measured lettuce leaves and roots separately using a four-digit analytical balance. The whole fresh weight was calculated by the sum of leaves and roots tissues. The measurements were conducted on two harvest dates including week 2 and week 4 after transplanting. Further details are described in **Chapter 2.3.5**. Fresh weight measurements of leaf and root tissues at week 2 and week 4 are shown in Figure 3.10, with week 2 data displayed in the upper graph and week 4 data in the lower graph. Although lettuces treated with B_{12} tended to have higher fresh weights compared to the control group, these differences were not statistically significant ($p > 0.05$). Trends indicated that higher B_{12} concentration (especially 10 mg / L) were associated with increased biomass in both leaves and roots, but variability among samples limits the strength of these conclusions. Fresh weight data at week 4, representing final harvest quality, are shown in the lower graph of Figure 3.10. Compared to the control group without B_{12} treatment, lettuces treated with B_{12} generally showed higher total fresh weight, except for the 2.5 mg / L treatment. Lettuces treated with B_{12} 5 mg / L had the highest value of total fresh weight (266.40 ± 28.41 g) and fresh leaf weight (240.14 ± 25.59 g). Interestingly, the highest fresh root weight (26.65 ± 4.18 g) was recorded in lettuces treated with B_{12} 10 mg / L, followed by the 5 mg / L treatment. However, these differences were not statistically significant ($p > 0.05$).

Overall, the present study found that B_{12} treatment contributed to an increase in the final harvest quality of lettuce compared to the control group without B_{12} treatment. Particularly, B_{12} treatments at 5, 7.5, and 10 mg / L increased fresh weights of lettuce leaf and root tissues, while lettuces treated with B_{12} 2.5 mg / L had the lowest fresh weights. These results indicate that relatively higher concentration of B_{12} (5, 7.5, and 10 mg / L) may enhance cells elongation and consequently increase harvest weights, suggesting a potential role of B_{12} in improving overall plant health and development. However, these differences were not statistically significant, which could be attributed to biological variability among individual plants or a limited sample size. Additionally, since plants do not inherently require B_{12} for their growth and development, the response to B_{12} application may be subtle or inconsistent. This is further supported by the morphological and physiological measurement conducted using PlantEye, which showed no substantial structural differences between control and B_{12} -treated groups.

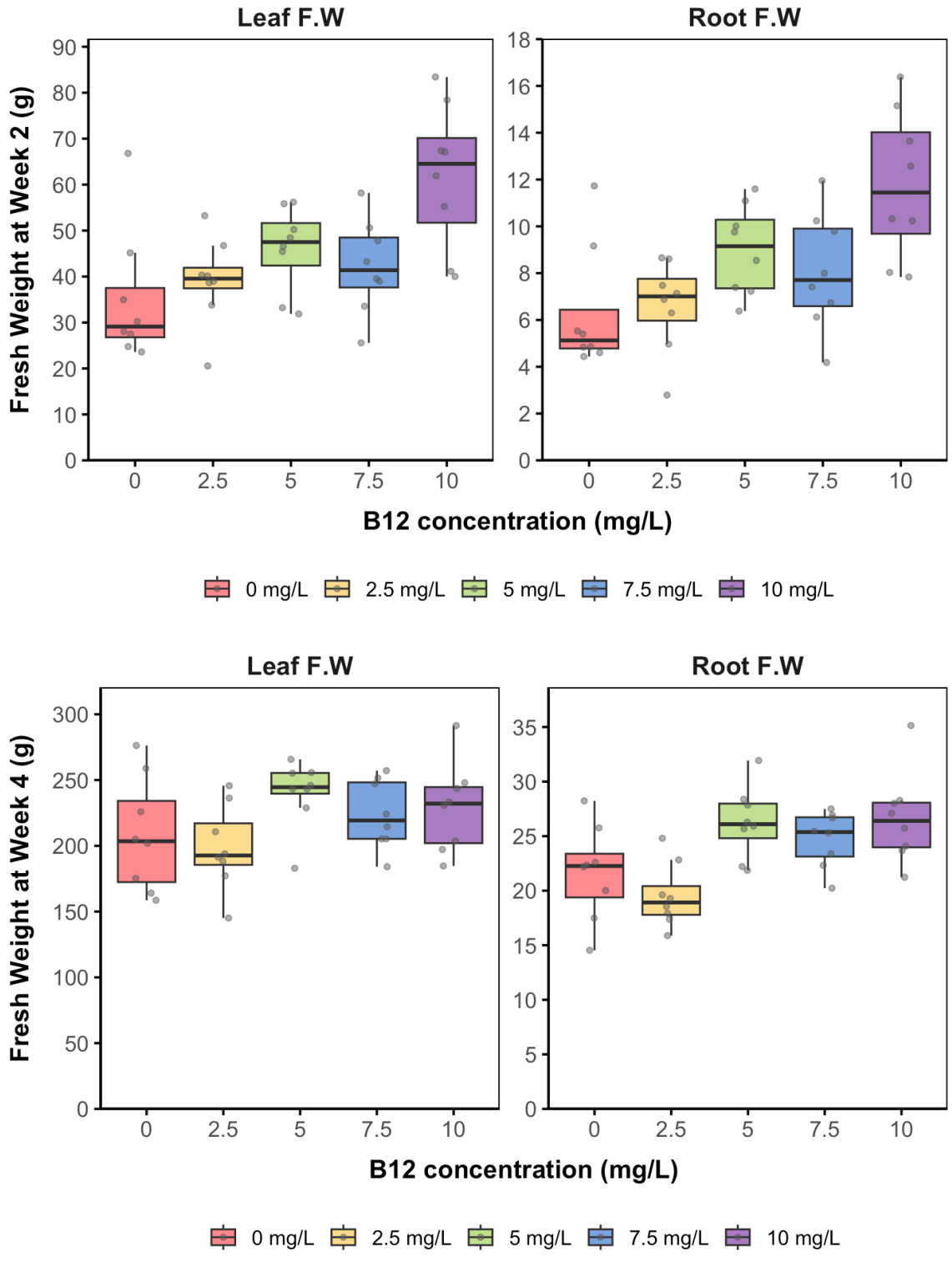


Figure 3.10 Harvest quality – Fresh weight (g) of lettuces at week 2 and week 4.

Coloured box plots indicate total, leaf, and root fresh weight within each B_{12} treatment group (0, 2.5, 5, 7.5, and 10 mg / L). (A)Fresh weight at week 2. (B)Fresh weight at week 4. No statistically significant differences were observed between treatments ($p > 0.05$).

3.1.3.5 Elemental analysis: B_{12} quantification

As an elemental analysis, ICP-MS was conducted to quantify the exact amount of vitamin B_{12} uptake, translocate and accumulated in lettuce tissues by leaf application of B_{12}. The ICP-MS outcomes are presented as milligram of B_{12} in the gram of dried lettuce tissues including leaf and root tissues (mg / g). The details of the ICP-MS analysis are described in **Chapter 2.3.1**. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**. Cobalt (Co) content, determined by ICP-MS, was used to estimate vitamin B_{12} concentration in plant tissues. Given that each B_{12} molecule contained one cobalt atom, the molecular weight ratio of vitamin B_{12} to cobalt (1,355.37 / 58.93) was applied to convert Co values into corresponding B_{12} levels. As the hydroponic nutrient solution did not include cobalt other than through B_{12} supplementation, it is assumed that all measured cobalt originated from the applied B_{12}.

Figure 3.11 depicts the quantification of B12 using ICP-MS analysis from the harvested samples at week 2 and week 4 including mean and standard deviation. The B_{12} content in leaf tissues harvested on week 2 showing a positive correlation with increasing B_{12} treatment levels. In both leaf and root tissues, lettuces treated with B_{12} 10 mg / L contained the highest value of B_{12} compared to the control group

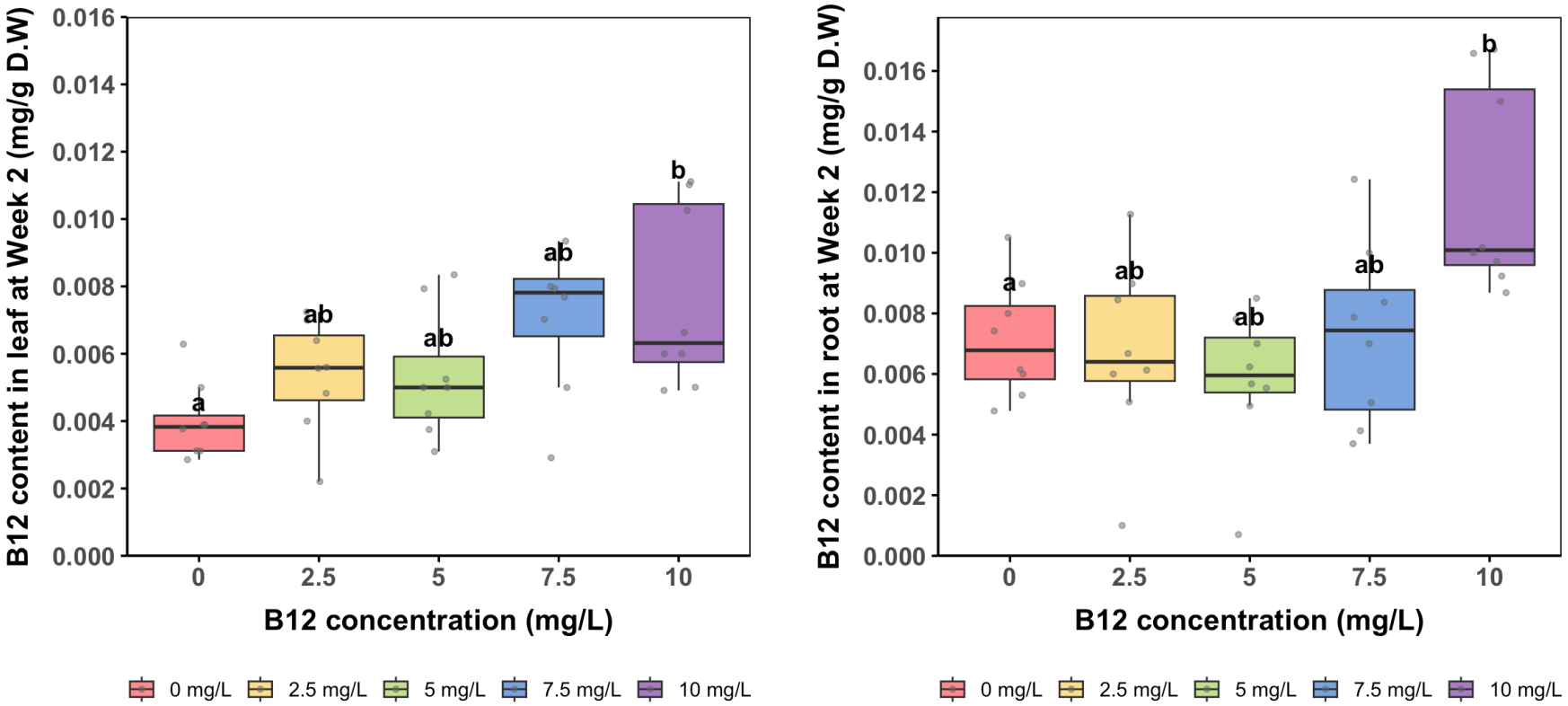


Figure 3.11 Quantification of vitamin B_{12} in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 2.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups. **(A)** B_{12} content in leaf tissues at week 2. **(B)** B_{12} content in root tissues at week 2.

As a measure of final harvest quality, lettuces harvested at week 4 were highlighted. Both leaf and root tissues exhibited a positive correlation with increasing B_{12} concentration. As shown in Figure 3.12, B_{12} quantification in leaf tissues was particularly notable. The highest B_{12} concentration was recorded in the group treated with B_{12} 10 mg / L (0.010 ± 0.003 mg / g), which was statistically significantly higher than the lettuces treated with the control (without B_{12} treatment). Specifically, the B_{12} content in the 10 mg / L group was 130 % higher than the control (B_{12} 0 mg / L, 0.004 ± 0.004 mg / g), and 118 % higher than the 2.5 mg / L treatment. These differences were statistically supported, as indicated by the different letter groupings in the figure ($p < 0.05$). The root tissues analysis evaluated the B_{12} translocation following leaf application of B_{12}. The highest B_{12} concentration was quantified in the group treated with B_{12} 10 mg / L (0.009 ± 0.003 mg / g). This difference was statistically significant, as indicated by distinct letter groups in the figure ($p < 0.05$).

Overall, incremental increases in B_{12} concentrations were observed in both leaf and root tissues, indicating enhanced B_{12} uptake, translocation, and accumulation as the applied B_{12} concentration increased. No evidence of restriction in B_{12} uptake or translocation was observed in lettuce tissues, even at higher B_{12} application level such as 10 mg / L. The B_{12} found in the control group without B_{12} application can be explained by following reason: (1) ICP-MS has the lowest detection limits compared to ICP-OES which is also known as ICP-AES. For instance, ICP-MS can quantify cobalt approximately 1 to 5000 nmol/L interpretated as 0.059 ppb as the lowest detectable concentration from the sample analysis [167]. Considered the sensitivity of ICP-MS readings, it would be possible to detect the traceable amount of B_{12} contained in lettuce tissues; (2) Cobalt can detect in lettuce grown in soil naturally, as it can be contained in soils or bacteria. Although the study was conducted hydroponic cultivation, the lettuce seed was sown in soil medium for seedling phase. This can address the potential reasons behind the samples harvested on week 2 contain higher level of cobalt in the control group compared to the lettuces harvested on week 4; (3) Cross contaminations can be also potential reasons which can be occurred during the foliar spray application of B_{12} to different groups in the growth room. However, the propagators for control groups were placed different benches. The same water resources were supplied for hydroponic tank which could be contained cobalt naturally. Furthermore, during the sample homogenisation, the contamination can be happened, although the control samples were prepared prior to the samples from B_{12} treated group.

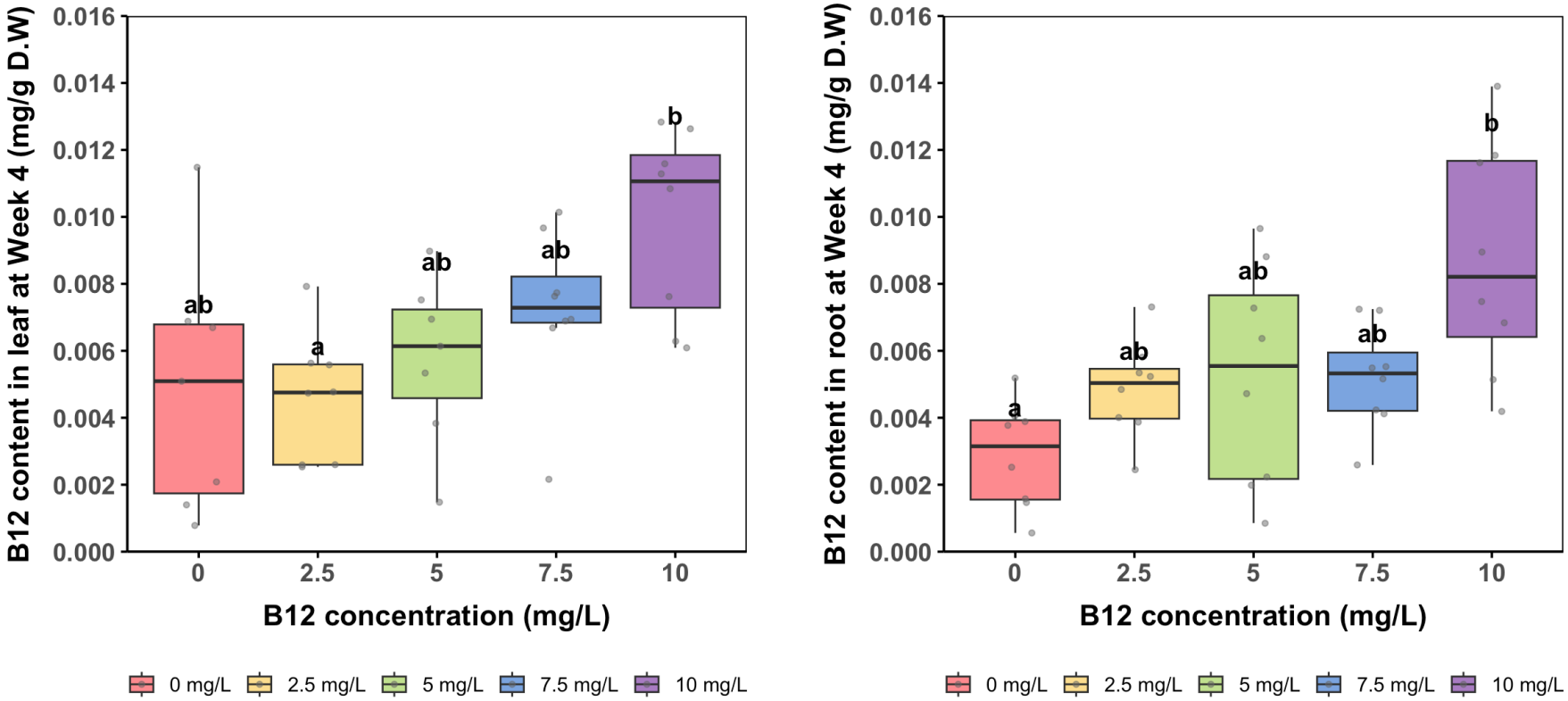


Figure 3.12 Quantification of vitamin B_{12} in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups. **(A)** B_{12} content in leaf tissues at week 4. **(B)** B_{12} content in root tissues at week 4.

3.1.3.6 Secondary metabolites analysis

To assess the effect of leaf-treated B_{12} application on secondary metabolites in lettuce, total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) were measured. The detailed methodology for sample preparation and analysis is described in **Chapter 2.4**. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**.

Total Phenolic Content (TPC)

Total phenolic content (TPC) in lettuce leaves was determined using Folin-Ciocâlteu reagent with the gallic acid equivalence method (GAE). Phenolic compounds play an important role in plant defence mechanisms by contributing to stress resistance and potentially prolonging postharvest shelf life [168]. The TPC results, described as milligram of gallic acid by gram of fresh weight (mg GAE / g) of fresh lettuce leaves, are presented in Figure 3.13.

Lettuces treated with B_{12} 5 mg / L treatment had the highest TPC value (0.460 ± 0.075 mg GAE / g), which was approximately 6 % higher than the control group without B_{12} treatment. This may suggest that B_{12} 5 mg / L treatment could be associated with increased phenolic compound accumulation, which in turn may contribute to improved oxidative stress tolerance. This is broadly consistent with the lower PSRI (plant senescence reflectance index) values measured by PlantEye, which can indicate delayed senescence or reduced stress signals. The lowest TPC value (0.416 ± 0.053 mg GAE / g) was observed in lettuces treated with B_{12} 7.5 mg / L. However, there was no statistical significance between the B_{12} treatment groups ($p > 0.05$), suggesting that the variations observed may be attributable to biological variability rather than a consistent treatment effect.

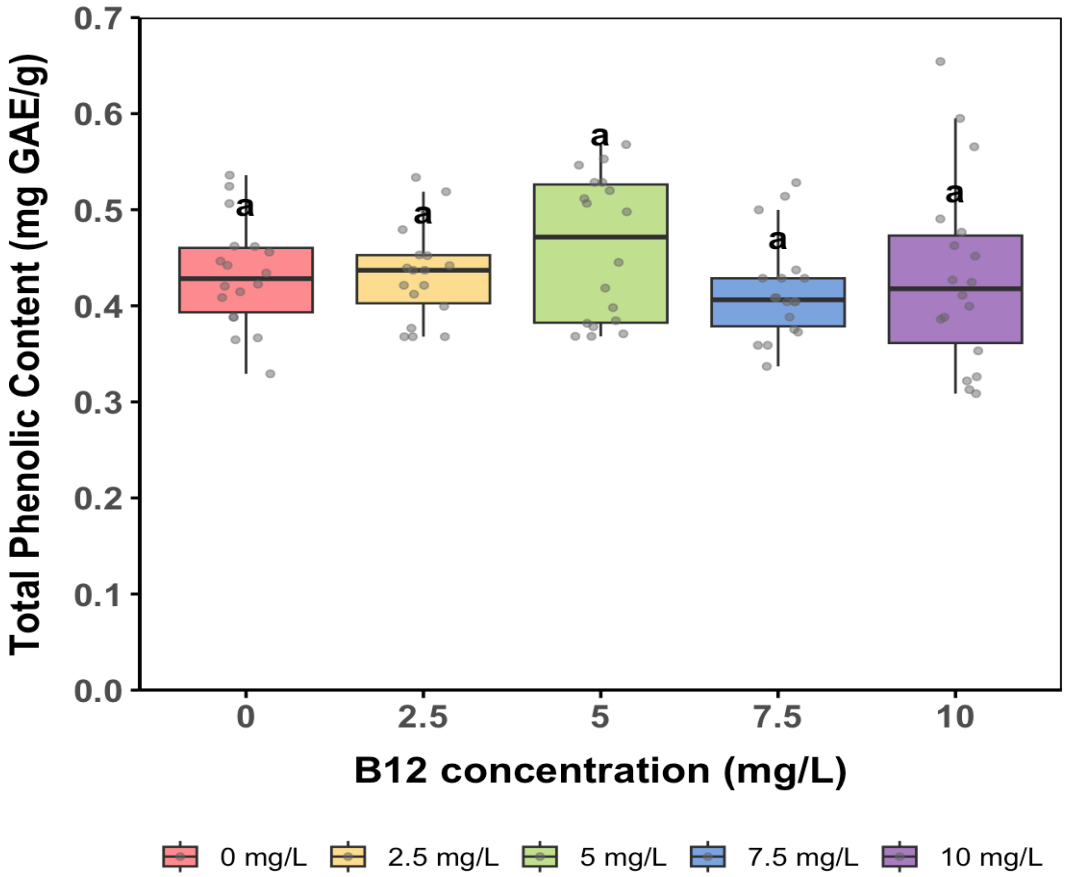


Figure 3.13 Total Phenolic Content (mg GAE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p > 0.05$). No statistically significant differences were observed between treatments ($p > 0.05$).

Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined by an aluminium chloride colorimetric assay, following the Rutin equivalence (RE) method [164,169]. Flavonoids play essential roles in plant physiology, including regulation of cell growth, protection against biotic and abiotic stress, contribution to pigment (e.g., chlorophyll and carotenoids), and involvement in plant defence mechanism [170]. The TFC results are described as milligram of Rutin by gram (mg RE / g) of fresh lettuce leaves. As shown in Figure 3.14, the control group (without B_{12} treatment) exhibited the highest TFC values (0.123 ± 0.022 mg RE / g). The TFC of the control group was statistically significantly higher ($p < 0.05$) than those of all B_{12} -treated groups (2.5 to 10 mg / L).

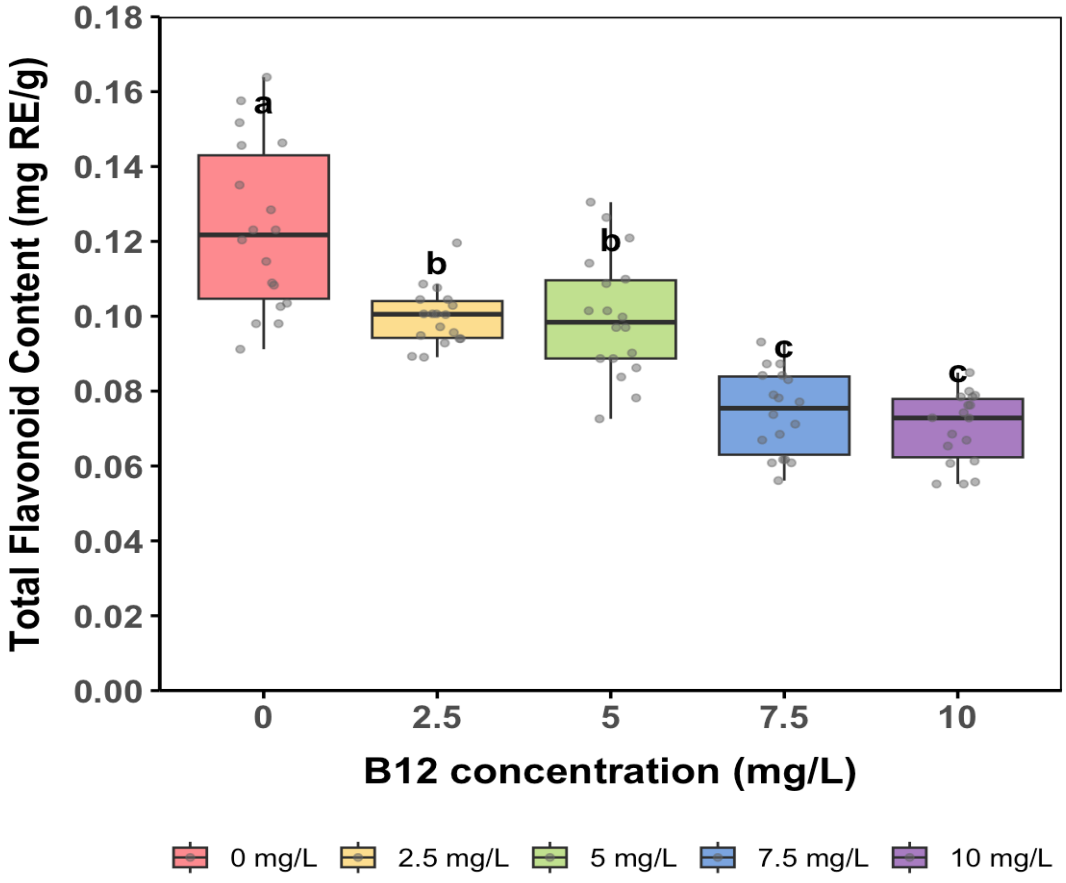


Figure 3.14 Total Flavonoid Content (mg RE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Total Antioxidant Activity (TAA)

Total antioxidant activity (TAA) was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay [164,169]. Trolox® equivalent antioxidant capacity (TEAC) was used as a standard reference to quantify antioxidant activity, expressed as milligrams of TEAC per gram of fresh weight of lettuce (mg TEAC / g). Phenolic compounds are known to contribute to antioxidant activity by providing free radical scavenging capacity [171]. As shown in Figure 3.15**,** the highest TAA was recorded in the B_{12} 5 mg / L treatment group (4.545 ± 0.179 mg TEAC / g), which was approximately 6 % higher than the control group (4.277 ± 0.193 mg TEAC / g). However, statistical analysis revealed no significant differences in TAA between treatment groups, as indicated by the shared letter groupings in the figure (p > 0.05). This suggests that although a numerical increase was observed at 5 mg / L, it was not statistically supported.

Overall, B_{12} treatment via leaf application affected TPC, TFC, and TAA in lettuce tissues. The TPC showed a modest response, with the highest content observed at 5 mg / L, although statistical differences among treatments were not substantial. In contrast, TFC significantly decreased as B_{12} concentration increased, with the control group showing the highest flavonoid content. For TAA, a slight numerical increase was observed at B_{12} 5 mg / L compared to the control group, but the differences were not statistically significant across treatments, as indicated by the overlapping letter groups. These results suggest that while B_{12} application may induce some changes in secondary metabolites, particularly phenolic compounds, its effect on antioxidant activity appears minimal and not dependent on B_{12} concentration. The reduction in flavonoid content with increasing B_{12} concentration may suggest a shift in the lettuce's secondary metabolites profile under higher B_{12} application.

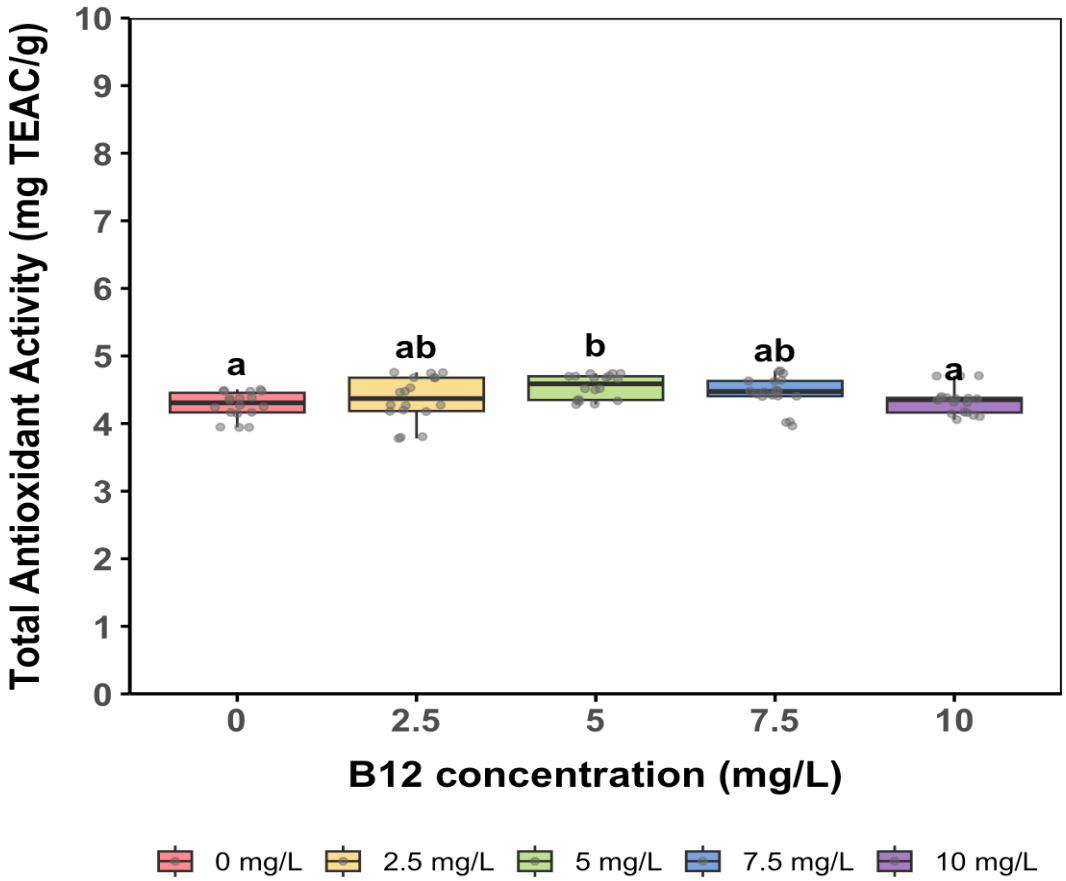


Figure 3.15 Total Antioxidant Activity (mg TEAC / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

3.2 Vitamin B_{12} fortification - Root application

3.2.1 Material and method

The materials and growing conditions are described in **Chapter 2.2.** The present experiment aimed to determine the effect of B_{12} the root application along with different concentrations of B_{12} including the control (0 mg / L), 2.5, 5, 7.5, and 10 mg / L. Vitamin B_{12} was weighed for the application into total volume of 10 litres of each propagator. B_{12} was dissolved with type I water for serial dilutions using 50 mL conical centrifuge tubes which was foiled to block light until the application. The treatments were applied once a day for four weeks throughout the growing period into the root zone in hydroponic nutrient tanks. For harvest sample collection, the entire lettuces were washed multiple times with running water to minimise the residues from the treatment application.

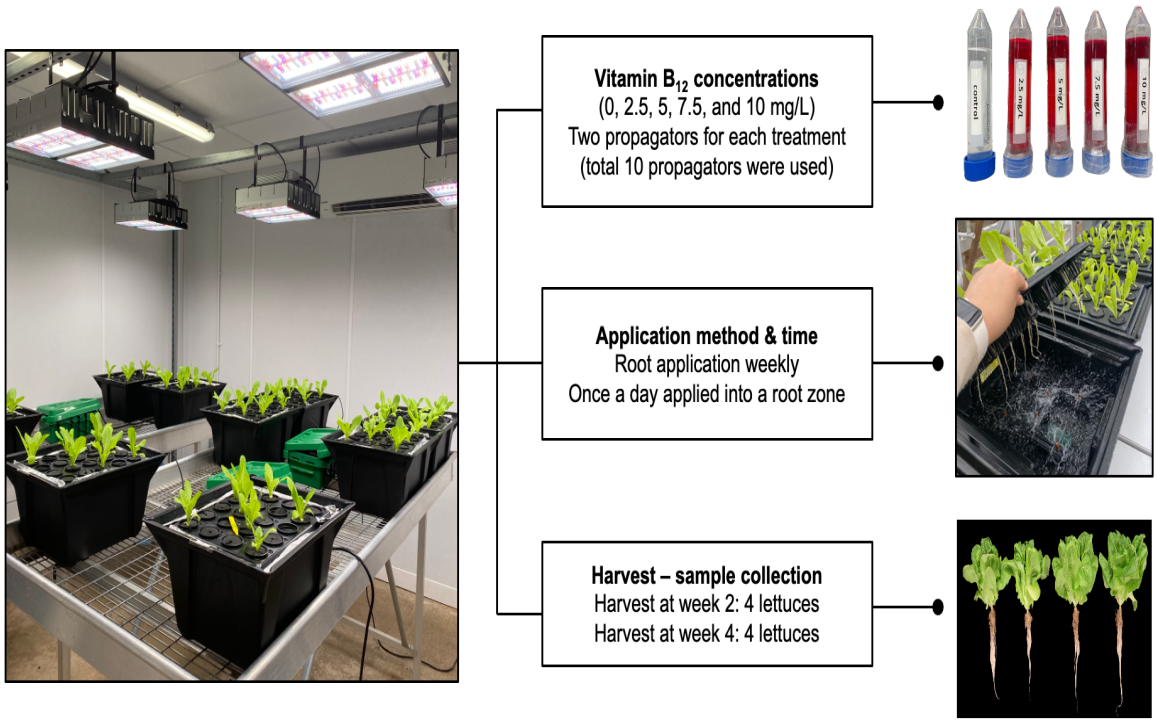


Figure 3.16 representation of growing condition for B_{12} fortification via root application.

Vitamin B_{12} concentrations (0, 2.5, 7.5, and 10 mg / L); Root application method (using foliar spray method), and harvest details.

3.2.2 Data collection and statistical analysis

This experiment has eight biological replicates per different concentration vitamin B_{12} treatment. Five different treatments of B_{12} were applied for two propagators each and total 10 propagators were used for this experiment which shown in Figure 3.16. A portion of the lettuces were harvested on week 2 for sample measurements and week 4 to quantify B_{12} uptake and translocate in lettuce tissues via elemental analysis.

The data was obtained via morphological, physiological, quality measurement, and elemental analysis in the same way conducted previous experiment and it was described in **Chapter 3.1.** For statistical analysis, the data was analysed using PERMANOVA. If the results of PERMANOVA presented significance differences, multiple permutational comparison test (no. permutation = 9,999) was conducted to present statistical significance between the group.

3.2.3 Results

This section investigates the effect of B_{12} fortification via root application on hydroponically grown lettuce. Unlike leaf application, root application introduces B_{12} via the nutrient solution in hydroponic/aeroponic tanks, allowing uptake through the root systems and potential translocation to the edible leaf tissues. This method may offer a more systemic pathway for B_{12} absorption and distribution throughout the plant. The aim of this chapter is to determine the effect of B_{12} application on the growth, physiology, and nutritional quality of hydroponically grown lettuce. The objectives are: (1) to quantify the uptake, translation, and accumulation of root applied B_{12} in lettuce leaves and root tissues using ICP-MS; and (2) to assess the effects of varying B_{12} concentrations (0, 2.5, 5,7.5, 10 mg / L) on morphological traits such as leaf area, biomass, physiological parameters (photosynthetic gas exchange, water management, and relative chlorophyll content, and nutritional quality including B_{12} content, secondary metabolites.

3.2.3.1 Growth and development measurements

Lettuce growth and morphological parameters, presented as mean ± standard deviation, were measured using the PlantEye F600 system. Further details of the methods and parameters are described in **Chapter 2.3.2**. Statistical analyses were conducted to evaluate the significance among the different B_{12} treatment groups using PERMANOVA, followed by pairwise multiple permutational comparisons with 9,999 permutations. Figure 3.17 presents the weekly measurements of plant height (cm) and leaf area (cm^2), illustrating the morphological development of lettuce over the growth period. The Leaf area parameters showed an overall increase throughout the weeks, with comparable variation observed between week 3 and week 4. However, no statistically significant differences were observed among the different B_{12} treatment concentrations based on permutational pairwise tests using 9,999 permutations.

At the final harvest (week 4), the B_{12} 5 mg / L treatment resulted in the greatest mean plant height (17.65 ± 0.66 cm), followed closely by the B_{12} 2.5 mg / L treatment group (17.45 ± 1.23 cm), shown in Figure 3.17. Regarding leaf area (cm^2), the largest mean value was observed in lettuces treated with B_{12} 2.5 mg / L recorded the largest leaf area (984 ± 112 cm^2), followed by the control group (905 ± 86 cm^2). However, no statistically significant differences were observed among the B_{12} treatment groups for either parameter.

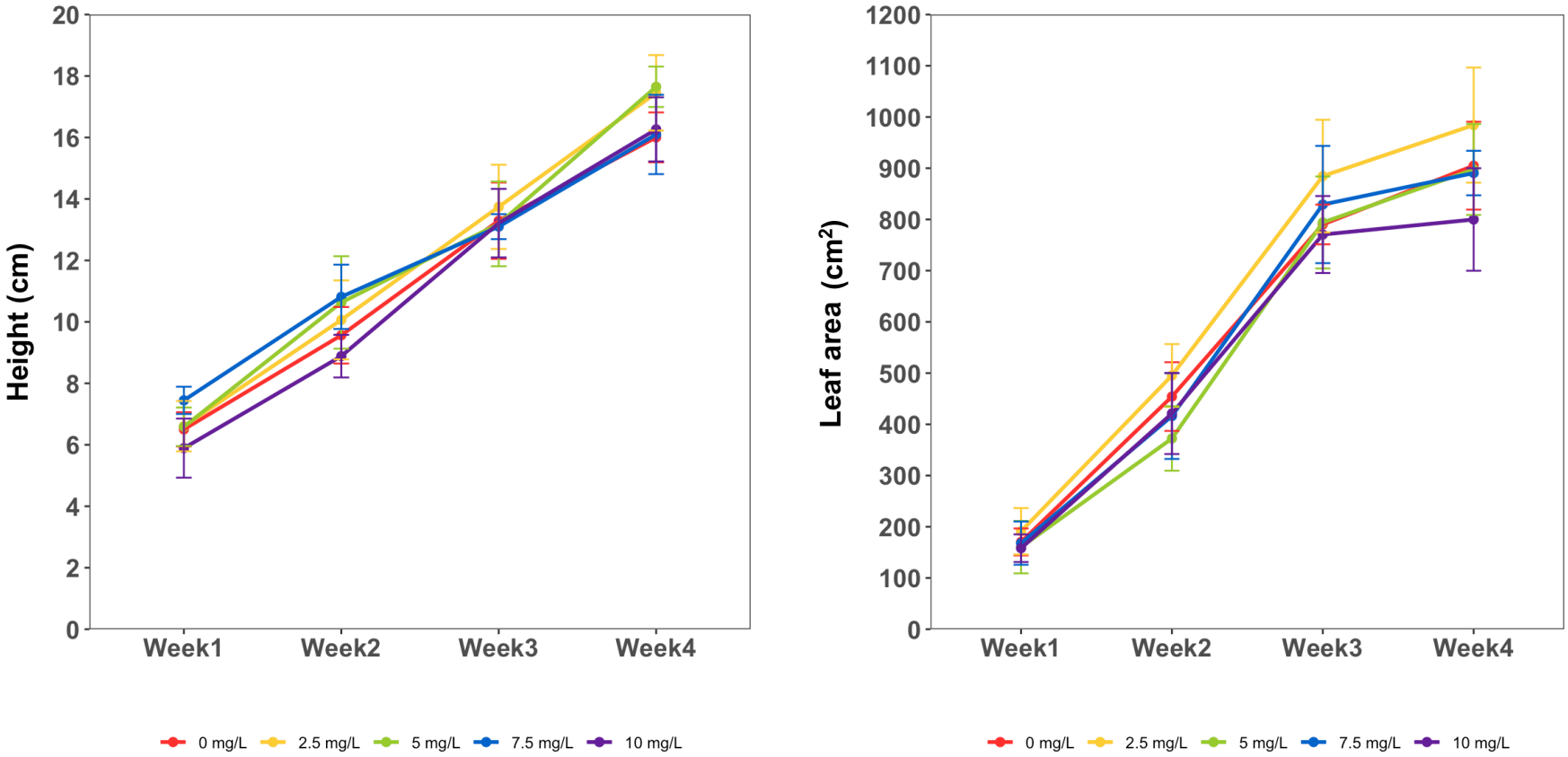


Figure 3.17 Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

As shown in Figure 3.18, digital biomass increased steadily across all B_{12} treatment groups through the growth period. Notable variations in digital biomass were observed between week 3 and week 4, while hue values remained relatively stable over time. The highest mean digital biomass value was recorded in the B_{12} 2.5 mg / L group (17,158 ± 2,065 cm^3), while the lowest was observed in the B_{12} 10 mg / L (12,973 ± 1,446 cm^3). Overall, lettuces treated with the B_{12} 2.5 mg / L group exhibited the most pronounced growth and morphological development compared to the control group. However, no statistically significant differences were found among the treatment groups in plant height, leaf area, and digital biomass (p > 0.05).

Hue measurements, ranging from 0 to 360 º, represent colour and can serve as an indicator of the morphological health of lettuce. Hue values between 90 º to 140 º correspond to green hues, typically associated with healthy plants. Throughout the growth period, hue values across all treatment groups remained within this healthy green range. The highest mean hue value was recorded in the B_{12} 2.5 mg / L treatment (101.48 ± 1.86 º), while the lowest was observed in the B_{12} 10 mg / L treatment (100.33 ± 1. 61 º). However, these differences were not statistically significant (p > 0.05), indicating the B_{12} application did not have a measurable impact on plant morphological development.

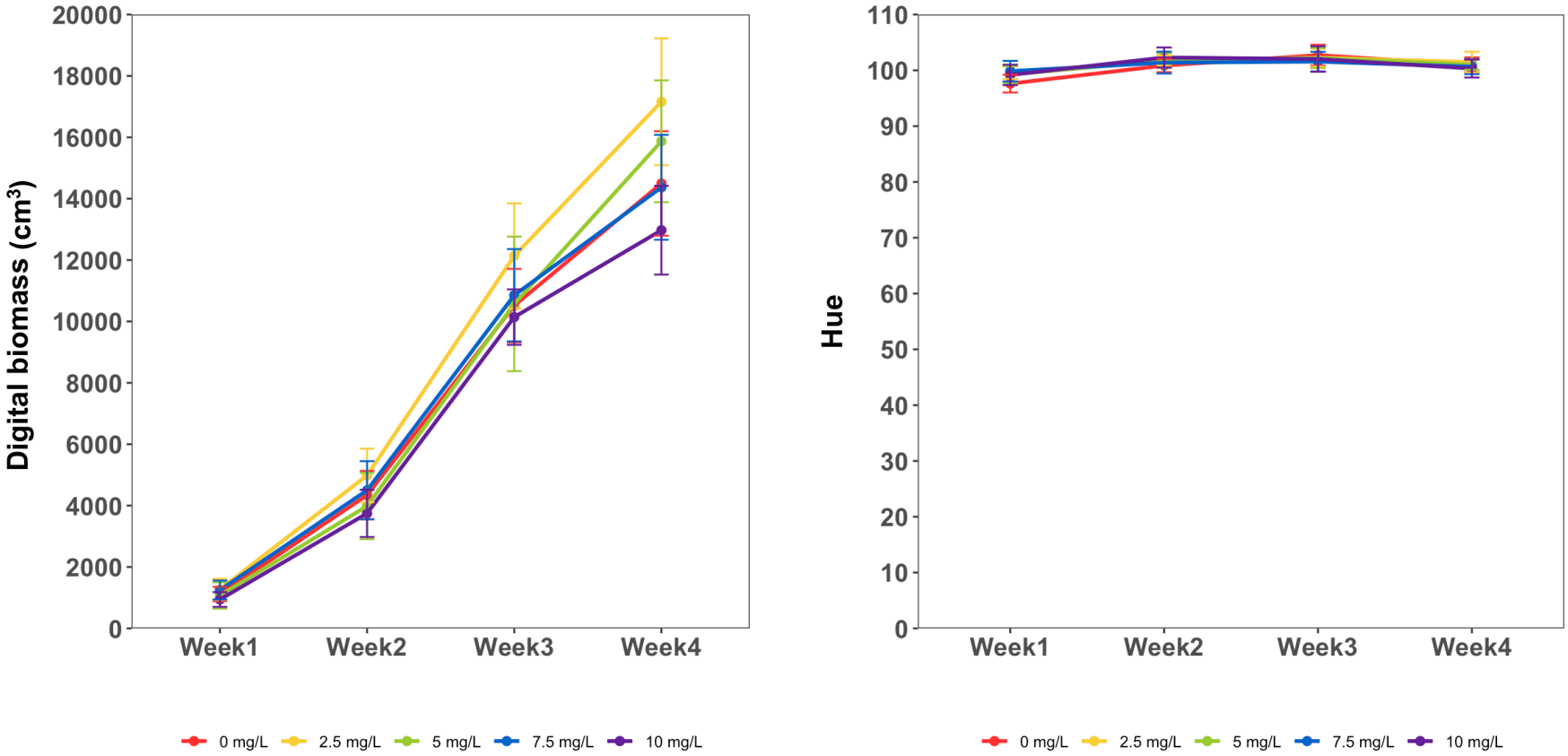


Figure 3.18 Digital biomass (cm^3) and Hue (°) of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

As shown in Figure 3.19**,** no notable variations were observed in either greenness or the Normalised Vegetation Index (NDVI) across B_{12} treatment groups. Greenness, ranging from -1 to 1, reflects the green leaf index, with higher values indicating greater greenness typically associated with healthy leaves. At the final harvest (week 4), the B_{12} 2.5 mg / L treatment recorded the highest mean greenness value (0.46 ± 0.00), closely followed by the control group without B_{12} treatment (0.46 ± 01). However, there were no statistically significant differences among B_{12} treatments for greenness ($p > 0.05$), indicating that B_{12} treatments did not have a significant effect on leaf colour or pigmentation.

NDVI is an indicator of vegetation health and relates to the interception of photosynthetically active radiation. Healthy vegetation generally exhibits NDVI values ranging from 0.6 to 0.9. Across all B_{12} treatment groups, NDVI values remained within this healthy range throughout the growth period. At the final harvest, B_{12} 2.5 mg / L treatment group recorded the highest mean NDVI value (0.66 ± 0.01), followed closely by the B_{12} 5 mg. L treatment (0.66 ± 0.01). However, there were no statistically significant differences among the treatment groups ($p > 0.05$), indicating that B_{12} application did not significantly affect vegetation health as measured by NDVI.

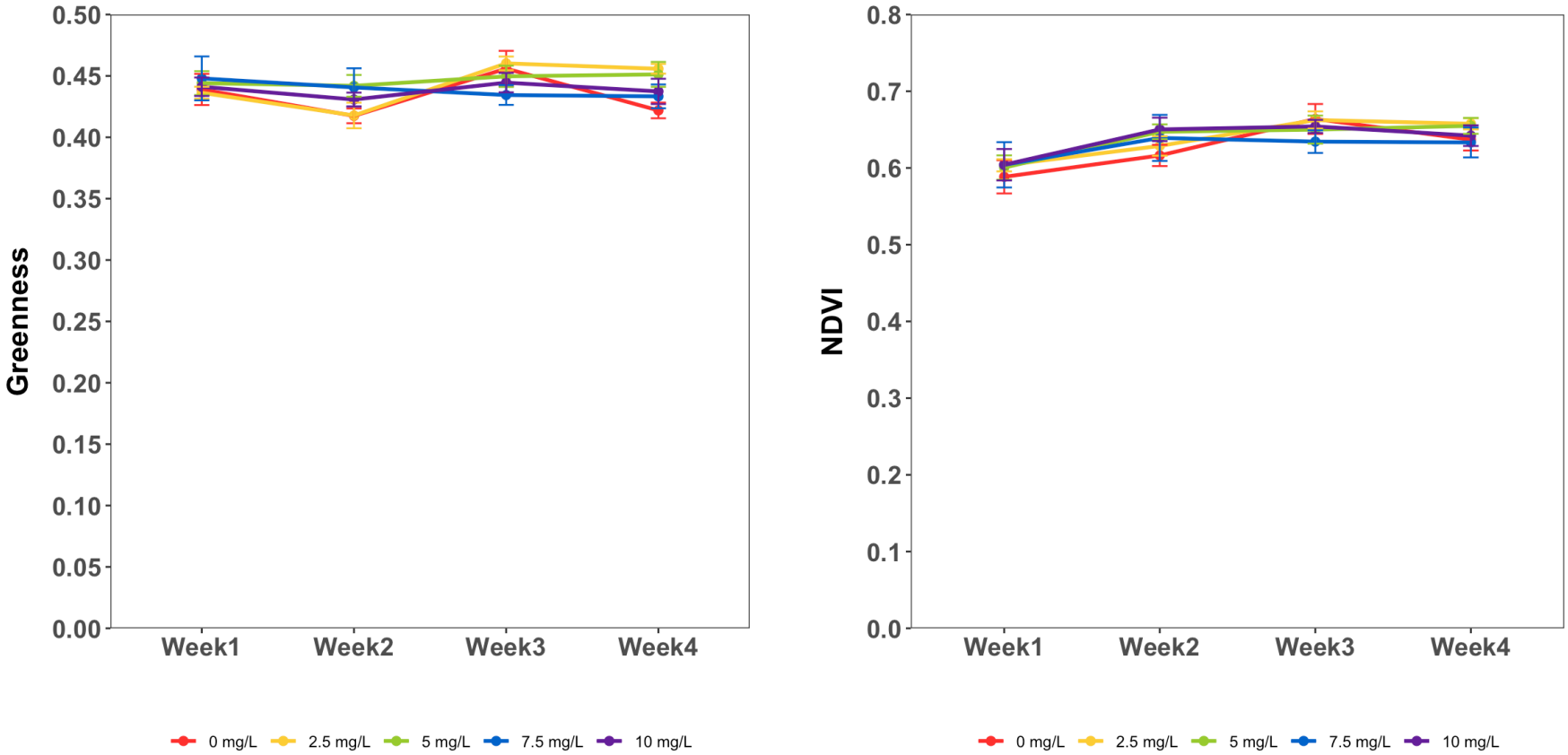


Figure 3.19 Greenness and NDVI of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

Figure 3.20 shows similar trends across B_{12} treatment throughout the growth period for both the Normalised Pigment Chlorophyll Ratio Index (NPCI) and Plant Senescence Reflectance Index (PSRI). NPCI, which accounts for chlorophyll content, showed the highest mean value of 0.33 ± 0.03 in lettuces treated with B_{12} 2.5 mg / L. However, there were no statistically significant differences in chlorophyll content between the B_{12} treatment groups ($p > 0.05$). PSRI, which measures plant senescence through the ratio of carotenoid and chlorophyll, had the lowest mean value of 0.10 ± 0.01 in lettuces in control group (without B_{12} treatment), indicating relatively lower senescence. However, PSRI values did not differ statistically significantly across treatment ($p > 0.05$), suggesting that B_{12} treatment may not have a significant impact on lettuce senescence. Overall, physiological parameters including chlorophyll content, pigment ratios, and senescence levels remained consistent across B12 treatments, demonstrating that B_{12} concentration had no significant impact on plant vigour or stress status.

In conclusion, B_{12} treatment via root application did not significantly affect the morphological or physiological development of lettuces grown hydroponically. Lettuce treated with B_{12} 2.5 mg/L resulted in the most favourable growth and morphological development among B_{12} treatments, although these improvements were not statistically significant compared to the control group (without B_{12} treatment). Conversely, the higher concentration of B_{12} 10 mg/L tended to restrict growth and development, suggesting that low concentrations of B_{12} may enhance certain growth parameters measured by PlantEye. However, the overall impact of B_{12} treatment on lettuce development remains limited ($p > 0.05$). These findings align with the results from the leaf application experiment presented in **Chapter 3.1.3.1**. Both studies support the biological understanding that lettuce do not require exogenous B_{12} for growth and development, as it typically lack B_{12}-dependent metabolic pathways. This likely explains the minimal physiological responses observed. Additionally, the tested B_{12} concentrations may have been insufficient to initiate the physiological responses that elicit measurable growth effects. It is also possible that the endogenous B_{12} levels in lettuces were already adequate to sustainable normal growth, thereby limited the benefits of external B_{12} treatments.

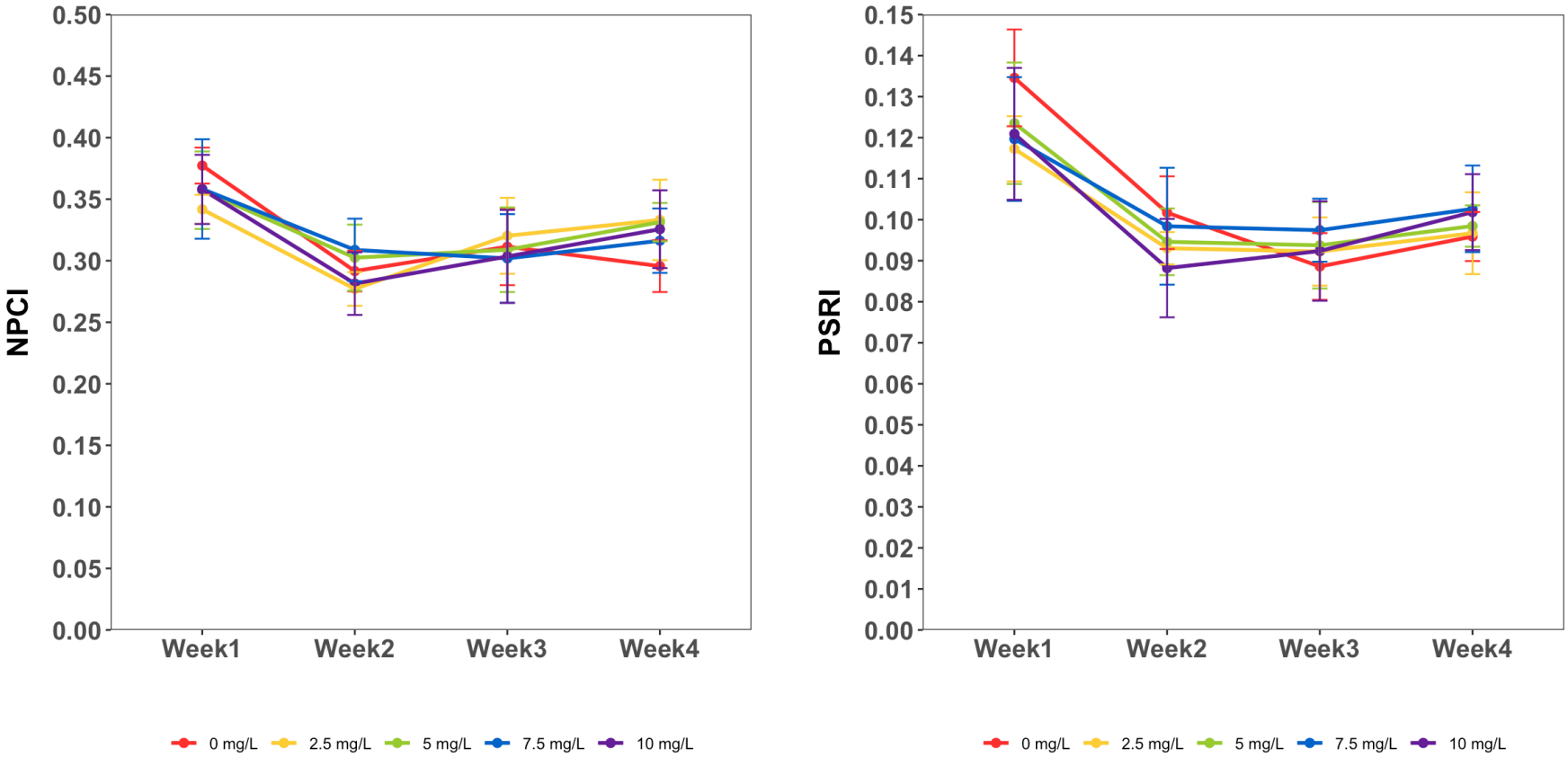


Figure 3.20 NPCI and PSRI of lettuces measured weekly via PlantEye.

Coloured lines indicate B_{12} concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend. Data are shown as mean ± standard error (n = 8).

3.2.3.2 Water management and gas exchange

The efficiencies of water management and gas exchange were measured by LI-COR (LI-6800, LICOR Bioscience, Lincoln, UK). Although measurements were taken biweekly at weeks 2 and 4 of the growth stage, only the data from week 4 are presented. The parameters assessed included Water Use Efficiency (*WUE*), Intrinsic water Use Efficiency (*iWUE*), and Stomatal Limitation (L_s). Further methodological details are provided in **Chapter 2.3.3**.

Water Use Efficiency (WUE)

Water Use Efficiency (*WUE*), which represents the ratio of carbon dioxide assimilation to water loss through transpiration, was measured at week 4 and is highlighted in Figure 3.21. Lettuces treated with B_{12} 5 mg / L exhibited the highest mean WUE values compared to the control group at light intensities 200 and 500, µmol m^2s^{-1}, with values recorded at 2362 and 3175 µmol CO_2 / mmol H_2O, respectively. At a higher light intensity of 1800 µmol m^2s^{-1}, the B_{12} 7.5 mg / L treatment resulted the highest mean *WUE* values of 2799 µmol CO_2 / mmol H_2O. These results suggest that the B_{12} 5 mg / L treatment group demonstrated better water use efficiency, potentially supporting higher photosynthetic rates per unit of water lost through transpiration, particularly up to light intensity of 500 µmol m^2s^{-1}. However, it is also possible that the increased *WUE* resulted not from enhanced photosynthetic capacity but from reduced stomatal conductance, which limits water loss. This interpretation highlights the need to consider stomatal behaviour alongside *WUE* in assessing physiological performance.

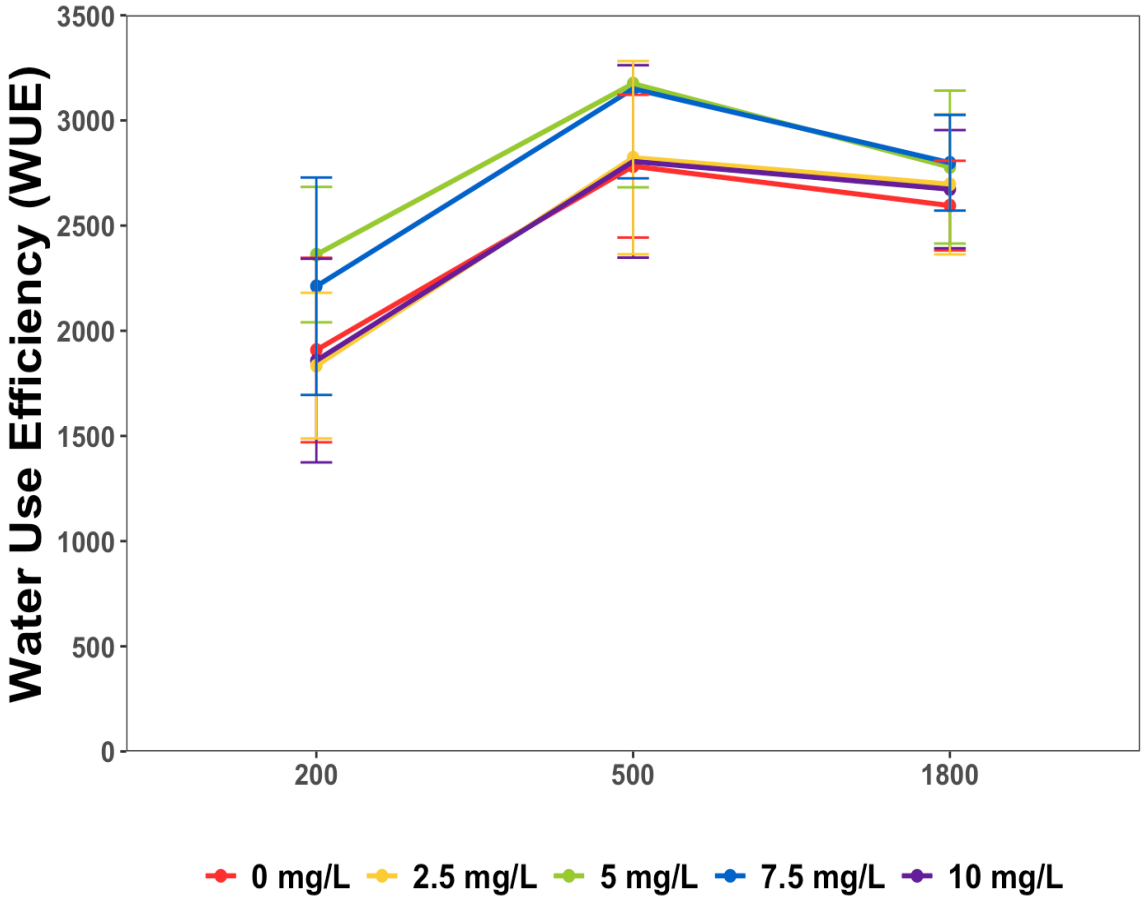


Figure 3.21 Water Use Efficiency (WUE) measured using LI-COR at Week 4.

Water use efficiency (WUE), calculated as net assimilation rate (A) divided by transpiration rate (E), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

Intrinsic Water Use Efficiency (iWUE)

As a direct parameter of stomatal activity including opening and closing of stomata, the results of *iWUE* measured at week 4 were highlighted. Interestingly, while most B_{12}-treated groups showed higher *iWUE* values than the control, the 2.5 mg / L treatment was an exception. The B_{12} 5 mg / L treatment recorded the highest *iWUE* values at light intensities of 200, 500, and 1800 µmol m^2s^{-1}, respectively (49.59, 73.55, and 77.42 µmol CO_2 / mmol H_2O, respectively). These results suggest that B_{12} treatment, especially 5 mg / L may promote more effective internal regulation of gas exchange under high light conditions (above 500 µmol m^2s^{-1}), potentially maintaining photosynthetic activity with relatively slower stomatal conductance and reduced water loss. Alternatively, the elevated *iWUE* observed may not necessarily indicate improved efficiency water use but rather reflect a condition where stomatal apertures are more restricted limiting CO_2 entry into the leaf (Figure 3.22).

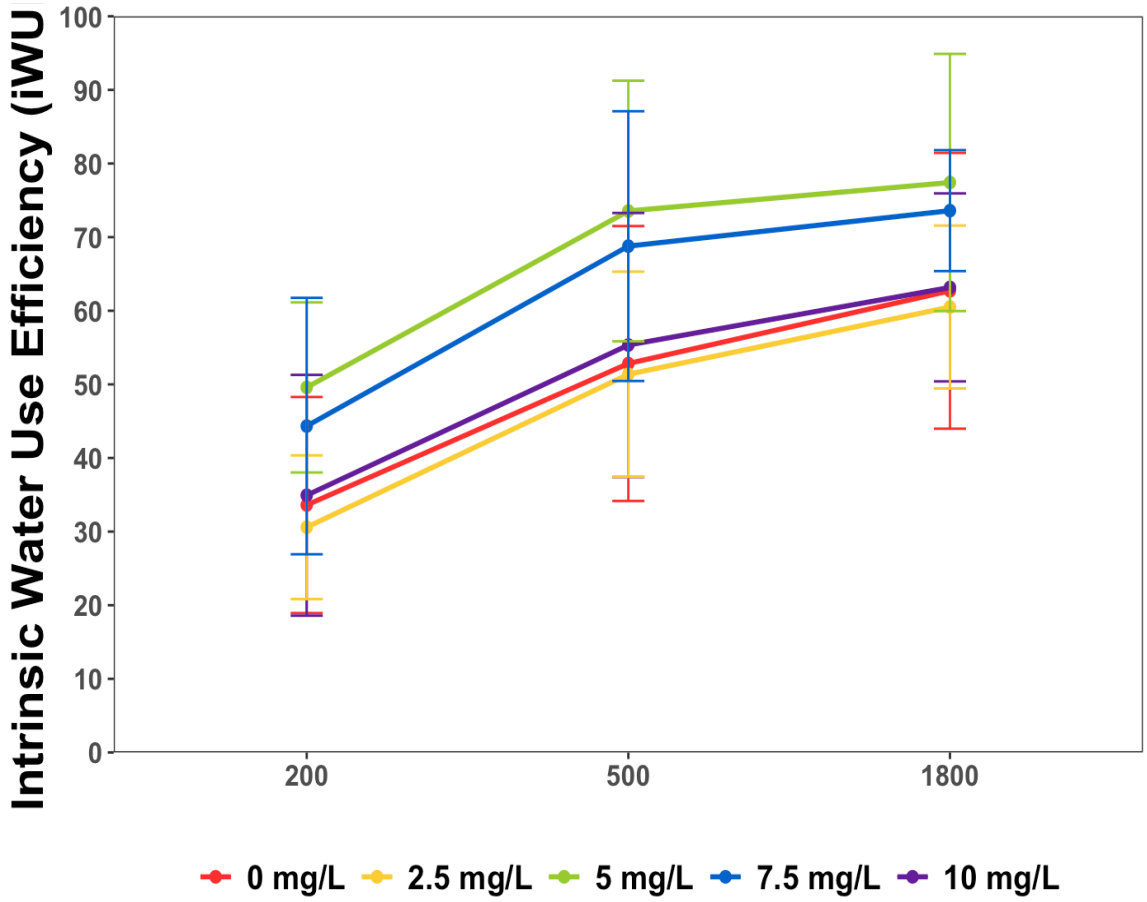


Figure 3.22 Intrinsic Water Use Efficiency (iWUE) measured using LI-COR at Week 4.

Intrinsic Water use efficiency (iWUE), calculated as net assimilation rate (A) divided by stomatal conductance to H_2O (gsw) , plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured line represents B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

Stomatal Limitation (L_s)

Stomatal limitation (L_s) measures the extent to which stomata limit CO_2 diffusion into the leaf. Low stomatal limitation indicates that the stomata are open, allowing CO_2 to enter freely. In contrast, high stomatal limitation reflects more closed stomata, restricting CO_2 intake and likely reducing photosynthesis. As shown in Figure 3.23**,** the B_{12} 5 mg / L treatment exhibited the highest L_s values at light intensities of 200, 500, and 1800 µmol m^2s^{-1} (0.25, 0.36, and 0.38, respectively). These results suggest that the 5 mg / L treatment appeared to induce stomatal limitation, which may have restricted photosynthetic performance despite high *iWUE*.

In summary, the B_{12} 5 mg / L treatment showed the highest values for *WUE*, *iWUE*, and L_s, suggesting a physiological tendency toward conserving water through reduced stomatal conductance. This may reflect a strategy to maintain photosynthetic activity with reduced water loss. However, no visible differences in overall plant growth or performance were observed among B_{12} treatments. It should be noted that statistical analyses were not performed on the LI-COR data due to the characteristics of the dataset. Therefore, these physiological trends, including water use efficiency and gas exchange related to photosynthetic activity, should be interpreted with caution as preliminary observations.

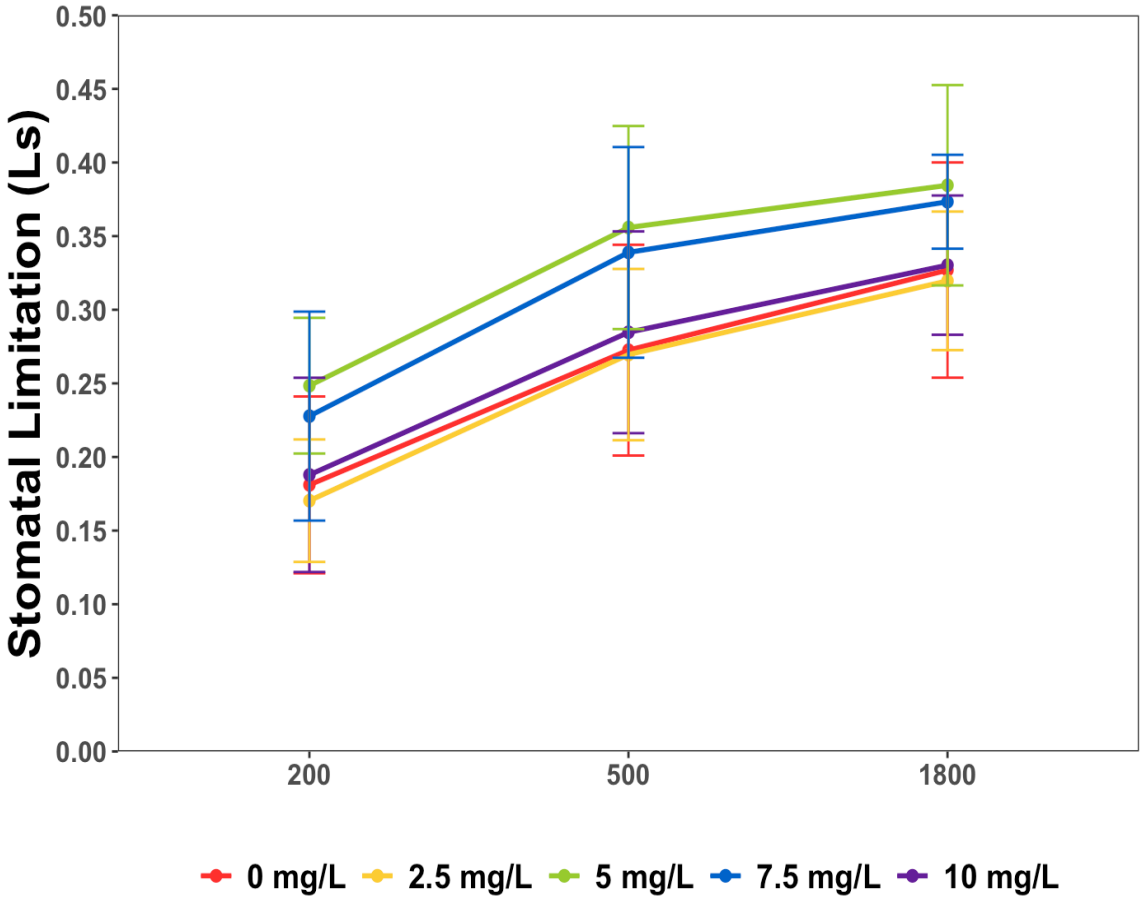


Figure 3.23 Stomatal Limitation (Ls) measured using LI-COR at Week 4.

Stomatal Limitation (L_s), calculated as 1- (intercellular CO2 concentration (Ci) divided by ambient CO_2 concentration (Ca), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured line represents B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

3.2.3.3 Relative chlorophyll measurements

Relative chlorophyll measurements were conducted using SPAD-502Plus (Konica Minolta Ltd., Osaka, Japan) biweekly on week 2 and week 4. Details are described in **Chapter 2.3.4**. SPAD value correlates with chlorophyll content, which plays an important role in photosynthesis. Higher SPAD values indicate healthier plant with more chlorophyll.

Figure 3.24 shows the SPAD measurement results at week 2 and 4, with overall SPAD values increasing over time. At week 2, lettuces treated with B_{12} except 2.5 mg / L group exhibited higher relative chlorophyll, with the B_{12} 10 mg / L treatment showing the highest value of 19.8 ± 1.7 SPAD values. For the final harvest at week 4, SPAD results are highlighted, where the B_{12} 2.5 and 10 mg / L groups had higher values compared to the control (without B_{12} treatment). The highest SPAD value of 21.3 ± 1.9 was recorded in the B_{12} 10 mg / L group which was 5 % higher than the lowest value was found in B_{12} 7.5 mg / L (20.2 ± 2.0). However, there these differences were not statistically significant ($p > 0.05$), indicating that B_{12} treatment did not have a significant effect on chlorophyll content.

Overall, the present study found that B_{12} 2.5 and 10 mg / L treatments contribute increasing chlorophyll content in lettuce leaf tissues compared to the control group without B_{12} treatment. Particularly, B_{12} 10 mg / L treatment appeared to enhance chlorophyll content, while lettuces treated with B_{12} 7.5 mg / L exhibited the lowest SPAD values. Although, these differences were not statistically significant ($p > 0.05$), the trend suggests that the highest concentration B_{12} (10 mg / L) applied via root application may potentially support chlorophyll production or retention in lettuce. This could imply that B_{12} plays a potential role in improving photosynthetic capacity and overall plant health. Since the SPAD measurement taken at week 4 reflect final harvest quality, these results cautiously suggest that plant treated with B_{12} 10 mg / L may have slightly improved harvest quality due to their high chlorophyll content compared to the control group.

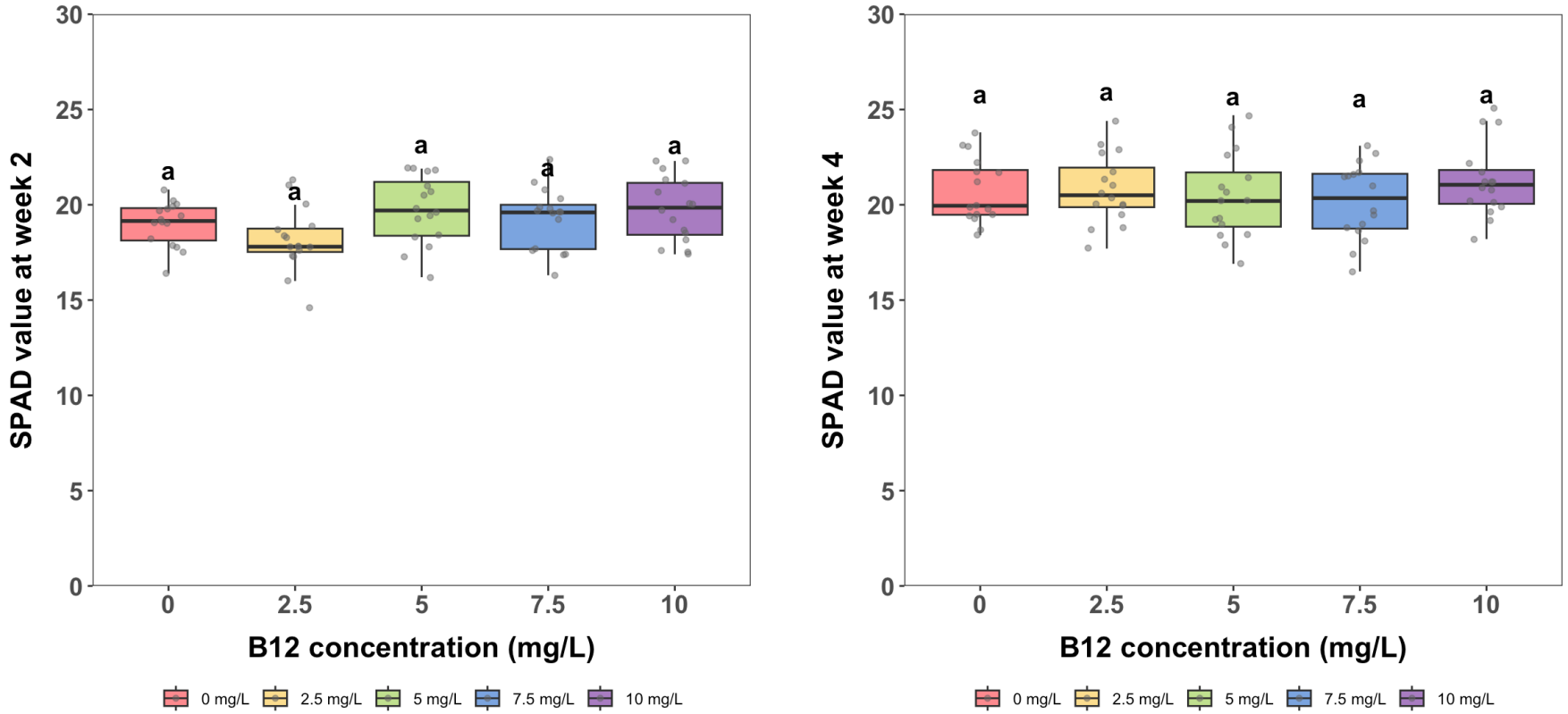


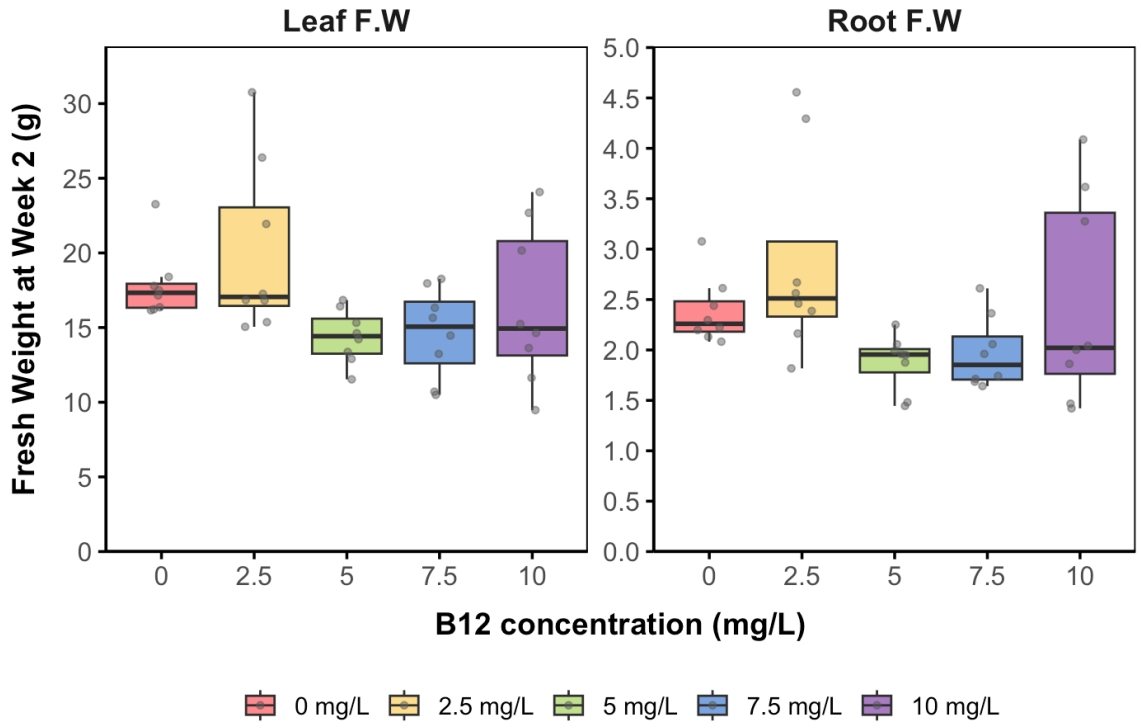
Figure 3.24 Relative chlorophyll content measured by SPAD meter at Week 2 and Week 4.

Coloured box plots indicate B_{12} treatment concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as shown in the legend. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). No statistically significant differences were observed between treatments ($p > 0.05$).

3.2.3.4 Harvest quality: fresh weights

Figure 3.25 shows the fresh weight measurements of total, leaf, and root tissues at the first harvest (week 2). Lettuces treated with B_{12} 2.5 mg / L had highest value of total fresh weight (22.92 ± 6.53 g) including leaf weight (20.05 ± 5.78 g), and root weight (2.86 ± 1.00 g). Particularly, B_{12} 5 mg / L treated lettuces had the lowest fresh weight in both leaf (14.40 ± 1.79 g) and root tissues (1.88 ± 0.28 g). However, these results do not show statistical significance between the B_{12} concentration ($p > 0.05$). As an indicator of final harvest quality, the measurements taken at week 4 are highlighted (Figure 3.25). Lettuces treated with B_{12} 2.5 mg / L exhibited the highest whole fresh weight (193.64 ± 9.19 g) which was 6 % higher than the lowest values from the lettuces treated with B_{12} 5 mg / L (177.64 ± 14.32 g). However, these differences were not statistically significant based on the results of the multiple permutational comparison test ($p > 0.05$).

Overall, the present study found that B_{12} treatment may contribute to improved final harvest quality in lettuce compared to the control group without B_{12} treatment. Particularly, B_{12} treatment with 2.5, 7.5, and 10 mg / L increased the fresh weights of both leaf and root tissues, whereas the B_{12} 5 mg / L treatment resulted in the lowest fresh weights. Although statistical analysis did not reveal significant differences ($p > 0.05$), these trends may indicate a potential role for B_{12} 2.5, 7.5, and 10 mg / L treatments may promote cell elongation and biomass accumulation, ultimately enhancing harvest yield. This supports the potential role of B_{12} in improving overall plant health and developmental performance.



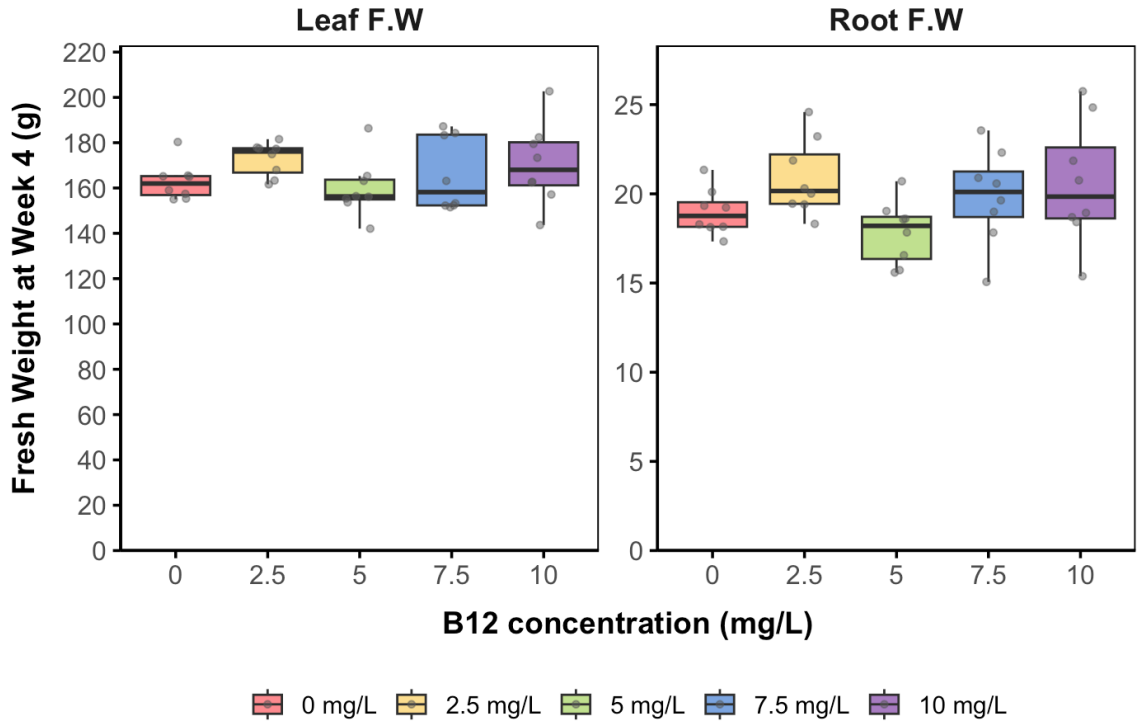


Figure 3.25 Harvest quality – Fresh weight (g) of lettuces at week 2 and week 4.

Coloured box plots indicate leaf, and root fresh weight within each B_{12} treatment group (0, 2.5, 5, 7.5, and 10 mg / L). No statistically significant differences were observed between treatments ($p > 0.05$).

3.2.3.5 Elemental analysis: B_{12} quantification

The ICP-MS outcomes are presented as milligram of B_{12} in the gram of dried lettuce tissues including leaf and root tissues (mg / g). The details of the ICP-MS analysis are described in **Chapter 2.3.1**. Figure 3.26 shows the quantification of B_{12} using ICP-MS analysis from the harvested samples in week 2 and week 4 including mean and standard deviation. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**. Cobalt (Co) content, determined by ICP-MS, was used to estimate vitamin B_{12} concentration in plant tissues. Given that each B_{12} molecule contained one cobalt atom, the molecular weight ratio of vitamin B_{12} to cobalt (1,355.37 / 58.93) was applied to convert Co values into corresponding B_{12} levels. As the hydroponic nutrient solution did not include cobalt other than through B_{12} supplementation, it is assumed that all measured cobalt originated from the applied B_{12}.

The B_{12} content in leaf tissues harvested at week 2 increased with B_{12} application, showing a general upward trend except in the B_{12} 7.5 mg / L group. The highest B_{12} content was observed in the B_{12} 10 mg / L group (0.018 ± 0.012 mg / g) in lettuce leaf tissues, which was significantly higher than the control group (B_{12} 0 mg / L), showing the lowest value ($p < 0.05$). Treatment with B_{12} 2.5 and 10 mg / L were grouped under the same significance category ('b'), while the B_{12} 7.5 mg / L treatment was labelled 'ab', indicating intermediate significance (Figure 3.26).

In the root tissues at week 2, the B_{12} 10 mg / L treatment resulted in the highest B_{12} accumulation in root tissues (0.051 ± 0.020 mg / g) and was statistically distinct from all lower concentration (B_{12} 0, 2.5 and 5 mg / L) ($p < 0.05$). The control group ('a') showed the lowest B_{12} content, while the B_{12} 10 mg / L group ('d') showed the highest. Intermediate B_{12} concentrations between 2.5 and 7.5 mg / L fell into overlapping statistical grouping ('b', 'abc', and 'cd'), reflecting a progressive and statistically supported in B_{12} uptake and accumulation. These results confirm that B_{12} was more efficiently accumulated in root tissues than leaves, consistent with the root application method. The lettuces treated with B_{12} 10 mg / L contained the highest B_{12} content in both leaf and root tissues, forming a distant statistical group.

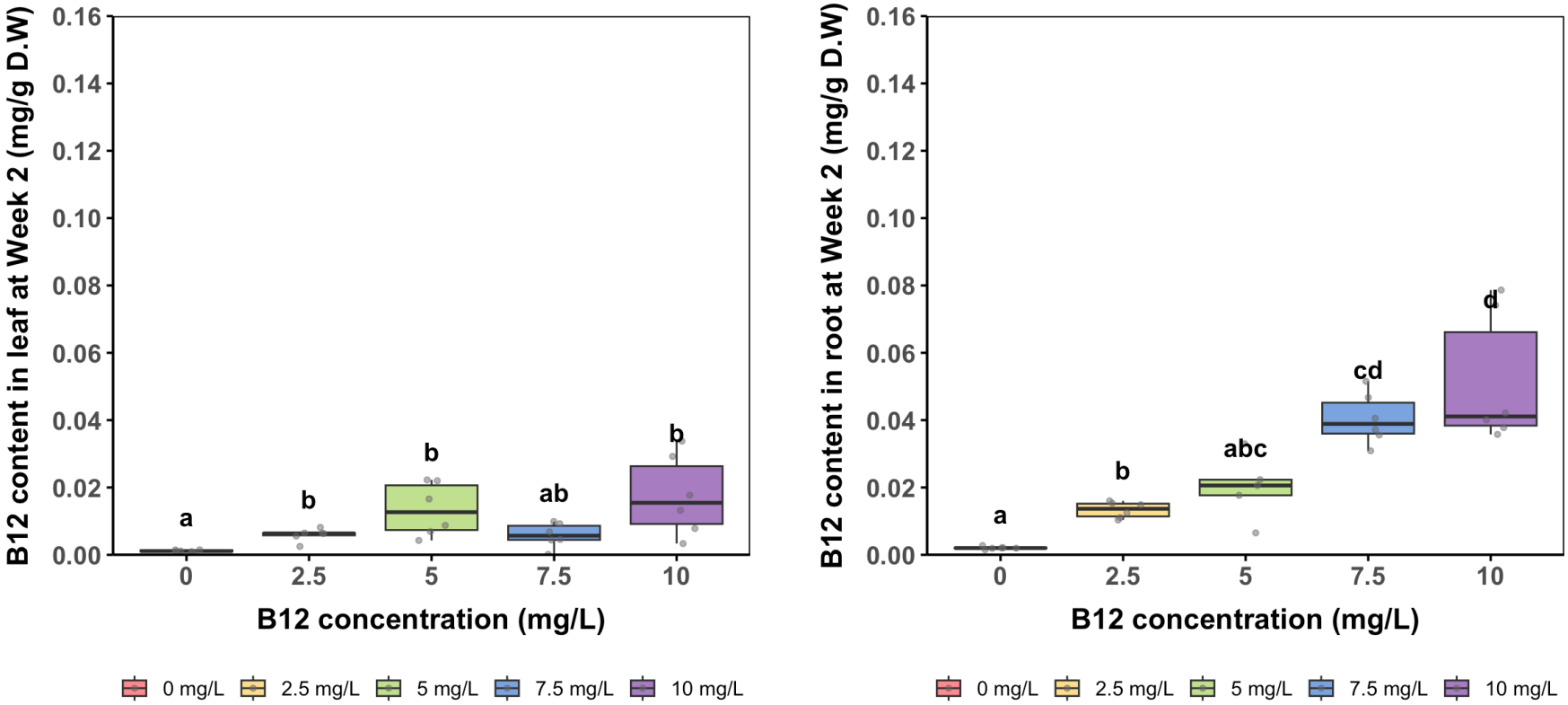


Figure 3.26 Quantification of vitamin B_{12} in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 2.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

As an indicator of final harvest quality, the B_{12} content in lettuce tissues harvested in week 4 is presented in Figure 3.27. There was a consistent increase observed with elevated levels of B_{12} concentrations, although the group treated with B_{12} 10 mg / L showed a decline compared to B_{12} 7.5 mg / L. The highest B_{12} content in lettuce tissues was recorded in the B_{12} 7.5 mg / L group (0.028 ± 0.007 mg / g), which was statistically significantly higher than both the control group (B_{12} 0 mg / L; 0.001 ± 0.000 mg / g, 'a') and the B_{12} 2.5 mg / L group (0.010 ± 0.002 mg / g, 'b') ($p < 0.05$), demonstrating a progressive pattern of B_{12} uptake and accumulation, with statistically distinct groupings reinforcing the observed trend. In root tissues, a similar trend was observed. The highest B_{12} content found in the group of B_{12} 7.5 mg / L (0.141 ± 0.025 mg / g) which was significantly higher than the control group (B_{12} 0 mg / L; 0.002 ± 0.001, 'a') and 2.5 mg / L group (0.060 ± 0.026 mg / g, 'b') ($p < 0.05$).

Overall, the data demonstrate a positive correlation between increasing B_{12} application and its uptake and accumulation in both leaf and root tissues. However, the treatment with the highest B_{12} concentration (10 mg / L) did not result in further increases and instead showed slightly reduced accumulation compared to the B_{12} 7.5 mg / L treatment. This result suggests a possible saturation threshold or physiological restraint in uptake and translocation at higher application rates through root application.

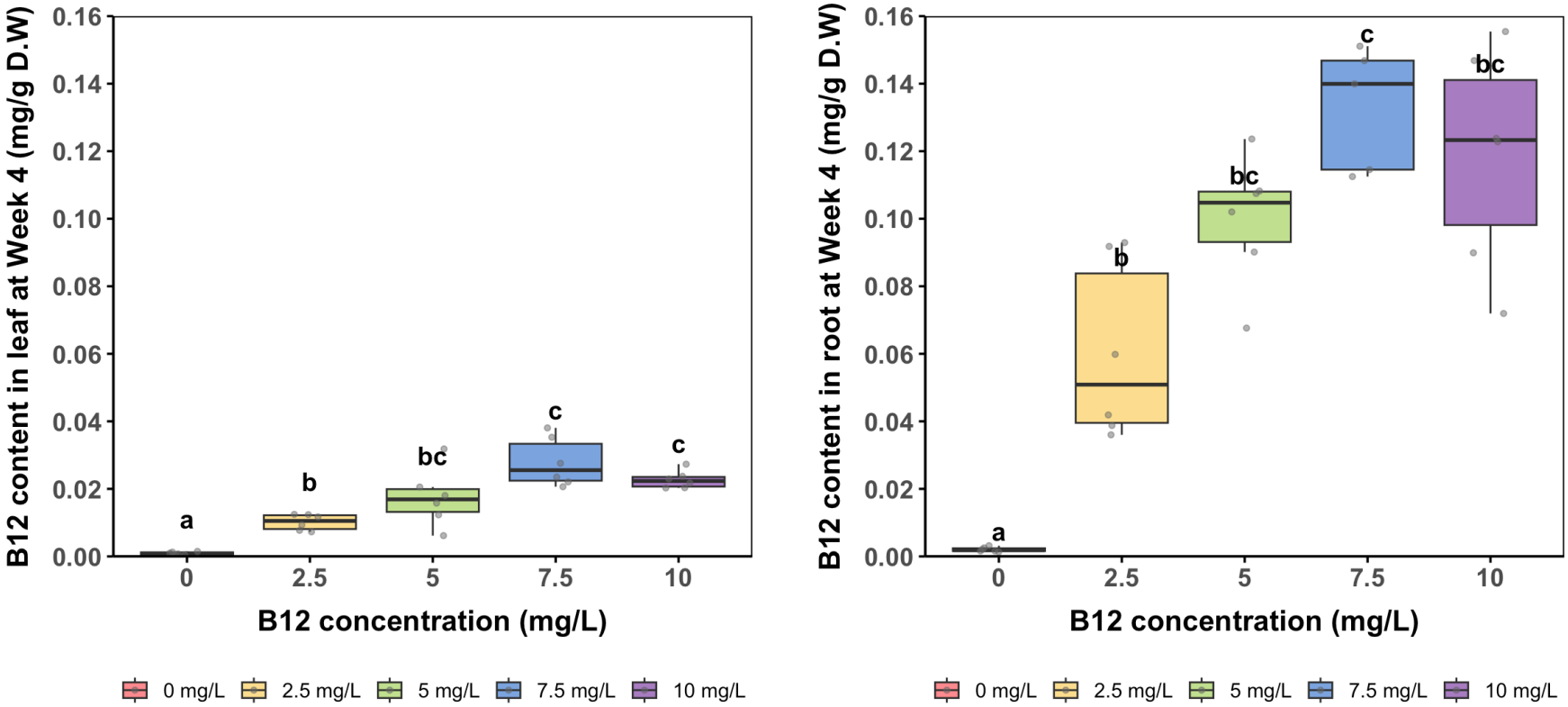


Figure 3.27 Quantification of vitamin B_{12} in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

3.2.3.6 Secondary metabolites analysis

To assess the effect of root-treated B_{12} application on secondary metabolites in lettuce, total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) were measured. The detailed methodology for sample preparation and analysis is described in **Chapter 2.4**. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**.

Total Phenolic Content (TPC)

Total phenolic content (TPC) in lettuce leaves was determined using Folin-Ciocâlteu reagent with the gallic acid equivalence method (GAE). Phenolic compounds play an important role in plant protection mechanisms by prolonging shelf life after harvest and improve stress resistance [168]. The results of TPC are described as milligram of gallic acid by gram of fresh weight of the final harvested lettuce (mg GAE / g) which shown in Figure 3.28. Overall, B_{12}-treated lettuces exhibited higher TPC than the control group (without B_{12} treatment).

The highest TPC value was observed in the B_{12} 10 mg / L (0.228 ± 0.137 mg GAE / g, 'ab'), which followed by B_{12} 7.5 mg / L group (0.219 ± 0.050 GAE / g, 'b') ($p < 0.05$). Although B_{12} 10 mg / L had the numerically highest TPC, this difference was not statistically significant compared to the control due to high variability within the group. In contrast, the B_{12} 7.5 mg / L group showed a statistically significant increase in TPC relative to the control group ($p < 0.05$), indicating a meaningful enhancement. The lowest TPC was recorded in the control group (0.174 ± 0.043 mg GAE / g, 'a'). These results suggest that root-applied B_{12} treatment, particularly at 7.5 mg / L, can enhance antioxidant-related compounds such as phenolics in lettuce.

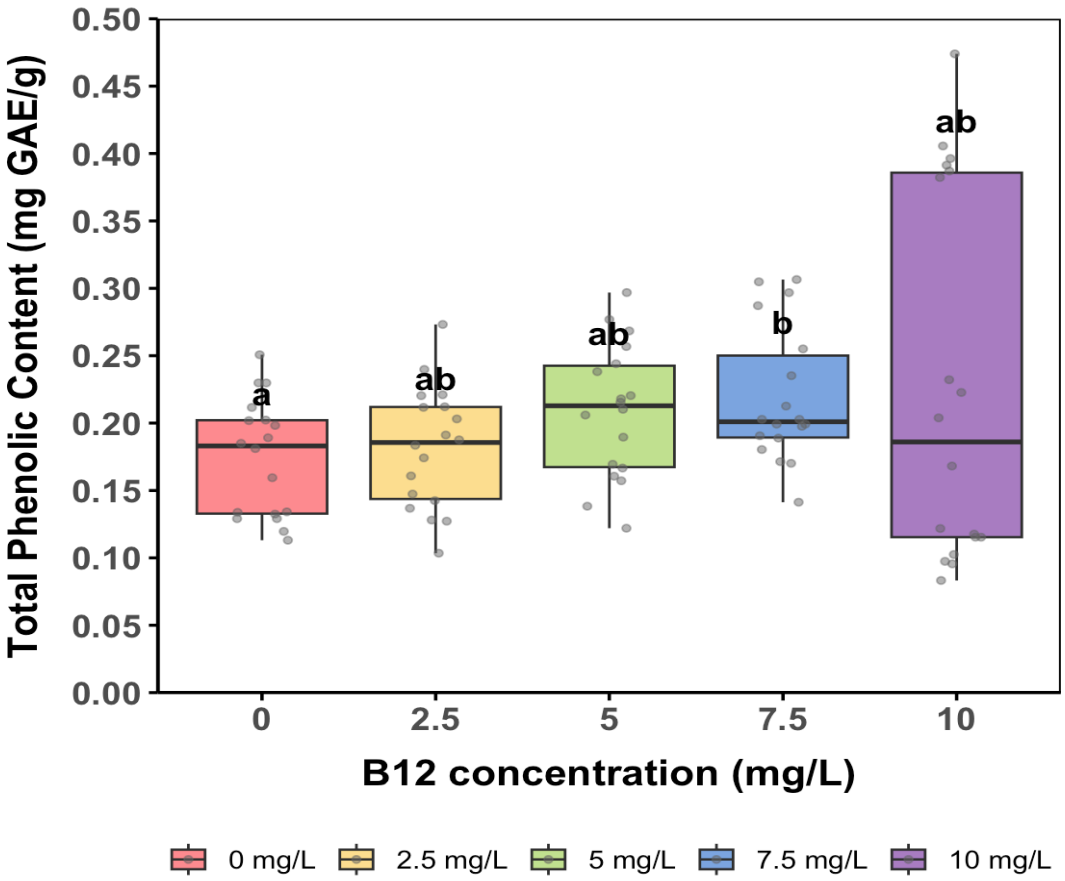


Figure 3.28 Total Phenolic Content (mg GAE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using the aluminium chloride assay, with results expressed as milligrams of Rutin equivalents per gram of fresh lettuce (mg RE / g) [164,169]. Flavonoids play critical roles in plants, including regulating cell growth, attracting pollinators, contributing to pigments production such as chlorophyll and carotenoids, and enhancing plant stress tolerance to both biotic and abiotic stress [170]. As shown in Figure 3.29, the TFC exhibited a non-linear pattern in response to increasing B_{12} concentration.

The highest TFC value was observed in B_{12} 2.5 mg / L group (0.140 ± 0.038 mg RE / g, 'c'), which was statistically significantly higher than the control group (B_{12} 0 mg / L, 0.092 ± 0.017 mg RE / g, 'ab'), as well as the 7.5 mg / L (0.080 ± 0.011 mg RE / g, 'b', and 10 mg / L (0.080 ± 0.016 mg RE / g, 'b') groups ($p < 0.05$). The TFC in the 2.5 mg / L group represented an increase of 53 %, 34 %, 76 %, and 76 % compared to the B_{12} 0, 5, 7.5, and 10 mg / L treatments, respectively. In contrasts, both 7.5 and 10 mg / L treatments exhibited the lowest TFC values, suggesting possible inhibition of flavonoid synthesis at higher B_{12} concentrations.

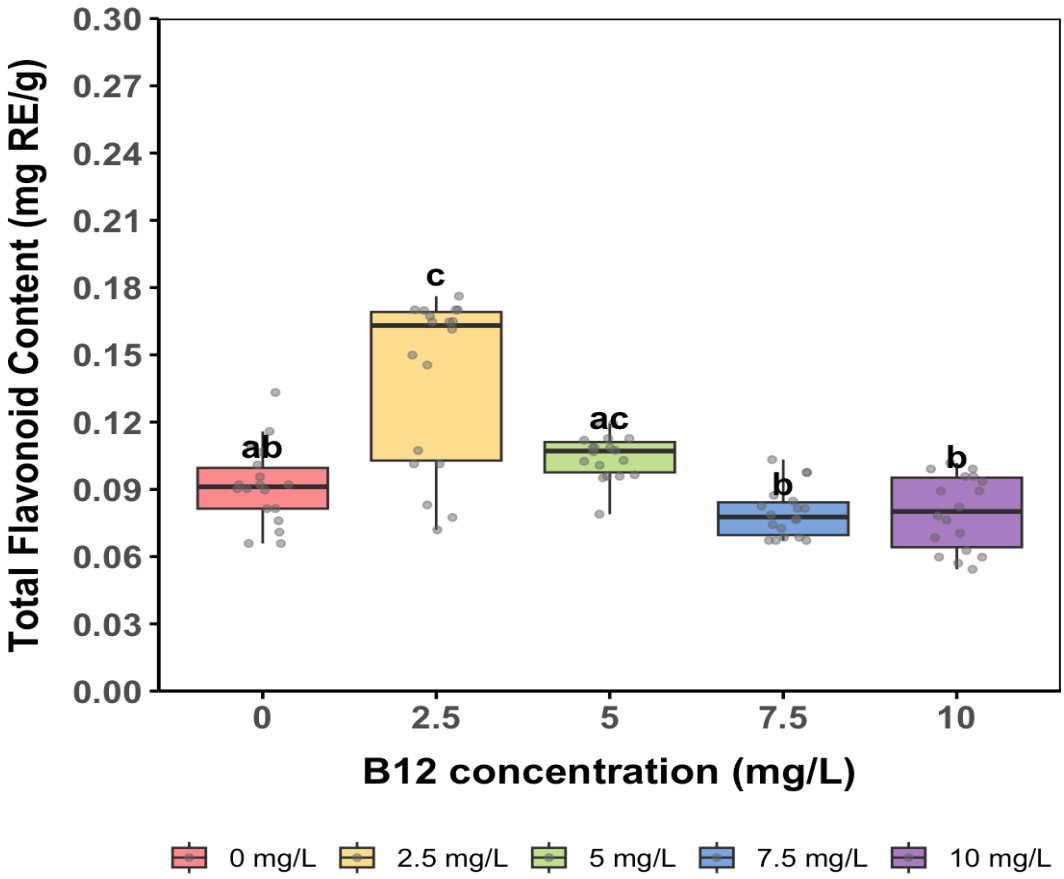


Figure 3.29 Total Flavonoid Content (mg RE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Total Antioxidant Activity (TAA)

Total antioxidant activity (TAA) was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method [164,169]. Trolox® equivalent antioxidant capacity (TEAC) was used as a standard reference to quantify antioxidant activity. TAA results are described as milligram of TEAC per gram of fresh lettuce weight (mg TEAC / g). As shown in

, all B_{12}-treated lettuce contained higher TAA values compared to the control group without B_{12} treatment. The highest TAA value recorded in the B_{12} 5 mg / L group (4.543 ± 0.097 mg TEAC / g, 'b'), which was statistically significantly higher than the control group (0 mg / L, 4.315 ± 0.200 mg TEAC / g, 'a') ($p < 0.05$). The distinct statistical groupings indicate a significant enhancement in antioxidant activity at B_{12} 5 mg / L, whereas the other B_{12}-treated group (2.5, 7.5 and 10 mg / L) showed intermediate values ('ab'), that were not significantly different from either extreme.

In summary, the results of secondary metabolites demonstrate that different concentration of B_{12} treatments via root application had variable effects on TPC, TFC, and TAA in lettuce. An overall increasing trend in TPC was observed in rising B_{12} concentration, reaching its highest numerical value at B_{12} 10 mg / L. However, due to variability, statistical significance was only achieved at B_{12} 7.5 mg / L, suggesting that moderate B_{12} levels may enhance phenolic accumulation, while the highest concentrations (B_{12} 10 mg / L) did not result in further statistically consistent improvement.

Notably, the B_{12} 2.5 mg / L treatment recorded the highest flavonoid content, which was statistically significantly greater than both the control and higher B_{12} concentrations. Higher B_{12} concentration (7.5 and 10 mg / L) were associated with a significant decline in flavonoid levels, indicating a potential inhibitory effect of excess B_{12} on flavonoid biosynthesis pathway. TAA results showed that the B_{12} 5 mg / L treatment recorded the highest antioxidant activity, which was statistically significantly greater than the control group (without B_{12} treatment). Although phenolic and flavonoid compounds contribute to antioxidant activity, the current findings show that the peaks of TPC, TFC, and TAA did not align across B_{12} treatments. For instance, the highest TPC was recorded in B_{12} 10 mg / L, whereas TFC peaked at 2.5 mg / L, and TAA was highest at 5 mg / L. This lack of direct correspondence suggests that antioxidant capacity in lettuce is not solely dependent on phenolic and flavonoid concentrations.

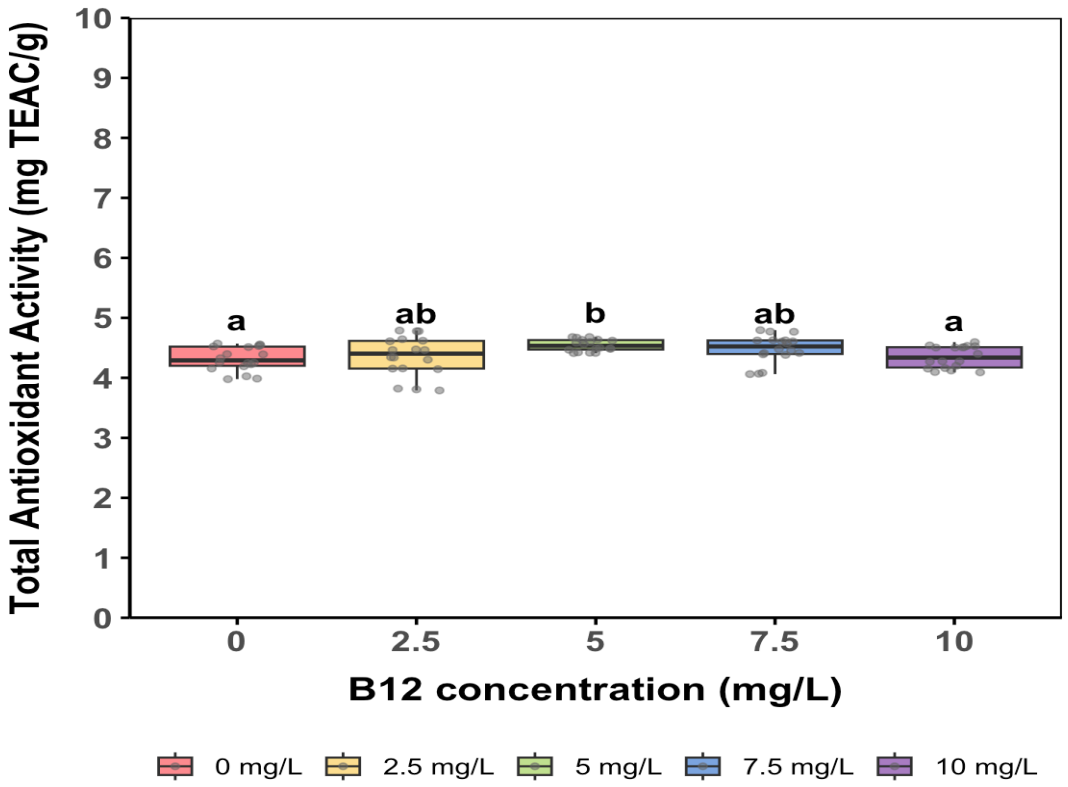


Figure 3.30 Total Flavonoid Content (mg RE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent B_{12} concentration (0, 2.5, 5, 7.5, and 10 mg / L Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

3.3 Discussions

3.3.1 Practical and economic implication of B_{12} fortification strategies

As described in the result section, B_{12} concentrations were estimated from cobalt content measured by ICP-MS, assuming that all detected cobalt originated from the supplemented B_{12} due to the absence of other cobalt sources in the hydroponic solution. This estimation was based on the molecular ratio of vitamin B_{12} to cobalt. Based on this elemental analysis, the B_{12} concentration in lettuce tissues reached up to 0.010 mg / g via leaf application and 0.028 mg / g via root application, expressed per gram of dried lettuce. Given that the recommended daily intake (RDI) of B_{12} for adults is 2.4 µg[1], the concentrations measured in this study were 10 µg / g via leaf application and 28 µg / g (root application), both of which substantially exceed the RDI on a per gram dry weight basis. Considering the lettuce typically contains more than 95 % moisture, these values correspond to approximately 0.5 µg / g via leaf application and 1.4 µg / g on a per gram of fresh weight basis[172]. As shown in Table 3.1, these results suggest that B_{12} -fortified lettuce, via both root and leaf applications, is highly effective in meeting the RDI of B_{12}. Even with the modest consumption such as above 4.8 g for leaf-treated and 1.71 g for root-treated lettuce, the intake would exceed the adult RDI of 2.4 µg per day. This highlights the strong potential of hydroponic B_{12} fortification as a practical dietary intervention.

Table 3.1 Elemental analysis of vitamin B_{12} in biofortified lettuce tissues: dry and fresh weight (µg / g)

Leaf application				
	B_{12} content in D.W (µg / g D.W)		**B_{12} content in F.W (µg / g F.W)**	
Vitamin B_{12}	**Leaf (µg / g)**	**Root (µg / g)**	**Leaf (µg / g)**	**Root (µg / g)**
0 mg / L	4	3	0.20	0.15
2.5 mg / L	5	5	0.25	0.25
5 mg / L	7	5	0.35	0.25
7.5 mg / L	7	5	0.35	0.25
10 mg / L	**10**	9	**0.50**	0.45
Root application				
	B_{12} content in D.W (µg / g D.W)		**B_{12} content in F.W (µg / g F.W)**	
Vitamin B_{12}	**Leaf (µg / g)**	**Root (µg / g)**	**Leaf (µg / g)**	**Root (µg / g)**
0 mg / L	1	2	0.05	0.10
2.5 mg / L	10	60	0.5	3
5 mg / L	17	100	0.85	5
7.5 mg / L	**28**	141	**1.4**	7.05
10 mg / L	23	118	1.15	5.90

Overall, B_{12} fortification via root application showed significantly higher B_{12} content in lettuce leaf and root tissues compared to those treated via leaf application. The final sample analysis indicated that B_{12} levels in leaf tissues were twice as high when treated via root application compared to leaf application, and root tissues contained 16 times more B_{12} via root application than leaf application. However, in the present study, root application required approximately 100 times more B_{12} than the leaf application in terms of total solution volume, resulting in substantially higher input costs. While root application proved to be more efficient in terms of B_{12} uptake, translocation, and accumulation, especially in root tissues, it may be economically less viable for leafy crops due to the increased resource demands. Nevertheless, this method may be better suited to root vegetables such as potato, carrot, or radish, where biofortification of root is desirable and economically justifiable. In contrast, leaf application is more cost-efficiency in terms of B_{12} usage, requiring significantly smaller volumes of the nutrient. However, this method may incur additional labour or automation costs due to the need for precise leaf spraying and possible repeated applications to achieve sufficient B_{12} accumulation, particularly in crops with larger or denser canopies. Despite these limitations, leaf application remains a practical option for biofortifying leafy vegetables and fruit crops, especially if the B_{12} concentration is increased (e.g., above 10 mg / L) or applied across multiple treatment intervals.

3.3.2 Determination of uptake, translocation, and accumulation of B_{12} in lettuce

The present study aimed to investigate the uptake, translocation, and accumulation pathway of B_{12} in lettuce using elemental analysis. Root and leaf tissues were analysed respectively to determine their uptake and translocation within lettuce following different application methods, specifically leaf and root treatments. Additionally, a time-series elemental analysis was conducted to evaluate B_{12} accumulation in lettuce tissues at two key growth stages: week 2 and week 4. The results of the elemental analysis demonstrated that B_{12} treatment significantly increased B_{12} concentration in both leaf and root tissues compared to the untreated control, regardless of the application method. However, in lettuces treated via leaf application, no significant change in B_{12} content was observed between week 2 and week 4 in either tissue type ($p > 0.05$). This suggests that the concentration used or the frequency of B_{12} application via leaf application may have been insufficient to achieve continued uptake or accumulation as the lettuce matured.

At the final harvest (week 4 of the growth stage), leaf tissues from root-treated lettuces had an average B_{12} content of 0.016 mg / g, which was approximately 137 % higher than in lettuces treated via leaf application. This pronounced difference may be attributed to the continuous exposure of root systems to the B_{12} -enriched hydroponic solution, in contrast to the limited and intermittent delivery from leaf application twice daily. The high B_{12} accumulation in roots following root application also suggests limited phloem mobility of B_{12}, as only a portion was translocated to the aerial parts. However, the leaf application method still effectively led to direct accumulation of B_{12} in the edible leaf tissues as shown in Table 3.1. Since lettuce is consumed for its leaves, this direct uptake through foliar feeding remains a viable and practical strategy for enhancing dietary B_{12} intake. However, further optimisation of the leaf application method is needed, including adjustment to B_{12} concentrations, application frequency, and automation of the process to reduce labour requirements.

Despite the successful quantification of B_{12} accumulation and preliminary observations of translocation in lettuce tissues in the present study, as well as similar observations reported in previous research [68,69], the specific mechanisms behind B_{12} uptake and translocation in plants remain largely unexplored. Unlike well-studied mineral nutrients such as iron, whose uptake involves characterised transporters and chelators [173,174], B_{12} presents unique structural properties that may require specialised uptake pathways. However, given that plants do not naturally require B_{12} for their growth and development, such mechanisms may be absent, limited or non-specific in plants. Therefore, future research should explore transcriptomic and proteomic analyses in B_{12}-fortified plants to explore candidate transport proteins. Additionally, isotope-labelled B_{12} analogues could allow detailed tracing of uptake routes [136].

3.3.3 Effect of B_{12} treatment in lettuce growth and development

Effect of B_{12} treatment in lettuce morphology and physiology

The present study aims to clarify the effect of B_{12} treatment on plant growth, development, and further nutritional quality. In the leaf application experiment, the control group without B_{12} showed the highest values in plant height, digital biomass, and greenness, whereas the B_{12} 5 mg / L treatment recorded the highest values in hue, leaf area, and normalised difference vegetation index (NDVI) measured by PlantEye. Notably, the B_{12} 2.5 mg / L group showed the lowest

performance in growth and development according to PlantEye measurements. Interestingly, these results align with the harvest weight measurement indicating that the lettuces treated with B_{12} 5, 7.5, and 10 mg / L had heavier fresh weights for both leaf and root weights compared to the control group with no B_{12} treatment, while those treated with B_{12} 2.5 recorded the lowest values in fresh leaf and root weight. However, there were no statistically significant differences in PlantEye and harvest biomass analysis ($p > 0.05$). Lastly, results of relative chlorophyll content measured by SPAD meter suggest that the B_{12} treatment enhanced chlorophyll content in lettuce leaf, particularly B_{12} 10 mg / L compared to B_{12} 2.5 mg / L and control group (without B_{12} treatment) ($p < 0.05$). The B_{12} 2.5 mg / L treatment resulted the lowest value of chlorophyll content. Although numerical values for morphology or physiological growth parameters, except chlorophyll content ($p < 0.05$), were higher in B_{12}-treated lettuces compared to the control group (without B_{12} treatment), these differences were not statistically significant ($p > 0.05$), indicating that these trends should be interpreted with caution.

Similar results were reported by Abd El-Bary (2017), where combined treatments of B_{12} and gibberellins at 4 mg / L improved the growth, harvest yield and nutritional quality in pear trees [78]. Furthermore, higher treatment concentrations were associated with increased chlorophyll content, which aligns with the findings of the present study. These results suggest that the chloroplasts may be involved in the biosynthesis pathway of B_{12}, as they are the site of major biosynthesis for compounds such as chlorophyll, fatty acids, and amino acids [28]. Additionally, Keshavarz and Sanavy (2015) found that B_{12} enhanced water use efficiency and better secured chlorophyll, and photosynthesis activity compared to the control group without B_{12} seed priming [74]. Particularly, common bean seeds primed with B_{12} 22 µM showed increased chlorophyll content relative to untreated control. Their studies suggested that B_{12} plays an important role in carbohydrate enhancement by activating Krebs cycle (also known as citric acid cycle) and Pentose phosphate pathway, leading to continuous carbohydrate accumulation. This increase in carbohydrate subsequently resulted in greater shoot diameter and expanded leaf area [74].

The investigation led by Abd El-Bary (2017) demonstrated that B_{12} fortification via leaf application at concentrations of 2 and 4 mg / L resulted in statistically significant improvements in shoot length and diameter, leaf area, relative chlorophyll content, final yield, fruit yield, and biochemical traits of the fruit [78]. In contrast, the present study showed statistically significant differences only in elemental analysis for B_{12} and secondary metabolites with B_{12} treatment, while

morphological and physiological parameters exhibited no significant changes, except for relative chlorophyll content measured by SPAD. This difference may be attributed to several factors: (1) Differences in plant species and growth periods, such as mature pear tees (approximately 10 years old) versus hydroponically grown lettuce cultivated over a short experimental period; (2) Differences in growing conditions, with pear trees planted in clay soil compared to lettuce grown in a controlled hydroponic environment. Overall, lower B_{12} concentrations (0, 2.5, and 5 mg / L) were associated with relatively better morphological and physiological development in lettuce, while higher concentrations (7.5 and 10 mg / L) tended to show reduced development performance based on PlantEye measurements. However, these differences were not statistically significant ($p > 0.05$).

According to PlantEye measurement for the B_{12} fortification via root application, the B_{12} 2.5 and 5 mg / L treatments had higher values in height, digital biomass, hue, greenness, NDVI, NPCI and PSRI compared to the control group without B_{12} treatment. However, these differences were not statistically significant ($p > 0.05$). Similarly, harvest weight measurement suggests that lettuces treated with B_{12} application resulted heavier leaf and root tissues, except the B_{12} 5 mg / L group, but these differences were also not statistically significant. Relative chlorophyll content measured by SPAD meter suggest that the B_{12} treatment enhanced chlorophyll content in lettuce leaf, particularly B_{12} 2.5 and 10 mg / L, yet statistical significance was not reached. Overall, while B_{12} treatments, particularly at 5, 7.5, and 10 mg / L showed trends toward improved lettuce morphology, physiology, and chlorophyll content, these results should be interpreted cautiously due to the lack of significant differences. The observed trends may indicate that B12 root application promotes lettuce growth and development by stimulating cell enlargement or division [74], as reflected by the heavier fresh root weights in B_{12} treated plants, except for the B_{12} 5 mg / L group.

Effect of B_{12} treatment in lettuce water management and gas exchange

The results from both leaf and root application experiment indicated that lettuce treated with B_{12} 5 mg / L demonstrated more efficient water management along with effective gas exchange. This suggests that B_{12} 5 mg / L treatment may help sustain higher photosynthetic rates relative to the amount of water loss through transpiration. However, it is also possible that the observed increase in water use efficiency (*WUE*) results from reduced stomatal conductance, which limit water loss rather than actively enhancing photosynthetic capacity. Overall, relatively

minor variations were observed among B_{12} concentrations in intrinsic water use efficiency (*iWUE*) and stomatal limitation (L_s), compared to broader differences in *WUE* across increasing light intensities. Higher *iWUE* values generally indicate maximised net carbon assimilation with minimal water loss, and the 5 mg / L treatment appears to enhance integral regulation of gas exchange without additional water loss compared to the control. Slightly higher Ls values were found in the 5 mg / L treatment for both application methods.

These combined results from *WUE*, *iWUE*, and L_s measurements indicate that B_{12} treatment, particularly 5 mg / L in both leaf and root applications, enhances stomatal efficiency and water use in lettuce, while also showing relatively higher stomatal limitation. These findings suggest that B_{12} treatment may improve the balance between gas exchange and water conservation, potentially contributing enhanced photosynthetic efficiency and water use under varying light conditions, ranging from low light (200 µmol m^2s^{-1}) to near-saturating condition (1800 µmol m^2s^{-1}). However, it is important to note that statistical analysis was not conducted on the LI-COR data due to dataset limitations. Therefore, these observed physiological trends related to water use efficiency and gas exchange should be considered preliminary observations. Additionally, further investigation into long-term physiological responses a photosynthetic assimilation rates is needed to fully determine the mechanisms.

Effect of B_{12} treatment in lettuce secondary metabolites

The present study profiled key plant-derived functional metabolites, including total phenolic, flavonoid and antioxidant activity. Secondary metabolites play crucial roles in plant physiology by enhancing tolerance to various environmental stress, both biotic and abiotic. Moreover, these compounds are important for human health due to their antioxidant properties and ability to scavenge free radicals [175]. Overall, the secondary metabolites analysis revealed that the B_{12} leaf application at 5 mg / L resulted in the highest levels of total phenolic content (TPC) and total antioxidant activity (TAA), while the control group (without B_{12} treatment) exhibited the highest total flavonoid content (TFC). These findings align with the work of Pitzschke et al. (2015), who reported that vitamin B group activates the plant antioxidant system by stimulating the biosynthesis of phenolics and proline, thereby enhancing stress tolerance [176].

Consistently, the B_{12} 5 mg / L group showed the lowest plant senescence reflectance values measured by PlantEye, suggesting that the B_{12} 5 mg / L

treatment may induce plant protection mechanisms that improve plant resilience to stress [168]. Additionally, the B_{12} 5 mg / L treatment was associated with improved water use efficiency and better harvest quality in the present study. Supporting these results, Supapvanich et al. (2020) demonstrated that B_{12} treatment like 0.1 µM (0.136 mg / L) enhanced antioxidant activity and increased phenolic and flavonoid content in sunflower and daikon sprouts during storage [177]. Overall, these findings suggest that B_{12} 5 mg / L treatment promotes phenolic biosynthesis within plant cell, which may contribute to enhanced growth, development, and stress resistance in lettuce.

Interestingly, the TAA showed a negative correlation with relative chlorophyll and hue measurement. This aligns with the study by Hameed et al. (2023), which reported higher antioxidant levels in green lettuce varieties [178]. Additionally, TFC analysis revealed that the control group (without B_{12} treatment) had the highest greenness values, suggesting that flavonoids may play a role in pigment development in lettuce leaf tissues. Keshavarz and Sanavy (2015) concluded that B_{12} application can stimulate antioxidant enzyme activity, which helps mitigate reactive oxygen species (ROS) by inducing endogenous phenolic compounds in common beans [74]. Their study showed that B_{12} stimulates phenolic compound synthesis in common beans, resulting in improved antioxidant activity in the seedlings [74]. These findings are consistent with the present study, where lettuces treated with B_{12} 5 mg / L via leaf application exhibited the highest TPC and TAA values. Interestingly, the present study also found that B_{12} leaf application resulted in significantly higher TPC values compared to root application. This may be due to phenolic biosynthesis being more actively stimulated in leaf tissues, making foliar B_{12} treatment more effective at increasing phenolic content in lettuce leaves. However, no significant difference between the application methods for TFC and TAA in the leaf application experiment.

The results of B_{12} fortification via root application presented an increasing trend in TPC with rising the B_{12} concentrations. This suggests that higher B_{12} concentration may stimulate phenolic biosynthesis within lettuce cells. Interestingly, the B_{12} 2.5 mg / L treatment applied through the root resulted in significantly higher flavonoid content in lettuce leaf tissues. According to Lavecchia et al. (2013), flavonoids are involved not only stress tolerance mechanisms but also contribute to plant pigmentation [175]. This supports the present study's findings, where lettuces treated with B_{12} 2.5 mg / L via root application exhibited the highest TFC values, which corresponded with increased hue, greenness, and vegetation index values, and optimal harvest quality.

General overview

The comprehensive results of B_{12} fortification via leaf application indicate that the B_{12} 5 mg / L treatment produced the most optimal outcomes in morphology, physiology, phytochemical attributes. The high level of total phenolic and antioxidant activity corresponded with the lowest plant senescence index, as well as efficient water management and stomatal function supporting photosynthetic activity. This enhanced photosynthesis and phytochemical profile contributed to improved harvest quality with better visual appeal and nutritional value. In contrast, the B_{12} 2.5 mg / L via leaf application showed relatively lower photosynthetic performance, with low performance stomatal regulation. This treatment also recorded lower hue and relative chlorophyll values, along with the highest plant sentence index. The B_{12} 10 mg / L treatment resulted in reduced biomass development, despite a high accumulation of B_{12} in both leaf and root tissues. Additionally, lettuces treated with B_{12} 10 mg / L exhibited relatively lower flavonoid levels, suggesting that this higher concentration may induced stress in the plants and negatively correlated with chlorophyll content.

Throughout the comprehensive investigation of B_{12} fortification via root application, the B_{12} 2.5 mg / L treatment emerged as the most favourable in terms of plant morphology, physiological performance, and phytochemical development. In particular, this treatment resulted in high levels of total flavonoid content, which is associated with enhanced stress tolerance. This aligns with the observed improvement in stomatal regulation in lettuces treated with B_{12} 2.5 mg / L via root application, contributing to more efficient photosynthetic activity. Improved photosynthesis, in turn, supported enhanced structural development, such as increased leaf area and healthy green pigmentation, ultimately leading to better harvest quality and higher fresh weight in both leaf root tissues.

Conversely, the B_{12} 5 mg / L treatment via root application resulted in relatively lower fresh weight at harvest. Interestingly, this treatment showed elevated antioxidant activity in leaf tissues, which may have contributed to improved water management and stomatal regulation during photosynthesis. However, this did not appear to correlate with chlorophyll content or normalised pigment concentration values. The varying statistical significance observed across the measured traits highlights that B_{12} effects on lettuce development is not uniform but rather selectively enhanced certain physiological or biochemical responses depending on concentration and application method.

3.4 Conclusions

The comprehensive investigation into B_{12} fortification via both leaf and root application has provided valuable insights into B_{12} uptake, translocation and accumulation within the lettuce, as well as its effect on plant growth, development, and quality. and analyse the effects of B_{12} on lettuce growth, development, and quality. Based on the findings, the following conclusions can be drawn:

- B_{12}-fortified lettuce grown hydroponically via both leaf and root application showed B_{12} content high enough to exceed the adult recommended daily intake (2.4 µg / day), even with small consumption amounts of around 1.7 g of fresh lettuce for root-treated and 4.8 g for leaf treated lettuce. These findings demonstrate the strong potential of B_{12} biofortification as a practical dietary strategy to address B_{12} deficiency.

- The higher concentration of B_{12} application showed increases in uptake and translocate of B_{12} in lettuce tissues for both leaf and root tissues. For instance, B_{12} 10 mg / L via leaf application presented the highest level of B_{12} in both leaf and root tissues, while the lettuces treated with B_{12} 7.5 mg / L via leaf application recorded the highest value of B_{12} in lettuce tissues.

- Overall, significant amount of B_{12} were quantified in lettuce leaf and root tissues via root application compared to leaf application. However, leaf application is more cost effective considering the total amount of B_{12} has been applied.

- Between the leaf and root application for B_{12} fortification, lettuces treated B_{12} via root application contained significantly higher B_{12} content in leaf and root tissues compared to those treated via leaf application. However, leaf application method is more cost-effective method to fortify B_{12} in the targeted plant tissues as it requires 100 times less amount of B_{12} for final treatment volume. Considering edible part of the plant, the application method can be viable as; B_{12} fortification via leaf application for the leafy vegetables or fruits, while root application can be considered for root vegetables.

- Although, there was no statistical significance in the B_{12} concentration, the comprehensive analysis for the present study indicated that B_{12} 5 and 10 mg / L treatments promoted growth and development performances via both

leaf and root application. These results also aligned with their marketable quality and high level of B_{12} content in lettuces leaf tissues.

- Lettuces treated with B_{12} 2.5 mg / L via leaf application may contribute to enhancing phenolic and antioxidant activity in lettuce compared to the control group without B_{12} treatment. This implies that B_{12} fortified lettuces may offer numerous health benefits to human such as better antioxidant attributes.

- Vitamin B_{12} supplements are commonly available in tablets and liquid formulation, but they reach a limited number of targeted demographic, geographic and socioeconomic groups. In contrast, this study suggests the potential B_{12} fortification in food crops, as it can be applied to various types of food crops (e.g., leafy vegetables, root crops and fruiting crops) and disseminated across diverse demographic, geographic and socioeconomic populations.

Chapter 4. Iron Fortification Using Fe_3O_4 NPs

In the present chapter, the study aims to investigate the applicability of iron oxide nanoparticles (Fe_3O_4 NPs) fortification as compared with conventional iron fortification (Fe-EDTA) and their effect on plant growth and development. Fe-EDTA is the most widely used iron chelate for hydro-based cultivations due to their beneficial effects of iron chelates [179,180]. Iron nano-fertiliser is hypothesised to offer various advantages over the conventional iron fertiliser such as Fe-EDTA due to their attributes: (1) Simple process of manufacture; (2) Physiologically safe as it can be controlled by magnetic fields inside human body; (3) Fulfil smart delivery purpose as it can be detected by magnetic resonance imaging technique; (4) Biocompatible and biodegradable with moderate amount of application [118].

This study adopted different synthesis methods to achieve favourable iron oxide nanoparticle for plant applications including co-precipitation method and the combined method of co-precipitation and sol-gel process using spinning disc reactor. Synthesis method plays a critical role in nanoparticles characteristics involving the chemical properties, magnetism, size, surface, and shapes of the particles. Along with the synthesis method, coating agents also impact on nanoparticle properties by modifying structural or conductivity properties [121]. This study used potato starch, L-lysine, and vitamin B_{12} as biodegradable capping agents to enhance stability of Fe_3O_4 NPs[181]. The detailed synthesis and capping procedures are described in **Chapter 2.1.2** and **Chapter 2.1.3**.

4.1 Outcomes of synthesis and characterisation

4.1.1 Size determination

Size is one of the key factors of nanoparticle applications in plants. The aggregation of nanoparticles hinders the uptake and translocation of the nanoparticles into the plant as the size of the nanoparticles restricts the penetration through cell wall in plants, therefore their size must be restricted to 5 to 20 nm [126]. This study conducted transmission electron microscopy (TEM) and dynamic light scattering (DLS) analysis to determine the size and size distribution of Fe_3O_4 NPs. TEM analysis provides the diameter of the metallic nanoparticles due to the high sensitivity of electron-dense regions [160]. Meanwhile, DLS analysis allows

observation of the hydrodynamic size of the nanoparticles corresponding not only to the inorganic core of the nanoparticles but also to the capping agents and adsorbed solvent outer layer [182,183].

The size of the Fe_3O_4 NPs determined by TEM are presented in Figure 4.1. Additionally, the diameters of the Fe_3O_4 NPs evaluated by several hundred particle measurements ranging from 225 to 610 to achieve statistically significant results using Image J software (NIH, USA) [182] . Figure 4.1A presents Fe_3O_4 NPs without capping agents with average value of 5.961 ± 2.029 nm. The results shown in Figure 4.1B indicated that Fe_3O_4 NPs capped with L-lysine had an average value of 5.51 ± 2.08 nm. As shown in Figure 4.1C, Fe_3O_4 NPs capped with starch had an average value of 8.48 ± 3.53 nm. Lastly, Figure 4.1D depicts Fe_3O_4 NPs capped with both starch and B_{12} had an average value of 10.02 ± 4.12 nm. TEM outcomes show the shape, size, and size distribution with synthesised Fe_3O_4 NPs indicating that agglomeration occurs on Fe_3O_4 NPs capped with starch and starch and B_{12}. Furthermore, the results of the morphological traits show both nanorods and sphere shapes topologies.

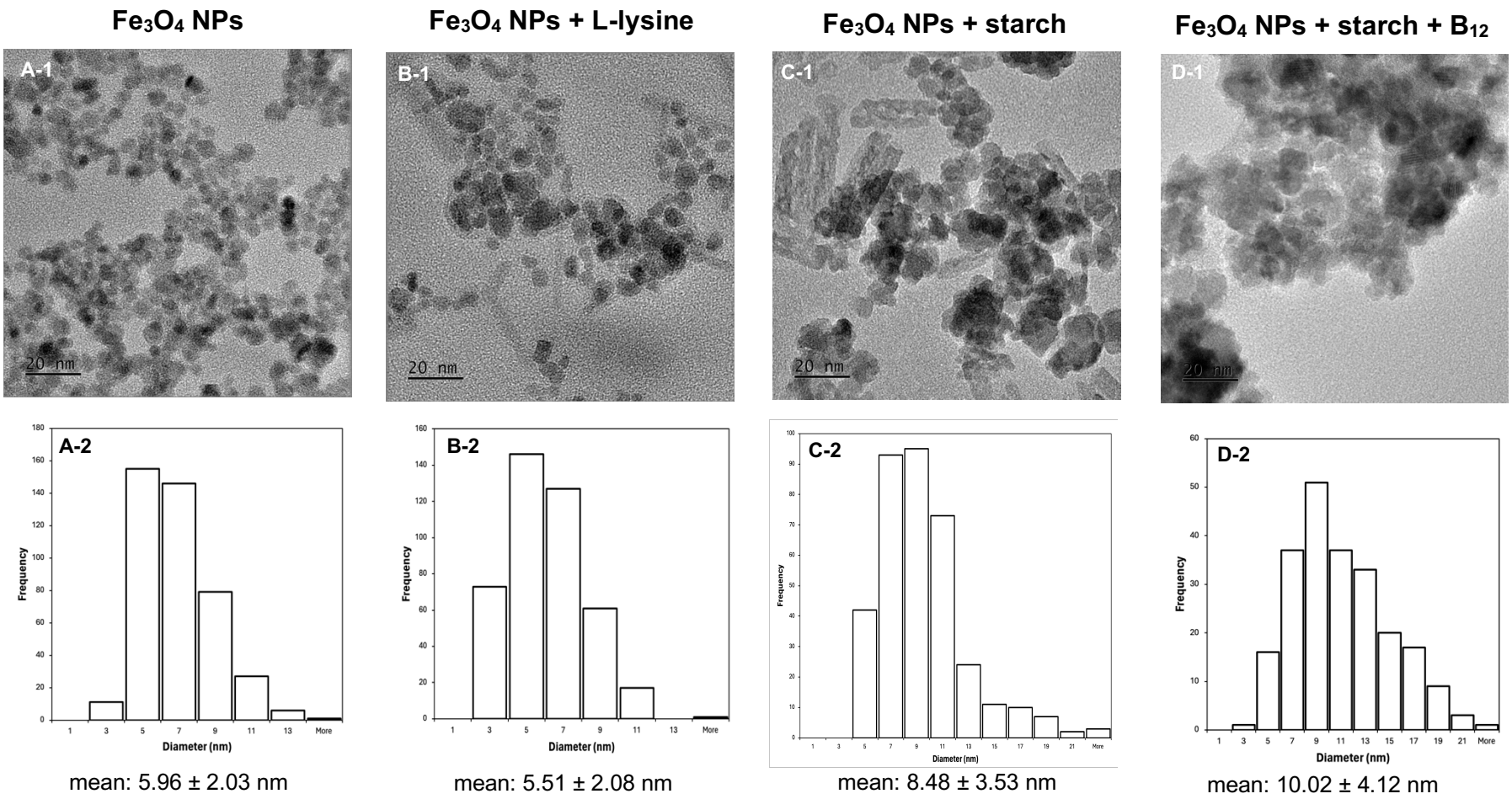


Figure 4.1 TEM images of Fe_3O_4 NPs and corresponding histograms of the size distribution.

A. pure Fe_3O_4 NPs without capping agents **B.** Fe_3O_4 NPs capped with L-lysine **C.** Fe_3O_4 NPs capped with starch **D.** Fe_3O_4 NPs capped with starch and B_{12}. The size distribution = Mean ± SD.

All the nanoparticle samples were dispersed and measured in Type I ultrapure water during characterisation, including dynamic light scattering (DLS) analysis. DLS analysis measurements of the hydrodynamic size distributions of Fe_3O_4 NPs are shown in Figure 4.2A-D, which display intensity (%) and polydispersity index (PDI) data. Most Fe_3O_4 NPs formulations exhibited two to four distinct size populations, with peak intensities generally occurring above 100 nm, except for the L-lysine capped Fe_3O_4 NPs. Notably, the L-lysine capped with Fe_3O_4 NPs (Figure 4.2B) showed the highest intensity peak (87.5 %) at a diameter of 74.89 nm, indicating a narrower and smaller particle distribution compared to other treatments.

Figure 4.2E-H presented the number-based (%) size distribution of Fe_3O_4 NPs, showing the mean particle diameter and standard deviation of the dominant peak in each synthesised nanoparticle. The results indicate that most Fe_3O_4 NPs had average size below 100 nm, except for the Fe_3O_4 NPs capped with both starch and B_{12}. Specifically, uncapped Fe_3O_4 NPs had an average diameter of 59.85 ± 37.84 nm (Figure 4.2E), while those capped with L-lysine had a smaller size of 18.87 ± 6.06 nm (Figure 4.2F). Fe_3O_4 NPs capped with starch measured 41.53 ± 22.37 nm (Figure 4.2G). In contrast, Fe_3O_4 NPs capped with both starch and B12 exhibited a much larger average size of 236.7 ± 240.8 nm (Figure 4.2H), with a broader distribution. These results suggest that the majority of nanoparticles, by number, were below 100 nm in aggregation or complex formation may have occurred.

The overall particle sizes measured by DLS differed from those observed using TEM. This discrepancy can be explained by several factors: (1) DLS measures the hydrodynamic diameter, which includes the particle core along with any surface-bound molecules or solvation layers, rather than the actual core size observed by TEM; (2) The Fe_3O_4 NPs were not perfectly monodispersed, leading to a broader size distribution; (3) The DLS results were heavily affected by the presence of larger particles, which can disproportionately influence the measurement due to their higher scattering intensity. Although they may be fewer in number, they can skew the overall size distribution [184]; (4) Partial aggregation, particularly in Fe_3O_4 NPs capped with both starch and B_{12}, may have contributed to the increase hydrodynamic diameter observed, further distorting the DLS results.

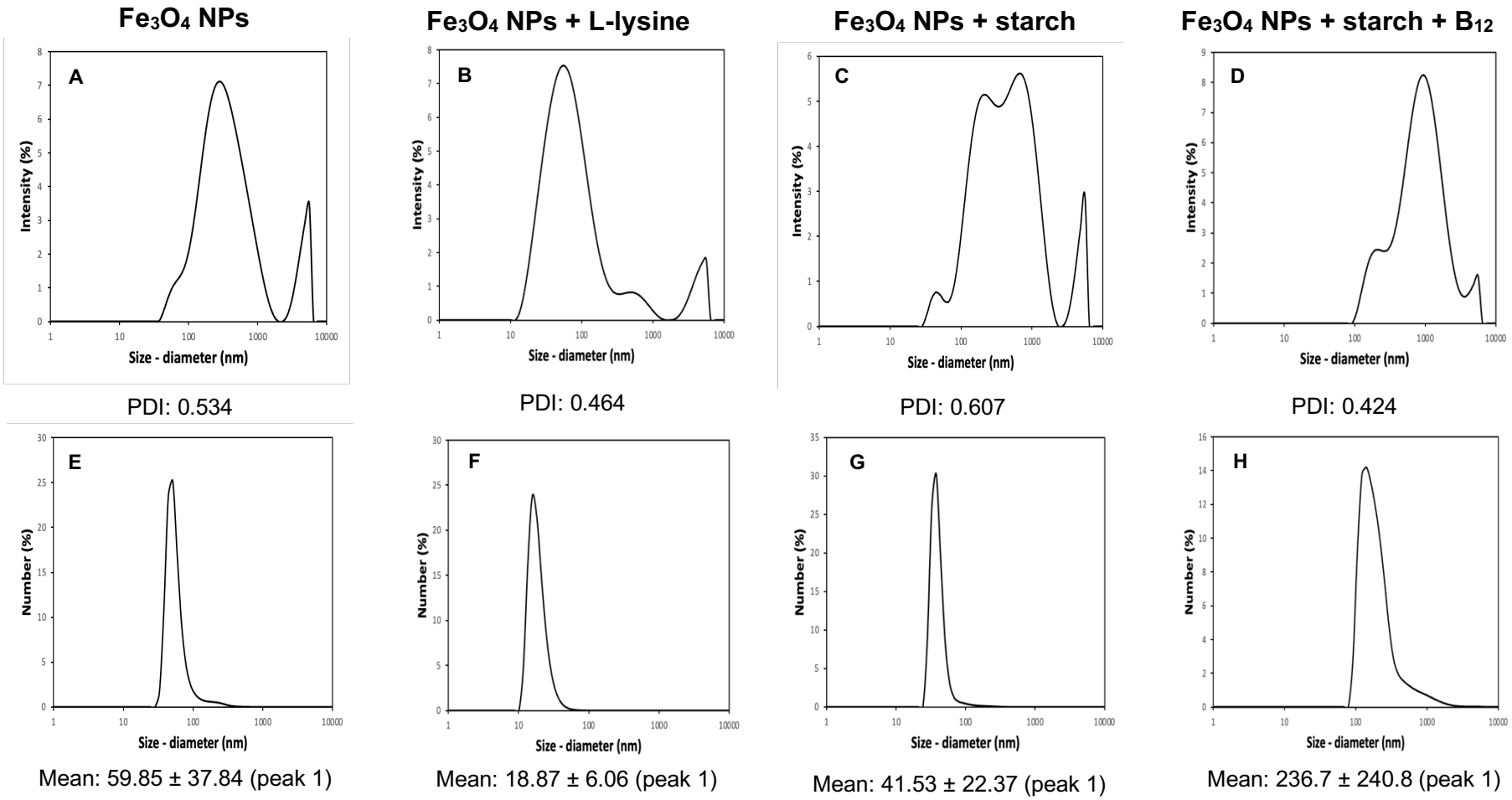


Figure 4.2 Size distribution of Fe_3O_4 NPs by number and intensity determined by DLS.

Polydispersity index (PDI) and z-potentials**.** The graphs showing the size and number (%) of nanoparticles present the mean ± SD of the highest peak. Fe_3O_4 NPs are included: **A.** pure Fe_3O_4 NPs without capping agents, **B.** Fe_3O_4 NPs capped with L-lysine, **C.** Fe_3O_4 NPs capped with starch, and **D.** Fe_3O_4 NPs capped with starch and B_{12}.

4.1.2 Surface characteristics

One of the important roles of the capping agents for Fe_3O_4 NPs is enhancing the stability for further application to plants. Once the nanoparticles are coated with capping agents, the surface chemistry is altered. This prevents aggregation of the particles and reduces the chance of nanoparticle agglomeration[183]. The zeta potential of the Fe_3O_4 NPs was measured by DLS to determine the electrical potential and chemistry. **Table 4.1** shows the zeta potential results with standard deviation (mV). All the nanoparticle samples were dispersed and measured in Type I ultrapure water during characterisation, including both zeta potential and DLS analysis.

All the nanoparticles coated with capping agents show positive zeta potentials: Fe_3O_4 NPs capped with L-lysine (35.8 ± 4.33 mV); Fe_3O_4 NPs capped with starch (29.2 ± 4.51 mV); and Fe_3O_4 NPs capped with starch and B_{12} (16.1 ± 3.04 mV). The electrical potential of the surface of the uncoated Fe_3O_4 NPs was -6.23 mV and deviation of 3.37 mV. The outcomes of electrical potentials and chemistry of the surface presented that the capping agents including L-lysine and starch improved the stability of NPs as it increased the electrostatic repulsions. The changes of the zeta potentials indicated the surface of NPs were changed due to capping agents.

Table 4.1 Zeta potentials of Fe_3O_4 NPs (mV): mean of Z-potentials ± SD

Sample name	Zeta potentials (mV)	SD (mV)
Fe_3O_4 NPs	-6.2	3.37
Fe_3O_4 NPs + L-lysine	35.8	4.33
Fe_3O_4 NPs + starch	29.2	4.51
Fe_3O_4 NPs + starch + B_{12}	16.1	3.04

As a spectroscopic technique, Fourier transform infrared (FT-IR) spectroscopy was used to determine the surface characteristics depicting the infrared spectra of Fe_3O_4 NPs and the capping agents (pure L-lysine, potato starch, vitamin B_{12}) (Figure 4.3). The interactions between Fe_3O_4 NPs and the capping agents on the surface of nanoparticles present as shifts in the wavelengths. The potato starch presents bands between 3800 and 2930 cm^{-1} representing O-H bond stretching while the peaks lower than 1076 cm^{-1} represent C-H bond bending[185]. A band at 1295 cm^{-1} suggested some part of amylopectin and the region of 1150 to 800 cm^{-}

[1] indicated the formation of polymeric chains [186]. FT-IR absorbance observed for vitamin B_{12} were presented at the peaks of 3329 and 3191 cm^{-1} due to N-H stretching in amid I. The peaks between 2974 and 2932 cm^{-1} might present aliphatic C-H stretching and the peak at 1659 cm^{-1} presents C=O stretching in the amide I of the propionamide side chains of the corrin ring. The peak at 1572 cm^{-1} presenting the breathing mode of the corrin ring, the peaks between 1397 to 1347 cm^{-1} presenting C-H deformation, and the peaks at 1213, 1142 and 1058 cm^{-1} presenting C-O stretching vibration [187,188].

For Fe_3O_4 NPs capped with starch and starch + B_{12}, the peak appeared at the lower band between 700 cm^{-1} and 550 cm^{-1} indicating the Fe-O stretching band of Fe_3O_4 NPs [137,189]. The peak at 1615 cm^{-1} presents the water layer absorbance by depicting the vibrational mode of water. The FT-IR spectra of Fe_3O_4 NPs capped with starch and B_{12} present some absorbance peaks at 1615, 1148, 1078, 1023 and 550 cm^{-1}. The band of peaks at 550-627 cm^{-1} and 994 - 1023 cm^{-1} seems to have resulted from the interaction between Fe_3O_4 NPs and starch in both Fe_3O_4 NPs capped with starch only and starch and B_{12}. However, there was no vibration was observed above 1950 cm^{-1} in Fe_3O_4 NPs capped with starch and B_{12} compared to the vitamin B_{12} spectrum. This can be explained by the following reasons: (1) Vitamin B_{12} was not fully coated with Fe_3O_4 NPs, and it was washed off after the capping process; (2) The amount of vitamin B_{12} coated with Fe_3O_4 NPs was too small to be detected by FT-IR which also accounted for ICP-MS outcomes.

Figure 4.3 depicts FT-IR spectra of Fe_3O_4 NPs capped with pure L-lysine provided by Dr. Gareth Cave, Nottingham Trent University. Compared to Fe_3O_4 NPs capped with starch and starch and B_{12}, the FT-IR capped with L-lysine presented distinct peaks between 1407 to 1618 cm^{-1} and 2580 to 3350 cm^{-1}. The spectrum of Fe_3O_4 NPs capped with L-lysine indicated that the absorbance peaks at 3348 cm^{-1} present the vibrations of N-H stretching overlapped by OH stretching. The peak at 2920 cm^{-1} indicates the vibrations of C-H stretching of methylene groups and the peaks at 1618 cm^{-1} shows the vibration of C=O stretching vibration. The peaks of below 1407 cm^{-1} indicated C-O stretching vibration[190]. The L-lysine spectrum depicts that NH_2 stretching is observed at 3400 to 3500 cm^{-1}. The absorbance band between 1402 and 1625 cm^{-1} presents asymmetric and symmetric stretching vibration of Carboxylate (COO^-)[190,191]. The overall results indicated the interaction of Fe_3O_4 NPs and L-lysine presented at the band of peaks at 549-559 cm^{-1}, 1407-1618 cm^{-1}, 2858-2937 cm^{-1} and 3470-3500 cm^{-1}.

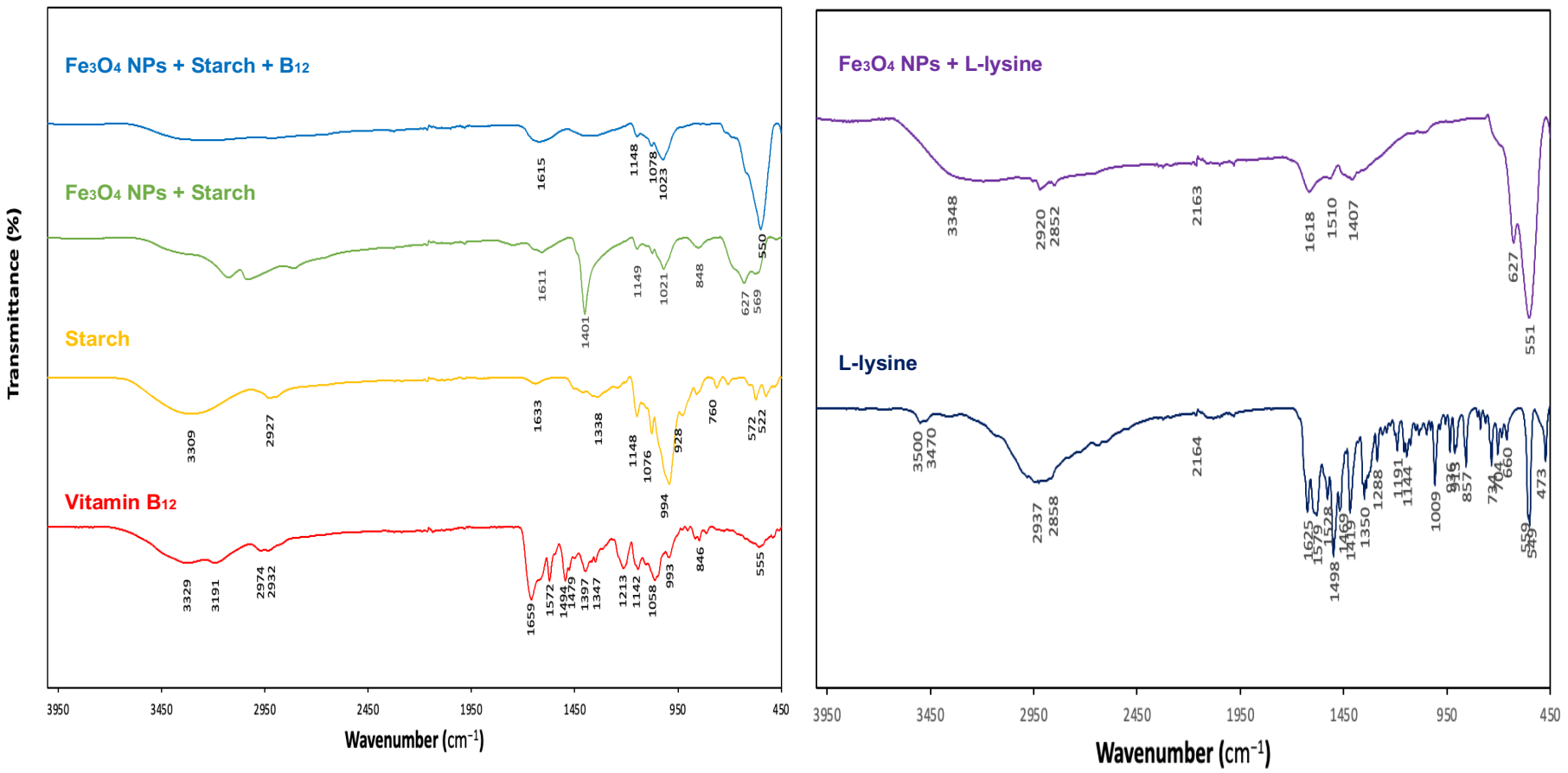


Figure 4.3 FT-IR of Fe_3O_4 NPs capped with starch and vitamin B_{12}.

Coloured lines indicate: Red = B_{12}, Yellow = Starch, Green = Fe_3O_4 NPs + Starch, and Blue = Fe_3O_4 NPs + Starch + B_{12} Navy = L-lysine, Purple = Fe_3O_4 NPs + L-lysine.

4.1.3 Crystalline structure

The crystalline structure of Fe_3O_4 NPs was determined by powder x-ray diffraction (p-XRD). As shown in Figure 4.4 depicts, all the Fe_3O_4 NPs samples presented characteristic difference peaks corresponding to the cubic spinel structure of magnetite (Fe_3O_4), consistent with International Centre for Diffraction Data (ICDD), Card No. 9002319 from Rigaku database. The primary diffraction peaks were observed around 2Θ = 30 - 36 ° and 62 - 63 °, which can be indexed to the (220), (311), and (511) crystal planes.

Fe_3O_4 NPs capped with starch and B12 exhibited distinct peaks at 2Θ = 30.3 ° , 35.7 °, 43.4 °, 57.3 °, and 62.9 °, corresponding to the (220), (311), (400), (511), and (440) planes, confirming a well0defined cubic structure with Fd-m space group symmetry. Fe_3O_4 NPs capped with starch also displayed peaks at 2Θ = 30.1 ° (220), 35.6 ° (311), 43.3 ° (400), and 62.8 ° (440). Uncoated Fe_3O_4 NPs showed a less resolved pattern, with a major peak at 2Θ = 57.9 ° corresponding to the (511) plane. Fe_3O_4 NPs capped with L-lysine exhibited peaks at 2Θ = 35.9 ° (311), 57.4 ° (33), and 62.8 ° (440).

These results confirm that all the Fe3O4 NPs retained the magnetite crystalline phase, although the clarity and intensity of diffraction peaks varied depending on the capping agent, suggesting potential differences in crystallinity or particle surface structure.

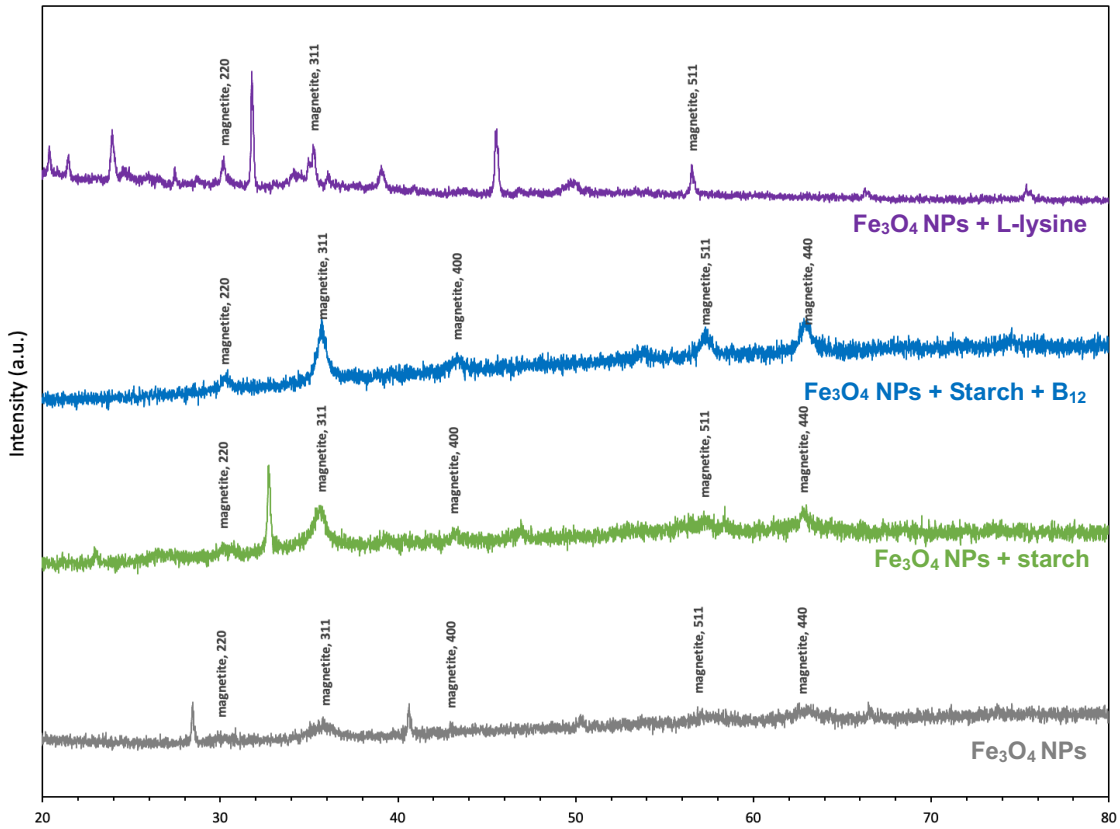


Figure 4.4 p-XRD outcomes of Fe_3O_4 NPs.

Coloured lines indicated: Grey = Fe_3O_4 NPs, Green = Fe_3O_4 NPs + starch, Blue = Fe_3O_4 NPs +starch + B_{12}, Purple = Fe_3O_4 NPs + L-lysine.

4.1.4 Conclusions of nanoparticle characteristics

In the present study, Fe_3O_4 NPs were successfully synthesised using co-precipitation method and a combined approach involving co-precipitation and sol-gel process. To enhance dispersion stability and potential functionality for plant application, the nanoparticles were surface-modified with several biocompatible capping agents, including L-lysine, potato starch, and vitamin B_{12}. These capping agents were selected due to their ability to stabilise the high surface energy of magnetic Fe_3O_4 NPs, which are otherwise tend to aggregation. Additionally, they offer potential nutritional benefits when applied to edible crops, such as enhanced nutrient bioavailability.

Table 4.2 Summary of Fe_3O_4 NPs synthesis and characterisation

Fe_3O_4 NPs with capping agents	Synthesis method	Size distribution (nm)	Hydro-dynamic number-based size % (nm)	Surface charge (mV)	Crystalline structure
Fe_3O_4 NPs without capping	Co-precipitation	5.96 ± 2.02	59.85 ± 37.84	-6.2 ± 3.37	magnetite
L-lysine	Co-precipitation & Sol-gel	5.51 ± 2.08	18.87 ± 6.06	35.8 ± 4.33	magnetite
Potato starch	Co-precipitation	8.48 ± 3.53	41.53 ± 22.37	29.2 ± 4.51	magnetite
Potato starch + B_{12}	Co-precipitation	10.02 ± 4.12	236.7 ± 240.8	16.1 ± 3.04	magnetite

Comprehensive characterisation techniques were used to analyse particle size, surface chemistry, crystalline structure, and the successful attachment of the capping agents. TEM revealed that the core sizes of the Fe_3O_4 NPs ranged from 5.51 and 10.01 nm, confirming their nanoscale dimensions, although some degree of aggregation was observed. DLS was used to access the hydrodynamic size distribution by both intensity and number (%). All nanoparticle samples, except those capped with both starch and B_{12}, exhibited hydrodynamic sizes within the nanoscale range (18.87 to 59.85), consistent with TEM results. The significant larger hydrodynamic size observed in the Fe_3O_4 NPs capped with starch and B_{12} suggests a higher degree of aggregation or larger surface coating.

FT-IR confirmed the presence of characteristic functional groups from the capping agents, indicating successful surface modification. Zeta potential measurement using DLS further supported improved dispersion stability due to increased

electrostatic repulsion. All Fe_3O_4 NPs retained the magnetite crystalline phase, with characteristic peaks corresponding to the cubic spinel structure according to p-XRD analysis.

Among the synthesised Fe_3O_4 NPs samples with different capping agents, a series of preliminary trails were conducted to optimise application methods for hydroponic cultivation. These pilot studies focused initially on Fe_3O_4 NPs without capping agents and Fe_3O_4 NPs capped with both starch and B_{12}. However, the total volume of uncoated Fe_3O_4 NPs synthetised via the co-precipitation method was found to be insufficient for use in subsequent experiments, as the final yield was limited and a large quantity of nanoparticles was required for hydroponic cultivation. Fe_3O_4 NPs capped with both starch and B_{12} were applied in the subsequent pilot trial to assess B_{12} uptake and accumulation in lettuce. However, elemental analysis using ICP-MS revealed no detectable level of B_{12} in either root or leaf tissues, and thus this nanoparticle formulation was excluded from further experiments.

Fe_3O_4 NPs capped with potato starch were selected to evaluate their applicability in **Chapter 4.2**, based on their favourable characteristics and comparatively higher final yield. In **Chapter 4.3**, the performance of Fe_3O_4 NPs capped with L-lysine was evaluated related to conventional iron fertilisers, including Fe-EDTA.

4.2 Iron fortification using Fe_3O_4 NPs

4.2.1 Materials and methods

The present experiment was designed to investigate the effect of Fe_3O_4 NPs on lettuce. The Fe_3O_4 NPs were synthesised via co-precipitation method, as detailed in **Chapter 2.1**. Fe_3O_4 NPs capped with potato starch were selected based on their favourable physiochemical characteristics rand relatively high final yield, as summarised in the characterisation results. Potato starch, as a capping agent, offers biocompatibility and biodegradability while enhancing stability they of the nanoparticles. Additionally, it improves water dispersibility and may provide potential nutritional benefits for further plant application. Following synthesis, the nanoparticles underwent post-synthesis process, including rinsing and drying, to minimise the residual impurities. The resulting highly concentrated solution of Fe_3O_4 NPs capped with potato starch was analysed by ICP-MS to quantify the iron concentration prior to application.

As previously described in **Chapter 2.2.3,** the Fe-EDTA and Fe_3O_4 NPs treatments were prepared based on the molecular weights, accounting for the iron fraction in the molecule to achieve equivalent elemental iron concentrations across treatments. All treatments were standardised to deliver a final concentration expressed as milligrams of elemental iron per litre. However, for data presentation, concentrations are reported as milligram per litre (mg / L). Based on these elemental analysis results, serial dilutions of Fe_3O_4 NPs were prepared using deionised water for subsequent application to lettuce.

In the present study, the Fe_3O_4 NPs capped with potato starch were applied at concentrations of 0.625, 1.25, 2.5 and 5 mg / L. As a control treatment, lettuces plants were treated with Fe-EDTA 2.5 mg / L, prepared and applied according to the Hoagland nutrient solution protocol [163]. Fe-EDTA 2.5 mg / L was chosen as the control because it represents the conventional iron source commonly used in hydroponic cultivation. Including Fe-EDTA as a benchmark enabled a comparative assessment of the applicability and effectiveness of Fe_3O_4 NPs under typical fertilisation condition. All Fe_3O_4 NPs treatments were applied weekly to the root zone in hydroponic tanks alongside Hoagland nutrient fertilisers. Further details on materials and growth conditions are described in **Chapter 2.2**.

4.2.2 Data collection and statistical analysis

This experiment used twelve biological replicates per Fe_3O_4 NPs treatment concentration. A total 10 propagators were utilised, as shown in Figure 4.5. Lettuces plants were harvested at the end of the four-weeks growing period for quantification of iron content in tissues via elemental analysis. Data analysis was conducted as described in **Chapter 2.5.**

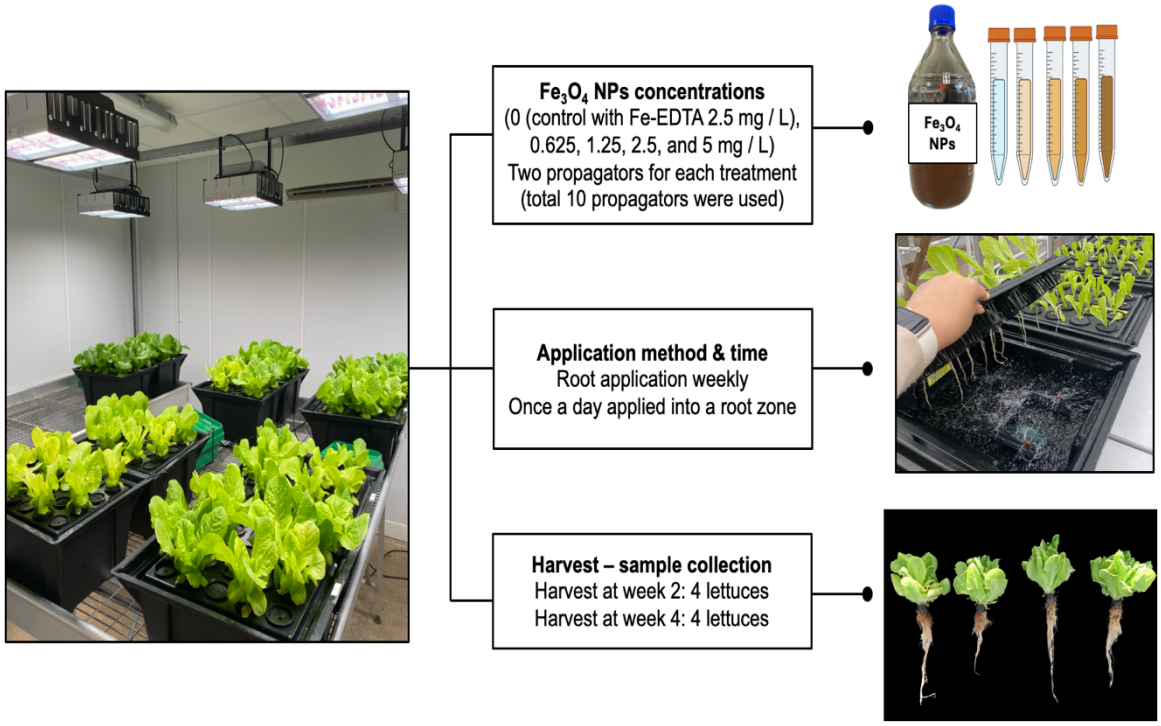


Figure 4.5 Schematic representation of growing condition for Fe fortification via root application.

Fe_3O_4 NPs concentrations (0 (control with Fe-EDTA 2.5 mg / L), 0.625, 1.25, 2.5, and 5 mg / L); Root application method; and harvest details.

4.2.3 Results

This section examines the effect of iron fortification on hydroponically grown lettuce through root application of Fe_3O_4 NPs capped with potato starch. Due to their favourable physiochemical properties, including enhanced dispersive stability and high bioavailability, Fe_3O_4 NPs are hypothesised to deliver iron more effectively to lettuce plants compared to conventional iron fertilisers such as Fe-EDTA. Their nano-size may confer enhanced mobility within the plant system, facilitating more efficient uptake through the roots and translocation to aerial tissues such as leaves. This contrasts with conventional iron fertilisers, which often exhibit limited mobility and bioavailability, resulting in less efficient iron delivery to edible parts of the plant.

The aim of this chapter is to evaluate the applicability of Fe_3O_4 NPs in lettuce cultivation compared to conventional iron fertiliser Fe-EDTA, and to determine the effect of the Fe_3O_4 NPs treatment in lettuce growth, physiology, and nutritional quality. The objectives are: (1) to quantify the uptake, translation, and accumulation of root applied Fe_3O_4 NPs in lettuce leaves and root tissues using ICP-MS; and (2) to assess the effects of varying concentrations of Fe_3O_4 NPs (0.625, 1.25, 2.5, 5 mg / L), in comparison to a control group (Fe-EDTA 2.5 mg / L), on morphological traits, physiological parameters, water management, and nutritional quality such as iron content, secondary metabolites levels.

4.2.3.1 Growth and development measurement

Figure 4.6 show the growth and morphological development of lettuce over the four-week cultivation period following transplanting. Considerable variation was observed across the entire growing phase, reflecting the dynamic effects of Fe_3O_4 NPs capped with potato starch treatments over time. A further notable increase was observed between week 3 and week 4, except in the Fe_3O_4 NPs 0.625 mg / L treatment. The leaf area steadily increased through the growing period for all treatments except the control (Fe-EDTA 2.5 mg / L). A gradual increase in digital biomass over time. Notably, the Fe_3O_4 NPs 2.5 mg / L treatment exhibited a pronounced increase in biomass from week 3 to week 4. In contrast to the growth parameters, the hue and greenness measurements showed a general decline throughout the growing period. The Fe_3O_4 NPs 0.625 and 1.25 mg / L treatment exhibited a continuous in hue across all weekly measurements.

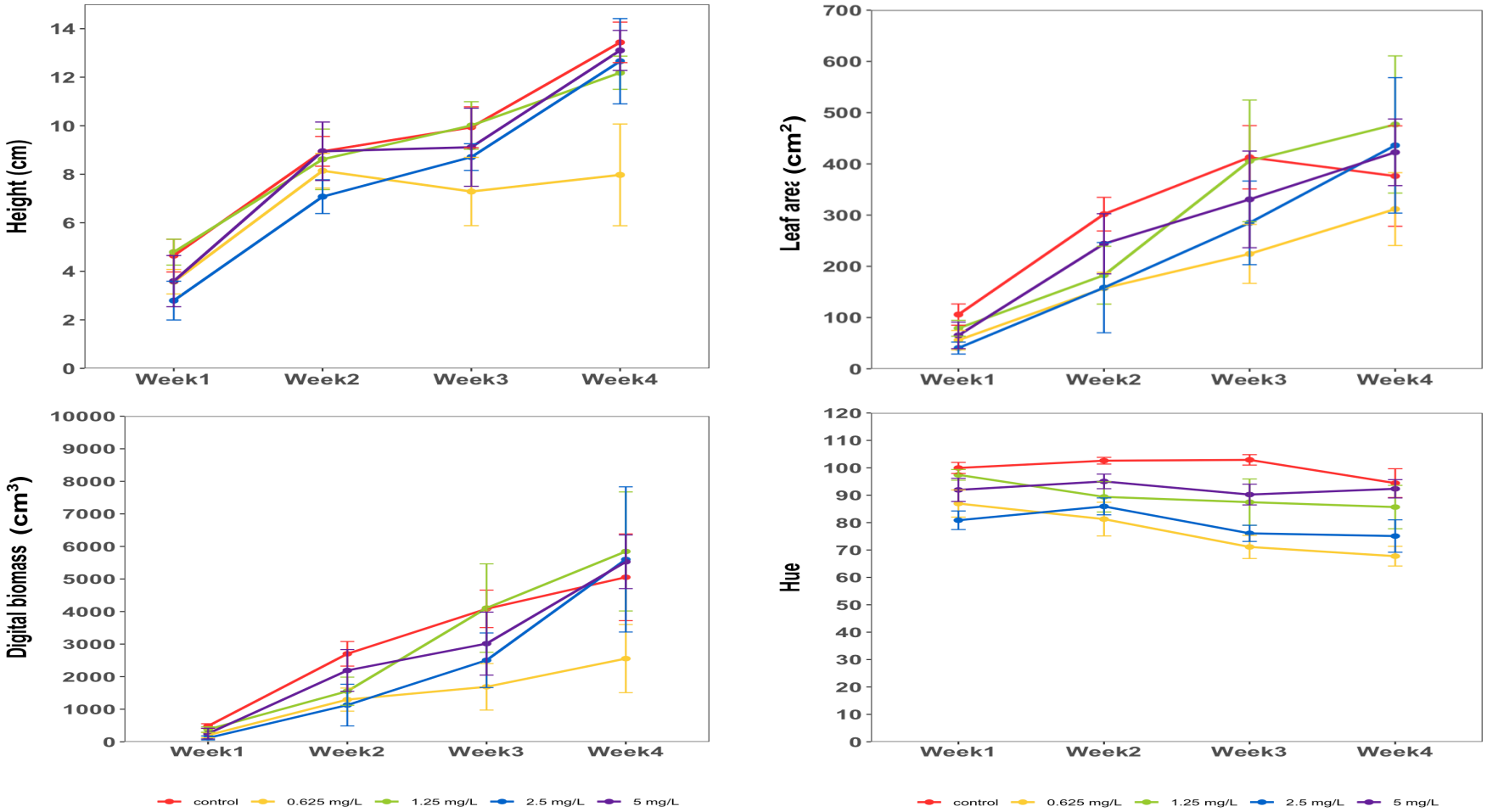


Figure 4.6 Growth and morphological development of lettuces measured weekly four times by PlantEye.

Coloured lines indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg Fe/ L), 0.625, 1.25, 2.5, and 5 mg / L). Data are shown as mean ± standard error (n = 8).

For the greenness, a slight increase was observed between week 1 and week 2 in the Fe_3O_4 NPs 0.625 and 1.25 mg / L treatments. However, from week 2 to week 4, a gradual decline was observed, particularly in the lower Fe_3O_4 NPs concentrations (0.625 to 2.5 mg / L). As shown in Figure 4.7, notable variations were observed in Normalised Differential Vegetation Index (NDVI) which reflects the absorption of photosynthetically active radiation. A continuous decline in NDVI was observed in the Fe_3O_4 NPs 0.625 and 1.25 treatments from week 1 to week 4. In contrast, Fe_3O_4 NPs 2.5 and 5 mg / L treatments showed an initial increase from week 1 and week 2, followed by a steady decline through to week 4.

The Normalised Pigment Chlorophyll Index (NPCI) is an indicator of vegetation health and relates to the interception of photosynthetically active radiation. Healthy vegetation generally exhibits NDVI values ranging from 0.6 to 0.9. The results present that Fe_3O_4 NPs 0.625, 2.5 and 5 mg / L treatments all exhibited a continuous decrease in chlorophyll content between week 2 to week 4.

The plant senescence reflection index (PSRI), which measures plant senescence through the ratio of carotenoid and chlorophyll, was varied considerably across treatment groups throughout the growing period. Weekly PSRI measurement suggest that lettuce plants treated with Fe_3O_4 NPs 0.625 mg / L exhibited greater signs of aging or stressed compared to the control group treated with Fe-EDTA 2.5 mg / L.

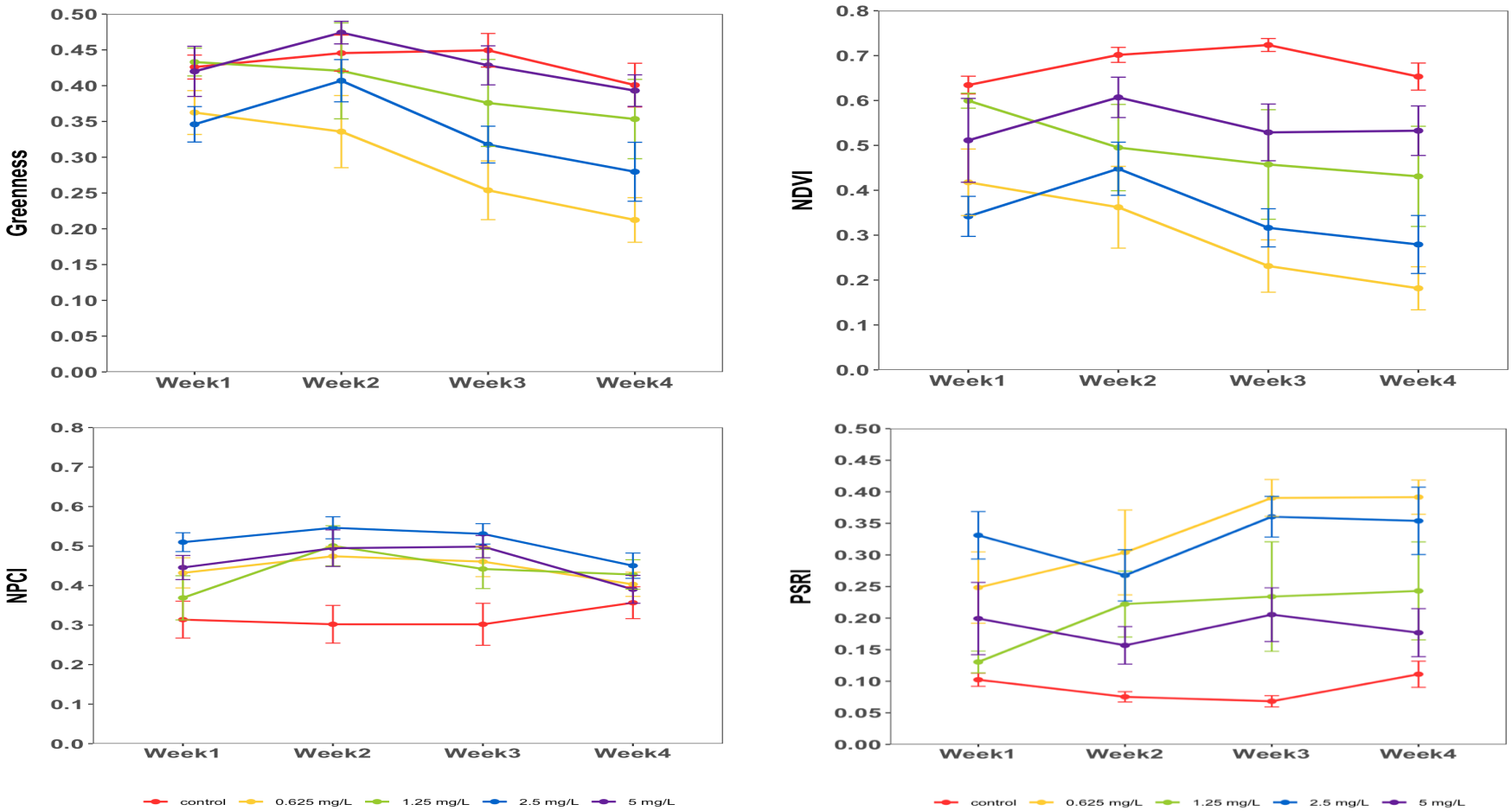


Figure 4.7 Growth and morphological development of lettuces measured weekly four times by PlantEye.

Coloured lines indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L), 0.625, 1.25, 2.5, and 5 mg / L). Data are shown as mean ± standard error (n = 8).

Following the weekly growth assessments, this section focusses on growth and morphological parameters measured at the final harvest (week 4). Statistically significant differences among treatment groups are indicated by different letters, based on PERMANOVA followed by pairwise comparisons using 9,999 permutations.

Figure 4.8 shows plant height (cm^2) and leaf area (cm^3) measured at the final harvest (week 4). Plant height result indicated that the control group (Fe-EDTA 2.5 mg / L) produced the tallest lettuce plants (13.44 ± 0.83 cm), which was 68 % higher than those treated with Fe_3O_4 NPs 0.625 mg / L treatment. The lowest concentration of Fe_3O_4 NPs (0.625 mg / L) treatment resulted the shortest plants (7.97 ± 2.10 cm), which was significantly lower than all other groups ($p < 0.05$). Statistical analysis grouped the treatment into two distinct categories: the control (Fe-EDTA 2.5 mg / L) and Fe_3O_4 NPs at 1.25, 2.5, and 5 mg / L, which were not significantly different ('a', $p > 0.05$), while the Fe_3O_4 NPs 0.625 mg /L treatment, which formed a separate, significantly lower group ('b', $p < 0.05$).

For leaf area, lettuces treated with Fe_3O_4 NPs 1.25 mg / L exhibited the highest mean leaf area value (477 ± 134 cm^2). However, statistical analysis indicated no significant differences in leaf area among the Fe_3O_4 NPs treatment groups ($p > 0.05$).

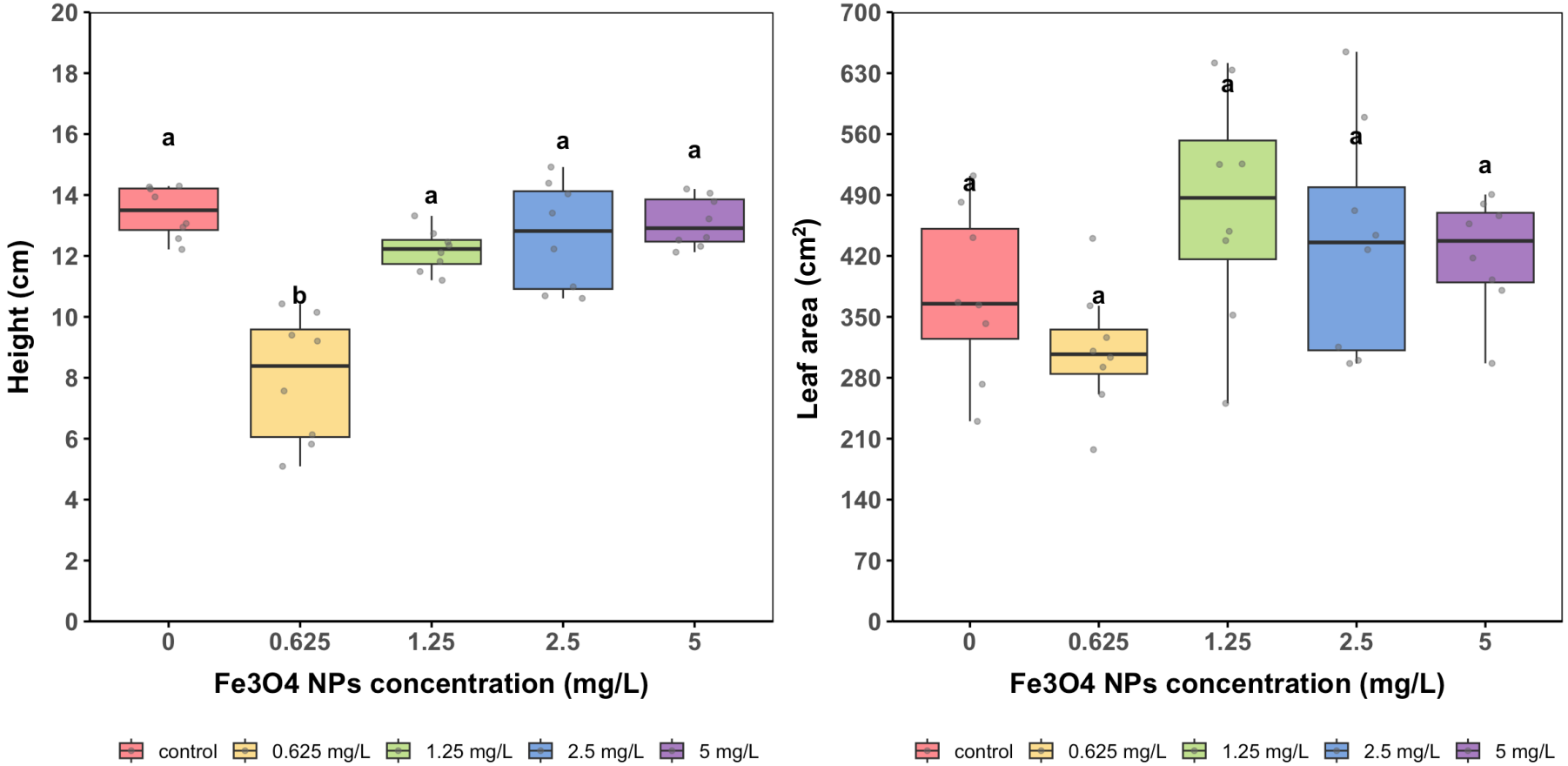


Figure 4.8 Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L), 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). No statistically significant differences were observed between treatments ($p > 0.05$).

Figure 4.9 presents digital biomass (cm3) and hue (º) measured at the final harvest (week 4). Digital biomass is a volumetric estimate generated by the PlantEye scanner, reflecting a combination of plant height and projected leaf area to represent overall lettuce growth. The Fe_3O_4 NPs 1.25 mg / L treatment produced the highest mean digital biomass value (5,846 ± 1,828 cm^3). Statistical analysis grouped the Fe_3O_4 NPs 1.25 mg / L treatment and control (Fe-EDTA 2.5 mg /L) as not significantly different (denoted 'b and 'ab', respectively, $p > 0.05$). In contrast, the Fe_3O_4 NPs 0.625 mg /L treatment formed a distinct, significantly lower group ('a', $p < 0.05$). The Fe_3O_4 NPs 2.5 and 5 mg / L were also grouped with the higher biomass treatment ('b'), indicating that the lowest Fe_3O_4 NPs concentration (0.625 mg /L) had a consistently negative impact on morphological growth compared to other treatments.

Hue measurements, ranging from 0 to 360 º, represent colour and can serve as an indicator of the morphological health of lettuce. Hue values between 90 º to 140 º correspond to green hues, typically associated with healthy plants. Relatively higher average hue values were observed during the early growth stages (week 1 to week 2) compared to the later stages (week 3 to week 4). The highest hue mean value was observed in the control group treated with Fe-EDTA 2.5 mg / L (94.41 ± 5.25 º, $p < 0.05$, 'a'), which was significantly higher than those of the Fe_3O_4 NPs 0.625 ('b'), and 2.5 mg / L treatments ('bc'), corresponding to increases of 29 % and 26 %, respectively.

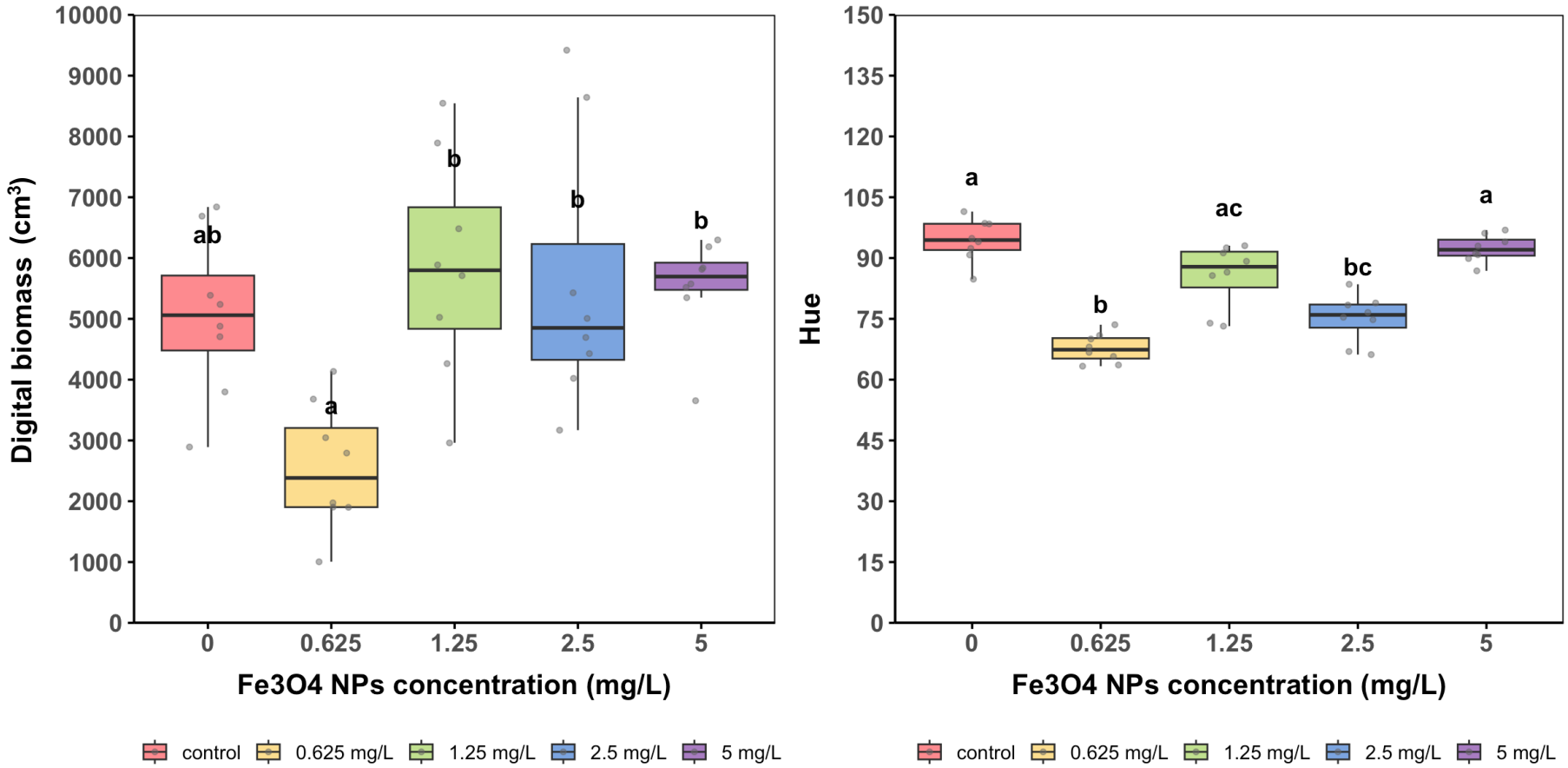


Figure 4.9 Digital biomass (cm^3) and Hue (°) of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L), 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Figure 4.10 presents greenness and Normalised Vegetation Index (NDVI) measured on week 4. Greenness, ranging from -1 to 1, reflects the green leaf index, with higher values indicating greater greenness typically associated with healthy leaves. The highest mean greenness value was observed in the control group treated with Fe-EDTA 2.5 mg / L (0.40 ± 0.03, 'a', $p < 0.05$), which was significantly greater than the values for Fe_3O_4 NPs 0.625 ('b') and 2.5 mg / L ('bc'), corresponding to increases of 89 % and 43 %, respectively. Overall, colour measurements indicate that the control group treated with Fe-EDTA 2.5 mg / L exhibited the healthiest green hues, followed closely by Fe_3O_4 NPs 5 mg / L treatment. In contrast, Fe_3O_4 NPs 0.625 mg / L showed the lowest hue and greenness values, corresponding to a more yellowish coloration and suggesting reduced chlorophyll content.

NDVI is an indicator of vegetation health and relates to the interception of photosynthetically active radiation. Healthy vegetation generally exhibits NDVI values ranging from 0.6 to 0.9. In the present study, the control group (Fe-EDTA 2.5 mg / L) exhibited the highest value (0.65 ± 0.03), which was significantly greater than all other treatment ('a', $p < 0.05$,). Compared to the control, the Fe_3O_4 NPs treatment groups showed decreases of 260 %, 52 %, 134 %, and 23 %, corresponding to 0.625 mg /L ('b'), 1.25 mg /L ('cd'), 2.5 mg /L ('bc'), and 5 mg / L ('d'), respectively. Among the nanoparticle treatments, the Fe_3O_4 NPs 5 mg /L group was significantly higher than the other nanoparticle treatments (0.53 ± 0.06, $p < 0.05$).

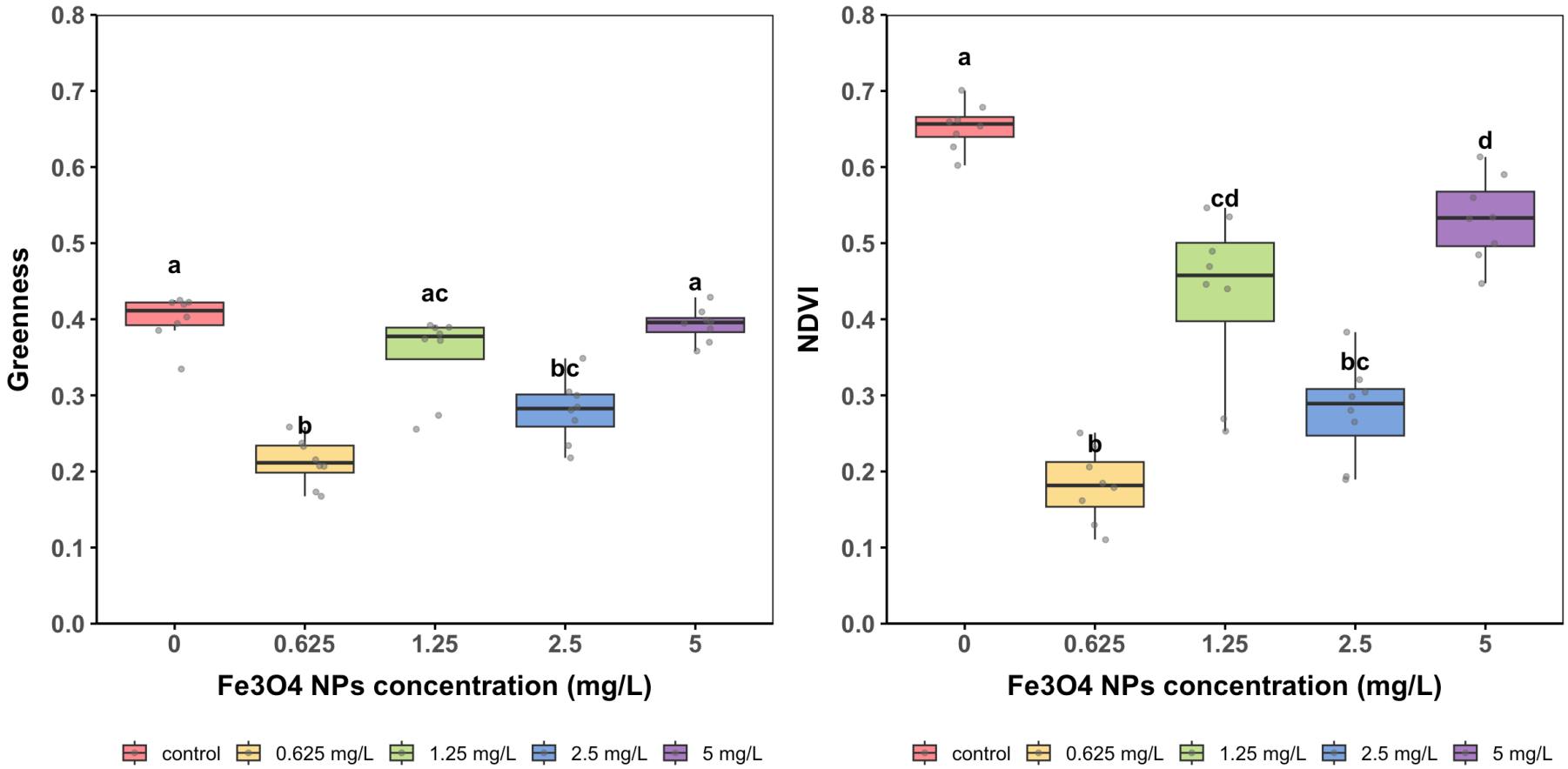


Figure 4.10 Greenness and NDVI of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L), 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Figure 4.11 presents both Normalised Pigment Chlorophyll index (NPCI) and Plant Senescence Reflectance Index (PSRI) parameters. NPCI measurements reflect the ratio between chlorophyll and carotenoids, providing an indicator of the plant's physiological status or stress condition. The highest mean NPCI value was observed in lettuces treated with Fe_3O_4 NPs 2.5 mg / L group (0.45 ± 0.03, 'b'), which was significantly higher than the control group (0.36 ± 0.04, 'a', $p < 0.05$). The control group ('a') differed significantly from the Fe_3O_4 NPs treatments at 1.25 and 2.5 mg /L (both 'b'), which did not differ significantly from each other ($p > 0.05$).

PSRI assess plant senescence and stress by estimating the relative content of carotenoid to chlorophyll, as measured by the PlantEye scanner. The PSRI results showed that the Fe_3O_4 NPs 0.625 mg / L treatment had the highest mean value (0.39 ± 0.03), which was significantly greater than the group of control (Fe-EDTA 2.5 mg / L, 'a'), Fe_3O_4 NPs 1.25 ('cd'), and 5 mg / L ('d'), with increases of 252 %, 61 % and 121 %, respectively ($p < 0.05$). The control group exhibited the lowest PSRI values, indicating relatively better plant health, followed by the Fe_3O_4 NPs 5 mg / L treatment.

Overall, the growth and morphological measurements suggested that the lettuces treated with the lowest concentration of Fe_3O_4 NPs (0.625 mg / L) resulted in significantly lower performance across most parameters, including plant height, leaf area, digital biomass, NDVI and colour indices ($p < 0.05$), indicating iron deficiency symptoms such as yellowing leaves. In contrast, the control group treated with Fe-EDTA 2.5 mg / L generally performed comparably to the higher Fe_3O_4 NPs concentration (1.25 to 5 mg / L), with no statistically significant differences observed in plant height, leaf area, digital biomass, hue, greenness, and NPCI between the control and the Fe_3O_4 NPs 5 mg / L treatment ($p > 0.05$). These results suggest that while the lowest nanoparticle concentrations were insufficient, higher concentration of Fe_3O_4 NPs (above 1.25 mg /L) could support similar growth and physiological responses as the conventional Fe-EDTA treatment. However, the variability and reduced performance at the lowest concentration may point to challenges in bioavailability.

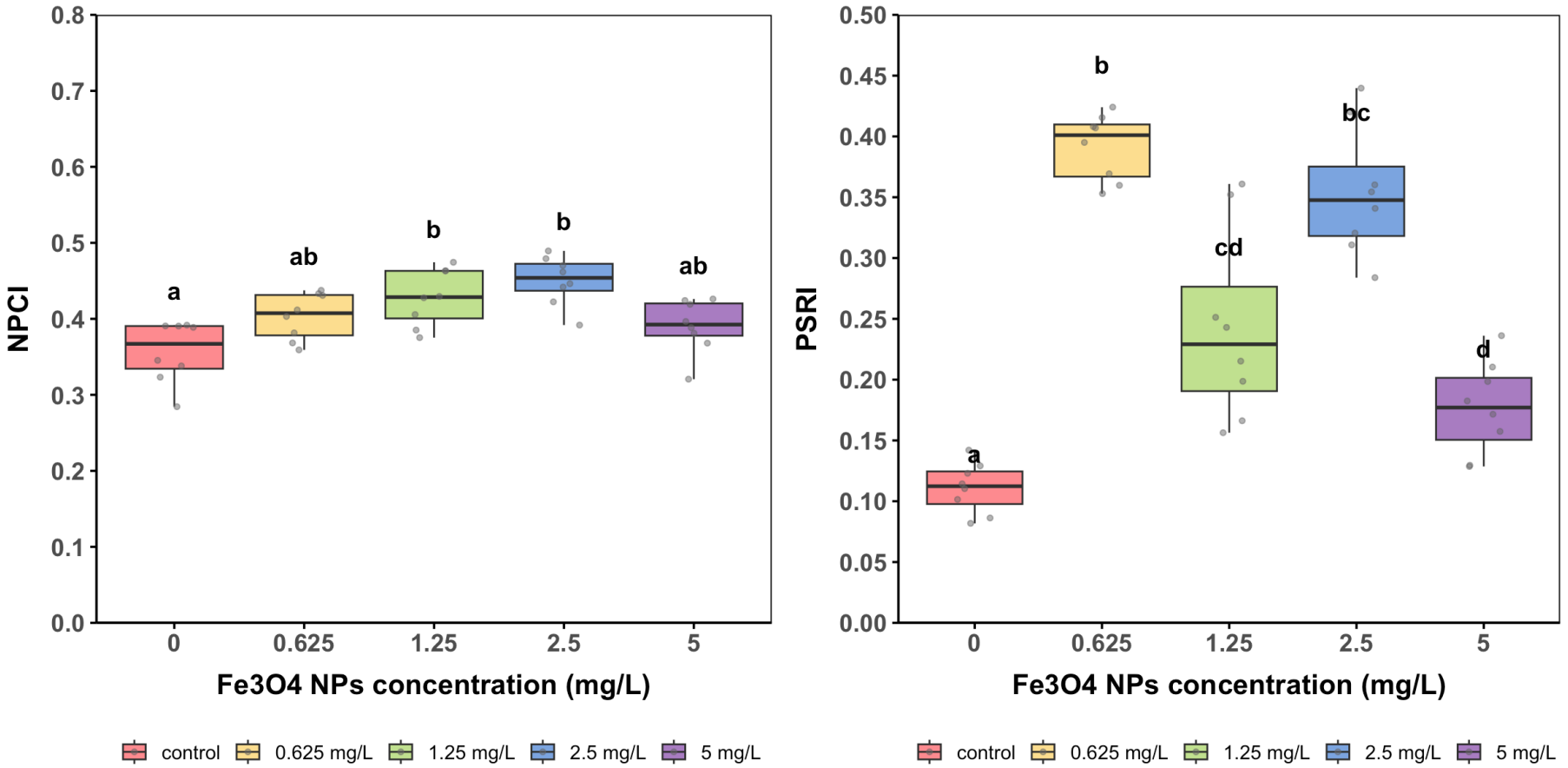


Figure 4.11 NPCI and PSRI of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L, 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

4.2.3.2 Water management and gas exchange

The final (week 4) measurements of water management efficiency and gas exchange in lettuce, including Water Use Efficiency (*WUE*), Intrinsic Water Use Efficiency (*iWUE*) and Stomatal Limitation (L_s), were obtained using LI-COR system. Although measurements were recorded weekly throughout the growth period, only the week 4 data are highlighted in this section to reflect the final physiological state of the plants at harvest, offering insights into the cumulative treatment effects on water use efficiency and gas exchange dynamics by the end of the experiment.

Water Use Efficiency (WUE)

Water Use Efficiency (*WUE*), which represents the ratio of carbon dioxide assimilation to water loss through transpiration, was measured at week 4 and is highlighted in Figure 4.12. The highest *WUE* values (1961, 2868, and 2792 µmol CO_2/mmol H_2O) were observed in the Fe_3O_4 NPs 5 mg / L treatment as light intensities increased from 200 to 1800 µmol m^2s^{-1}. These findings suggest that lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited improved use efficiency compared to those treated with Fe_3O_4 NPs 0.625 and 2.5 mg / L treatments. However, it is also possible that the increased *WUE* resulted not from enhanced photosynthetic capacity but from reduced stomatal conductance, which limits water loss. This interpretation highlights the need to consider stomatal behaviour alongside *WUE* in assessing physiological performance.

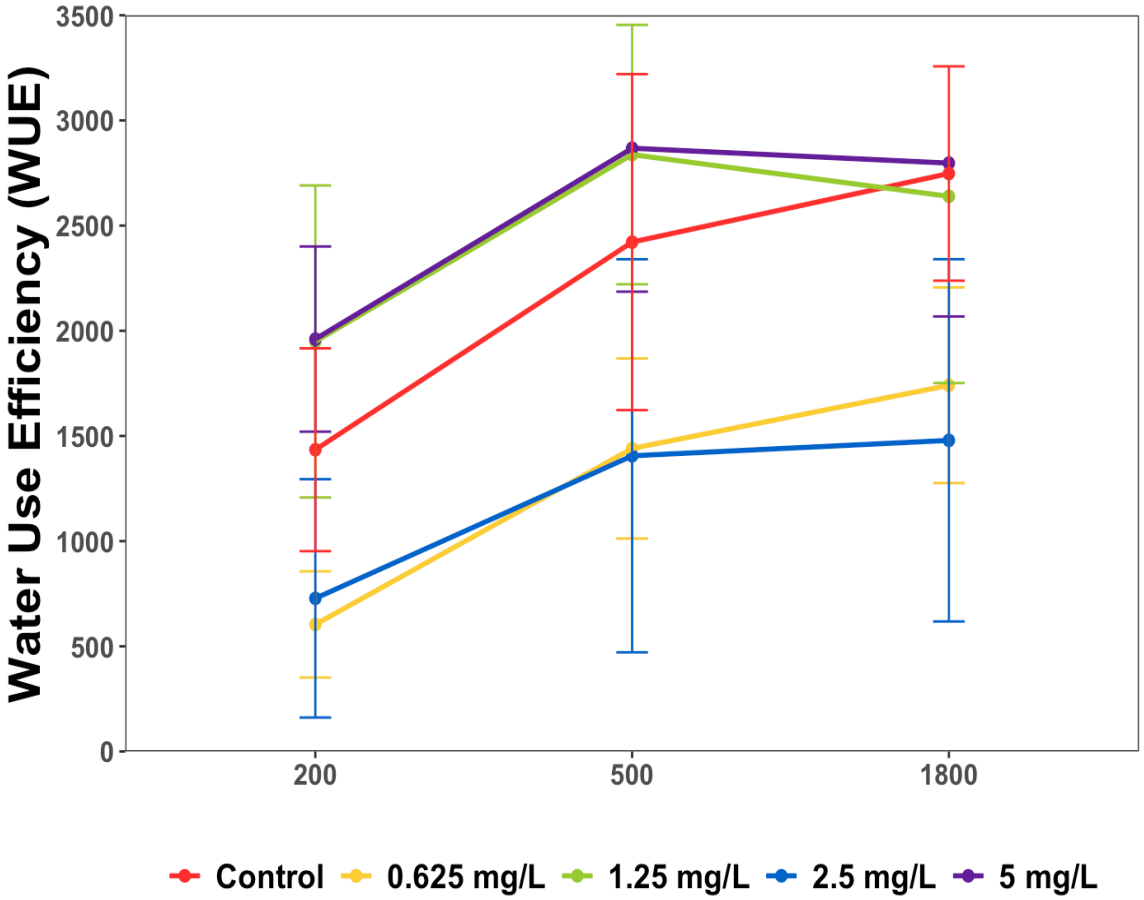


Figure 4.12 Water Use Efficiency (WUE) measured using LI-COR at Week 4.

Water use efficiency (WUE), calculated as net assimilation rate (A) divided by transpiration rate (E), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg Fe/ L), 0.625, 1.25, 2.5, and 5 mg / L).

Intrinsic Water Use Efficiency (iWUE)

Intrinsic Water Use Efficiency (*iWUE*), a direct indicator of stomatal activity reflecting the balance between carbon assimilation and water loss through stomatal opening and closing, was measured weekly, but only the week 4 results are highlighted in this chapter (Figure 4.13). The Fe_3O_4 NPs 1.25 mg / L treatment showed the highest *iWUE* values of 43.13 and 67.34 µmol CO_2/mmol H_2O) at light intensities of 200 and 500 µmol m^2s^{-1}, respectively. At the light intensity of 1800 µmol m^2s^{-1}, the Fe_3O_4 NPs 5 mg / L group recorded the highest *iWUE* value (76.74 µmol CO_2/mmol H_2O). These results suggest that at harvest, lettuces treated with Fe_3O_4 NPs 1.25, and 5 mg / L maintained better stomatal regulation and potentially reduced water loss during photosynthesis compared to the control treated with Fe-EDTA 2.5 mg /L and other treatment (Fe_3O_4 NPs 0.625 and 2.5 mg / L). Alternatively, the elevated *iWUE* could indicate a stomatal limitation on gas exchange, where carbon assimilation is sustained despite a reduction in stomatal aperture.

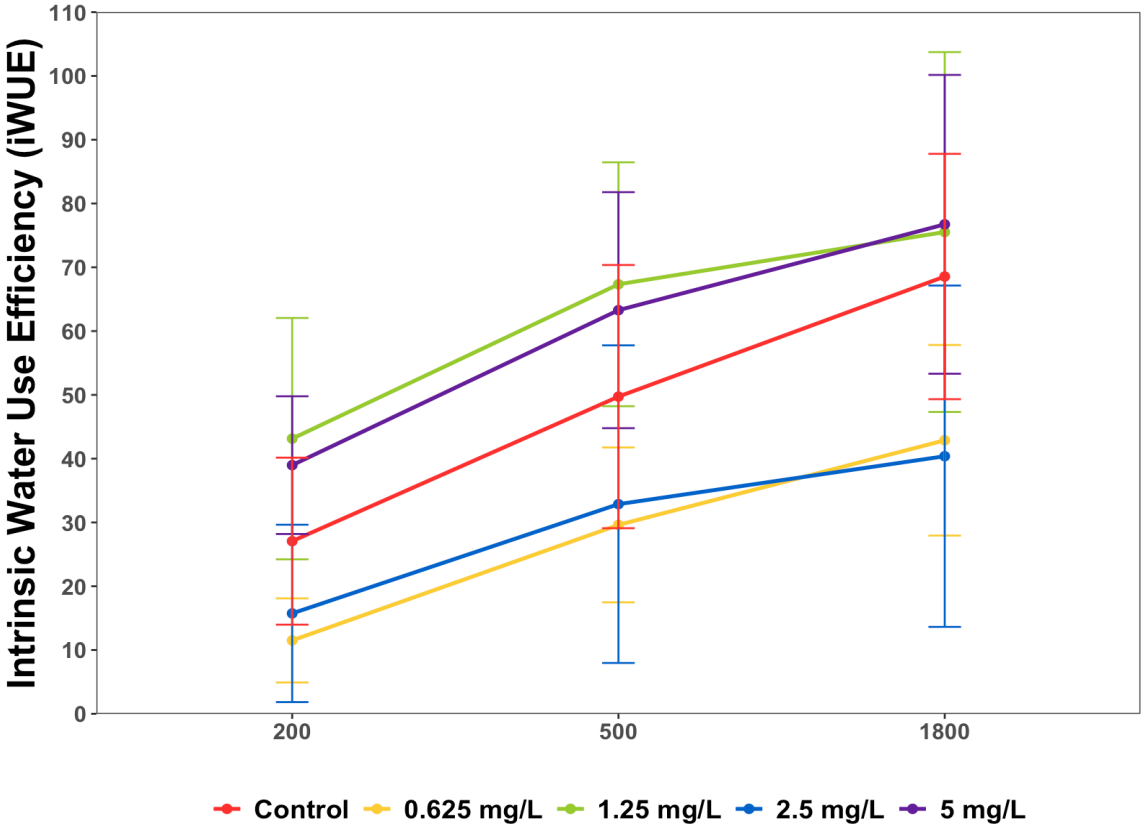


Figure 4.13 Intrinsic Water Use Efficiency (iWUE) measured using LI-COR at Week 4.

Intrinsic Water use efficiency (iWUE), calculated as net assimilation rate (A) divided by stomatal conductance to H_2O (gsw), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg Fe/ L), 0.625, 1.25, 2.5, and 5 mg / L).

Stomatal Limitation (L_s)

Stomatal limitation (L_s) measures the extent to which stomata limit CO_2 diffusion into the leaf. Low stomatal limitation indicates that the stomata are open, allowing CO_2 to enter freely. In contrast, high stomatal limitation reflects more closed stomata, restricting CO_2 intake and likely reducing photosynthesis. Figure 4.14 presents that the Fe_3O_4 NPs 0.625 mg / L treatment exhibited the lowest restriction of stomatal activity. However, despite this, the lettuces treated with Fe_3O_4 NPs 0.625 mg / L showed suboptimal development, including symptoms of iron deficiency, which may have contributed to poor water management and inadequate stomatal response to environmental conditions. In contrast, the highest stomatal limitation was observed in Fe_3O_4 NPs 5 mg / L, suggesting that this treatment may reduce stomatal conductance, thereby limiting water loss.

The combined results from *WUE*, *iWUE*, and *Ls* measurements suggest that Fe_3O_4 NPs treatments influenced lettuce physiological performance in a Fe_3O_4 NPs concentration-dependent manner. Notably, Fe_3O_4 NPs 5 mg / L treatment showed the highest WUE values across increasing light intensities, suggesting enhanced water conservation under high irradiance (above 500 µmol m^2s^{-1}). Similarly, *iWUE* was also elevated at 5 mg / L, particularly under near-saturating light (1800 µmol m^2s^{-1}), closely followed by the Fe_3O_4 NPs 1.25 mg / L, indicating efficient internal CO_2 assimilation relative to water loss. However, this treatment also exhibited the highest stomatal limitation, implying that the improved water use metrics may have resulted from reduced stomatal conductance rather than enhanced photosynthetic capacity. Conversely, the Fe_3O_4 NPs 0.625 mg / L treatment showed the lowest stomatal limitation, indicating more open stomata. However, this group also displayed the lowest *WUE* and *iWUE* values which is likely due to greater water loss without proportional photosynthetic gain, possibly reflecting suboptimal plant health and iron deficiency symptom.

Overall, intermediate concentration (1.25 and 2.5 mg / L) appeared to show a more balanced physiological response, exhibiting moderate to high *WUE* and *iWUE*, along with moderate stomatal limitation. This suggest that these concentrations may represent a potential optimal range for maintaining effective gas exchange while limiting excessive water loss. It is important to note that no statistical analysis was conducted on these physiological parameters due to dataset limitations. As such, these results should be interpreted as preliminary observations reflecting trends in water use and stomatal behaviour in response to Fe_3O_4 NPs under varying light conditions.

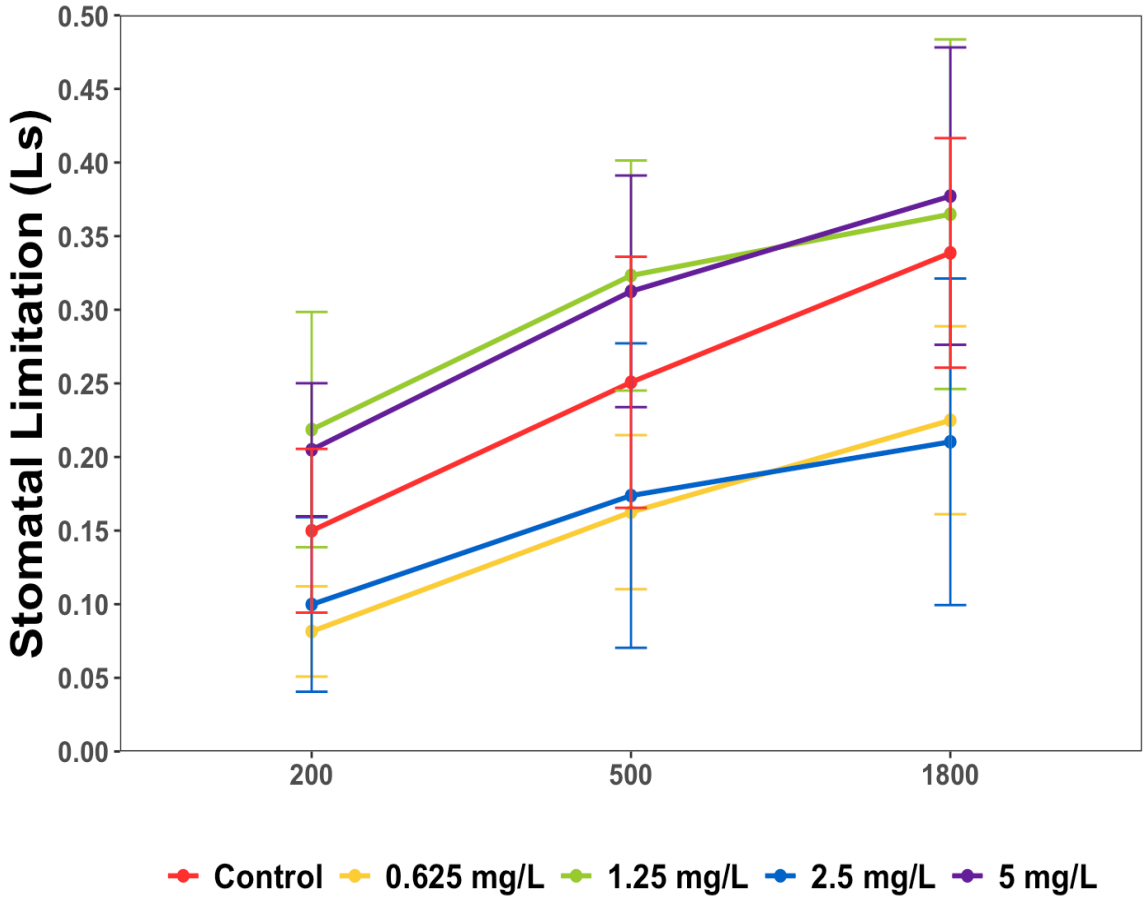


Figure 4.14 Stomatal Limitation (Ls) measured using LI-COR at Week 4.

Stomatal Limitation (L_s), calculated as 1- (intercellular CO2 concentration (Ci) divided by ambient CO_2 concentration (Ca), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg Fe/ L), 0.625, 1.25, 2.5, and 5 mg / L).

4.2.3.3 Harvest quality: Fresh weight

Fresh weight measurements were conducted at two harvest points including week 2 and week 4 (14 days and 28 days after transplanting) to evaluate the effect of Fe_3O_4 NPs on lettuce growth. The detailed methodology is provided in **Chapter 2.3.5**. Briefly, fresh weights were recorded biweekly using a four-digit precision scale. Figure 4.15 presents the fresh weights leaf and root tissues harvested at week 2, expressed in grams. Overall, no substantial differences were observed between the two harvest dates, with both time points showing a consistent growth trend across treatments. Lettuces plants from the control group (Fe-EDTA 2.5 mg / L) recorded the highest total fresh weight (60.56 ± 7.15 g), including the highest leaf fresh weight (50.30 ± 5.78 g).

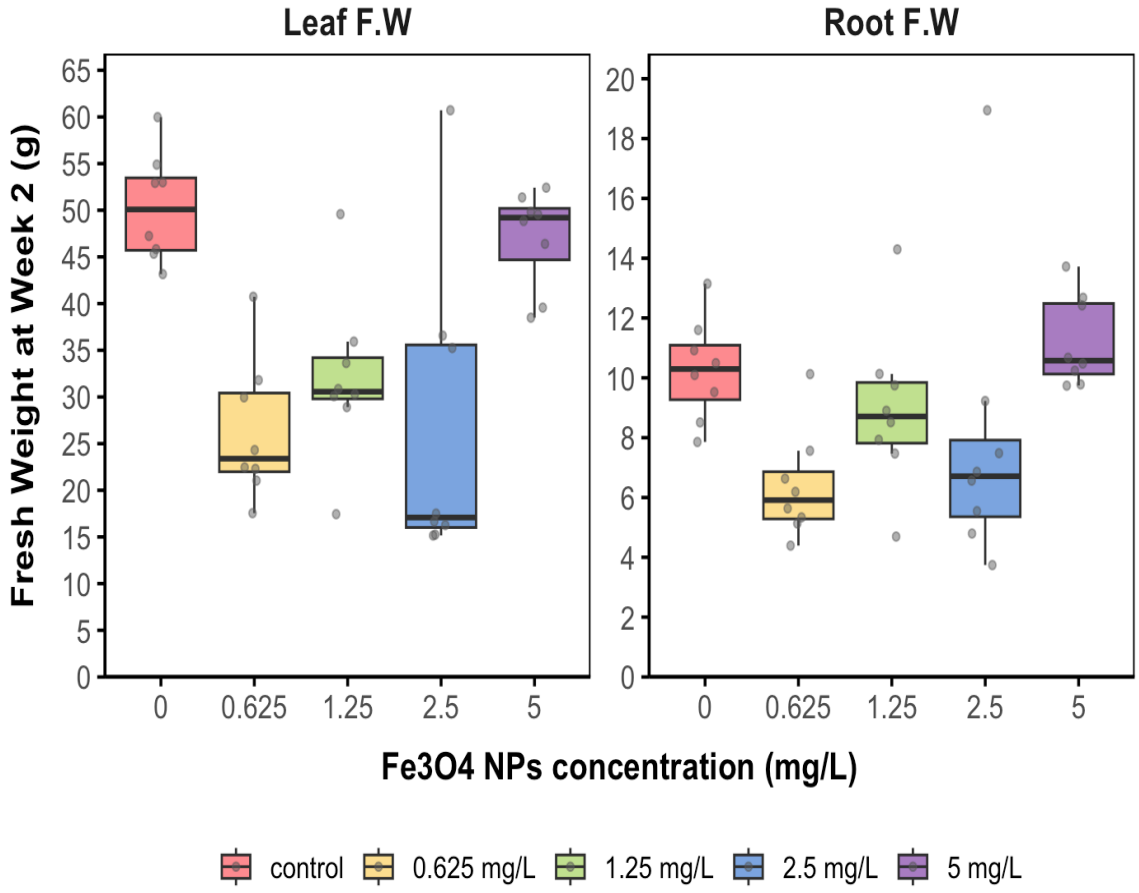


Figure 4.15 Harvest quality – Fresh weight (g) of lettuces harvested at week 2.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L, 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). No statistically significant differences were observed between treatments ($p > 0.05$).

Final harvest quality measurements at week 4 are presented in Figure 4.16. illustrating fresh leaf weight and fresh root weight across Fe_3O_4 NPs treatment. Total fresh weight, calculated as the sum of leaf and root fresh weights, was highest in the control group (Fe-EDTA 2.5 mg / L) (194.08 ± 45.20 g).

For fresh leaf weight, the control group showed the highest value (166.59 ± 42.71 g), which was significantly greater than the Fe_3O_4 NPs 0.625 mg / L treatment by approximately 239 % ($p < 0.05$). However, there no statistically significant difference was observed among the other treatment (Control group treated with Fe-EDTA 2.5 mg / L, Fe_3O_4 NPs 1.25, 2.5, and 5 mg /L) ($p > 0.05$).

Interestingly, fresh root weight showed a different trend, where the highest value was observed in Fe_3O_4 NPs 5 mg /L treatment (27.73 ± 6.72 g). While this was not significantly different from the control treated with Fe-EDTA 2.5 mg / L (‘a’) and Fe_3O_4 NPs 1.25 mg / L treatments (‘ac’), it was significantly greater than the Fe_3O_4 NPs 0.625 (‘b’) and 2.5 mg / L treatments (‘c’) ($p < 0.05$).

Overall, the fresh weight data suggest that while control group treated with Fe-EDTA 2.5 mg / L resulted in the highest total and leaf biomass at harvest, the application of Fe_3O_4 NPs 5 mg / L also produced comparable fresh weight, with no statistically significant difference from the control ($p > 0.05$), indicating its potential as an effective alternative iron source.

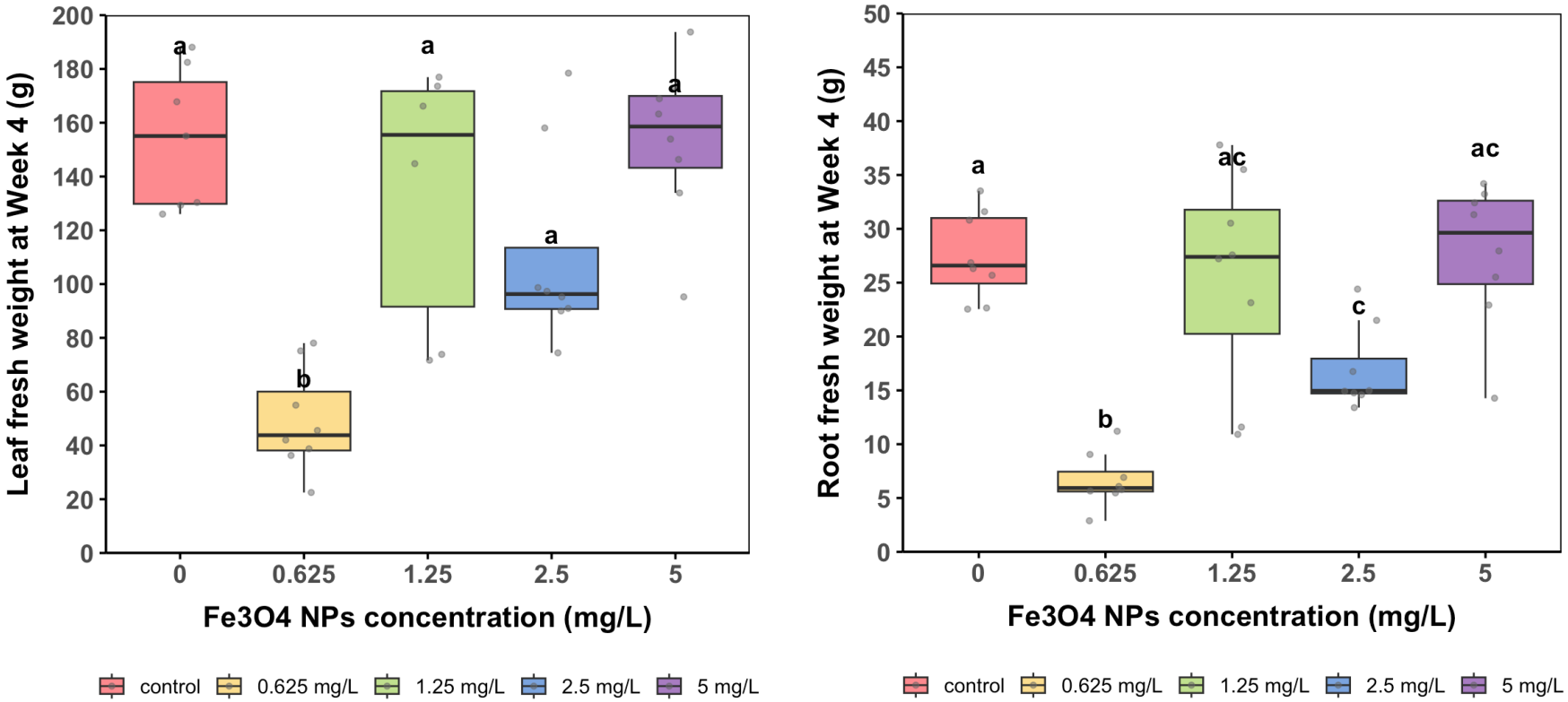


Figure 4.16 Harvest quality – Fresh weight of lettuce harvested at week 4.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L, 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

4.2.3.4 Elemental analysis: Fe quantification

Elemental analysis was performed using the ICP-MS, and results are presented as milligram of iron per gram of dried lettuce tissues (mg / g), including leaf and root tissues. The ICP-MS quantification was based on the Fe56 isotope, which is most widely used due to its natural abundance and analytical stability. It should be noted that the ICP-MS measurements reflect total elemental concentrations and do not provide molecular speciation of iron, as such analyses were beyond the scope of this study. Methodological details are provided in **Chapter** 2.3.1. Figure 4.17 presents the quantification iron concentration from samples harvested at week 2. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**.

Notably, iron content in lettuces harvested at week 2 was approximately 12-fold higher in both leaf and root tissues compared to week 4. The highest iron concentration in leaf tissue was observed in lettuces treated with the Fe_3O_4 NPs 5 mg / L treatment (0.229 ± 0.171 mg / g, 'ab'), closely followed by the control group (Fe-EDTA 2.5 mg / L) (0.225 ± 0.033 mg / g, 'a'). Although the Fe_3O_4 NPs 5 mg / L treatment showed the greatest numerical iron content, it did not differ significantly from the Fe_3O_4 NPs 0.625, 1.25, or 2.5 mg / L treatment (all 'b'), likely due to high variability within Fe_3O_4 NPs 5 mg / L group. On average, root tissues contained approximately 11 times more iron than the leaf tissues. The highest iron concentration in roots (6.534 ± 1.423 mg / g) was detected in the control group (Fe-EDTA 2.5 mg / L) and was significantly greater than all Fe_3O_4 NPs treatment ('a', $p < 0.05$). The Fe_3O_4 NPs 5 mg / L treatment had the second-highest root iron content (2.084 ± 0.094 mg / g), although it was not significantly different from other nanoparticle treatment ('ab').

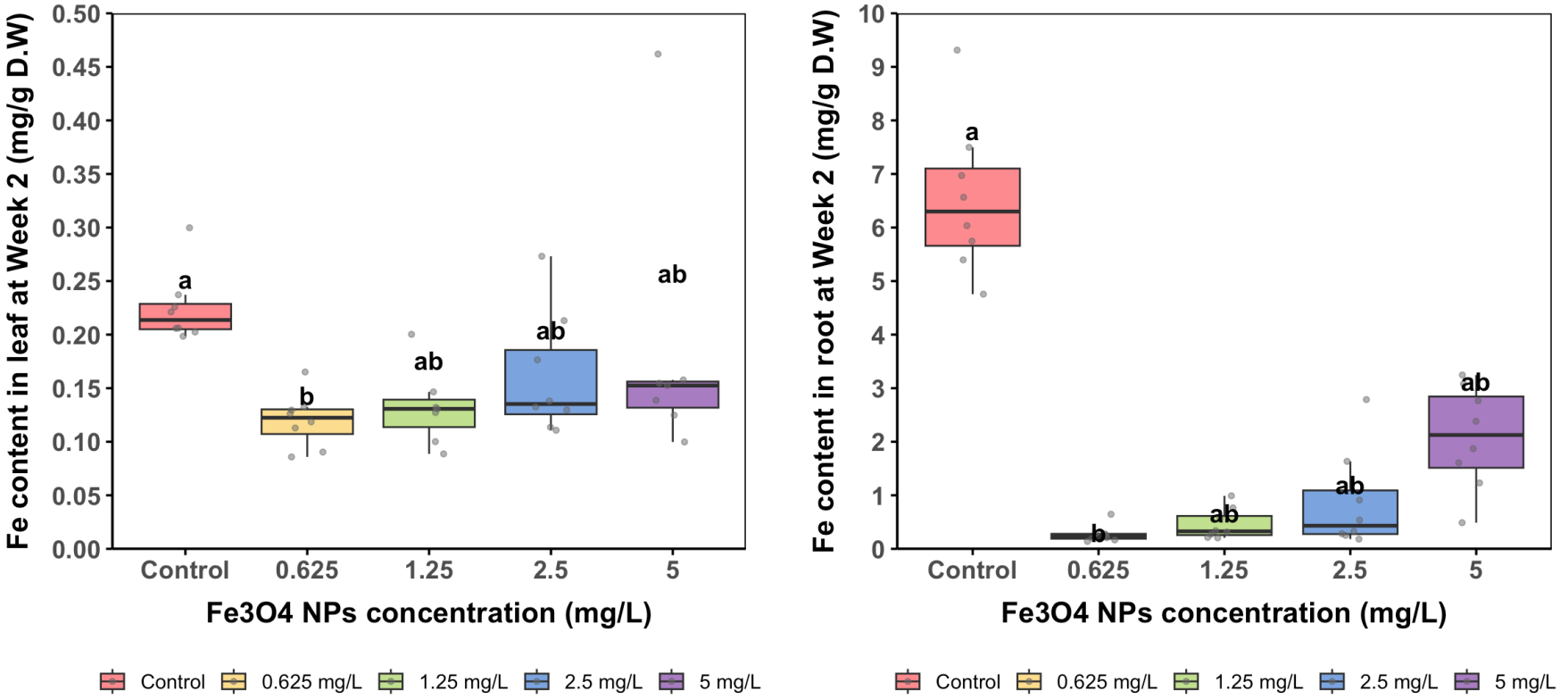


Figure 4.17 Quantification of iron in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 2.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L, 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

A comparison between week 2 and week 4 revealed a marked decline in iron accumulation, with significantly lower iron concentrations measured in both leaf and root tissues at week 4. In the final harvest samples collected at week 4, the highest iron concentration in leaf tissues (0.029 ± 0.012 mg / g) was observed in the control group treated with Fe-EDTA 2.5 mg / L, which was significantly higher than all Fe_3O_4 NPs-treated groups ($p < 0.05$). Compared to the control, iron concentrations in the Fe_3O_4 NPs 0.625, 2.5, and 5 mg / L treatments were lower by 147 %, 159 %, and 219 %, respectively (all 'b'). The Fe_3O_4 NPs 1.25 mg / L treatment recorded the highest iron concentration among the nanoparticles-treated group (0.018 ± 0.013 mg / g, 'ab'), indicating it was not statistically different from other Fe_3O_4 NPs treated group ($p > 0.05$) (Figure 4.18). For the root tissues, the highest iron concentration was recorded in the control group treated with Fe-EDTA 2.5 mg / L (0.687 ± 0.095 mg / g), which was significantly higher than all other Fe_3O_4 NPs treatment ('a', $p < 0.05$). Among the Fe_3O_4 NPs group, the high iron content (0.094 ± 0.054 mg / g) was observed in the Fe_3O_4 NPs 5 mg / L group ('b'), which was significantly higher than the lowest iron concentration found in the Fe_3O_4 NPs 0.625 mg / L treatment (0.020 ± 0.008 mg / g, 'b', $p < 0.05$).

As a result, the control group treated with Fe-EDTA 2.5 mg / L showed the highest iron concentrations in both leaf and root tissues at both week 2 and week 4 ($p < 0.05$). This indicates that the bioavailability of Fe-EDTA was higher than all Fe_3O_4 NPs treatments for lettuce uptake. Compared to week 2, iron uptake and accumulation were significantly reduced by week 4 in both root and leaf tissues. Overall, a relatively higher amount of iron was quantified in the younger seedlings at the early growth stage (week 2) compared to the lettuces at the final harvest (week 4).

The comparison between leaf and root iron content further suggests that iron may have translocated from root to leaf, particularly in the Fe-EDTA and Fe_3O_4 NPs-treated groups. Among the Fe_3O_4 NPs treatments, except for the control group, lettuces treated with Fe_3O_4 NPs 5 mg / L showed the highest iron concentration in both leaf and root tissues on week 2, although the differences were not always statistically significant due to high sample variability. By week 4, this trend reversed as has the highest iron concentration within Fe_3O_4 NPs treatments was observed in the Fe_3O_4 NPs 1.25 group. In contrast, root tissues at week 4 showed the highest iron concentration in the Fe_3O_4 NPs 5 mg / L group, followed by the 1.25 and 2.5 mg /L treatment, while Fe_3O_4 NPs 0.625 mg / L group showed the lowest iron content in root tissues.

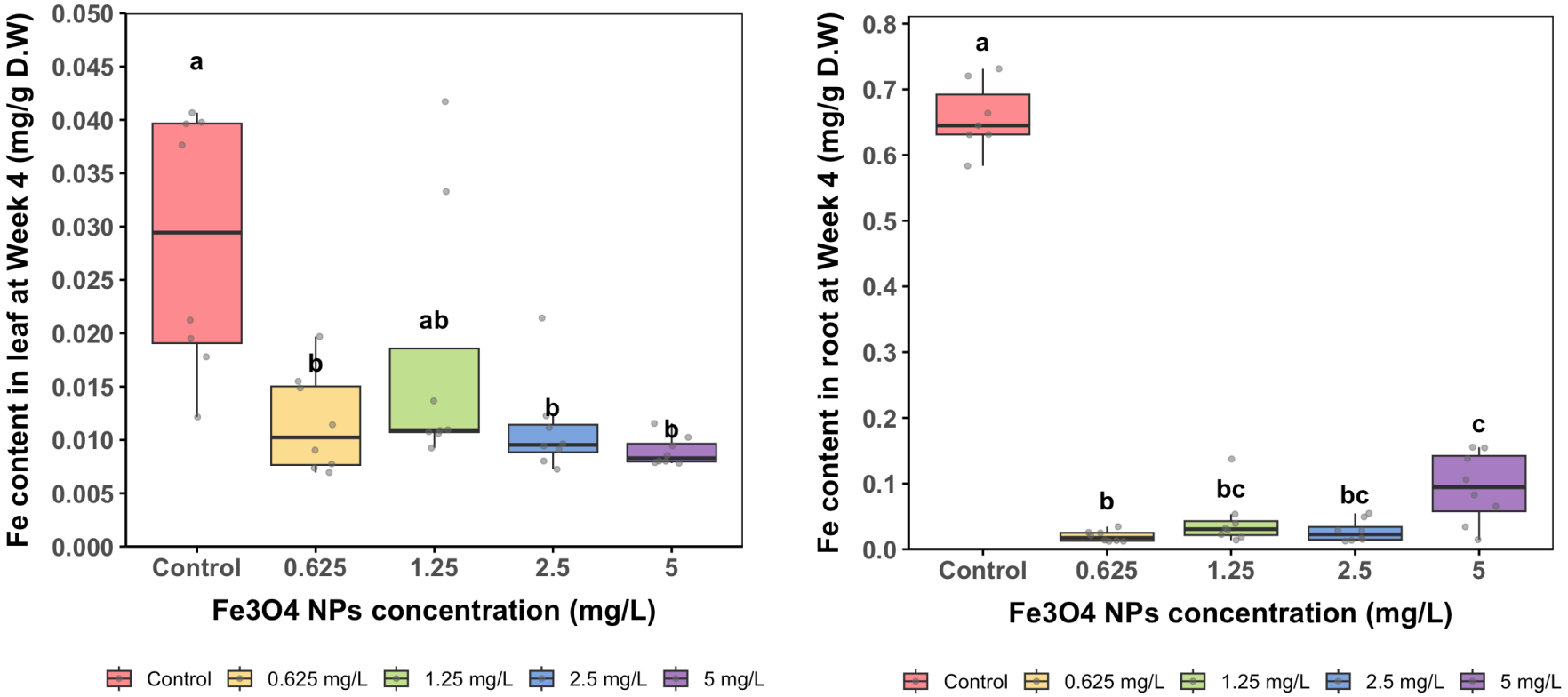


Figure 4.18 Quantification of iron in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs concentrations (control: Fe-EDTA 2.5 mg / L, 0.625, 1.25, 2.5, and 5 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

4.3 Iron fortification: Fe_3O_4 NPs vs. Fe-EDTA

The key finding from the previous experiment was that lettuces treated with Fe_3O_4 NPs exhibited relatively poor morphological and physiological performance, along with lower iron bioavailability, when compared to those treated with control group (Fe-EDTA 2.5 mg / L). However, morphological and physiological analyses indicated that the Fe3O4 NPs 5 mg / L treatment supported comparable plant growth and development to the control, suggesting that this concentration may offer functional equivalence in terms of plant health. Several factors may explain the overall lower iron uptake from Fe_3O_4 NPs treatments: (1) the concentrations of Fe_3O_4 NPs applied may have been insufficient for effective plant uptake due to their limited bioavailability; (2) the use of potato starch as a capping agent may have affected the stability and dispersion of Fe_3O_4 NPs in hydroponic solutions; and (3) additional post-synthesis processing steps may be necessary to enhance the performance and plant compatibility of Fe_3O_4 NPs in nutrient delivery system.

In light of these findings, the current experiment employed Fe_3O_4 NPs capped with a different stabilising agent, L-lysine, to improve the stability and biocompatibility of the nanoparticles. L-lysine, an essential amino acid, was selected for its ability to bind effectively with magnetic nanoparticles, as well as for its additional health benefits, including roles in bone development, collagen synthesis, antibody production, and calcium absorption [192,193]. **Chapter 4.1.2** indicates that Fe_3O_4 NPs capped with L-lysine exhibited moderate stability compared to uncapped Fe_3O_4 NPs. Additionally, according to the DLS analysis described in **Chapter 4.1.1**, the average hydrodynamic particle size of Fe_3O_4 NPs capped with L-lysine was 18.87 nm, which is approximately three times smaller than that of Fe_3O_4 NPs capped with potato starch. Therefore, this study hypothesises that Fe_3O_4 NPs capped with L-lysine may offer a comparable alternative to conventional iron fertilisers like Fe-EDTA, due to their improved stability, smaller particle size, and potential for enhanced bioavailability.

4.3.1 Materials and methods

The materials and growing conditions are described in **Chapter 2.2**. The present experiment was conducted to determine the effect of Fe-EDTA and Fe_3O_4 NPs capped with L-lysine on lettuce across comparable iron concentration. Fe_3O_4 NPs

capped with L-lysine were provided by Dr. Gareth Cave, Nottingham Trent University, which were synthesised via a SDR method. The L-lysine capped Fe_3O_4 NPs were processed with the post-synthesis procedure including rinsing and drying to minimise the by-product from the synthesis, then the Fe_3O_4 NPs were quantified for further application which described in **Chapter 2.1.4.6**. As previously described in **Chapter 2.2.3,** the Fe-EDTA and Fe_3O_4 NPs treatments were prepared based on the molecular weights, accounting for the iron fraction in the molecule to achieve equivalent elemental iron concentrations across treatments. All treatments were standardised to deliver a final concentration expressed as milligrams of elemental iron per litre. However, for data presentation, concentrations are reported as milligram per litre (mg / L), without specifying elemental iron explicitly, as the iron fraction was accounted for during preparation.

Based on these elemental analysis results, serial dilutions of Fe_3O_4 NPs were prepared using deionised water for subsequent application to lettuce. Based on the elemental analytical results, the serial dilutions of Fe_3O_4 NPs and Fe-EDTA were prepared for lettuce application using deionised water. The concentration of the serial dilutions are as follows; 0, 2.5, 5, 7.5 and 10 mg / L. The serial dilutions Fe_3O_4 NPs were sonicated for 15 minutes until no debris was observed. Within equivalent concentrations, Fe-EDTA treatments were prepared, and details are described in **Chapter 2.2.2**. Both Fe_3O_4 NPs and Fe-EDTA treatments were applied to the root zone located in hydroponic tanks along with Hoagland nutrient fertilisers.

4.3.2 Data collection and statistical analysis

This experiment used three biological replicates per different concentration Fe_3O_4 NPs and Fe-EDTA treatments. Each treatment was applied in two different propagators and total 20 propagators were used for the present experiment **(**Figure 4.19). The control group was treated no iron either Fe_3O_4 NPs or Fe-EDTA treatment. The lettuces were harvested at the final harvest date on week 4 to quantify multielement in lettuce tissues via elemental analysis such as iron (Fe), potassium (K), and magnesium (Mg). Further secondary metabolites analysis was conducted for the present study. Compared to previous experiments, the current experiment presented the outcomes of the descriptive statistical analysis due to limited number of replicates of the experiment with three biological replicates for each treatment. Therefore, neither PERMANOVA analysis nor multiple permutational comparison tests were conducted for the present study.

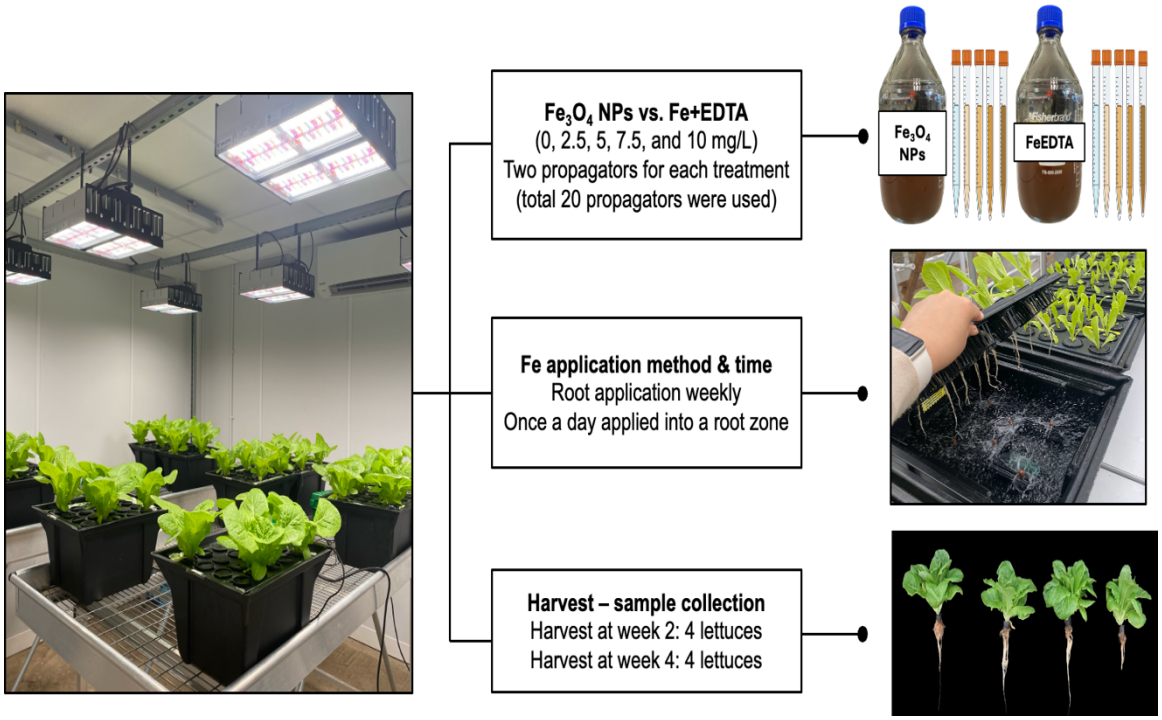


Figure 4.19 Schematic representation of growing condition for Fe_3O_4 NPs and Fe-EDTA fortification via root application.

Fe_3O_4 NPs and Fe-EDTA concentration (Control, 2.5, 5, 7.5, and 10 mg / L); Root application method; and harvest details.

4.3.3 Results

This section investigates the effect of iron fortification on hydroponically grown lettuce through root application of Fe_3O_4 NPs capped with L-lysine, in comparison with a conventional iron fertiliser, such as Fe-EDTA. Fe_3O_4 NPs capped with L-lysine possess several advantageous properties, including nano-scale particle size, enhanced dispersive stability, and improved bioavailability. It is therefore hypothesised that these nanoparticles will facilitate more efficient iron delivery to lettuce plants than Fe-EDTA. Furthermore, as an essential amino acid, L-lysine is expected to promote strong electrostatic interaction with the nanoparticle surface, reducing aggregation and maintaining colloidal stability in hydroponic solutions. Its biocompatibility and biodegradability, and potential nutritional benefit make L-lysine a promising capping agent for the development of plant-compatible nutritional nanofertiliser.

The aim of this chapter is to evaluate the performance of Fe_3O_4 NPs capped with L-lysine in lettuce cultivation, in comparison with the conventional iron fertiliser Fe-EDTA. This includes assessing their effect on lettuce growth, physiology, and nutritional quality. The objectives are: (1) to quantify the uptake, translation, and accumulation of root applied Fe_3O_4 NPs capped with L-lysine in lettuce leaves and root tissues using ICP-MS, alongside the multi-elemental analysis of essential nutrients; and (2) to assess the effects of varying concentrations of Fe_3O_4 NPs (0, 2.5, 5, 7.5, and 10 mg / L), compared to the equivalent concentration of Fe-EDTA, on morphological traits such as leaf area, biomass, physiological parameters including photosynthetic gas exchange, water management, and relative chlorophyll content, and nutritional quality such as iron content, secondary metabolites levels.

4.3.3.1 Growth and development measurement

The weekly measurements of growth and morphological development obtained by PlantEye system are presented in this section. Overall, distinct differences were observed between the control groups (without iron supplementation) and the Fe_3O_4 NPs and Fe-EDTA groups. Increases in plant height, leaf area and digital biomass were apparent in all treatment group except the control without iron treatment. The greenness, NDVI and NPCI values showed slight increases until week 3, followed by decreases at week 4. However, it is important to note that these measurements

were not subjected to statistical analysis, so the observed trends should be interpreted with caution. The following section will focus on a detailed examination of the measurements taken at week 4.

Figure 4.20 presents plant height (cm) and leaf area (cm^2) recorded at the final harvest (week 4). For height, lettuces treated with Fe-EDTA (13.10 ± 1.41 cm) were, on average, approximately 8 % taller than those treated with Fe_3O_4 NPs (12.14 ± 1.15 cm). Among Fe_3O_4 NPs groups, the highest mean height (14.36 ± 0.21 cm) was observed in lettuces treated with Fe_3O_4 NPs 7.5 mg / L. In contrast, the greatest average height among Fe-EDTA treatments was found in the 5 mg / L group (15 ± 1 cm).

Regarding leaf area (cm^2), lettuces treated with Fe_3O_4 NPs (387 ± 53 cm^2) exhibited, on average, a 15 % larger leaf area compared to those treated with Fe-EDTA (331 ± 50 cm^2). In Fe_3O_4 NPs group, the highest average leaf area (488 ± 65 cm^2) was observed in lettuces treated with Fe_3O_4 NPs 10 mg / L. For the Fe-EDTA treatments, the largest leaf area (402 ± 26 cm^2) was also recorded in Fe-EDTA 10 mg / L group.

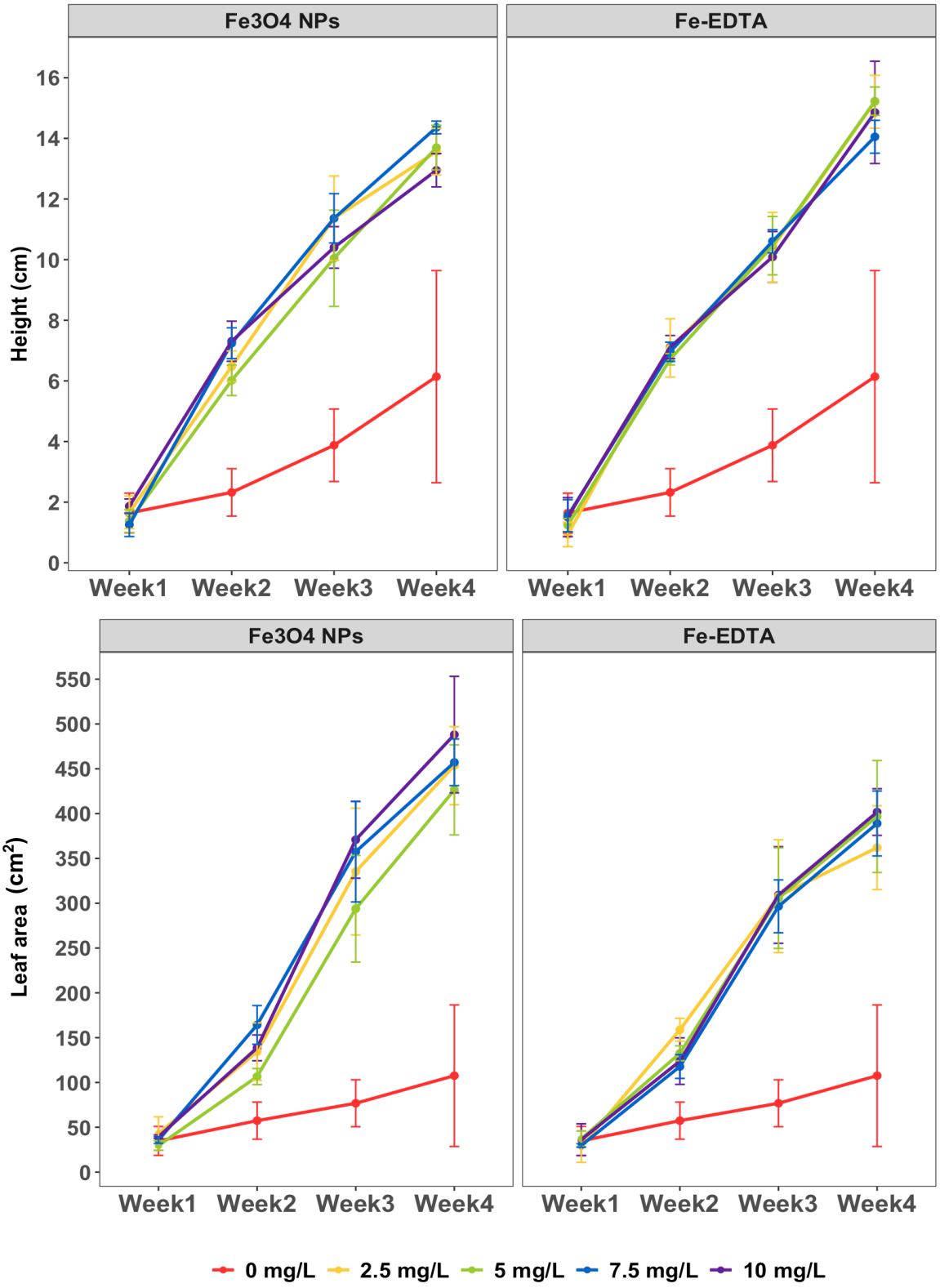


Figure 4.20 Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

Coloured lines indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Figure 4.21 presents the digital biomass (cm^3) and hue (º) measurements. As volumetric estimate, digital biomass integrates plant height and projected leaf area, providing an overall representation of lettuce growth. On average, lettuces treated with Fe_3O_4 NPs group exhibited a 7 % higher digital biomass (5131 ± 658 cm^3) compared to those treated with Fe-EDTA (4749 ± 846 cm^3). Within the Fe_3O_4 NPs treatment group, lettuces treated with 7.5 mg / L recorded the highest digital biomass (6,561 ± 276 cm^3). Among the Fe-EDTA treatment group, the greatest digital biomass was recorded in lettuce treated with 5 mg / L (6,060 ± 1,143 cm^3).

Overall, the morphological results indicated that the lettuces treated with Fe_3O_4 NPs showed slightly better growth and morphological development compared to those treated with Fe-EDTA. The control groups for both Fe_3O_4 NPs and Fe+ EDTA exhibited less performance in growth and development in the lettuce due to iron deficiency. However, due to the limited number of biological replicates, no statistical analysis was performed, and therefore these results should be interpreted with caution.

Hue values, which range from 0 to 360 º, represent colour and can serve as an indicator of the morphological health of plants. Hue values between 90 º to 140 º correspond to green hues, typically associated with healthy plants. On average, lettuces treated with Fe-EDTA exhibited higher hue mean values (98.39 ± 2.14 º), approximately 4 % greater than those treated with Fe_3O_4 NPs group (94.62 ± 2.03 º). Within the Fe_3O_4 NPs treatment, the 2.5 mg / L group recorded the highest hue value (99.02 ± 1.24 º), while the highest value in the Fe-EDTA group, was observed at 7.5 mg / L (103.35 ± 1.74 º). However, as no statistical analysis was performed, these observations should be considered preliminary.

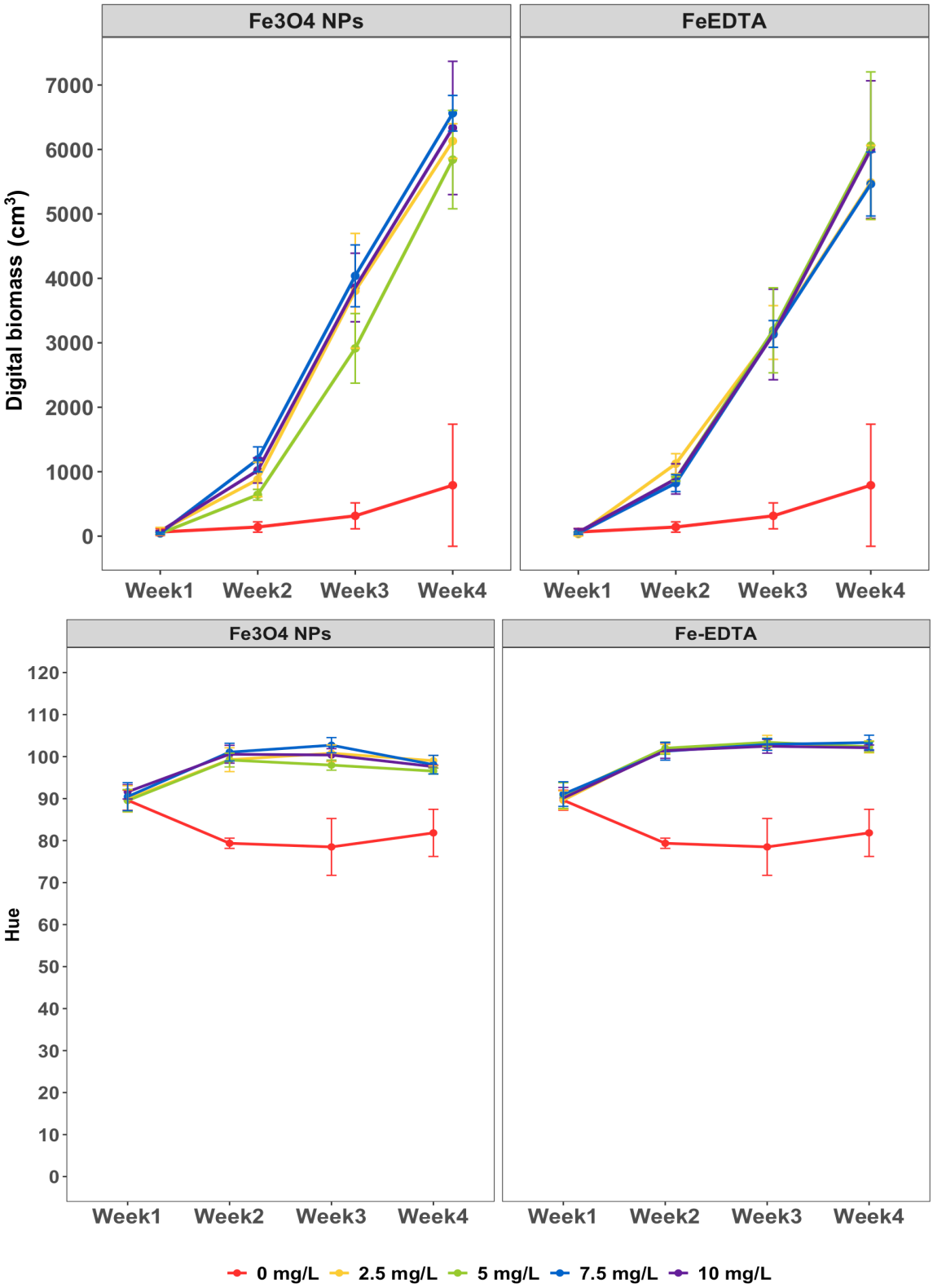


Figure 4.21 Digital biomass (cm^3) and Hue (°) of lettuces measured weekly via PlantEye.

Coloured lines indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Figure 4.22 presents the greenness and Normalised Differential Vegetation Index (NDVI) measurements obtained from PlantEye. Greenness, which ranges from -1 to 1, reflects the green leaf index, with higher values indicating greater greenness typically associated with healthy leaves. On average, the Fe-EDTA group (0.41 ± 0.02) exhibited slightly higher greenness, approximately 0.4 % greater, compared to the Fe_3O_4 NPs group (0.39 ± 0.02). Within the Fe_3O_4 NPs treatment, the highest value was recorded at 2.5 mg / L (0.43 ± 0.00). Among the Fe-EDTA treatments, the 10 mg / L group exhibited the highest greenness value (0.45 ± 0.01). These results suggest that higher concentration of Fe-EDTA (7.5 and 10 mg / L) were associated with improved chlorophyll content and overall plant health. In contrast, the control group without iron treatment exhibited visibly yellow foliage, indicative of severe iron deficiency symptoms. However, as no statistical analysis was performed, these observations should be considered preliminary.

The NDVI serves as an indicator of vegetation health and relates to the interception of photosynthetic activity, as it related to plant's ability to intercept photosynthetically active radiation (PAR). Typically, healthy vegetation display NDVI values between 0.6 to 0.9. In this study, the Fe-EDTA treatment group recorded higher NDVI values (0.61 ± 0.02), which were approximately 15 % greater than those of Fe_3O_4 NPs group (0.54 ± 0.02). Within the Fe_3O_4 NPs treatments, the 2.5 mg / L concentration exhibited the highest NDVI value (0.63 ± 0.01). Within the Fe-EDTA group, lettuces treated with 7.5 mg / L had the highest value (0.69 ± 0.01). However, as no statistical analysis was performed, these observations should be considered preliminary.

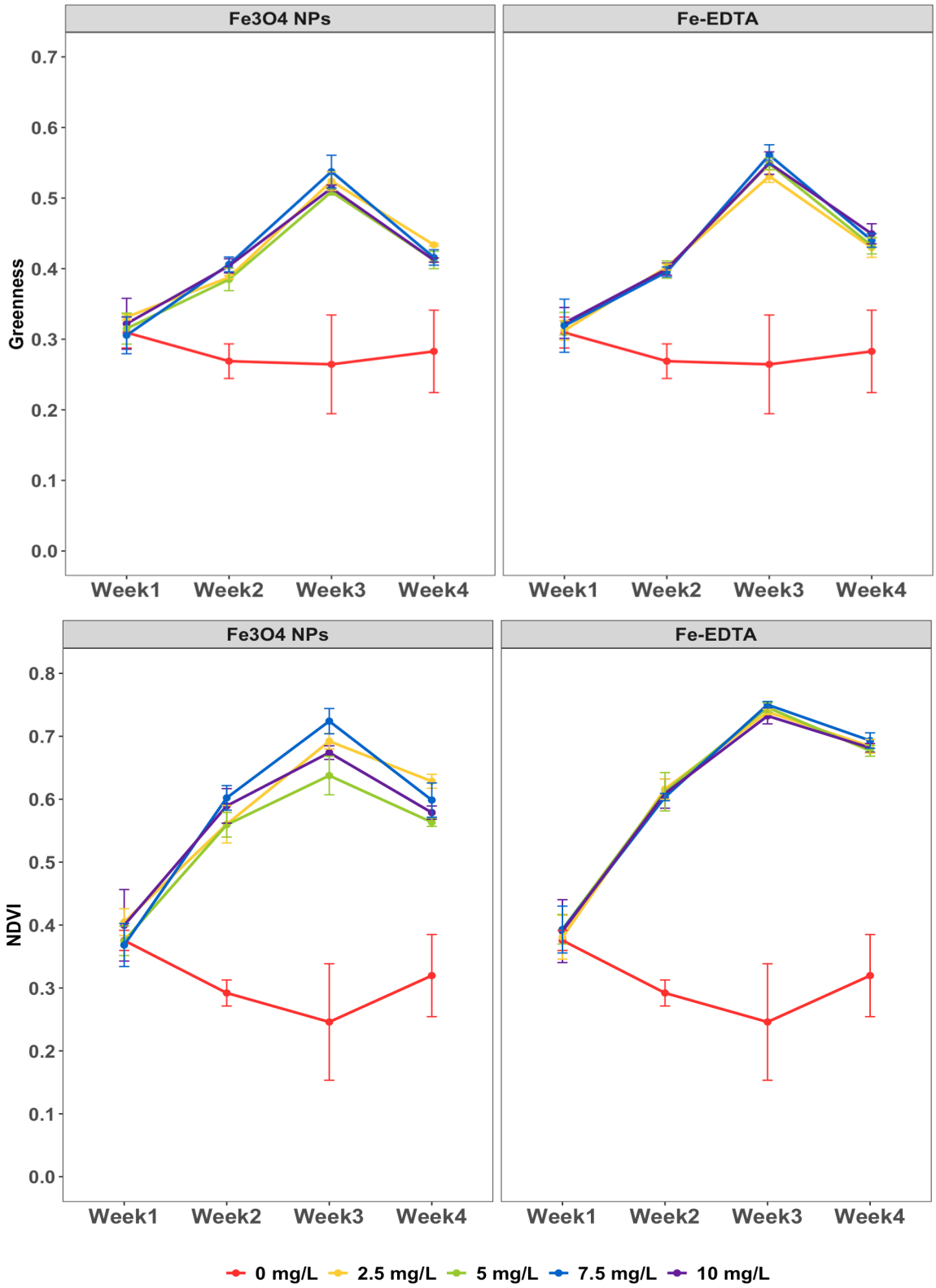


Figure 4.22 Greenness and NDVI of lettuces measured weekly via PlantEye.

Coloured lines indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

Figure 4.23 shows Normalised pigment chlorophyll index (NPCI) and Plant Senescence Reflectance Index (PSRI) measurement obtained by PlantEye. NPCI reflects the ratio between chlorophyll and carotenoids, providing an indicator of the plant's physiological status or stress condition. The Fe_3O_4 NPs treatment group exhibited an average NPCI value of 0.34 ± 0.03, which was approximately 15 % higher than the Fe-EDTA group (0.30 ± 0.02). Notably, among Fe_3O_4 NPs treatment, the control group (without iron supplementation) recorded the highest NPCI mean value (0.35 ± 0.05), suggesting greater physiological stress.

PSRI estimate the relative content of carotenoids to chlorophyll, providing a measure of plant senescence and stress. The Fe_3O_4 NPs treatment group showed an average PSRI of 0.15 ± 0.01, representing a f 38 % increase compared to the Fe-EDTA group (0.12 ± 0.01). Across all treatments, the control group (without iron) from both Fe_3O_4 NPs and Fe-EDTA treatments recorded the highest PSRI values (0.263 ± 0.015), indicating pronounced chlorophyll degradation due to iron deficiency. Overall, plants treated with the Fe-EDTA group exhibited lower PSRI, value, suggesting reduced levels of plant senescence or stress relative to those treated with Fe_3O_4 NPs group. However, these results should be considered preliminary results as no statistical analysis was conducted.

Overall PlantEye measurements suggest that the higher concentration of both Fe_3O_4 NPs and Fe-EDTA treatments (7.5 and 10 mg / L) may associated with improved growth and leaf colour compared to the control groups without any iron treatment. In contrast, lettuces grown without iron supplementation displayed limited growth and yellowing symptoms indicative of iron deficiency. However, as no statistical analyses were performed, these findings should be considered preliminary and interpreted with caution.

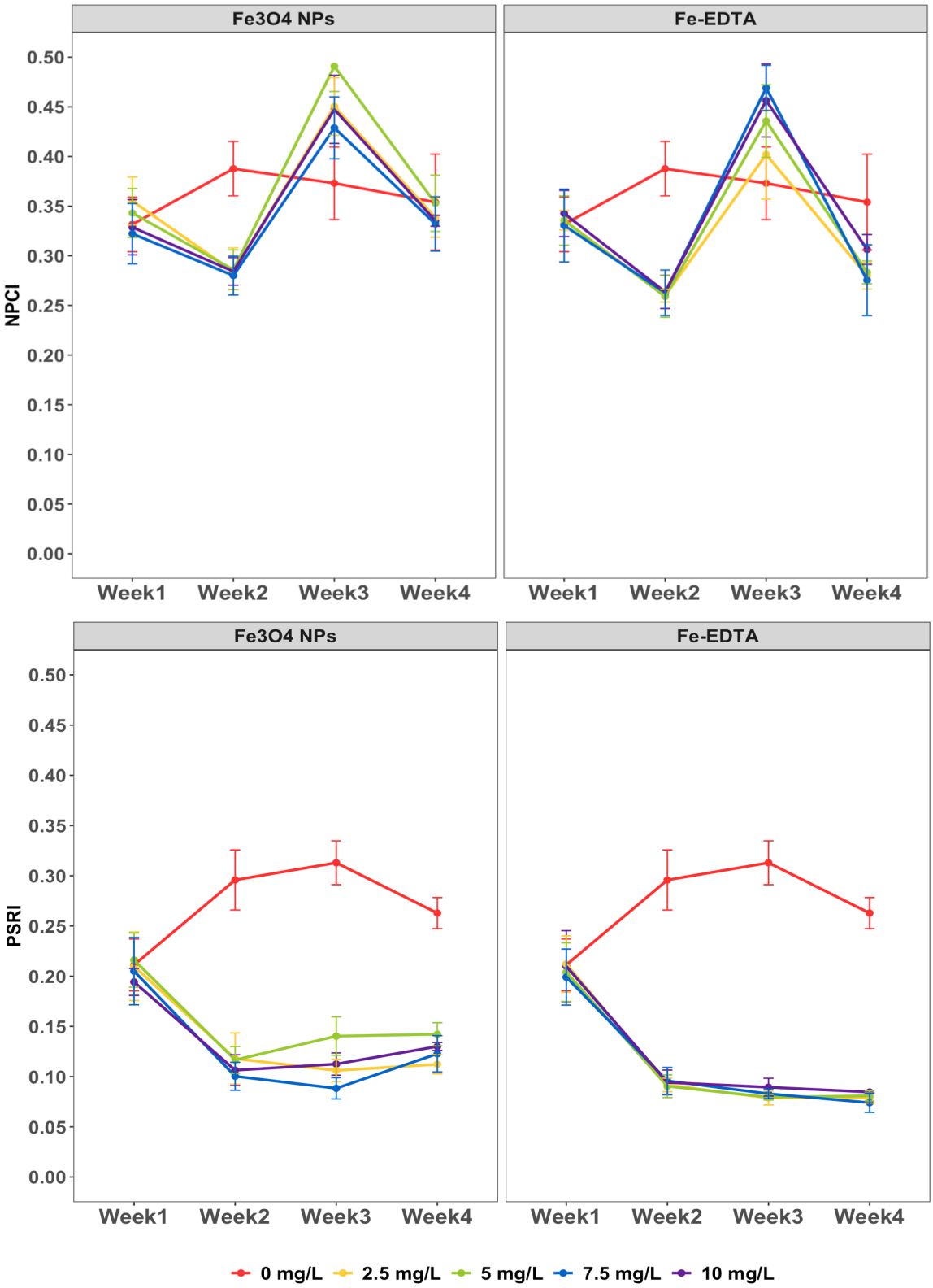


Figure 4.23 NPCI and PSRI of lettuces measured weekly via PlantEye.

Coloured lines indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Data are shown as mean ± standard error (n = 8).

4.3.3.2 Water management and gas exchange

The final measurements (week 4) of water management efficiency and gas exchange parameters in lettuce were obtained using LI-COR system. These include Water Use Efficiency (*WUE*), Intrinsic Water Use Efficiency (*iWUE*) and Stomatal Limitation (L_s). While these physiological traits were measured weekly throughout the experiment, only the week 4 data are highlighted in this section to reflect the final physiological state of the plants at harvest. This approach offers insights into the cumulative treatment effects of the treatments on water use efficiency and gas exchange dynamics by the end of the growth period. No statistical analysis was conducted for these measurements, and the results are intended to provide a descriptive overview of treatment-related physiological responses.

Water Use Efficiency (WUE)

Water Use Efficiency (*WUE*), which represents the ratio of carbon dioxide assimilation to water loss through transpiration, was measured at week 4 and is highlighted in Figure 4.24**.** The highest *WUE* values (1952, 3254, and 3581 µmol CO_2/mmol H_2O) were observed in the Fe-EDTA 10 mg / L treatment group as light intensities increased from 200 to 1800 µmol m^2s^{-1}. Overall, the Fe-EDTA treatments yield slightly higher average *WUE* values compared to Fe_3O_4 NPs treatments as light intensity increased. However, it is also possible that the increased *WUE* resulted not from enhanced photosynthetic capacity but from reduced stomatal conductance, which limits water loss. This interpretation highlights the need to consider stomatal behaviour alongside *WUE* in assessing physiological performance.

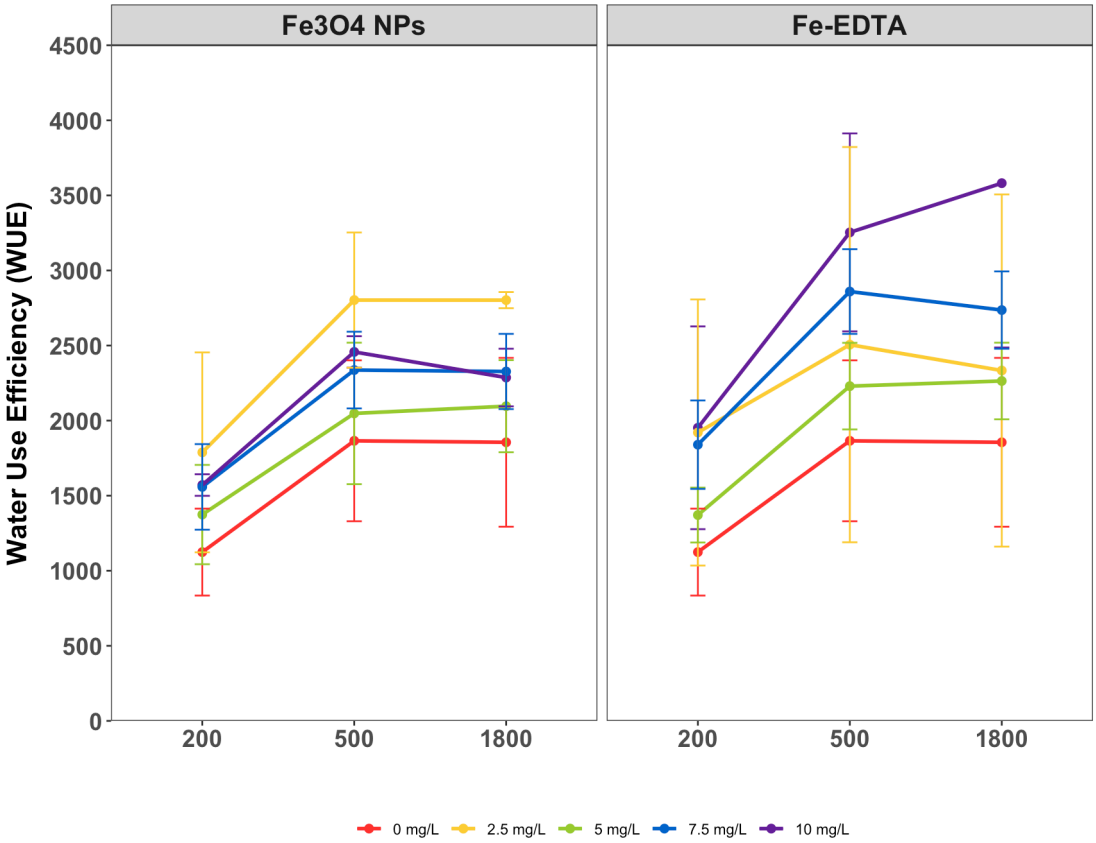


Figure 4.24 Water Use Efficiency (WUE) measured using LI-COR at Week 4.

Water use efficiency (WUE), calculated as net assimilation rate (A) divided by transpiration rate (E), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

Intrinsic Water Use Efficiency (iWUE)

Intrinsic Water Use Efficiency (*iWUE*), a direct indicator of stomatal activity reflecting the balance between carbon assimilation and water loss through stomatal opening and closing, was measured weekly, but only the week 4 results are highlighted in this chapter. Figure 4.25 shows that the Fe-EDTA 2.5 mg / L group indicated the highest *iWUE* value (35.37, 52.12, and 63.20 µmol CO_2/mmol H_2O) as light intensities were increased from 200 to 1800 µmol m^2s^{-1}. In summary, the Fe_3O_4 NPs treatment exhibited slightly higher average *iWUE* values compared to Fe-EDTA treatment, as light intensity increased. The elevated iWUE may reflect more than just improved carbon assimilation efficiency. It could also indicate stomatal limitation to gas exchange where carbon fixation is maintained despite reduced stomatal conductance.

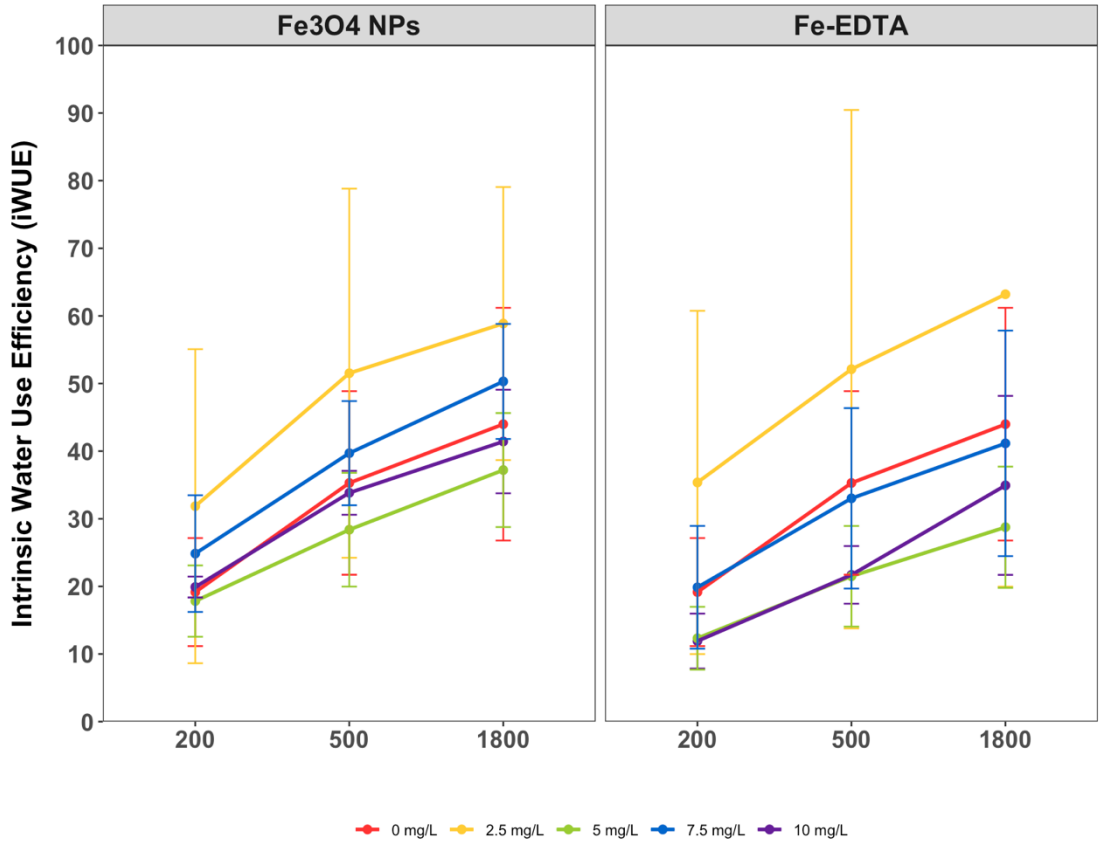


Figure 4.25 Intrinsic Water Use Efficiency (iWUE) measured using LI-COR at Week 4.

Intrinsic Water use efficiency (iWUE), calculated as net assimilation rate (A) divided by stomatal conductance to H_2O (gsw) , plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

Stomatal Limitation (L_s)

Stomatal limitation (L_s) measures the extent to which stomata limit CO_2 diffusion into the leaf. Low stomatal limitation indicates that the stomata are open, allowing CO_2 to enter freely. In contrast, high stomatal limitation reflects more closed stomata, restricting CO_2 intake and likely reducing photosynthesis. As shown in Figure 4.26**,** the Fe_3O_4 NPs 2.5 mg / L treatment exhibited the lowest overall stomatal restriction with L_s values (0.83, 0.73, and 0.68) as light intensities increased from 200 to 1800 μmol m^2s^{-1}. In contrast, slightly higher average L_s values were observed in the Fe-EDTA treatment group compared to Fe_3O_4 group as light intensity increased. These results suggest that the Fe_3O_4 NPs treatment imposed less restriction on stomatal activity, potentially allowing for improved gas exchange and, consequently, better photosynthetic performance than the Fe-EDTA treatment.

In summary, the combined data from LI-COR measurement indicate that Fe-EDTA and Fe_3O_4 NPs exhibited distinct physiological profiles in terms of water use efficiency and gas exchange, influenced by both the type of iron treatment and its concentrations. On average, Fe-EDTA treatments led to higher *WUE* values and greater stomatal limitation (L_s), suggesting more conservative water use strategy. In contrast, Fe_3O_4 NPs treatments showed higher *iWUE*, indicating more efficient CO_2 assimilation relative to stomatal conductance. Fe_3O_4 NPs treatment, especially at lower concentration (e.g., 2.5 mg / L) consistently showed lower L_s values, reflecting less stomatal restriction and potentially more favourable gas exchange condition. These findings suggest that both the type (Fe_3O_4 NPs or Fe-EDTA) and concentration of iron treatment influence physiological responses, and optimal outcomes may depend on balancing nutrient supply with stomatal behaviour. However, the results are intended to provide a descriptive overview of iron treatment-related physiological responses, as no statistical analysis was conducted for these measurements.

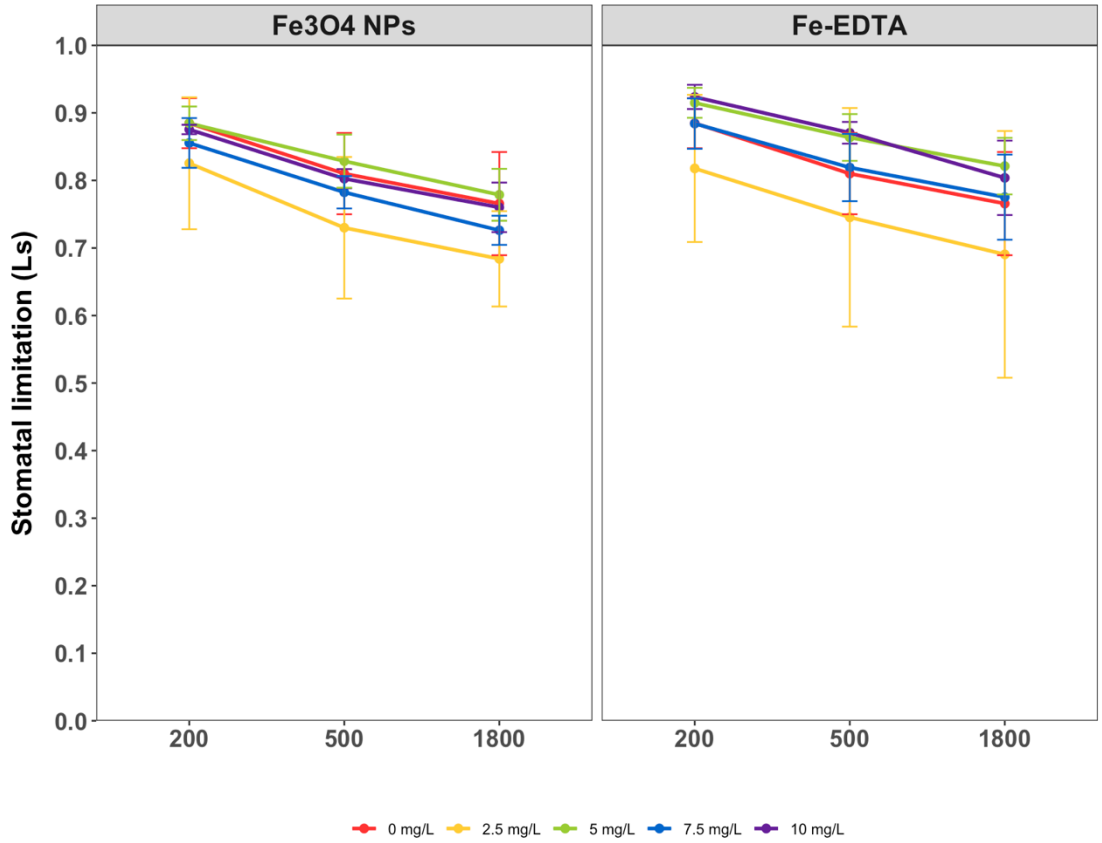


Figure 4.26 Stomatal Limitation (Ls) measured using LI-COR at Week 4.

Stomatal Limitation (L_s), calculated as 1- (intercellular CO2 concentration (Ci) divided by ambient CO_2 concentration (Ca), plotted against increasing light intensity (200 to 1800 µmol m^2s^{-1}). Coloured lines represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as indicated in the legend.

4.3.3.3 Relative chlorophyll measurement

Relative chlorophyll content was measured weekly for four weeks using SPAD-502Plus (Konica Minolta Ltd., Osaka, Japan). Details are described in **Chapter 2.3.4**. SPAD value correlates with chlorophyll content, which plays an important role in photosynthesis. Higher SPAD values indicate healthier plant with more chlorophyll. Figure 4.27 shows the weekly changes in relative chlorophyll content (SPAD values) of lettuce leaves under different iron treatments. Across all iron-treated groups, SPAD values increased between week2 and week 3 and were either maintained or slightly declined by week 4. In contrast, the control group (without Fe supplementation) consistently displayed significantly lower SPAD values throughout the growth period.

Lettuces treated with Fe_3O_4 NPs generally showed slightly higher SPAD values compared to those treated with Fe-EDTA. By week 4, representing the physiological status at the final harvest, the Fe_3O_4 NPs treatment yielded SPAD values approximately 18% higher than the Fe-EDTA group. The highest relative chlorophyll content was observed in the Fe_3O_4 NPs 2.5 mg / L group in (28.63 ± 2.40), followed by Fe_3O_4 NPs 10 mg / L (27.47 ± 2.10).

Throughout the growing period, the control group without any iron treatment consistently maintained visibly lower chlorophyll content, as also reflected in leaf colour. It should be noted that no statistical analysis was performed on these measurements. Therefore, these results provide a descriptive overview of iron treatment effects at harvest.

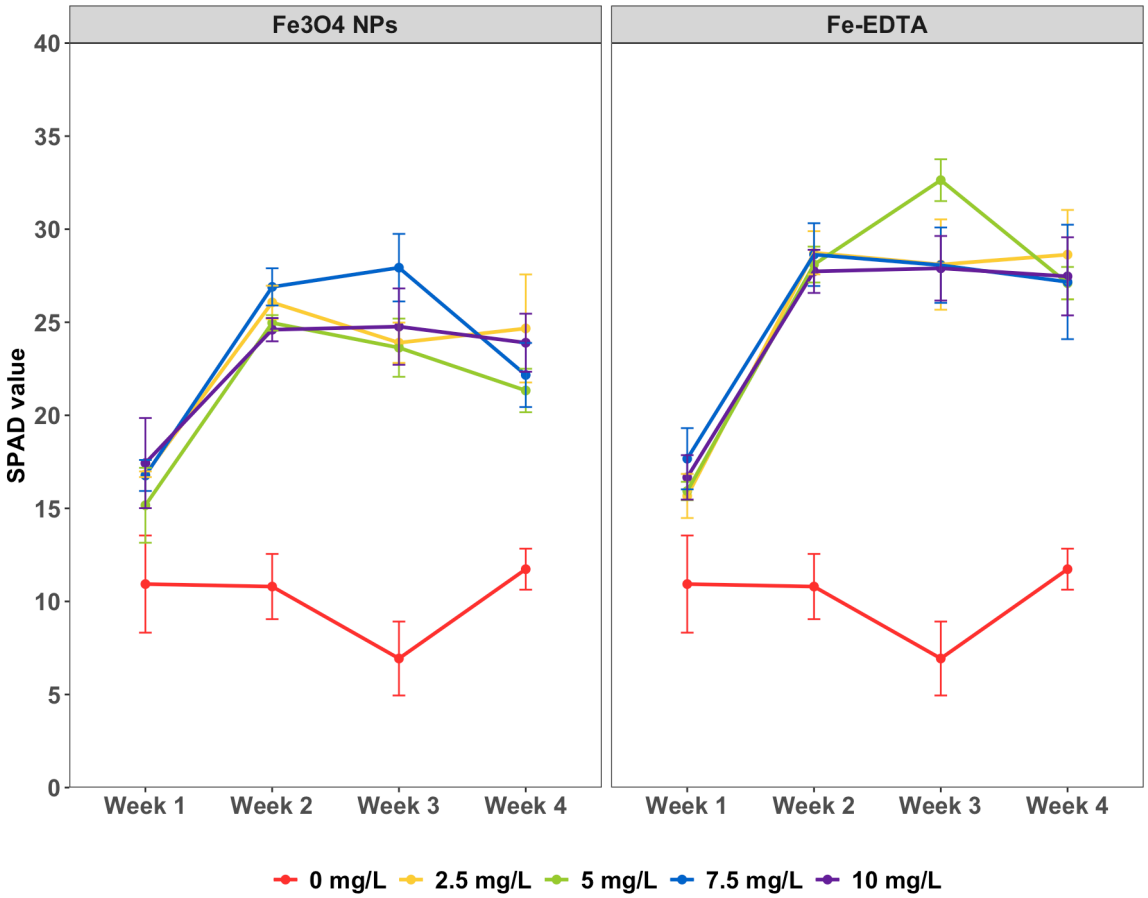


Figure 4.27 Relative chlorophyll content measured by SPAD meter from Week 1 to Week 4.

Coloured lines represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

4.3.3.4 Harvest quality: Fresh weight

Fresh weight measurements were conducted at two harvest points including week 2 and week 4 (14 days and 28 days after transplanting) to evaluate the effect of Fe_3O_4 NPs on lettuce growth. The detailed methodology is provided in **Chapter 2.3.5**. Briefly, fresh weights were recorded biweekly using a four-digit precision scale. Total fresh weight was calculated as the sum of leaf and root weights. Given the limited biological replication, the data are presented descriptively without statistical comparison.

Figure 4.28 presents the fresh weight measurements at week 2, showing that Fe-EDTA treatments resulted in 20 %, 24 %, and 7 % higher total, leaf, and root fresh weights, respectively, compared to the Fe_3O_4 NPs treatments. The highest total fresh weight (19.97 ± 4.84 g) was recorded in the Fe-EDTA 7.5 mg / L treatment, which also produced the greatest fresh leaf weight (15.93 ± 3.76 g). In contrast, the control group (without Fe supplementation) displayed the lowest total, leaf and root fresh weights due to severe iron deficiency. Among the Fe_3O_4 NPs treatment group, the highest total fresh weight was observed at 7.5 mg / L (18.43 ± 2.59 g), which also produced the highest fresh root weight (4.06 ± 0.53 g).

Overall, both Fe_3O_4 NPs and Fe-EDTA treatments resulted in clear increases in total, leaf, and root weights compared to control group (without Fe supplementation). However, a consistent does-dependent trend was not observed at week 2, particularly for Fe_3O_4 NPs treatments, suggesting that early biomass accumulation may not be strongly influenced by increasing Fe concentration within this range.

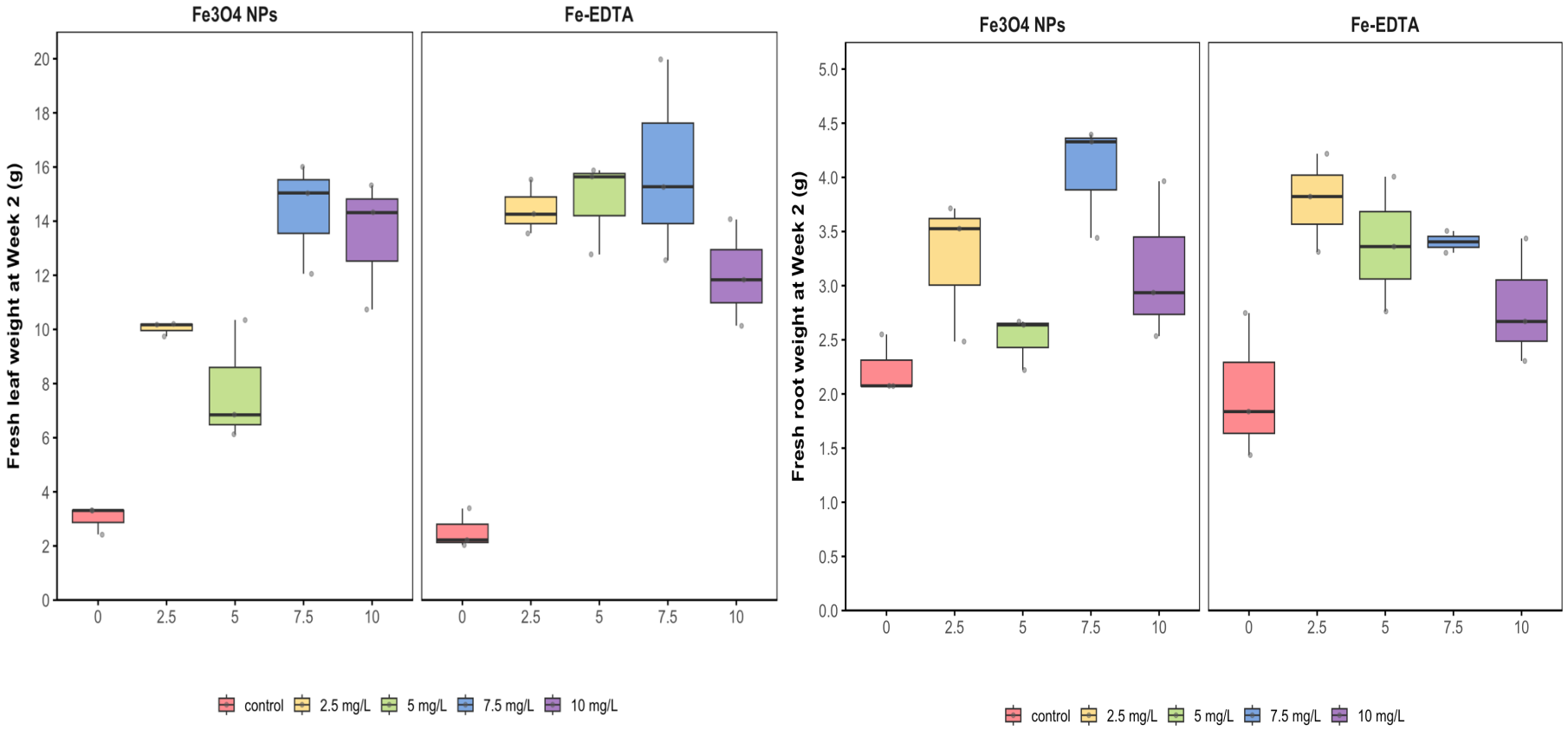


Figure 4.28 Harvest quality – Fresh weight (g) of lettuces harvested at week 2.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

The final harvest quality measurement at week 4 are presented in Figure 4.29. Overall, the Fe_3O_4 NPs treatment group exhibited slightly higher fresh weight for total biomass, leaves, and roots compared to the Fe-EDTA treatment group, with increases of approximately 6 %, 5 %, and 16 %, respectively. The highest total fresh weight (158.78 ± 9.38 g) was recorded in lettuces treated with Fe_3O_4 NPs 2.5 mg / L, which exceeded the yield of the control group (no Fe supplementation). Among the Fe-EDTA treatments, the greatest total fresh weight was recorded in Fe-EDTA 7.5 mg / L treatment (150.77 ± 8.57 g). The greatest leaf fresh weight found in the Fe_3O_4 NPs 2.5 mg / L treatment (141.12 g ± 11.13 g), while the highest root fresh weight was recorded in the Fe_3O_4 NPs 7.5 mg / L treatment (17.94 ± 4.78 g).

In summary, the Fe_3O_4 NPs treatment groups exhibited higher total fresh weights at the final harvest (week 4), including greater fresh leaf and root weights, compared to the Fe-EDTA treatment. While some variation across doses was evident, no clear correlation with dose was observed. It should be noted that no statistical analysis was conducted on these results due to limited biological replication. Therefore, the interpretation remains descriptive.

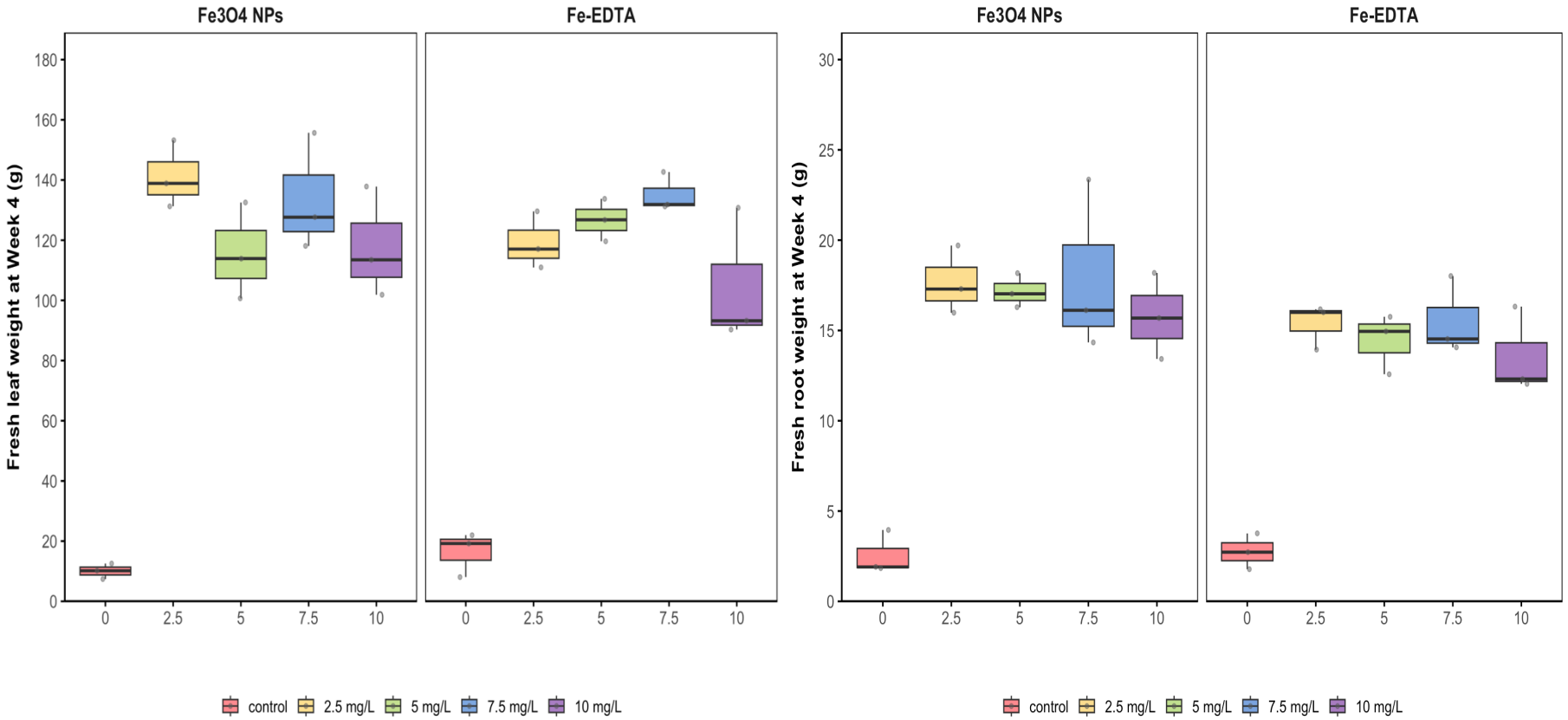


Figure 4.29 Harvest quality – Fresh weight (g) of lettuces harvested at week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

4.3.3.5 Elemental analysis

Multielement analysis was performed using ICP-MS to quantify iron (Fe), potassium (K) and magnesium (Mg) in lettuce leaf tissues harvested at week 2 and week 4. The ICP-MS methodology followed the procedure described previously in **Chapter 2.3.1**. The contents of Fe, K and Mg in both lettuce leaf and root tissues, expressed as milligram of each element per gram of dried lettuce tissues (mg / g).

Iron (Fe) quantification

Iron quantification was based on the Fe56 isotope, due to its high analytical stability and natural abundance. However, it should be noted that ICP-MS measures total elemental concentrations and does not distinguish between different oxidation states or molecular forms of iron (such as Fe^{2+} and Fe^{3+}). As a result, the data represent the total iron content in plant tissues without indicating chemical form or bioavailability.

Figure 4.30 presented the iron (Fe) quantification in lettuce leaf and root tissues at week 2. On average, the Fe-EDTA treatment led to 133 % higher Fe accumulation in leaf tissues compared to the Fe_3O_4 NPs group. The highest leaf Fe content was observed in the Fe-EDTA 7.5 mg / L treatment (0.757 ± 0.266 mg / g), followed by 10 mg / L treatment (0.550 ± 0.106 mg/ g). Within the Fe_3O_4 NPs group, the 2.5 mg / L treatment resulted in the highest Fe concentration in leaves (0.260 ± 0.113 mg / g), while the control group (no Fe treatment) had the lowest Fe concentration.

In contrast, root tissues analysis showed that Fe_3O_4 NPs treatments resulted in substantially greater Fe accumulation, with an average of 83 % higher root Fe content than Fe-EDTA treatments. The highest Fe concentration in root tissues was observed in lettuces treated with Fe_3O_4 NPs 10 mg / L (7.273 ± 0.950 mg / g). Among the Fe-EDTA group, lettuces treated with 10 mg / L group showed the highest Fe concentration in root tissues (4.574 ± 2.103 mg / g).

Overall, these results suggest that Fe-EDTA promotes greater translocation of Fe to the shoots, despite being applied via the root zone. In contrast, Fe_3O_4 NPs tend to accumulate more Fe in the roots at week 2. This indicates distinct uptake and distribution patterns between the two iron sources at early growth stages.

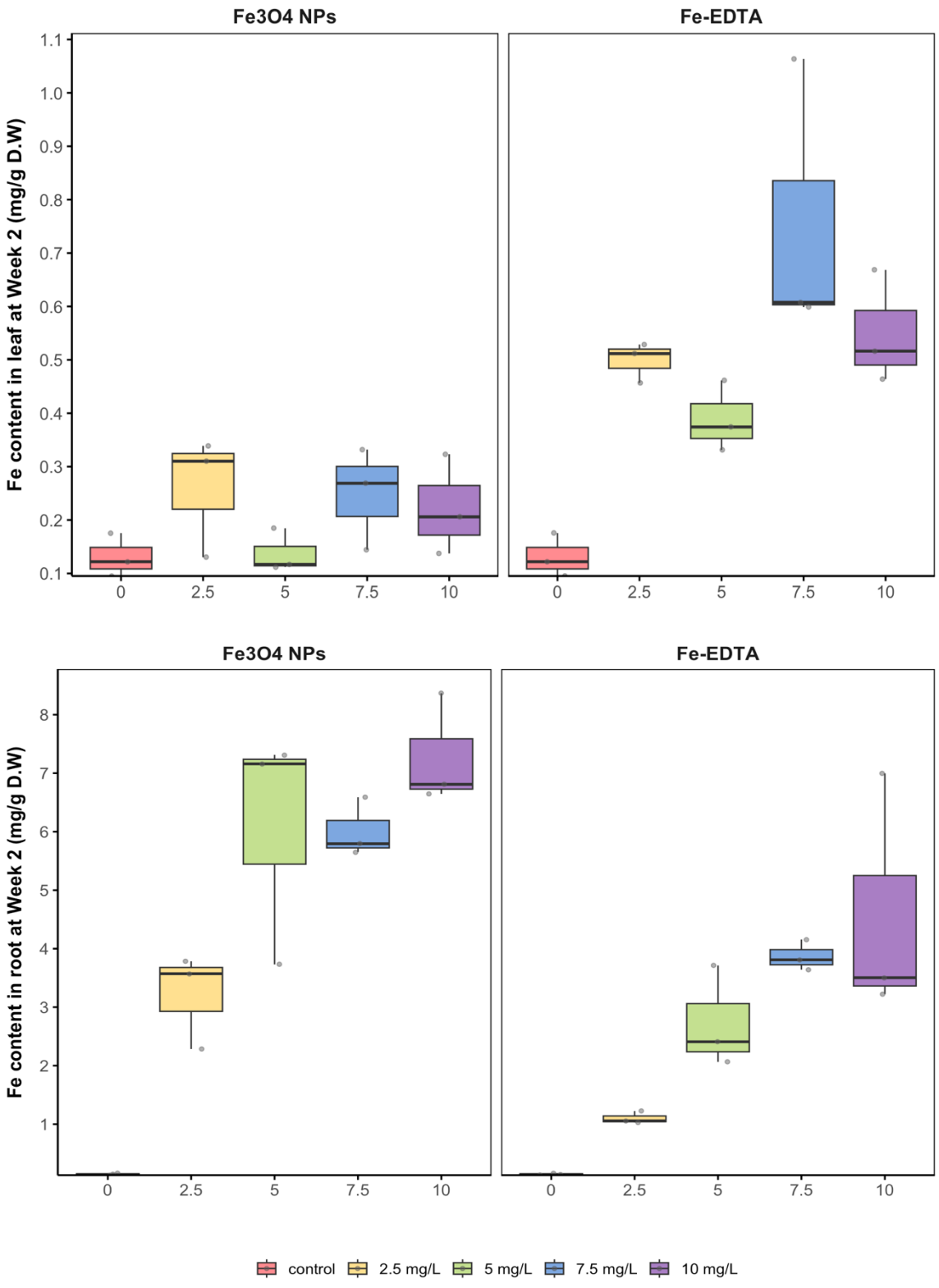


Figure 4.30 Quantification of iron in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 2.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Figure 4.31 presents iron (Fe) quantification in lettuce leaf and root tissues at week 4. Overall, Fe concentrations in leaf tissues were notably higher in the Fe_3O_4 NPs treatment group compared to Fe-EDTA group, with an average increase of 144 %. The highest Fe content was quantified in leaves was observed in lettuces treated with Fe_3O_4 NPs 2.5 mg / L (0.291 ± 0.076 mg / g), which was significantly higher than all other treatments. The lowest Fe concentration was recorded in the Fe EDTA 5 mg / L treatment group (0.064 ± 0.002 mg / g). In root analysis, lettuces treated with Fe_3O_4 NPs exhibited, on average, 5% higher Fe concentrations compared to those treated with Fe-EDTA. The highest Fe content in roots was measured in the with Fe_3O_4 NPs 10 mg / L treatment (4.517 ± 1.004 mg / g), followed by the Fe-EDTA 10 mg / L treatment (4.410 ± 0.913 mg / g).

To conclude, comparisons between week 2 and week 4 indicated limited translocation or accumulation of Fe in lettuces tissues over time, as Fe concentrations were lower in both leaf and root samples at week 4. Notably, lettuces treated with Fe_3O_4 NPs exhibited 68 % higher Fe content in root tissues at week 2 compared to week 4. These findings suggest that Fe uptake was more pronounced during the early growth stage (week 2), with minimal subsequent accumulation or movement within plant tissues.

Iron quantification in root tissues showed a positive correlation with increasing treatment concentration, with higher Fe content and greater variability, especially at 10 mg / L. This pattern suggests that no apparent uptake limitation at the highest concentration for either Fe_3O_4 NPs or Fe-EDTA treatments. Notably, while both 10 mg / L treatments led to the highest Fe accumulation in root tissues, the Fe levels in the leaves remained relatively low. This suggests that at higher iron concentration, Fe translocation from roots to shoots is limited. Overall, root tissues contained much higher Fe content (average 2.643 mg / g) than leaf tissues (average 0.158 mg / g), which is consistent with expectation given the root-based delivery method.

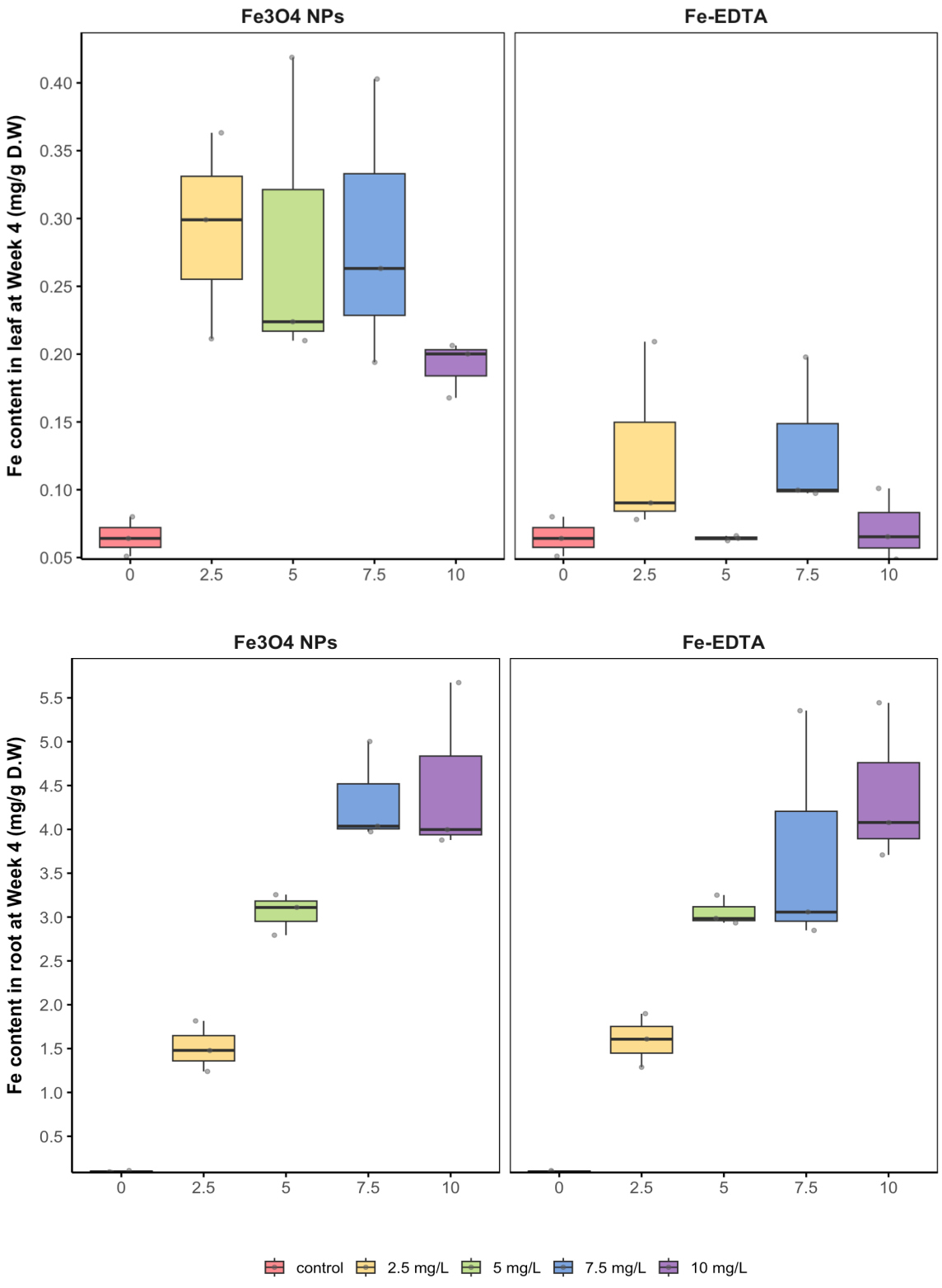


Figure 4.31 Quantification of iron in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Potassium (K) quantification

Comparison between the iron sources showed that Fe_3O_4 NPs treatments resulted in slightly higher potassium (K) content in both leaf and root tissues compared to Fe-EDTA treatments, as shown in Figure 4.32. In leaf tissues, lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited the highest K content (21.924 ± 1.677 mg / g), which was 58 % greater than the lowest K values measured in the control group without Fe treatment (13.895 ± 2.398 mg / g).

In contrast, root tissue analysis revealed that lettuces treated with Fe-EDTA had on average, 16 % higher K content in their root tissues compared to those treated with Fe_3O_4 NPs. The highest K content in roots was observed in the Fe-EDTA 5 mg / L treatment (18.839 ± 2.710 mg / g), representing an 87 % increase compared to the control group (10.092 ± 0.995 mg / g). Overall, leaf tissues contained approximately 30 % higher K concentrations than root tissues.

These findings suggest that iron supplementation may enhance K uptake or accumulation in lettuce tissues, particularly at moderate concentration (5 mg / L). However, no clear correlation was observed between increasing levels of Fe_3O_4 NPs or Fe-EDTA and nutrient accumulation in either leaf or root tissues.

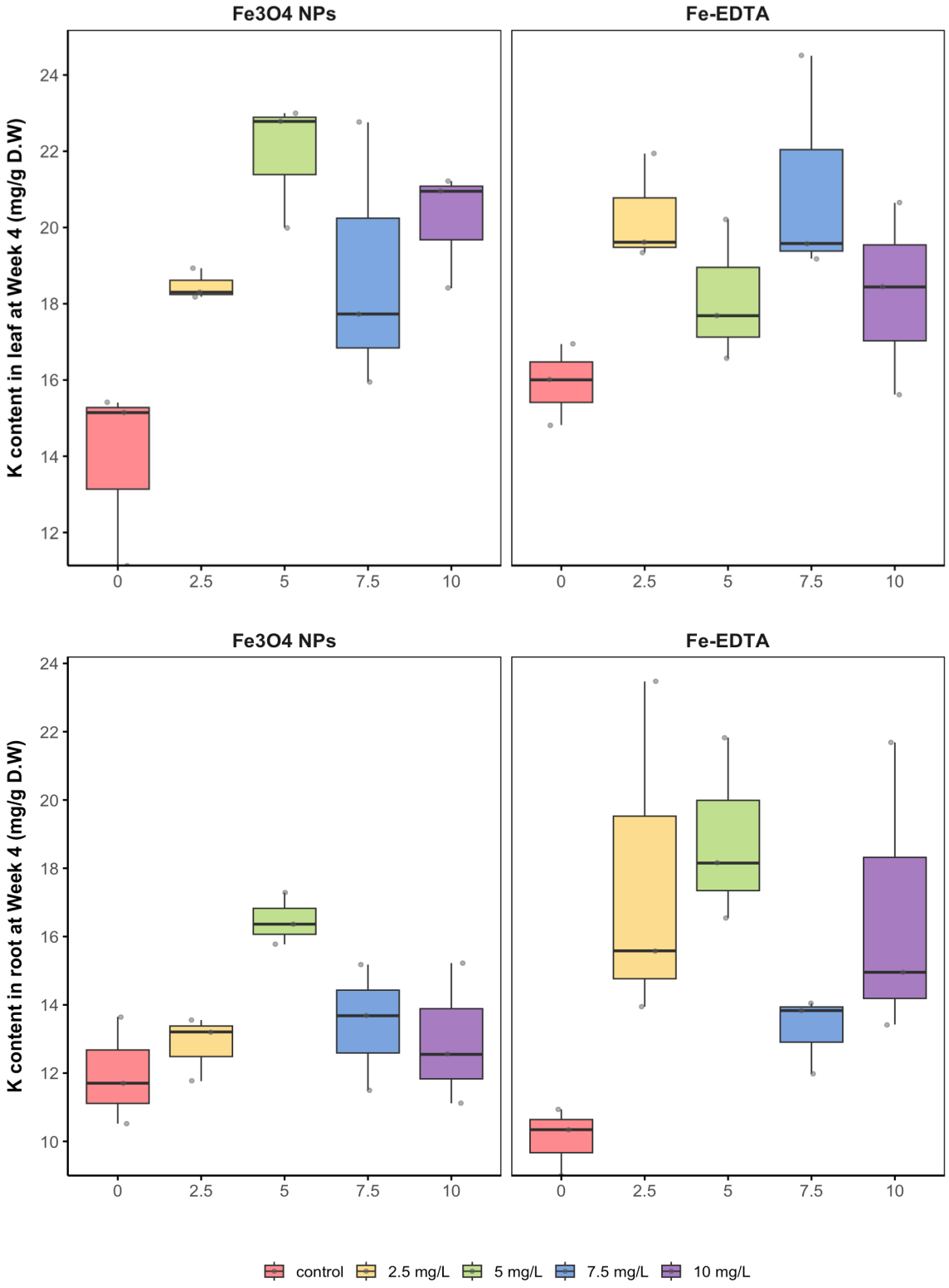


Figure 4.32 Quantification of potassium in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Magnesium (Mg) quantification

Overall, lettuces treated with Fe-EDTA exhibited slightly higher average magnesium (Mg) content in their leaf tissues, whereas those treated with Fe_3O_4 NPs showed marginally higher Mg accumulation in leaf tissues (Figure 4.33). The highest Mg concentration in leaf tissues was found in the control group (15.612 ± 5.522 mg / g), which was greater than all other treatments. The lowest leaf Mg content was observed in the Fe-EDTA 10 mg / L group (6.371 ± 2.029 mg / g). Overall, the Fe_3O_4 NPs group showed an 11% higher average Mg content in leaf tissues compared to Fe-EDTA.

For the root tissues analysis as shown in the bottom graph of Figure 4.33, the highest Mg uptake was recorded in the Fe-EDTA 10 mg / L treatment (5.464 ± 1.737 mg / g), while the lowest was in the Fe_3O_4 NPs 7.5 mg / L group (3.137 ± 0.296 mg / g). Overall, Fe-EDTA groups contained 31 % more Mg in root tissues compared to Fe_3O_4 NPs treatments. Notably, Mg content was substantially higher in leaf tissues than in root tissues across all treatments, with leaf concentrations averaging approximately three times greater than root values.

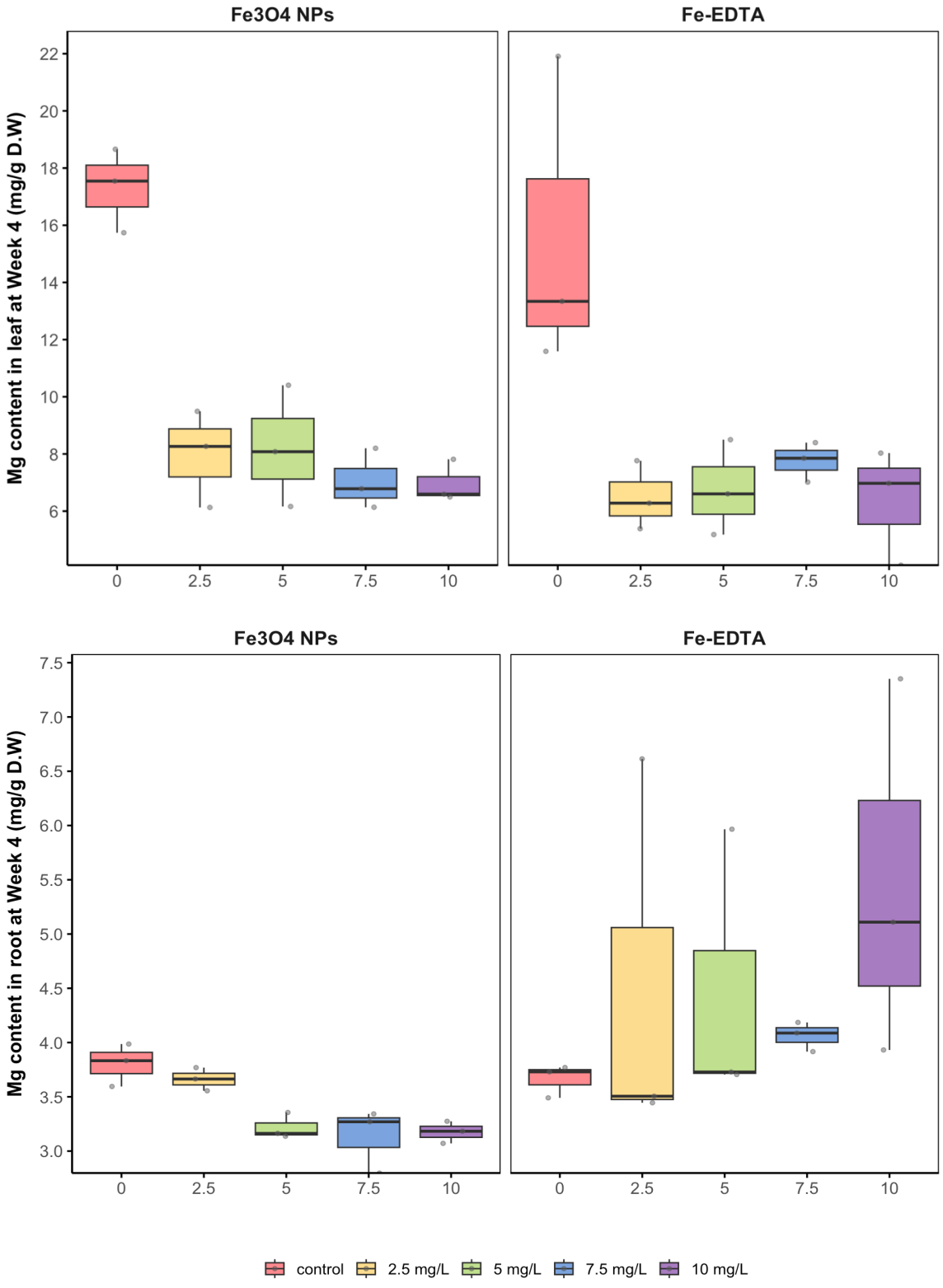


Figure 4.33 Quantification of magnesium in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Summary of multielement analysis

In summary, the multielement analysis demonstrated that lettuces treated with Fe_3O_4 NPs exhibited the highest Fe concentration in both leaf and root tissues compared to those treated with Fe-EDTA. These results suggest that Fe_3O_4 NPs offer enhanced bioavailability and uptake efficiency related to non-nano iron fertilisers (Fe-EDTA). A positive correlation in Fe accumulation was observed in the root tissues for both iron treatments. However, no statistical analysis was performed to determine whether differences between Fe_3O_4 NPs and Fe-EDTA were statistically significant. Fe quantification in leaf tissues showed higher Fe content in the Fe_3O_4 NPs group compared to Fe-EDTA treatment. Notably, Fe_3O_4 NPs 2.5 to 7.5 mg / L treatment resulted in an average Fe content of 0.287 mg / g in leaf tissues, while the 10 mg / L treatments showed comparatively lower accumulation.

In terms of K concentration, Fe_3O_4 NPs treatment generally resulted in slightly higher K concentrations in both leaf and root tissues compared to Fe-EDTA. The highest K content in leaf tissues was observed in the Fe_3O_4 NPs 5 mg / L group, while the Fe-EDTA 5 mg / L treatment led to the highest root K levels. Overall, leaf tissues accumulated approximately 30 % more K than root tissues across all treatments. Magnesium (Mg) analysis showed a different pattern. Although root Mg content was slightly higher in the Fe-EDTA group, the highest Mg concentration overall was found in the. Control group, which received no Fe treatments. Across all treatments, Mg levels were substantially higher in leaf tissues than root tissues, reflecting the key role of Mg in chlorophyll synthesis and leaf function.

These findings indicate that Fe_3O_4 NPs not only enhance Fe uptake and translocation but may also influence the absorption of other essential nutrients such as K and Mg, depending on tissue type and treatment concentration. However, due to the limited number of biological replicates per treatment, no statistical analysis was conducted to determine the significance of the difference between treatments. Therefore, while some correlation between treatment levels and nutrient uptake (Fe, K, and Mg) were observed, these should be interpreted with caution due the lack of statistical analysis to confirm significance.

4.3.3.6 Secondary metabolites analysis

To assess the effect of leaf-treated B_{12} application on secondary metabolites in lettuce, total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) were measured. The detailed methodology for sample preparation and analysis is described in **Chapter 2.4**. Statistical significance was evaluated using the PERMANOVA test, based on 9,999 permutations for multiple pairwise comparisons, as described in **Chapter 2.5**.

Total Phenolic Content (TPC)

The Total phenolic content (TPC), expressed as milligram of gallic acid by gram of fresh weight (mg GAE / g), is presented in Figure 4.34**.** On average, Fe_3O_4 NPs-treated lettuces (0.389 mg GAE / g) exhibited a 6 % higher TPC compared to those treated with Fe-EDTA (0.369 mg GAE / g). The highest TPC value (0.495 ± 0.054 mg GAE / g) was recorded in the control group, which was 76 % higher than the lowest value (0.282 ± 0.034 mg GAE / g) observed in the Fe-EDTA 2.5 mg / L treatment. Excluding the control group, the highest overall TPC was found in the Fe_3O_4 NPs 2.5 mg / L treatment (0.447 ± 0.052 mg GAE / g), followed by Fe3O4 NPs 7.5 mg / L treatment (0.392 ± 0.075 mg GAE / g). Overall, the TPC results suggest that control group exhibited the highest total phenolic content, likely as a result of stress-induced activation of antioxidant pathways. This response may reflect iron deficiency experienced by the control group, which receive no Fe application, as indicated by poor morphological and physiological traits and low harvest weight.

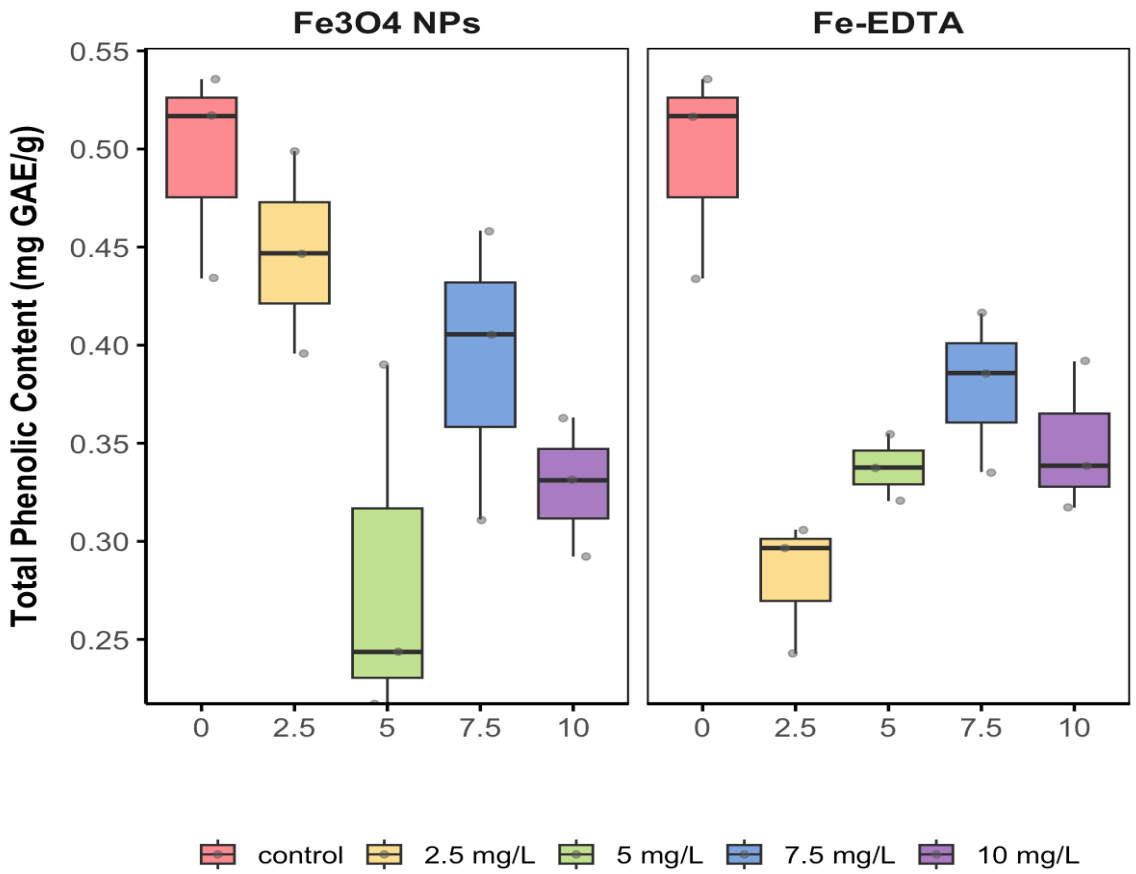


Figure 4.34 Total Phenolic Content (mg GAE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Total Flavonoid Content (TFC)

Figure 4.35 depicts total flavonoid content (TFC) in fresh lettuce tissues. The TFC results are expressed as milligram of Rutin equivalent (RE) per gram of fresh weight (mean ± standard deviation mg RE / g). On average, the Fe_3O_4 NPs treatments (0.088 mg RE / g) exhibited a slightly higher TFC compared to the Fe-EDTA treatments (0.086 mg RE / g). Among the Fe_3O_4 NPs group, the highest TFC values (0.106 ± 0.014 mg RE / g) was observed in the 2.5 mg / L treatment. In the Fe-EDTA group, the highest TPC value (0.119 ± 0.014 mg RE / g) was found in the group of Fe_3O_4 NPs 2.5 mg / L treatment. The control group, which did not receive any Fe treatment, exhibited the lowest flavonoid content, consistent with signs of iron deficiency. This deficiency likely impaired pigment development, such as chlorophyll and carotenoids, leading to reduced photosynthetic capacity.

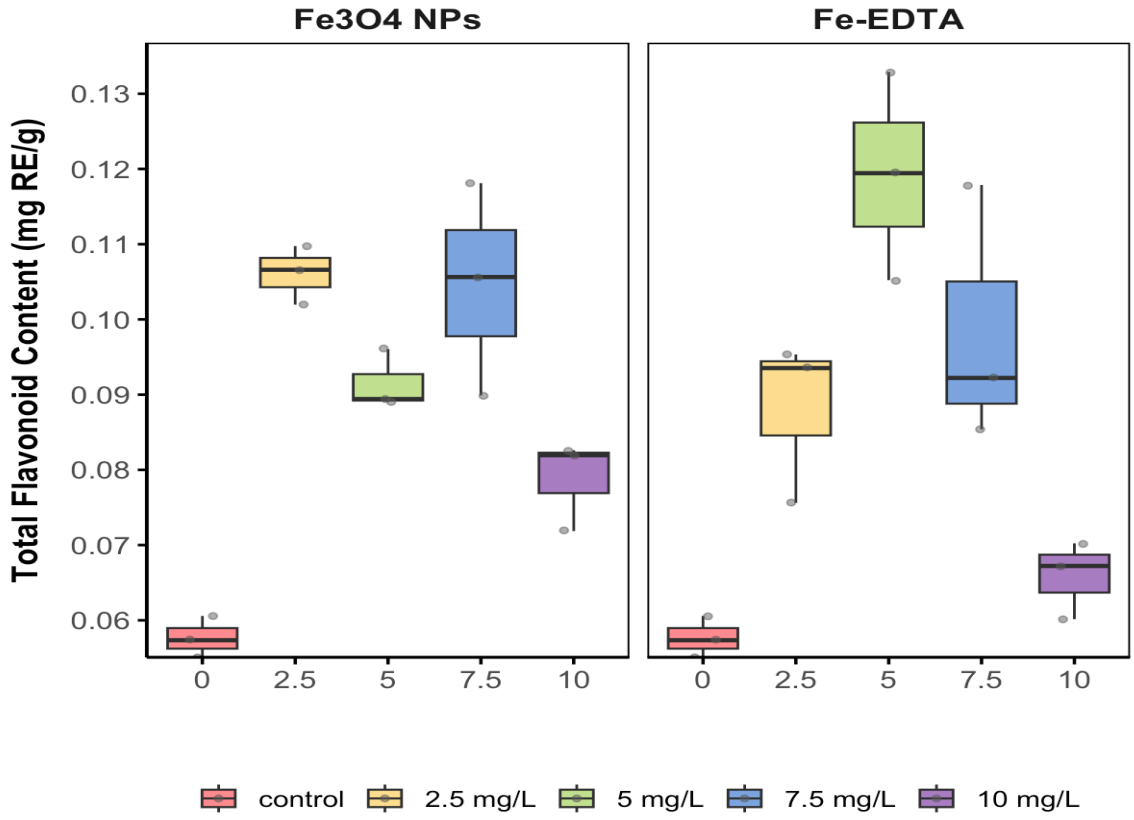


Figure 4.35 Total Flavonoid Content (mg RE / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Total Antioxidant Activity (TAA)

Total antioxidant activity (TAA) was measured using Trolox® equivalent antioxidant capacity (TEAC), expressed as milligram of TEAC per gram of a gram of fresh lettuce leaf tissues (mg TEAC / g), as shown in Figure 4.36**.** On average, Fe_3O_4 NPs treatments exhibited a slightly higher TAA (6.040 mg TEAC / g) compared to Fe-EDTA treatments (6.021 mg TEAC / g). Within the Fe_3O_4 NPs group, the highest value of TAA (6.156 ± 0.350 mg TEAC / g) resulted in lettuces treated with 7.5 mg / L treatment. Among the Fe-EDTA treatment group, the lettuces treated with Fe-EDTA 7.5 mg / L contained the highest TAA value (6.247 ± 0.419 mg TEAC / g). The lowest TAA value was recorded in Fe_3O_4 NPs 2.5 mg / L treatment (0.582 ± 0.311 mg TEAC / g). Overall, relatively low variation in antioxidant activity was observed among the Fe treatments. The Fe-EDTA 2.5 treatment showed the lowest antioxidant activity, which corresponded with the reduced phenolic compounds in the lettuce.

In summary, the results of secondary metabolites demonstrate that different types and concentrations of iron treatments had variable effects on TPC, TFC, and TAA in lettuce. Overall, the average levels of secondary metabolites showed negligible differences between Fe_3O_4 NPs and Fe-EDTA treatments, although Fe_3O_4 NPs group exhibited slightly higher average values across all three parameters. Particularly, there was no clear correlation was observed between increasing Fe concentrations and levels of secondary metabolites. Moreover, no consistent correlation was detected between TPC, TFC, and TAA measurements, suggesting that each parameter responded independently to the Fe treatments.

Notably, the control group without Fe treatment showed the highest TPC, likely reflecting stressed-induced phenolic accumulation due to iron deficiency. In contrast, the lowest TFC was observed in the control group, possible due to reduced pigment biosynthesis and photosynthetic activity under limited iron condition. However, it is important to note that due to the limited number of biological replicates, no statistical analysis was conducted to assess the significance of these differences between the treatments.

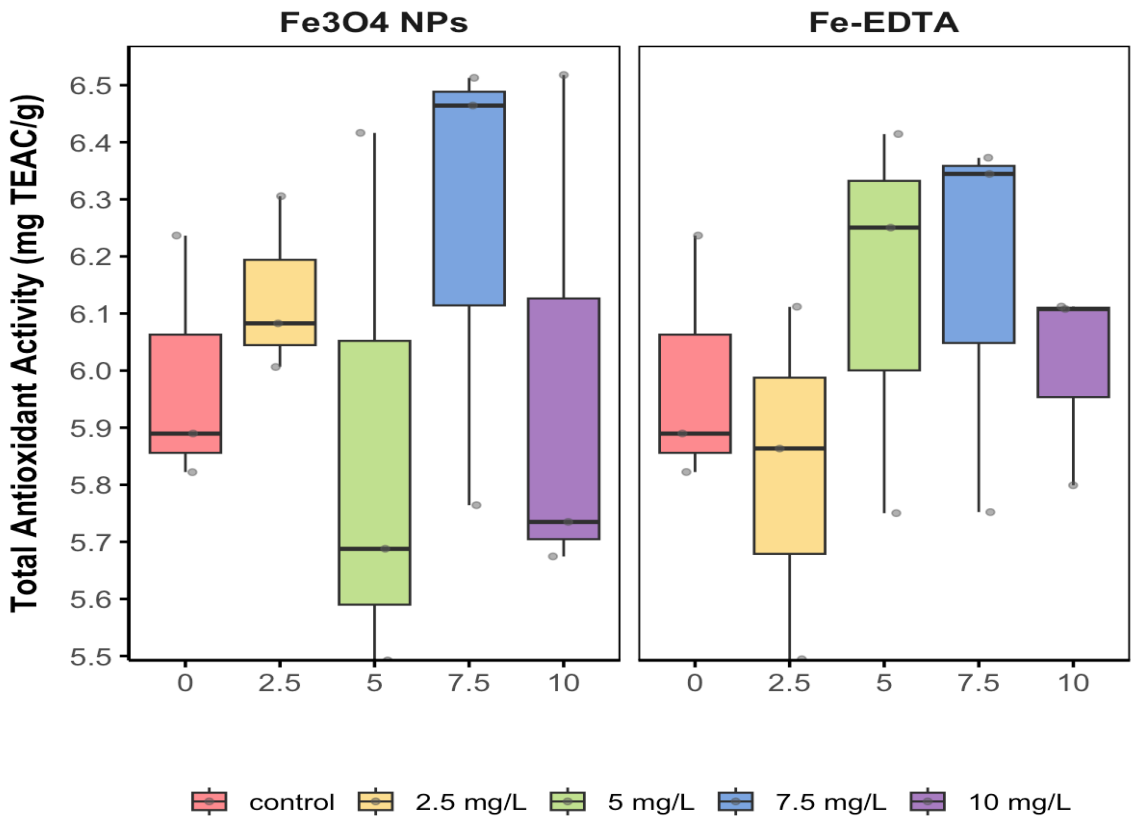


Figure 4.36 Total Antioxidant Activity (mg TEAC / g) in lettuce leaf tissues at Week 4.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

4.4 Discussions

4.4.1 Applicability of Fe_3O_4 NPs for iron fortification in lettuce

The study synthesised Fe_3O_4 NPs using different methods and capping agents to optimise their potential for iron fortification in hydroponically grown lettuce. In **Chapter 4.2**, the Fe_3O_4 NPs capped with potato starch were evaluated for their effects on lettuce growth and nutritional quality. Iron was applied at five different concentrations: 0, 0.625, 1.25, 2.5 and 5 mg / L. A control group Received a conventional iron fertiliser, Fe-EDTA at 2.5 mg / L, enabling direct comparisons between Fe-EDTA and Fe3O4 NPs at the same concentration (2.5 mg / L). In addition, Fe_3O_4 NPs were applied at lower concentration (0.625 and 1.25 mg / L) to investigate whether their theoretically enhanced uptake efficiency and bioavailability, which are attributed to their nanoscale dimension, could achieve comparable effectiveness at reduced dosages [141,145].

A higher concentration (5 mg / L) was also tested to investigate whether increased nanoparticle availability would improve uptake. Overall, the results of growth and morphological assessments indicated that the control group treated with Fe-EDTA 2.5 mg / L exhibited the better growth, morphological development, and harvest weight. In contrast, lettuces treated with the Fe_3O_4 NPs 0.625 mg / L treatment exhibited poor morphological development, low harvest weight, and visible symptoms of iron deficiency symptoms on leaf tissues, as seen in Figure 4.37.

Figure 4.37 Digital photograph of lettuces on week 4 harvest samples.

(A) Control group (Fe-EDTA 2.5 mg / L). (B) Fe_3O_4 NPs 0.625 mg / L.

The Fe_3O_4 NPs treatments at 5 mg / L and 1.25 mg / L exhibited better water management and gas exchange compared to the 0.625 mg / L treatment, indicating a dose-dependent effect of the nanoparticles on these physiological processes. Notably, the Fe_3O_4 NPs treatment at both lower (1.25 mg / L) and higher (5 mg / L) concentration resulted in improved *WUE*, *iWUE*, and L_s compared to the intermediate 2.5 mg / L treatment. In contrast, the lowest concentration treatment (0.625 mg / L) showed reduced *WUE*, likely due to insufficient iron availability hindering photosynthetic performance in lettuce. This consistent with the essential role of iron in key cellular process such as chlorophyll biosynthesis, chloroplast development, and overall photosynthetic activity, all of which directly affect the plant's ability to efficiently manage water use and gas exchange [145].

However, the application of Fe_3O_4 NPs 2.5 mg / L resulted in relatively lower values of *WUE*, *iWUE,* and L_s compared to both control group treated with Fe-EDTA at the same concentration (2.5 mg / L) and the lower concentration of Fe_3O_4 NPs treatment (1.25 mg / L). This reduction may be attributed to the potato starch capping agent used on the Fe_3O_4 NPs, which is thought to induce nanoparticle aggregation, thereby hindering their uptake by lettuce plants. As shown in Figure 4.38, the Fe_3O_4 NPs agglomeration of the Fe_3O_4 NPs was observed in higher Fe_3O_4 NPs treatments (2.5 and 5 mg / L), and such aggregation likely impedes the absorption of Fe_3O_4 NPs by lettuces.

The Fe_3O_4 NPs were capped with potato starch at a 1:1 (w/w) ratio of capping agent to Fe_3O_4 NPs, which suggests that excess starch not fully coated with nanoparticles could form hydrogen bonds with other starch molecules, promoting further. Notably, this aggregation was observed at an early stage of growth (week 1 only) in a Fe_3O_4 NPs 2.5 mg / L nutrient tank, which may have limited iron bioavailability of iron and contributed to the reduced physiological performance in the Fe_3O_4 NPs 2.5 mg /L treatments. This aggregation may have also influence elemental analysis results by restricting Fe uptake. Another possible explanation for the observed cluster could be microbial biofilms or bacterial colonisation, even though the experiment was conducted in a controlled environment. While contamination is unlikely due to sterilisation measures, the presence of biofilm in one tank cannot be completely disregard without further microbiological or microscopic analyses.

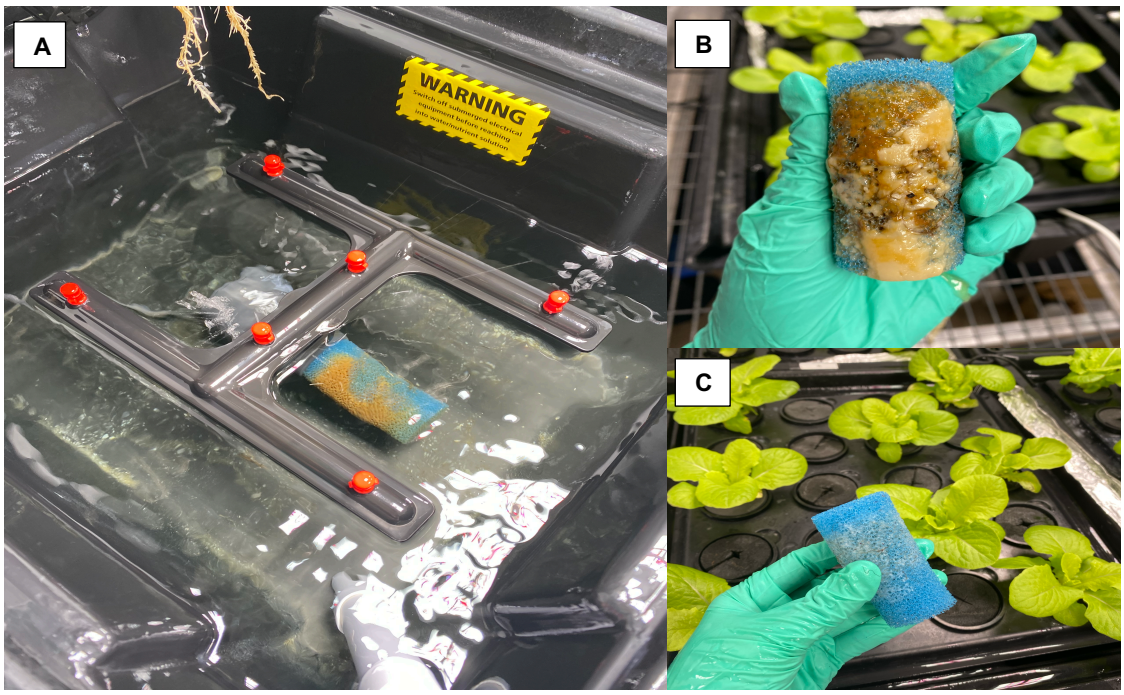


Figure 4.38 Hydroponic nutrient tank with potential Fe_3O_4 NPs aggregation.

Experiment conducted on Chapter 4.2. **A.** Hydroponic solution tank and the aggregation of Fe_3O_4 NPs was observed in submersible water pumps from nutrient tanks relatively higher concentration (2.5 and 5 mg / L) **B.** Potato starch cluster might cause Fe_3O_4 NPs agglomeration which eventually hinder the uptake of iron by plant**. C.** After gently squeeze the sponge, clustered Fe_3O_4 NPs were dissolved, unlike the residue of potato starch.

Elemental analysis at the final harvest (week 4) revealed that the control group treated with Fe-EDTA 2.5 mg / L had the highest uptake and translocate in both leaf and root tissues of lettuce. Interestingly, the Fe_3O_4 NPs 5 mg / L treatment showed the lowest Fe content in leaf, while the 0.625 mg / L treatment showed the lowest Fe content in root. These findings differ from those reported by Azizkhani et al. (2023) [141] who found that the Fe content in strawberry leaf and fruit increased when treated with Fe_3O_4 NPs coated with cysteine, even at concentrations 10 % lower than conventional iron fertilisers. In their study, 6 mg / L of conventional iron sulphate was compared with 0.06 and 6 mg / L of Fe_3O_4 NPs coated with cysteine. In contrast, the present study observed that a lower concentration of Fe_3O_4 NPs (0.625 mg / L) resulted in visible symptoms of iron deficiency in lettuce, such as poor development and leaf yellowing. This discrepancy may be attributed to differences in nanoparticle surface coating, plant species, or nutrient demands.

As discussed above, the reduced Fe uptake observed in the Fe_3O_4 NPs 2.5 mg / L treatment may be explained by the aggregation of nanoparticles, likely induced by excessive amounts of the capping agent (potato starch). This aggregation, whether of the nanoparticles themselves or the capping agent, can hinder not only the uptake of Fe_3O_4 NPs but also limit the availability of other essential nutrients. Elemental analysis via ICP-MS revealed that lettuces treated with Fe_3O_4 NPs 2.5 mg / L contained lower iron content in both leaf and root tissues, followed by the 0.625 mg / L treatment group. Notably, Fe_3O_4 NPs 2.5 mg / L treatment group did not exhibit clear adverse morphological symptoms such as chlorosis or stunted growth, unlike the 0.625 mg / L treatment.

In summary, several factors may have limited Fe uptake in this study: (1) The Fe_3O_4 NPs concentrations, particularly 0.625 and 0.125 mg / L, were likely insufficient compared to the conventional Hoagland fertiliser (Fe-EDTA 2.5 mg / L); (2) The post-synthesis preparation of Fe_3O_4 NPs may not have yielded optimally dispersed and bioavailable nanoparticles suitable for plant uptake; and (3) Potato starch capping agent may have promoted nanoparticle aggregation, reducing their stability and bioavailability.

Therefore, alternative capping agents or improved post-synthesis methods may be necessary to enhance nanoparticle stability and efficiency for hydronic application. Overall, this experiment suggests that Fe_3O_4 NPs capped with potato may need to be applied at concentrations equivalent to or higher than traditional iron fertilisers to ensure sufficient iron availability in lettuce. For context, the recommended Fe-EDTA concentration in Hoagland nutrient solution, depending on the plant species

grown hydroponically. Accordingly, Fe_3O_4 NPs treatment below 2.5 mg / L (e.g., 0.625 and 1.25 mg / L) may not provide adequate Fe to support optimal plant development.

However, these findings should be interpreted cautiously, especially in case of Fe_3O_4 NPs capped with potato starch at 2.5 mg / L treatment, which observed clusters in early week of growth (week 1). This highlights a key limitation when using potato starch as a capping agent in hydroponic system. Different stabilisers or capping agents may improve nanoparticle dispersion and stability, reduce aggregation and enhance nutrient uptake. Therefore, further optimisation process of post-synthesis processes and careful selection of capping agents are essential for improving the efficiency of Fe_3O_4 NPs in hydroponic application.

4.4.2 Comparability of Fe_3O_4 NPs to Fe-EDTA

Effect of Fe_3O_4 NPs treatment in lettuce morphology and physiology

This study aimed to compare the effectiveness of Fe_3O_4 NPs with Fe-EDTA, a widely used conventional iron fertiliser, in improving lettuce growth and nutritional quality. **Chapter 4.3** focuses on iron fortification using Fe_3O_4 NPs capped with L-lysine, applied in a series of dilutions. The experimental design included a control group that received no iron supplementation (0 mg / L) and treatment groups that received Fe_3O_4 NPs at concentrations of 2.5, 5, 7.5, and 10 mg / L. An equivalent amount of iron was applied using Fe-EDTA to allow a direct comparison between the nanoparticle and conventional formulations. This approach enabled a preliminary assessment of the applicability of Fe_3O_4 NPs relative to Fe-EDTA in hydroponic cultivation. This study hypothesises that Fe_3O_4 NPs capped with L-lysine may offer a comparable alternative to conventional iron fertiliser such as Fe-EDTA, due to their improved physiochemical stability, smaller particle size, and potential enhanced bioavailable.

The comprehensive iron fortification experiment revealed that the control group (Fe-EDTA 2.5 mg / L) exhibited clear signs of iron deficiencies, including poor growth, underdeveloped morphology, and reduced harvest weight. Apart from the control group with no iron application, both Fe_3O_4 NPs and Fe-EDTA treatments followed comparable trends throughout the experiment. The morphological and physiological results were consistent with the harvest weight. Lettuces treated with Fe_3O_4 NPs recorded higher fresh weights (total, leaf and root) at the final harvest compared to those treated with Fe-EDTA. Notably, the Fe_3O_4 NPs 2.5 mg / L

produced the highest fresh weight, followed by 7.5 mg / L treatment, indicating that Fe_3O_4 NPs capped with L-lysine could serve as a viable alternative to traditional iron fertiliser such as Fe-EDTA.

Overall, the Fe-EDTA treatment group exhibited greater height, more visibly vibrant green foliage, and a higher vegetation index, as measured by PlantEye. In contrast, the Fe_3O_4 NPs group demonstrated enhanced morphological development, including increased leaf area and digital biomass, as well as improved pigment content (e.g., higher chlorophyll levels) and greater harvest weight, reflected in heavier total, leaf and root biomass (Chapter 4.3.3.1). Similar results were observed by Azizkhani et al (2023), who reported that the Fe_3O_4 NPs coated with cysteine (0.06 and 6 mg / L) enhanced strawberry biomass, leaf area, and chlorophyll a, and b content, and harvest yield compared to the control group treated with non-nano iron sulphate at 6 mg / L [141]. Notably, the Fe_3O_4 NPs 6 mg / L treatment significantly improved overall the strawberry growth and development. In the present study, a positive correlation was found between biomass, leaf area, chlorophyll content, and foliar iron concentration. Although statistical analysis was limited due to a low number of biological replicates, these trends provide valuable preliminary insights into the potential benefits of Fe_3O_4 NPs iin promoting plant growth and physiological performance.

Effect of Fe_3O_4 NPs treatment in lettuce water management and gas exchange

The combination of high Water Use Efficiency (*WUE*) and elevated Stomatal Limitation (L_s) observed in Fe-EDTA treated lettuces suggests that water conservation was achieved primarily through increased stomatal closure, which likely restricted CO_2 uptake and potentially constrained photosynthetic carbon assimilation. In contrast, the higher Intrinsic Water Use Efficiency (*iWUE*) observed in Fe_3O_4 NPs treated lettuce indicates more efficient gas exchange, which may support enhanced photosynthetic activity. These findings imply that Fe_3O_4 NPs may enhance photochemical efficiency of photosystem II (PSII) by reducing energy dissipation via nonphotochemical quenching mechanisms [140]. Furthermore, potential interactions between Fe_3O_4 NPs and proteins may promote photosynthetic efficiency by influencing light reflection or emission process [140]. This is consistent with findings of Tombuloglu et al. (2024), who reported that optimal Fe_3O_4 NPs applications significantly improved photosystem II (PSII) photochemical efficiency and minimised energy loss via nonphotochemical regulation in tomato plants [140]. However, due to limited biological replicates for the present study,

statistical analysis was not performed, and therefore these results should be considered preliminary.

Effect of Fe_3O_4 NPs treatment in lettuce for multielement fortification

Elemental analysis using ICP-MS provides multielement quantification. In this study, iron quantification was based on the Fe56 isotope, due to its high analytical stability and natural abundance. However, it should be noted that ICP-MS measures total elemental concentrations and does not distinguish between different oxidation states or molecular forms of iron (such as Fe^{2+} and Fe^{3+}). As a result, the data represent the total iron content in plant tissues without indicating chemical form or bioavailability, both of which are critical for evaluating nutritional value and uptake efficiency.

Lettuces treated with Fe_3O_4 NPs exhibited greater iron uptake and translocate to both leaf and root tissues compared to those treated with Fe-EDTA. These results suggest that Fe_3O_4 NPs may provide enhanced iron availability and mobility within the plant, potentially due to their significantly smaller particle size compared to conventional iron sources such as Fe-EDTA[141,145]. Similar results were reported in a previous study by Azizkhani et al. (2023), which demonstrated Fe_3O_4 NPs significantly increased iron content in both the leaves and fruits of hydroponically grown strawberries [141]. The study found a positive correlation between elevated iron levels in strawberry tissues and improved morphological and physiological development. These findings are in accordance with the present study. Iron plays a critical role in photosynthetic processes and chlorophyll biosynthesis which are crucial metabolic processes for plant growth and development [93,94]. Therefore, enhanced uptake of Fe_3O_4 NPs may boost photosynthetic activity, as reflected in increased chlorophyll content in leaves, ultimately contributing to higher final harvest biomass.

Unlike the results observed for iron accumulation, there was negligible differences in the macronutrient contents of potassium (K) and magnesium (Mg) between the different iron treatments. Compared to the control group (without iron application), both Fe_3O_4 NPs and Fe-EDTA treatments showed relatively higher level of K in leaf and root tissues, with Fe-EDTA treated lettuces exhibiting slightly higher K concentrations than those treated with Fe_3O_4 NPs. Interestingly, the control group displayed a higher Mg concentration in leaf tissues compared to both iron-treated groups. This may be attributed to a compensatory response under iron deficiency, where more light energy is absorbed per chlorophyll molecule, potentially

stimulating increased Mg uptake to enhance chlorophyll biosynthesis and ferredoxin activity as an adaptive mechanism [194]. These findings highlight the plant's capacity to modulate nutrient uptake in response to micronutrient stress, particularly through mechanisms that support photosynthetic function. It is important to note that due to the limited number of biological replicates, statistical analysis was not performed. Therefore, these results should be considered preliminary and interpreted with caution.

Effect of B_{12} treatment in lettuce secondary metabolites

In the present study, the Fe_3O_4 NPs treatment group exhibited higher average levels of total phenolics content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) compared to Fe-EDTA group. These findings suggest that Fe_3O_4 NPs may positively influence the biosynthesis of secondary metabolites, potentially due to improved iron uptake and its role in enzymatic pathway associated with oxidative stress regulation [145]. Interestingly, the control group (without any Fe treatment) examined the highest phenolic content in lettuce leaves. This may indicate that lettuces experienced greater physiological stress due to iron deficiency. Such deficiency is known to trigger the accumulation of phenolic compounds, as these secondary metabolites play a protective role in mitigating oxidative stress under nutrient-limited condition [194]. As a stress response, plant often enhance the stimulate the biosynthesis of phenolics and proline to improve tolerance and survival in iron-deficient environments [176].

Compared to Fe-EDTA treatment group, the Fe_3O_4 NPs treatment resulted in higher flavonoid content in lettuce leaves. Interestingly, the control group, which exhibited significant iron deficiency, showed the lowest total flavonoid levels. These findings align with the relative chlorophyll measurement obtained by the SPAD meter, as flavonoids play a crucial role in pigment development in plant [170]. Supporting this, Holghoomi and Colagar (2024) reported that Fe NPs treatment induced a notable increase in secondary metabolites, including phenolic and flavonoids, in garden cress (*Lepidum sativum* L.) [195]. This effect may be attributed to nanoparticles inducing reactive oxygen species (ROS), particularly hydrogen peroxide and superoxide [195]. Additionally, Fe NPs-treated plants exhibited upregulated expression of jasmonate biosynthesis gene, which are key regulators involved in the production and accumulation of phytochemicals [195].

Although no significant correlation was overserved between TPC and TAA, the Fe-EDTA 2.5 mg / L treatment exhibited lowest value of both TPC and TAA,

respectively. This may because the Fe-EDTA 2.5 mg / L treatment is recommended by the Hoagland nutrient solution recipe for lettuce grown in hydroponic/aeroponic cultivation [163]. Therefore, this treatment likely provided an optimal growth environment, reducing the need for plants to activate self-defence mechanisms against stress. Consequently, the Fe-EDTA 2.5 mg / L treatment group showed the lowest accumulation of phenolics and antioxidant activity.

Collectively, these findings demonstrate that Fe_3O_4 NPs not only improve iron uptake but also enhance the accumulation of important secondary metabolites such as phenolics and flavonoids, which contribute to total antioxidant activity and potentially improve plant stress resilience and nutritional quality. This effect highlights the promise of Fe_3O_4 NPs as a multifaceted iron fertilisation strategy in hydroponic lettuce cultivation. However, given the preliminary nature of the current results, further research with larger sample sizes and detailed molecular analyses is warranted to fully elucidate the mechanisms underlying these responses and to confirm the broader applicability of Fe_3O_4 NPs in crop biofortification and stress management.

General overview

Thorough comprehensive experiments, this study evaluated the applicability of Fe_3O_4 NPs as an alternative conventional iron fertiliser such as Fe-EDTA. The characteristic of Fe_3O_4 NPs capped with different biodegradable capping agents, including potato starch and L-lysine, were thoroughly examined. The results demonstrated that Fe_3O_4 NPs hold strong potential as an effective iron source for lettuce cultivation. However, further optimisation is needed to enhance the stability of the capping agents, as the excessive potato starch may cause nanoparticle aggregation or potential microbiological contamination, which adversely impacted the iron uptake. Addressing these formulation challenges will be crucial to maximise the efficiency and practical application of Fe_3O_4 NPs in agricultural biofortification. On the other hand, Fe_3O_4 NPs capped with L-lysine emerged as a promising alternative to Fe-EDTA fertiliser. The use of L-lysine, a chemically and nutritionally beneficial amino acid, improved the dispersive stability and bioavailability of Fe_3O_4 NPs. Post-synthesis optimisation and characterisation confirmed that Fe_3O_4 NPs capped with L-lysine are well-suited for application in hydroponic and aeroponic cultivation system.

The present study demonstrated that the Fe_3O_4 NPs capped with L-lysine treatment enhanced lettuce growth and development, as shown in Chapter 4.3.3.1,

with increased leaf area, digital biomass, and final fresh weights. These improvements corresponded with higher iron concentrations measured in leaf tissues, particularly at Fe_3O_4 NPs 2.5 mg / L treatment. Additionally, this treatment elevated chlorophyll content and improved photosynthetic efficiency, contributing to better growth and enhanced nutritional quality in lettuces. Effective stomatal regulation, indicated by reduced stomatal limitation, was also observed in Fe_3O_4 NPs group and correlated with higher chlorophyll level. In contrast, Fe-EDTA treatment exhibited higher water use efficiency and increased stomatal limitation at elevated light intensities, suggesting a strategy of conserving water through stomatal closure. Overall, both Fe_3O_4 NPs and Fe-EDTA treatment promoted healthy growth but through different physiological responses related to water management and photosynthesis.

The Fe_3O_4 NPs treatment group exhibited slightly higher levels of phenolic, flavonoid and antioxidant activity compared to the Fe-EDTA treatment group. These results suggest that Fe_3O_4 NPs may stimulate the production of phytochemicals as a part of a self-defence mechanism, which could positively influence photosynthetic activity and overall plant developmental. Additionally, Fe_3O_4 NPs application resulted in increased iron content in both lettuce leaves and roots throughout the growing period. The elevated iron levels in leaves at final harvest indicate effective accumulation and translocation of iron from Fe_3O_4 NPs, whereas relatively lower iron concentrations were observed in leaves treated with Fe-EDTA treatment. This enhanced mobility is likely due to the nano-size of Fe_3O_4 NPs, which facilitates better transport within plant cellular pathways compared to Fe-EDTA. Along with the improved iron bioavailability, the Fe_3O_4 NPs treatment also increased potassium content compared to Fe-EDTA treatment. Interestingly, the control group without iron treatment showed higher magnesium levels in lettuce leaf tissues, suggesting that these plants may have increased Mg uptake to support photosynthetic activity under iron-deficient stress. These results should be considered preliminary, and further research with larger sample size is necessary to confirm the interaction among these elements and the broader effects of Fe_3O_4 NPs treatment on nutrient uptake.

This study is limited to determine the toxicity of Fe_3O_4 NPs for further application in food crops and human consumption. Excessive iron can generate reactive oxygen species (ROS), such as superoxide anion radicals ($O2^-$) and hydroxyl radicals (OH), which can cause oxidative damage [95]. Previous studies demonstrated toxic effect of Fe_3O_4 NPs, including reduced growth rate, and decreased viability in both plant and animal cells [196]. However, the potential toxicity

of Fe_3O_4 NPs to plants, animal, and the environment remains under- investigated [196]. In the present study, no phytotoxicity effects were observed across a range of Fe_3O_4 NPs concentration. Nonetheless, further research into the toxicity and ROS-related activities of Fe_3O_4 NPs is necessary to ensure their safe application in agriculture and beyond.

To estimate the cost efficiency of Fe_3O_4 NPs, expense details were provided by Dr. Gareth Cave, Nottingham Trent University, UK. The total cost of Fe_3O_4 NPs synthesis includes raw materials such as Fe^{2+} and Fe_{3+} salts, sodium hydroxide, hydrochloric acid, and coating agents (e.g., L-lysine), as well as operational expenses like equipment activation rate (e.g., flow rate per hour and running disc); and production rate (final yield of coating and production). The cost of the uncapped Fe_3O_4 NPs is approximately £ 3.62 per kilogram, whereas Fe_3O_4 NPs capped with L-lysine costs £ 18.15 per kilogram. For comparison, the conventional Fe-EDTA fertiliser, manufactured by Solufeed Ltd. (West Sussex, UK), costs approximately £17.50 per kilogram. Therefore, Fe_3O_4 NPs capped with L-lysine present a competitively priced and viable alternative iron source for hydroponically grown plants.

Additionally, both treatments (Fe_3O_4 NPs and Fe-EDTA) were prepared based on their molecular weights, with adjustments made to account for the iron fraction in each compound to ensure equivalent elemental iron concentrations across treatments. Fe_3O_4 NPs has a molecular weight of 231.53 g / mol, of which approximately 72.36 % (167.55 g / mol) is elemental iron. In contrast, the $FeSO_4 \cdot 7H_2O$ & EDTA $2Na \cdot 2H_2O$ complex has a combined molecular weight of 650.25 g / mol, with only 8.59 % (55.85 g / mol) attributable to elemental iron. Therefore, to achieve the same iron dosage, a larger quantity of Fe-EDTA compound is required relative to Fe_3O_4 NPs due to its lower iron fraction by weight. Consequently, Fe_3O_4 NPs can deliver higher iron bioavailability in a more cost-effective manner, requiring less materials to supply equivalent amounts of element iron.

4.5 Conclusions

Iron oxide nanoparticle (Fe_3O_4 NPs) have been useful to establish their applicability for lettuce grown hydroponic cultivation and analyse their effects of B_{12} on lettuce growth, development, and quality. Thus, the following conclusions can be made:

- The present study produced different forms of Fe_3O_4 NPs using several synthesis methods and capping agents. The sizes of the Fe_3O_4 NPs, both with and without capping agents, were all within the nanoscale range as confirmed by TEM and DLS analysis. Further characterisation confirmed the key features of Fe_3O_4 NPs including surface chemistry, shape, and their crystalline structure.

- In **Chapter 4.2**, this study found that the Fe_3O_4 NPs showed relatively limited performance in term of morphological and physiological development at the same concentration (2.5 mg / L) when compared to the control group within a Fe-EDTA (2.5 mg / L). However, at both lower (1.25 mg / L) and higher (5 mg / L) concentrations, the Fe_3O_4 NPs treatments resulted comparable response in morphological and physiological parameters. These results imply that further optimisation post-synthesis process is required for Fe_3O_4 NPs application into the plant as it shows aggregation of Fe_3O_4 NPs and capping agents potato starch.

- In **Chapter4.3**, the present study found that the treatment of the Fe_3O_4 NPs capped with L-lysine represent a promising alternative to iron fertiliser, showing enhanced morphological, physiological, harvest weight and phytochemical development, such as phenolic content and flavonoid content when compared to Fe-EDTA treatment. Notably, of Fe_3O_4 NPs 2.5 mg / L treatment resulted in highest relative chlorophyll content and fresh weight (total, leaf and root) observed at final harvest.

- The multielement analysis by ICP-MS confirmed that Fe_3O_4 NPs have higher bioavailability compared to Fe-EDTA possibly due to their great mobility within a nano-size. Interestingly, Fe_3O_4 NPs 2.5 mg / L treatment enhanced iron accumulation which were followed by 5 and 7.5 mg / L treatment, highlighting the improved bioavailability and growth promotion.

- Overall, the present study suggests that Fe_3O_4 NPs represent a viable alternative to traditional Fe fertiliser such as Fe-EDTA, offering higher

bioavailability and improved mobility within the plant cells, which leads to enhanced growth and increased final harvest weight. These promising results highlight the potential of Fe_3O_4 NPs in sustainable agriculture and hydroponic cultivation. Additionally, Fe_3O_4 NPs contained a substantially higher proportion of elemental iron by weight compared to Fe-EDTA complex. As a result, less Fe_3O_4 NPs is needed to deliver an equivalent iron dosage, making them a more efficient and cost-effective source of bioavailable iron for hydroponically grown lettuce.

- Further statistical analysis is required to confirm treatment differences, alongside comprehensive assessments of the potential toxicity and their environmental impacts of Fe_3O_4 NPs on plants, animals, and ecosystem.

Chapter 5. Vitamin B_{12} and Iron Fortification

The present chapter focuses on vitamin B_{12} (B_{12}) and iron fortification in lettuce using iron oxide nanoparticles (Fe_3O_4 NPs). The study aims to fortify B_{12} and Fe in lettuce and investigate their effect on lettuce growth and quality. This study focuses on the following research questions: (1) Between 2.5 and 5 mg / L of Fe_3O_4 NPs, which concentration of Fe_3O_4 NPs is better for iron fortification and growth and quality of the lettuce?; (2) Does the combined fortification of B_{12} and Fe_3O_4 NPs provide better overall quality compared to individual fortification?; and (3) What are the optimal treatment conditions for fortifying with B_{12} and Fe using Fe_3O_4 NPs?

An initial pilot study conducted prior to the present study revealed that leaf application (foliar spray) of uncapped Fe_3O_4 NPs (pure Fe_3O_4 NPs) was ineffective for iron fortification in lettuce (Figure 5.1). The lettuce treated with uncapped Fe_3O_4 NPs via leaf application exhibited visible symptoms of iron deficiency. This may be attributed to several factors: (1) the bidaily (twice-daily) application frequency may have been insufficient to meet the iron requirements of hydroponically grown lettuce; (2) the concentration of Fe_3O_4 NPs used may have been too low for effective leaf uptake; and (3) the leaf application method of Fe_3O_4 NPs likely requires further optimisation to improve efficacy, such as the use of surfactants smaller particle size, or chelating agents. Therefore, in the present study, the two concentrations of Fe_3O_4 NPs (2.5 and 5 mg / L) were instead delivered directly to the root zone via the nutrient solution tanks (root application).

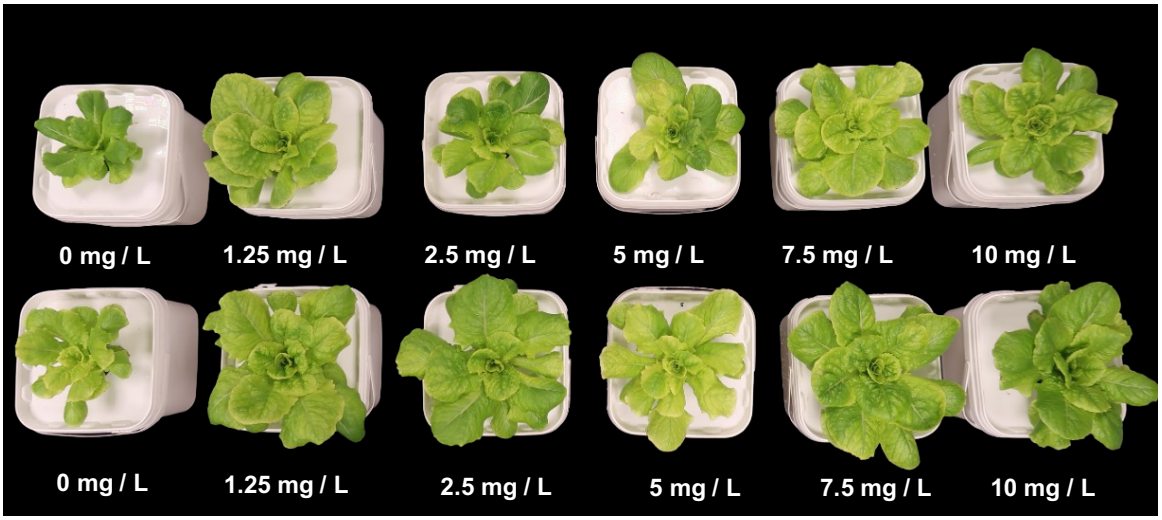


Figure 5.1 Pilot study for uncapped Fe_3O_4 NPs (pure Fe_3O_4 NPs) leaf application. Concentrations of Fe_3O_4 NPs includes: the control (no iron), 1.25, 2.5, 5, 7.5, and 10 mg / L.

The comprehensive experiments described in **Chapter 3** enabled a comparison between leaf and root application for B_{12} fortification, and the present chapter adopted the leaf application for B_{12} fortification. The rationale for this decision is: (1) Although B_{12} fortification via root application resulted in approximately three times higher B_{12} content in leaf tissues compared to leaf application, it is also led to a substantial accumulation of B_{12} in root tissues, which are not consumable; (2) The B_{12} fortification via root application required approximately 100 times more B_{12} than the leaf application to achieve the same treatment volume, making it significantly less cost-effective. Considering these factors and edible part of lettuce, the leaf application method was selected for this chapter. Five different B_{12} were tested via leaf application, including 0, 10, 50, 100 and 1000 mg / L.

5.1 Materials and method

The materials and growing conditions used in this experiment are described in **Chapter** 2.2. Figure 5.2 presents the treatment setup, including the number of propagators, application methods, treatment schedule, and sample collection details. In this study, two types of treatments were applied: Fe_3O_4 NPs capped with L-lysine and vitamin B_{12}. The Fe_3O_4 NPs capped with L-lysine were provided by Dr. Gareth Cave, Nottingham Trent University in Nottingham, UK and prepared according to the synthesis protocols outlined in **Chapter 2.1**. Two concentrations of Fe_3O_4 NPs (2.5 and 5 mg / L) were applied via root application. As previously described in **Chapter 2.2.3**, all Fe_3O_4 NPs treatments were prepared based on the molecular weights, accounting for the iron fraction in the molecule and a final concentration expressed as milligrams of elemental iron per litre. However, for data presentation, concentrations are reported as milligram per litre (mg / L) without specifying elemental iron explicitly, as the iron fraction was accounted for during preparation. Vitamin B_{12} was prepared in five concentrations (0, 10, 50, 100, and 1000 mg / L) and applied through leaf application (foliar spray).

The B_{12} solutions were initially prepared at a total volume of 50 mL for application during week 1 and week 2 and. To account for the increasing biomass of the lettuce plants, the total volume was increased to 100 in week 3 and 200 mL in week 4. The B_{12} treatments were applied twice daily, at 9 am and 5 pm, until harvest. The B_{12} solutions were sprayed evenly across the entire leaf surface of lettuce plants. During harvest and sample collection, all lettuce samples were thoroughly washed multiple times with running water to minimise any surface residues from the leaf application. Lettuces plants were harvested at two time points, week 2 and week

4 (14 days and 28 days after transplanting) for multielement analysis. Final harvest samples collected at week 4 were also analysed for harvest-related parameters (fresh and dry weight) and secondary metabolites, including total phenolic content, total flavonoid content, and total antioxidant content.

5.2 Data collection and analysis

Five different concentrations of B_{12} (0, 10, 50, 100, and 1000 mg / L) were applied in combination with two concentration of Fe_3O_4 NPs (2.5 and 5 mg / L), with each treatment replicated across two propagators, resulting in a total 20 propagators as outlined in Table 5.1. Four lettuce plants were harvested at week 2 and week 4 to enable multielement analysis during the growth period. Morphological and physiological development was monitored using PlantEye and SPAD. Harvest weight was assessed through fresh and dry weight measurements. In addition, secondary metabolite analysis was conducted to evaluate the effect of Fe_3O_4 NPs and B_{12} treatment on nutrition quality of the lettuces. The overall process for data collection and data analysis followed the same to methodology described in **Chapter 3** and **Chapter 4**.

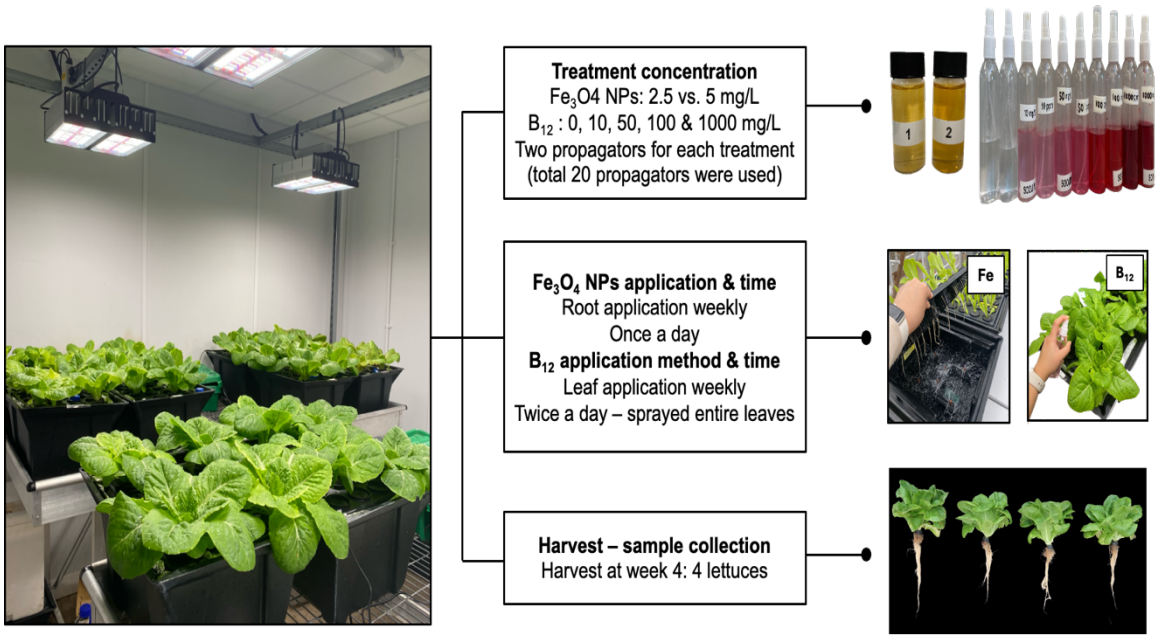


Figure 5.2 Schematic representation of growing condition for B_{12} and Fe fortification.

Fe_3O_4 NPs treatment (2.5 and 5 mg / L) via root application; Vitamin B_{12} treatment (0, 10, 50, 100, and 1000 mg / L) via leaf application; application method; and harvest details.

Table 5.1 Plant map - number of plant (total 8 of lettuces): harvested on week 2 and week 4 (4 lettuces each); Fe_3O_4 NPs concentration (2.5 and 5 mg / L); B_{12} concentration (0, 10, 50, 100, and 1000 mg / L)

No.	No. plant	Fe_3O_4NPs (mg / L)	B_{12} (mg / L)	No.	No. plant	Fe_3O_4NPs (mg / L)	B_{12} (mg / L)
1	8 lettuces 4 – week 2 + 4 – week 4	2.5	0	11	8 lettuces 4 – week 2 + 4 – week 4	5	0
2			0	12			0
3			10	13			10
4			10	14			10
5			50	15			50
6			50	16			50
7			100	17			100
8			100	18			100
9			1000	19			1000
10			1000	20			1000

5.3 Results

This chapter aims to fortify lettuce with both vitamin B_{12} and iron (Fe) and to investigate their combined effects on plant growth, physiology, and nutritional quality. Two different concentrations of Fe_3O_4 NPs (2.5 and 5 mg /L) were applied via root application to determine the optimal iron treatment, while five B_{12} concentrations (0, 10, 50, 100, and 10000 mg / L) were applied via leaf application to enhance B_{12} content specifically in the edible leaf tissues. The inclusion of higher B_{12} concentration (100 and 1000 mg / L) also aimed to assess any potential phytotoxic effect from excessive B_{12} exposure. The objectives of this chapter are: (1) to quantify the uptake, translation, and accumulation of leaf applied B_{12} in lettuce leaves and root tissues using ICP-MS; and (2) to evaluate the effects of varying B_{12} concentrations (0, 10, 50, 100, and 1000 mg / L) on morphological traits such as leaf area, biomass, physiological parameters (photosynthetic gas exchange, water management, and relative chlorophyll content, and nutritional quality including B_{12} content, secondary metabolites.

5.3.1 Growth and development measurements

The weekly progression of lettuce growth and morphological development throughout the 28 days cultivation period (Appendix C). Week 1 corresponds to 7 days after transplanting, and week 4 (28 days after transplanting) represents the final harvest. The results are grouped by the Fe_3O_4 NPs treatment (2.5 and 5 mg / L) across the five B_{12} concentrations (0, 10, 50, 100, and 1000 mg / L). Further methodological details are described in **Chapter 2.3.2**.

Weekly increases in plant height, leaf area, digital biomass, and NDVI were observed throughout the cultivation period (week 1 to week 4), regardless of Fe_3O_4 NPs concentrations (2.5 and 5 mg / L) and B_{12} concentrations (0, 10, 50, 100, and 1000 mg / L). In contrast, hue, greenness, NPCI, and PSRI values declined after week 2. Overall, plants treated with Fe_3O_4 NPs 5 mg / L showed higher average values for digital biomass, height, hue, and NDVI, compared to those treated with 2.5 mg / L, irrespective of B_{12} concentrations.

Morphological and physiological data collected at week 4 were highlighted to represent final harvest outcomes. The results are initially grouped by the two Fe_3O_4 NPs concentrations (2.5 and 5 mg / L), and within each group, response to the five B_{12} concentrations (0, 10, 50, 100, and 1000 mg / L) were further examined. Statistical significance was assessed using the PERMANOVA test with 9,999 permutations to evaluate differences among treatment combinations.

Figure 5.3 presents plant height (cm^2) and leaf area (cm^3) measured at the final harvest (week 4). Lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited an average height increase of 17 % compared to those treated with 2.5 mg / L, averaged across all B_{12} concentrations. The combination of Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L resulted in the tallest plants (16.25 ± 2.01 cm, 'c'), which was significantly greater ($p < 0.05$) than the height observed in the treatments combining Fe_3O_4 NPs 2.5 and B_{12} 0 ('ab'), 10 ('a), and 50 mg / L ('ab') treatments. Within the Fe_3O_4 NPs 2.5 mg / L group, the tallest plants were also those treated with B_{12} 1000 mg / L (13.55 ± 0.70 cm, 'c'). Overall, B_{12} treatments increased plant height compared to the control group without B_{12} application.

Regarding leaf area (cm^2), lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited a 12 % larger leaf area compared to those treated with 2.5 mg / L, averaged across all B_{12} concentrations. The highest leaf area mean value was observed in the control groups without B_{12} treatment for both Fe_3O_4 NPs 2.5 mg / L (1014 ± 164 cm^2, 'a') and 5 mg / L (905 ± 110 cm^2, 'a'). These values were significantly higher ($p < 0.05$) than those recorded in the B_{12} 1000 mg / L treatments within both Fe_3O_4 NPs 2.5 ('bc') and 5 mg / L treatments ('c'). However, except for B_{12} 1000 mg / L treatment, no significant differences were observed among the B_{12} treatments from 10 to 100 mg / L ($p > 0.05$).

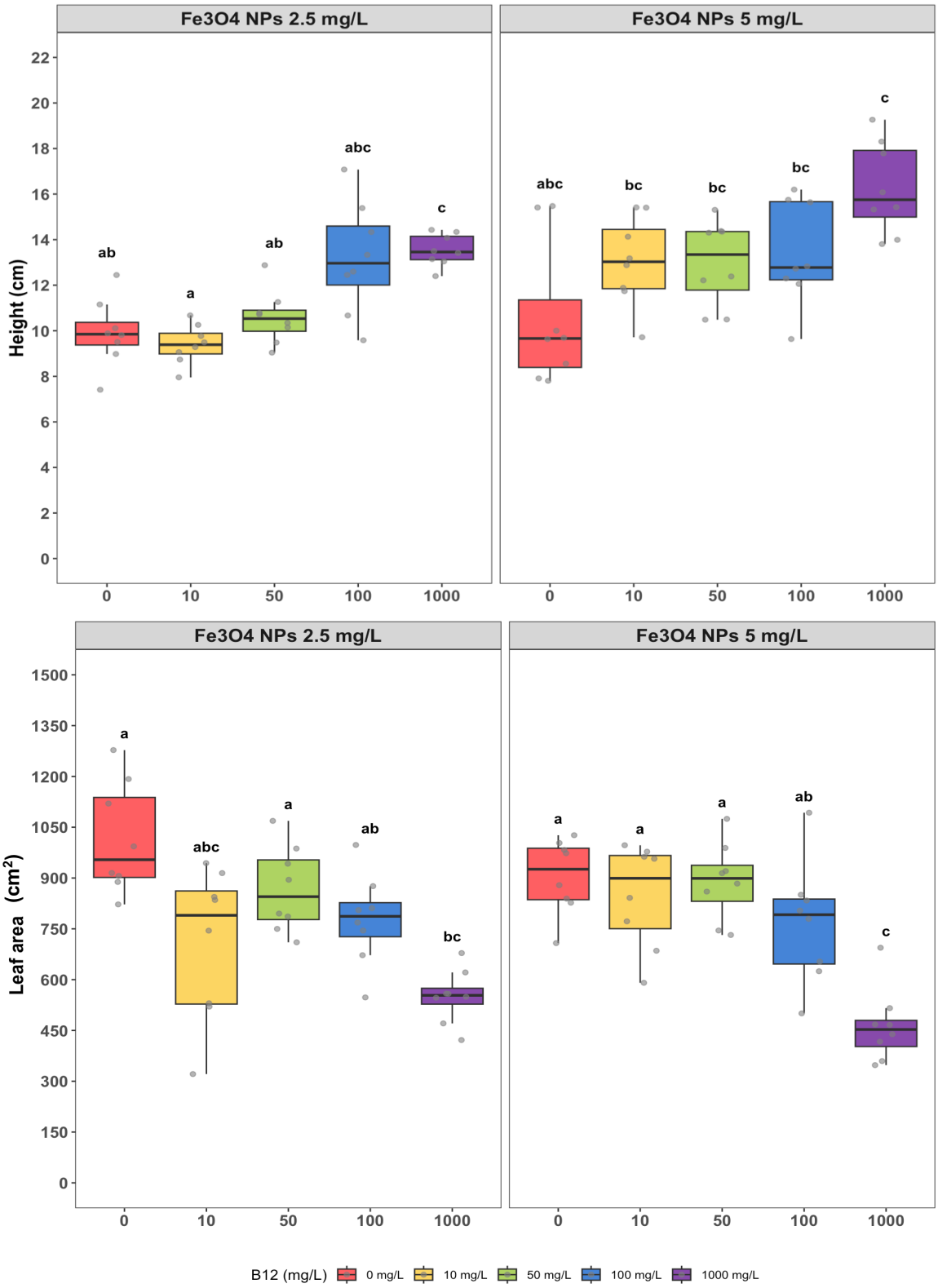


Figure 5.3 Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

The two Fe3O4 NPs group (2.5 and 5 mg / L) are shown separately, with coloured box plots indicating the B_{12} concentrations (control: 0, 10, 50, 100, and 1000 mg / L), represented by coloured box plots. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p <$ 0.05). Different letters denote statistically significant differences between groups.

Figure 5.4 presents digital biomass (cm^3) and hue (º) measured at the final harvest (week 4). Digital biomass is a volumetric estimate generated by the PlantEye scanner, reflecting a combination of plant height and projected leaf area to represent overall lettuce growth. Regarding digital biomass (cm^3), on average, 14 % higher in lettuces treated with Fe_3O_4 NPs 5 mg / L compared to those treated with 2.5 mg / L. The highest digital biomass was observed in the Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L treatment (11,487 ± 1,864 cm^3, 'b'), while the lowest was found in the Fe_3O_4 NPs 2.5 mg / L with B_{12} 1000 mg / L treatment (6,724 ± 2,327cm^3, 'b'). Despite the large numerical difference, the shared statistical label indicates no significant difference between these two treatments ($p > 0.05$). Within the Fe_3O_4 NPs 2.5 mg / L group, the highest digital biomass was recorded in the B_{12} 100 mg / L treatment (10,212 ± 2,376 cm^3, 'ab'), with no significant differences observed among other B_{12} treatments in this group ($p > 0.05$).

Hue measurements, ranging from 0 to 360 º, represent colour and can serve as an indicator of the morphological health of lettuce. Hue values between 90 º to 140 º correspond to green hues, typically associated with healthy plants. Negligible differences were observed in average hue measurements between Fe_3O_4 NPs 2.5 (103.74 ± 2.71 °) and 5 mg / L treatments (103.88 ± 2.35 °). The Fe_3O_4 NPs 2.5 mg / L with the B_{12} 50 mg / L treatment exhibited the highest hue (106.25 ± 1.50 °), which was significantly higher than the B_{12} 1000 mg / L treatments with both Fe_3O_4 NPs 2.5 and 5 mg / L treatment. However, except for B_{12} 1000 mg / L treatment, no significant differences were observed among the B_{12} treatments from 0 to 100 mg / L ($p > 0.05$).

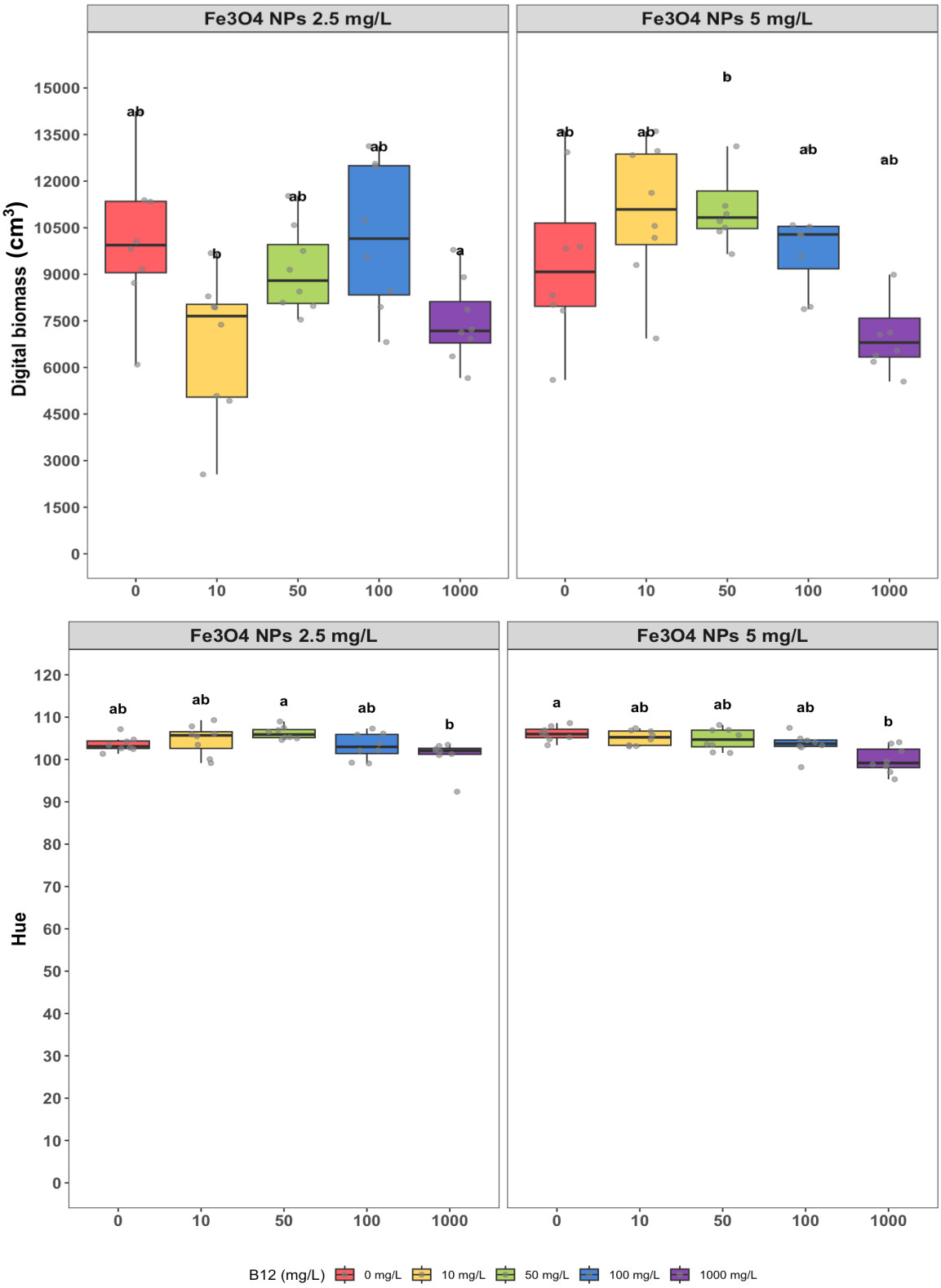


Figure 5.4 Digital biomass (cm^3) and Hue (°) of lettuces measured weekly via PlantEye.

The two Fe3O4 NPs group (2.5 and 5 mg / L) are shown separately, with coloured box plots indicating the B_{12} concentrations (control: 0, 10, 50, 100, and 1000 mg / L), represented by coloured box plots. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

Figure 5.5 presents the greenness and Normalised Differential Vegetation Index (NDVI) measurements obtained from PlantEye. Greenness, which ranges from -1 to 1, reflects the green leaf index, with higher values indicating greater greenness typically associated with healthy leaves. The highest greenness mean value was observed in the Fe_3O_4 NPs 2.5 mg / L with B_{12} 0 mg / L treatment (0.43 ± 0.01, 'a'), followed by B_{12} 10 mg / L treatment (0.43 ± 0.02, 'ab'), showing no statistically significant difference between them ($p > 0.05$). The lowest greenness was recorded in Fe 2.5 mg / L with B_{12} 1000 mg / L (0.38 ± 0.03, 'c'), which was significantly lower than other B_{12} treatments within the same Fe_3O_4 NPs group ($p < 0.05$), as well as significantly lower than the Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L treatment (0.41 ± 0.01, 'ab') ($p < 0.05$). These findings suggest that the B_{12} 1000 mg / L treatment resulted in less desirable greenness values, consistent with the hue results, across both Fe_3O_4 NPs 2.5 mg / L and 5 mg / L treatments.

The NDVI serves as an indicator of vegetation health and relates to the interception of photosynthetic activity, as it related to plant's ability to intercept photosynthetically active radiation (PAR). Typically, healthy vegetation display NDVI values between 0.6 to 0.9. Overall, all lettuces treatments exhibited NDVI values ranging from 0.6 to 0.8, indicating generally healthy vegetation status across treatments. The Fe_3O_4 NPs 5 mg / L treatment showed slightly higher average normalised differential vegetation index (NDVI) values compared to the 2.5 mg / L treatment, regardless of B_{12} concentrations. The highest NDVI was recorded in the Fe_3O_4 NPs 2.5 mg / L with B_{12} 50 mg / L treatment (0.72 ± 0.01, 'a'), which was significantly higher than the lowest value observed in the B_{12} 1000 mg / L treatment (0.65 ± 0.03, 'b') ($p < 0.05$). In contrast, no statistically significant differences were found among the B_{12} treatments in the Fe_3O_4 NPs 5 mg / L group, with all values either 'a' or 'ab' ($p > 0.05$).

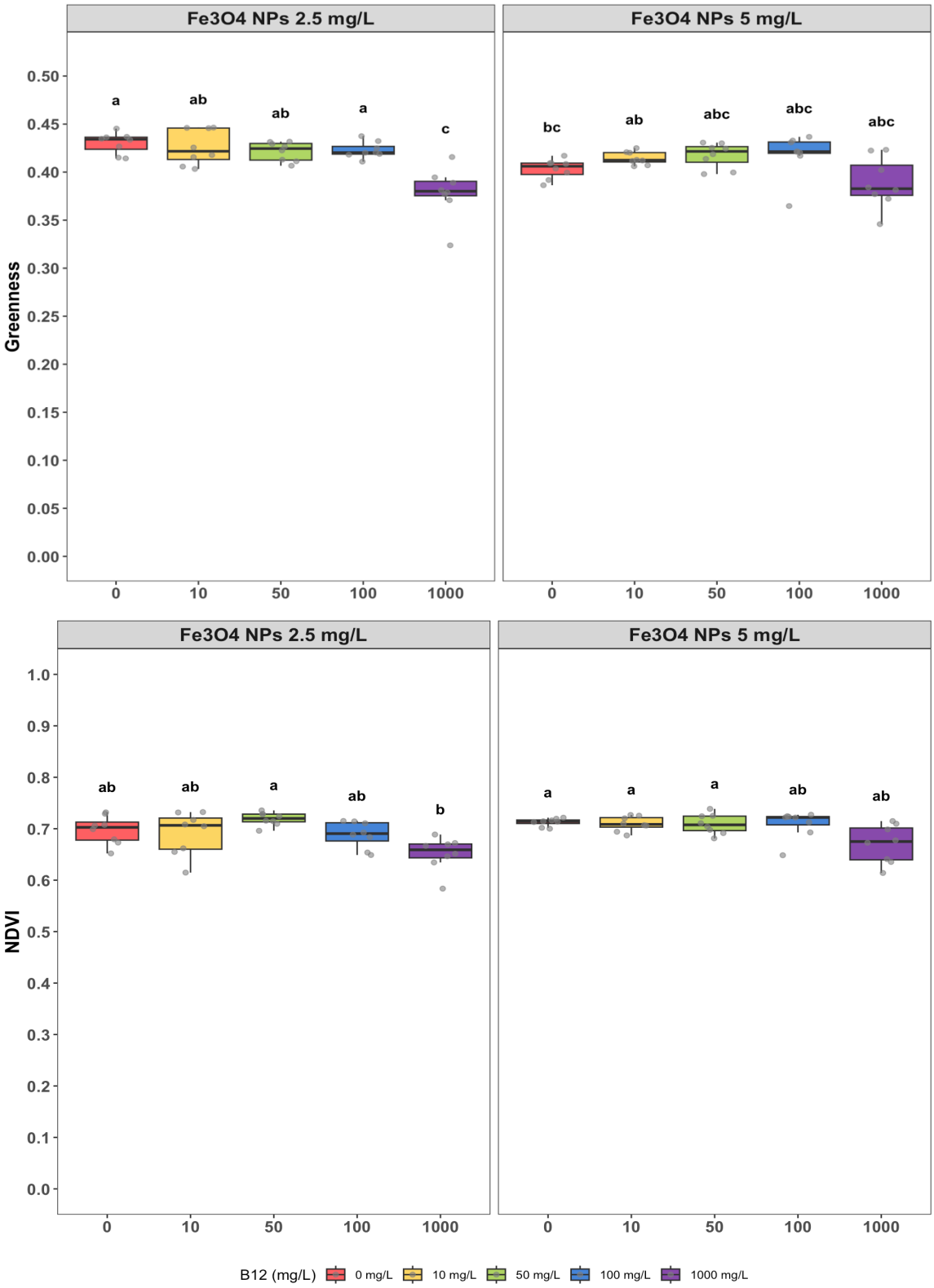


Figure 5.5 Greenness and NDVI of lettuces measured weekly via PlantEye.

The two Fe3O4 NPs group (2.5 and 5 mg / L) are shown separately, with coloured box plots indicating the B_{12} concentrations (control: 0, 10, 50, 100, and 1000 mg / L), represented by coloured box plots. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p <$ 0.05). Different letters denote statistically significant differences between groups.

Figure 5.6 presents both Normalised Pigment Chlorophyll index (NPCI) and Plant Senescence Reflectance Index (PSRI) parameters. NPCI measurements reflect the ratio between chlorophyll and carotenoids, providing an indicator of the plant's physiological status or stress condition. The highest normalised pigment chlorophyll ratio index (NPCI) was observed in lettuces treated with Fe_3O_4 NPs 2.5 mg / L with B_{12} 0 mg / L treatment (0.27 ± 0.03, 'ab'). No statistically significant differences were observed among B_{12} treatment within the Fe_3O_4 NPs 2.5 mg / L group (all 'ab'). However, within the Fe_3O_4 NPs 5 mg / L group, the B_{12} 0 mg / L ('a') and B_{12} 100 mg / L ('b') treatments showed a statistically significant difference ($p < 0.05$), Notably, although the B_{12} 1000 mg / L treatment exhibited one of the highest NPCI mean value, it was not statistically different from most other B_{12} concentrations ($p > 0.05$). Overall, there were a general increasing trend in NPCI values with increasing B_{12} concentration in the Fe_3O_4 NPs 5 mg / L group.

PSRI assess plant senescence and stress by estimating the relative content of carotenoid to chlorophyll. The average PSRI was higher in the Fe_3O_4 NPs 2.5 mg / L compared to the Fe_3O_4 NPs 5 mg / L group, indicating increase plant senescence and stress at the lower Fe concentration (2.5 mg / L). Within the Fe_3O_4 NPs 2.5 mg / L group, the highest PSRI was observed in the B_{12} 1000 mg / L treatment (0.08 ± 0.02, 'b'), which was significantly higher than the B_{12} 50 mg / L treatment ('a') ($p < 0.05$). The remaining B_{12} treatment (0, 10, and 100 mg / L) were labelled 'abc', showing no significant differences with either extreme ($p > 0.05$). In the Fe_3O_4 NPs 5 mg / L group, PSRI values tended to increase with rising B_{12} concentrations. The highest PSRI was again observed in the B_{12} 1000 mg / L treatment (0.08 ± 0.02, 'bc'), which was significantly higher than the lowest values exhibited in B_{12} 0 mg / L group ('a') ($p < 0.05$). These results suggest a B_{12} dose-dependent increase in plant senescence within the Fe_3O_4 NPs 5 mg / L treatment.

Overall, the Fe_3O_4 NPs 5 mg / L treatment group demonstrated superior morphological and physiological development compared to the Fe_3O_4 NPs 2.5 mg / L group. This was reflected in greater plant height, increased digital biomass, and a healthier vegetation profile, including a vibrant green colour and lower stress indices. In contrast, the Fe_3O_4 NPs 2.5 mg / L treatment group exhibited larger leaf area and higher greenness, alongside increased chlorophyll content. Within the B_{12} treatment (0 to 1000 mg / L), no significant differences were observed among the 0 to 100 mg / L range in most morphological and physiological parameters, indicating minimal effect at low to moderate B_{12} concentrations. However, a high B_{12} concentration (1000 mg / L) negatively affected lettuce health and quality across both Fe_3O_4 NPs treatments.

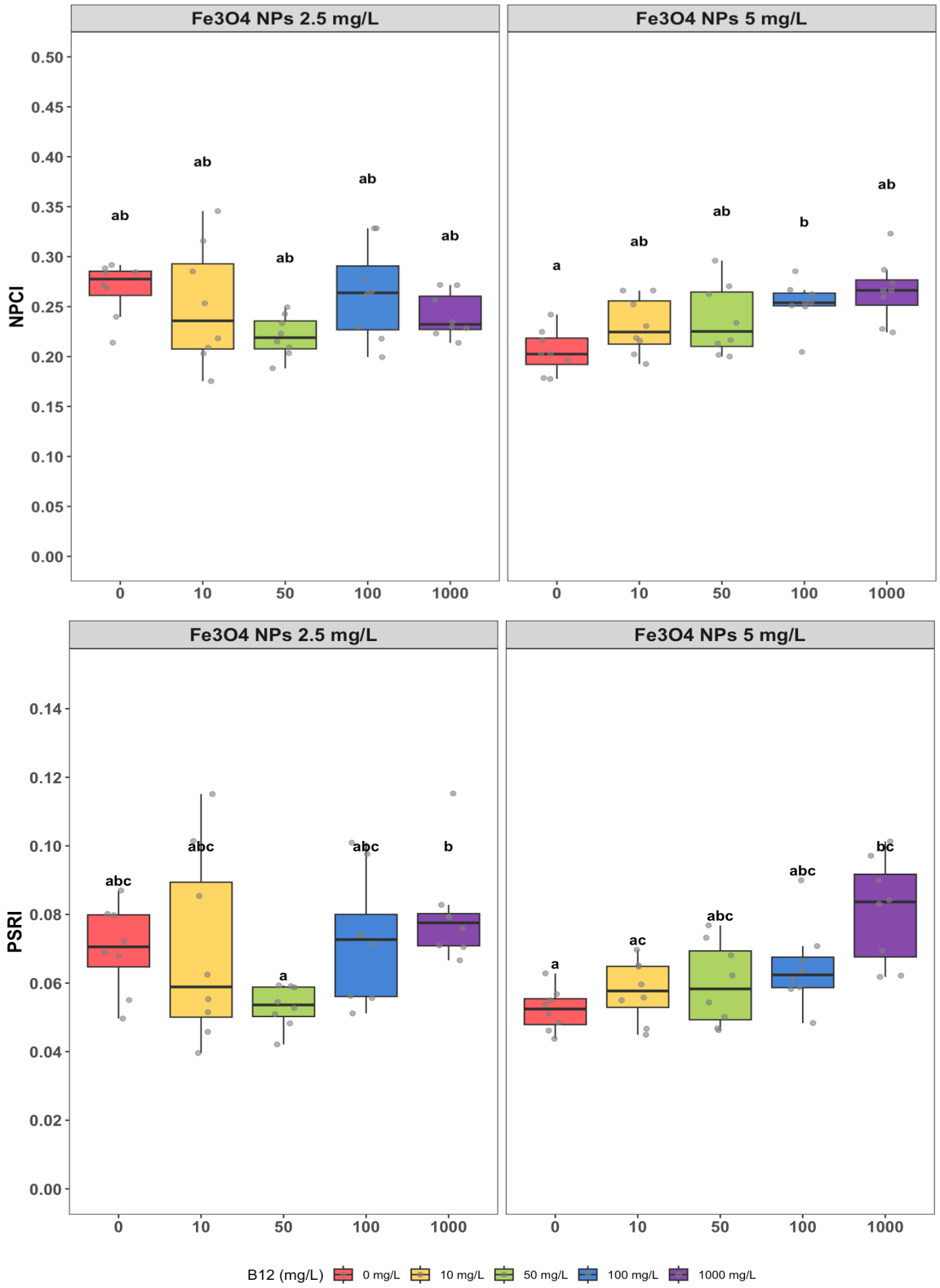


Figure 5.6 NPCI and PSRI of lettuces measured weekly via PlantEye.

The two Fe3O4 NPs group (2.5 and 5 mg / L) are shown separately, with coloured box plots indicating the B_{12} concentrations (control: 0, 10, 50, 100, and 1000 mg / L), represented by coloured box plots. Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p <$ 0.05). Different letters denote statistically significant differences between groups.

5.3.2 Relative chlorophyll measurement

Relative chlorophyll content was measured weekly for four weeks using SPAD-502Plus (Konica Minolta Ltd., Osaka, Japan). Details are described in **Chapter 2.3.4**. SPAD value correlates with chlorophyll content, which plays an important role in photosynthesis. Higher SPAD values indicate healthier plant with more chlorophyll. Figure 5.7 shows the SPAD measurements recorded weekly over a four-week period. Variations in SPAD values between the treatments became more pronounced in week 2 and week 3, indicating treatment effects during the mid-growth stage. By week 4, however, these differences diminished, and no statistically significant differences were observed among treatments within the Fe_3O_4 NPs 2.5 mg / L group (all 'ab'). Overall, lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited consistently higher SPAD values compared to those treated with 2.5 mg / L, suggesting improved chlorophyll content under the higher iron concentration.

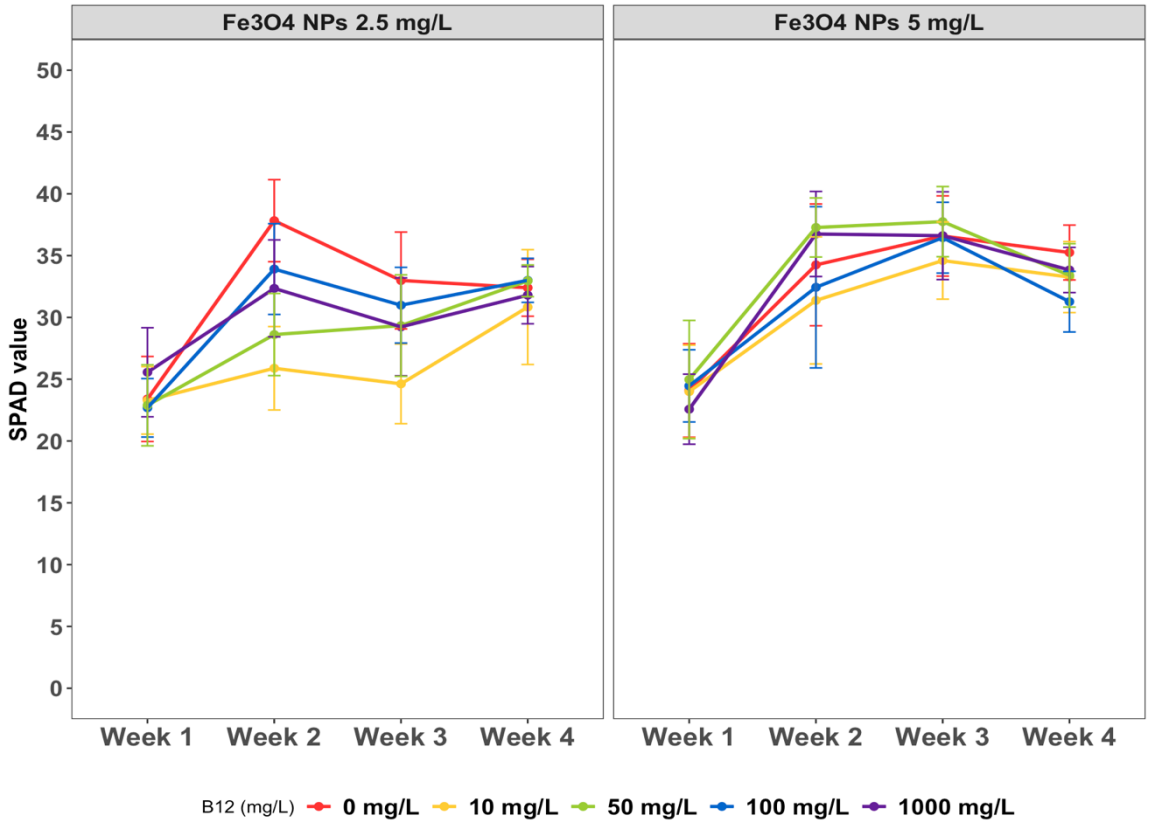


Figure 5.7 Relative chlorophyll weekly measurement by SPAD meter.

Coloured bars indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. weekly measured relative chlorophyll data.

Figure 5.8 presents the SPAD values measured at week 4, reflecting relative chlorophyll content at the final harvest. The highest SPAD value was recorded in the Fe_3O_4 NPs 5 with B_{12} 0 mg / L treatment (35.3 ± 2.2, 'a'), while the lowest value was found in the Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L (30.8 ± 4.7, 'ab'). Despite this numerical difference, the shared group label indicates that the difference was not statistically significant. The Fe_3O_4 NPs 5 mg / L with B_{12} 100 mg / L treatments (31.3 ± 2.5, 'b') exhibited significantly lower SPAD values than the B_{12} 0 mg / L treatments ('a') ($p < 0.05$). Overall, the SPAD results suggest that the Fe_3O_4 NPs 5 mg / L treatment slightly increased chlorophyll content in lettuce compared to 2.5 mg / L treatment. Within the Fe_3O_4 NPs 5 mg / L group, a significant difference between the control group (B_{12} 0 mg / L) and B_{12} 100 mg / L treatment ($p < 0.05$). In contrast, no significant differences were found among B_{12} treatments within the Fe_3O_4 NPs 2.5 mg /L group, as all were labelled the same group ('ab', $p > 0.05$).

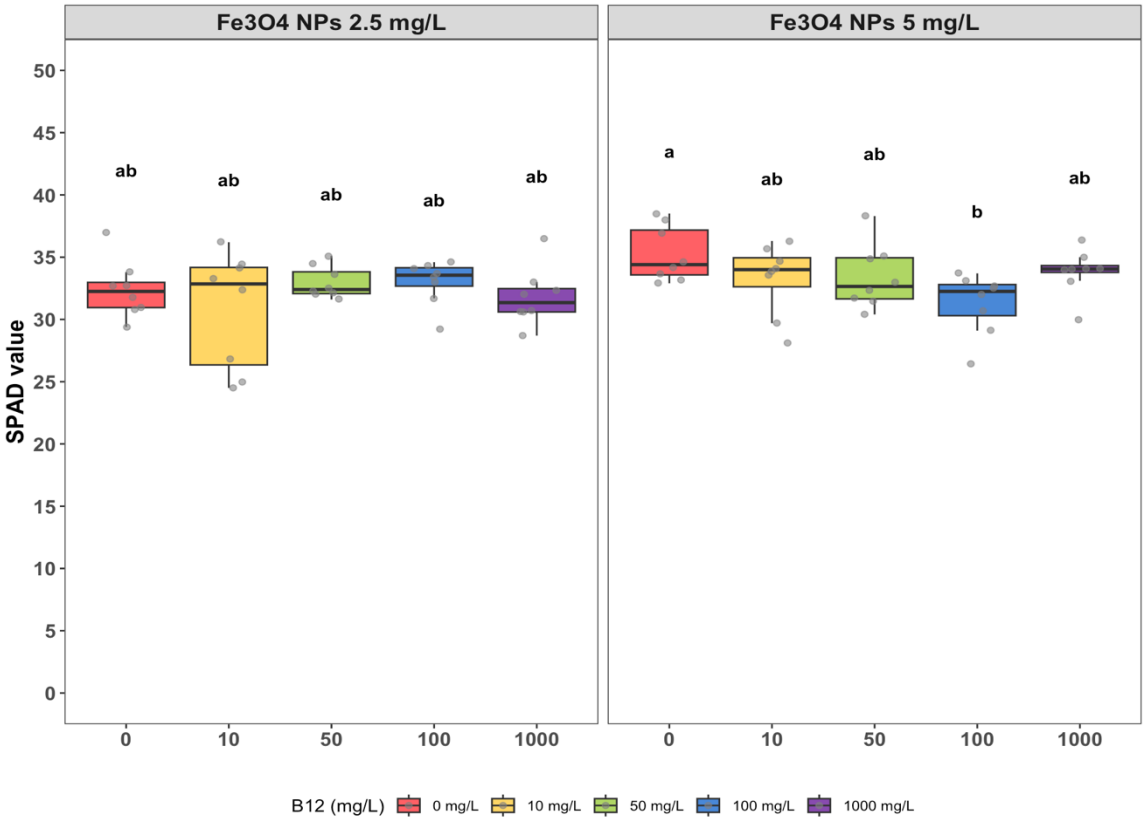


Figure 5.8 Relative chlorophyll measurement by SPAD meter at Week 4.

Coloured bars indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom.

5.3.3 Harvest quality: fresh and dry weights

For harvest quality analysis, fresh and dry weights were measured at final harvest (week 4). Leaf and root tissues were weighted separately using a four-digit analytical balance, and total weights were calculated as the sum of both tissues. The statistical analysis was conducted using multiple pairwise permutational comparisons alongside PERMANOVA with 9,999 permutations. Figure 5.9 shows the leaf, and root fresh and dry weights (g) across Fe_3O_4 NPs with B_{12} treatment groups. On average, lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited higher total fresh and dry weights than those treated with 2.5 mg / L, regardless of B_{12} concentration. Notably, within the Fe_3O_4 NPs 5 mg / L group, B_{12}- treated groups (10, 50, and 100 mg / L), except for the 1000 mg / L treatment, exhibited higher total fresh weights compared to the control group (B_{12} 0 mg / L). Similarly, the Fe_3O_4 NPs 5 mg / L with B_{12} 50 and 100 mg / L treatment resulted in higher dry weights than the control group.

As shown Figure 5.9, the fresh leaf weight (g) was highest in lettuces treated with Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L (198.19 ± 35.13 g), with no statistically significant differences observed among the B_{12} treatments within this group. However, this value was significantly greater than fresh leaf weights in lettuce treated with Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L (‘d’), 50 mg / L (‘abd’), 100 mg / L (‘abd’) and 1000 mg / L (‘ad’) ($p < 0.05$). The highest fresh root weight (g) was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L (21.55 ± 4.96 g, ‘b’). This weight was significantly higher than those in lettuces treated with Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L (‘cd’), 50 mg / L (‘ac’), 1000 mg / L (‘d’) and the treatment of with Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L (‘acd’) ($p < 0.05$).

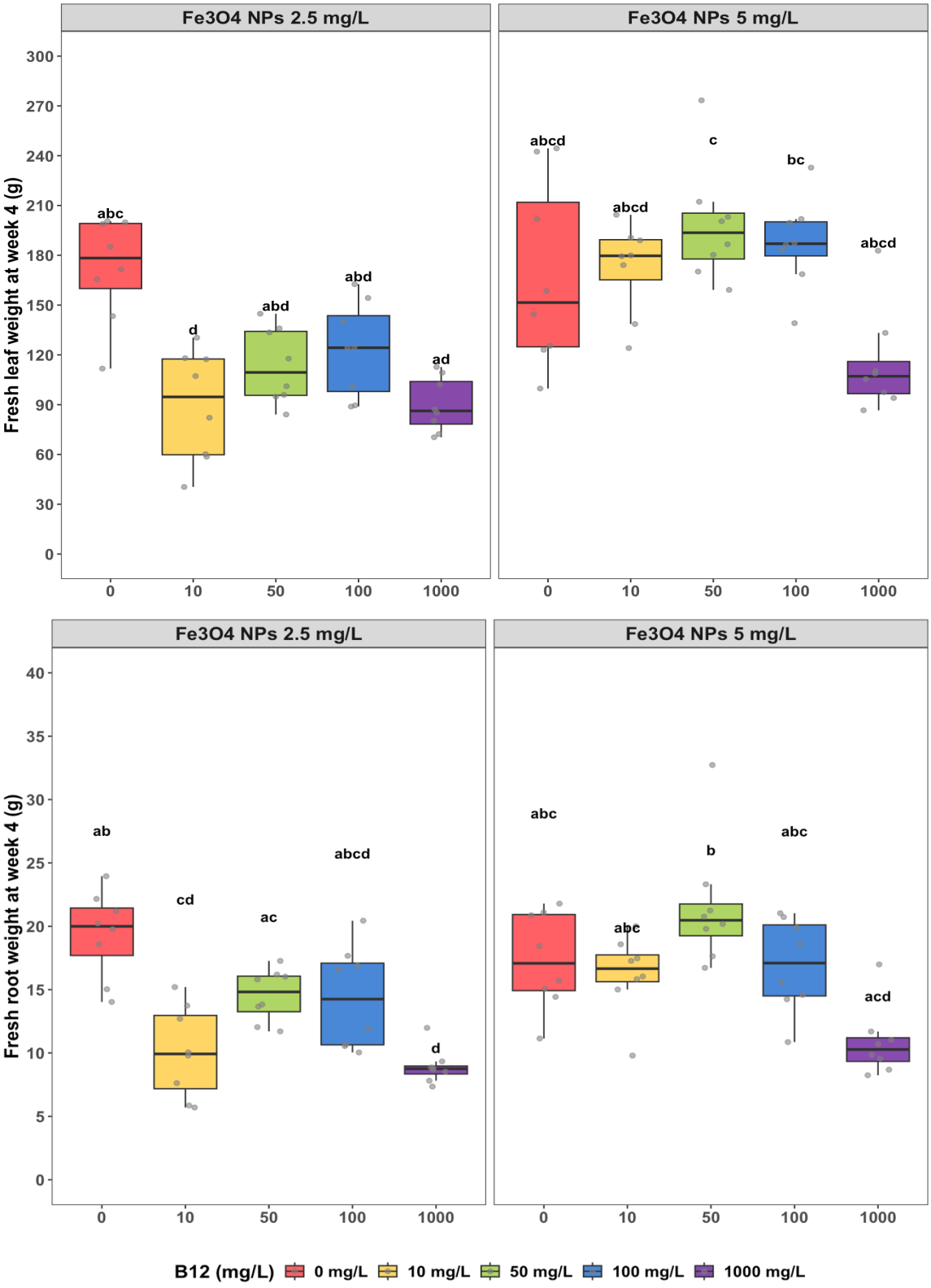


Figure 5.9 Harvest quality – Fresh weight (g) of lettuces harvested at week 2.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

Figure 5.10 presents dry weights (g) measured from fully dehydrated leaf and root tissues after drying at 65 °C until constant weight was achieved. The highest dry weight was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L (6.39 ± 1.51 g, 'a'), which was significantly higher than lettuces treated with Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L ('bc'), 50 mg / L ('b'), and 1000 mg / L ('c'), and Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L ('bc'), showing increases of 103 %, 56 %, 80 %, and 39 %, respectively ($p < 0.05$). Within the Fe3O4 NPs 2.5 mg / L group, the B_{12} 0 mg / L recorded the highest dry leaf weight (5.36 ± 0.95 g, 'a'), which was significantly higher than B_{12} 10 mg / L ('ab'), 50 mg / L ('b'), and 1000 mg / L ('c') ($p < 0.05$).

The highest dried root weight was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L combined with B_{12} 50 mg / L have the highest dried root weights (0.98 ± 0.23 g, 'be'), closely followed by the Fe_3O_4 NPs 2.5 mg /L and B12 0 mg / L (0.97 ± 0.18 g, 'ab'). Both values were significantly higher than those of lettuces treated with Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L ('cd'), and Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L ('cd'), with increases of 109 % and 84 %, respectively ($p < 0.05$). As a result, lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited higher harvest weights compared than those treated with Fe_3O_4 NPs 2.5 mg / L, showing an average increase of 41 % in total fresh weight and 38 % in total dry weight. Within the Fe_3O_4 NPs 2.5 mg / L group, the control (B_{12} 0 mg / L) had the highest leaf and root fresh and dry weights. In contrast, within the Fe_3O_4 NPs group, lettuce treated with B_{12} 50 mg / L achieved the highest leaf and root fresh and dry weights.

Overall, the concentration of Fe_3O_4 NPs treatment had a significant positive impact on lettuce fresh and dry weights, whereas B_{12} treatment showed no significant effect except at the highest concentration (1000 mg / L), which showed adverse effects. The best final harvest quality was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L, showing the highest fresh and dry weights in both leaf and root tissues, which is consistent with digital biomass measurements obtained via PlantEye. Conversely, Fe_3O_4 NPs 2.5 mg / L with B_{12} 1000 mg / L and 10 mg / L resulted in suboptimal harvest quality, with the lowest total fresh weight and total dry weight, respectively. Notably, within the Fe_3O_4 NPs 2.5 treatment, B_{12} 10 mg / L treatment showed limited leaf development, reflected in low fresh and dry weights, while B_{12} 1000 mg / L treatment led to underdeveloped root tissues as indicated by reduced fresh and dry weights.

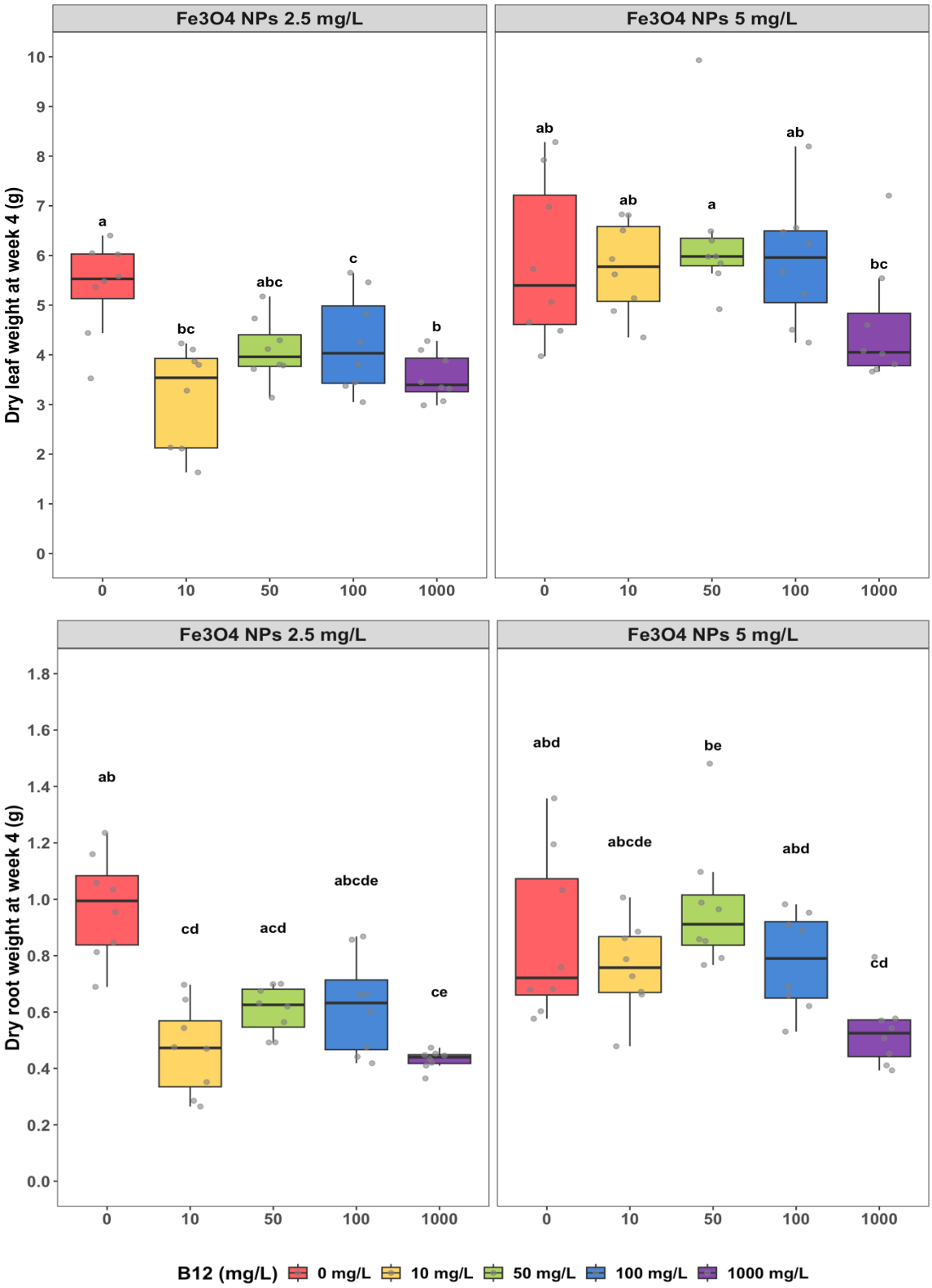


Figure 5.10 Harvest quality – Fresh weight (g) of lettuces harvested at week 2.

Coloured box plots represent Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L), as described in the legend.

5.3.4 Multielement analysis

5.3.4.1 Quantification of multielement

Multielement analysis was performed using ICP-MS to quantify vitamin B_{12} (B_{12}), iron (Fe), potassium (K), magnesium (Mg), calcium (Ca), and phosphorous (P) in lettuce leaf and root tissues harvested at week 4. The ICP-MS protocol followed the methodology previously described in **Chapter 2.3.1**. The concentrations of B_{12}, Fe, K, Mg, Ca, and P in both lettuce leaf and root tissues are presented as milligram of each element per gram of dried tissue (mg / g).

Cobalt (Co) content, determined by ICP-MS, was used to estimate vitamin B_{12} concentration in plant tissues. Given that each B_{12} molecule contained one cobalt atom, the molecular weight ratio of vitamin B_{12} to cobalt (1,355.37 / 58.93) was applied to convert Co values into corresponding B_{12} levels. As the hydroponic nutrient solution did not include cobalt other than through B_{12} supplementation, it is assumed that all measured cobalt originated from the applied B_{12}. Based on the elemental analysis using ICP-MS, the B_{12} concentration in lettuce tissues reached up to 0.010 mg / g via leaf application and 0.028 mg / g via root application, expressed per gram of dried lettuce.

Iron quantification was based on the Fe56 isotope, due to its high analytical stability and natural abundance. However, it should be noted that ICP-MS measures total elemental concentrations and does not distinguish between different oxidation states or molecular forms of iron (such as Fe^{2+} and Fe^{3+}). As a result, the data represent the total iron content in plant tissues without indicating chemical form or bioavailability.

Vitamin B_{12} (B_{12}) quantification

ICP-MS analysis revealed the vitamin B_{12} (B_{12}) content in the lettuce leaf and root tissues. As shown Figure 5.11, the group treated with Fe_3O_4 NPs 5 mg / L, regardless of B_{12} treatment, exhibited on average, higher B_{12} content in lettuce leaf tissues compared to those treated with Fe_3O_4 NPs 2.5 mg / L. Among the Fe_3O_4 NPs 5 mg / L treatments, the B_{12} 1000 mg / L group showed the highest leaf B_{12} content (0.537 ± 0.138 mg / g), which was significantly higher than the Fe_3O_4 NPs 2.5 mg / L with 10 mg / L treatment ('ab'), and Fe_3O_4 NPs 5 mg / L with 10 mg / L ('ac'), and 100 mg / L treatments ('bd') ($p < 0.05$). Similarly, in the Fe_3O_4 NPs 2.5

mg / L group, the highest B_{12} content was observed at B_{12} 1000 mg / L (0.292 ± 0.110 mg / g, 'abcde'), followed by the B_{12} 100 mg / L treatment (0.238 ± 0.079 mg / g, 'abcde'). However, no statistically significant differences were observed among B_{12} treatments within Fe_3O_4 NPs 2.5 mg / L, as indicated by overlapping group labels (all 'ab', 'abcde'). Notably, the B_{12} 0 mg / L in both Fe_3O_4 NPs 2.5 and 5 mg /L groups recorded the lowest B_{12} content in leaf tissues (both 'abcde'). No statistically significant difference was observed between the two B_{12} 1000 mg / L treatments ('abcde' and 'e'), based on 9,999 permutations tests ($p > 0.05$).

The lower part of Figure 5.11 shows the B_{12} content in root tissues. On average, the group treated with Fe_3O_4 NPs 2.5 mg / L (0.228 mg / g) exhibited higher B_{12} content in root tissues compared to those treated with 5 mg / L (0.171 mg / g), across all levels of B_{12} supplementation. Within the Fe_3O_4 NPs 2.5 mg / L treatment, the highest B_{12} content was observed in lettuces treated with B_{12} 1000 mg / L (0.596 mg / g, 'abcd'), followed by B_{12} 100 mg / L (0.275 ± 0.117 mg / g, 'abcd'). However, no statistically significant differences were observed among the B_{12} treatments, as indicated by overlapping group labels ($p > 0.05$).

Among the Fe_3O_4 NPs 5 mg / L group, lettuces treated with B_{12} 1000 mg / L exhibited the highest root B_{12} content (0.368 ± 0.094 mg / g, 'cd'), followed by B_{12} 100 mg / L (0.239 ± 0.045 mg / g, 'abcd'). Similar to the 2.5 mg / L group, no statistically significant differences were observed between B_{12} concentration, as indicated by overlapping group labels ($p > 0.05$). Notably, a statistically significant difference was observed between the Fe_3O_4 NPs 2.5 mg / L with B_{12} 1000 mg / L ('ac') and Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L ('cd') ($p < 0.05$). However, this pair does not reflect greatest numerical difference in B12 content, indicating that statistically significant doses not always aligned with absolute value differences among treatments based on pairwise permutation test.

A consistent dose-dependent response to B_{12} supplementation was observed in both Fe_3O_4 NPs 2.5 mg / L and 5 mg / L groups, suggesting that B_{12} concentration had a pronounced effect on B_{12} uptake. Notably, higher B_{12} treatment (100 and 1000 mg / L) resulted in significantly greater B_{12} accumulation in both tissues. Notably, the Fe_3O_4 NPs 5 group exhibited higher B_{12} content in leaf tissues, whereas the 2.5 mg / L group exhibited greater B_{12} accumulation in root tissues. Notably, root tissues contained approximately 1.5 times greater B_{12} content than leaf tissues. This difference may be attributed to excess B_{12} solution likely leaking into the hydroponic system, resulting in direct exposure of root tissues to B_{12} in the nutrient solution, which facilitated enhanced B_{12} uptake. In addition to this potential

contamination, foliar-applied B_{12} can be translocated via the phloem from leaves to the roots, contributing to higher accumulation in root tissues. This dual pathway of uptake could explain the unexpected pattern in which the Fe_3O_4 NPs 5 mg / L with B_{12} 100 mg / L treatment showed lower B_{12} content in leaf tissues compared to the Fe_3O_4 NPs 2.5 mg / L group, suggesting greater root translocation or uptake in the former.

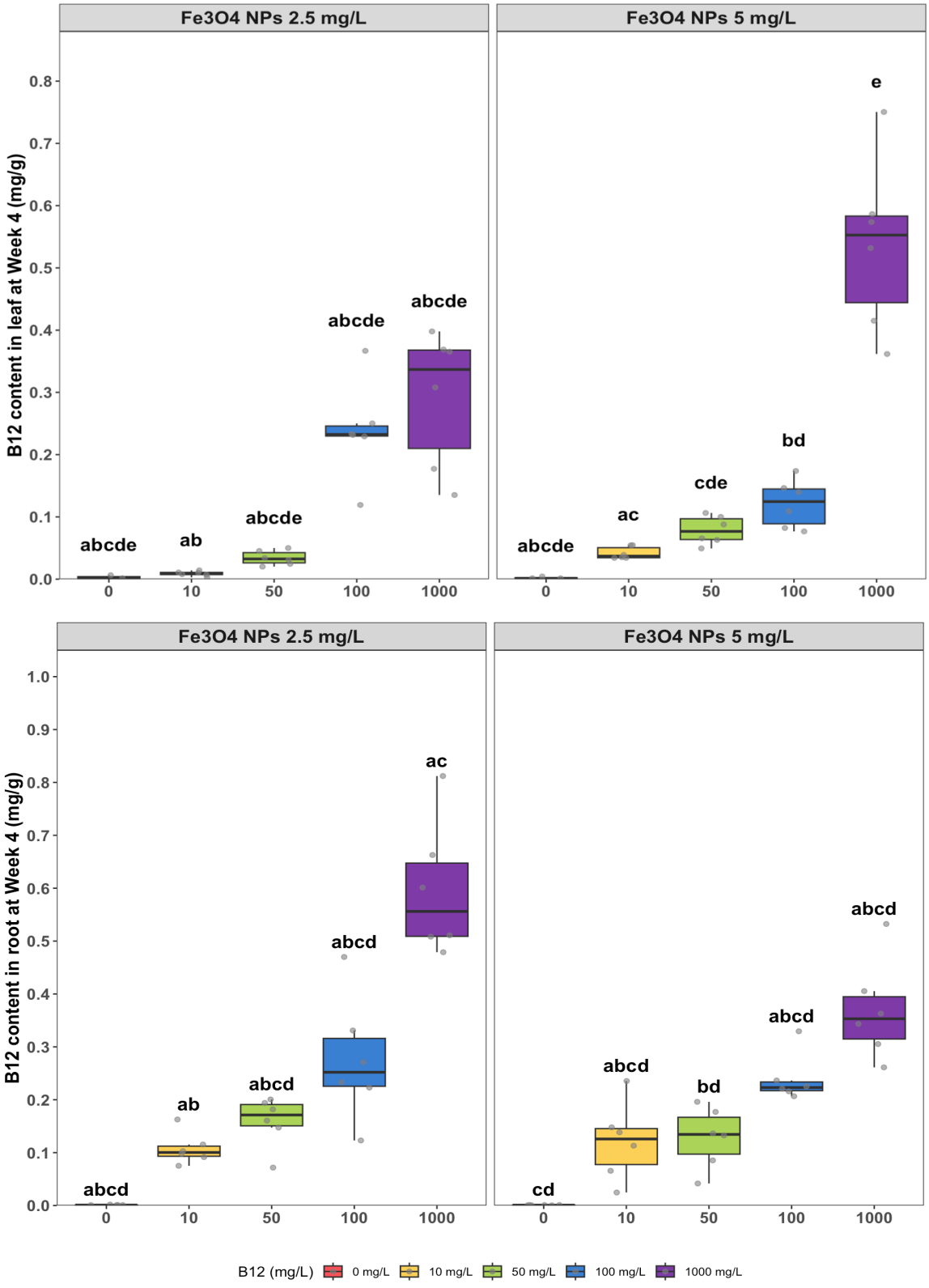


Figure 5.11 B_{12} quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Iron (Fe) quantification

Figure 5.12 presents the iron (Fe) quantification in both leaf and root tissue following root application of Fe_3O_4 NPs at concentration of 2.5 and 5 mg / L concentrations, in combination with different B_{12} concentrations (0 to 1000 mg / L). Higher average iron content in leaf tissues was recorded in the Fe_3O_4 NPs 5 mg / L treatment (0.133 mg / g) compared to the 2.5 mg / L group (0.101 mg / g), regardless of B_{12} concentrations. The highest iron content was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L without B_{12} supplementation (0.155 ± 0.090 mg / g), followed by Fe_3O_4 NPs 2.5 mg / L with B_{12} 0 mg / L (0.137 ± 0.046 mg / g). The lowest Fe concentrations were recorded in Fe_3O_4 NPs 2.5 mg / L group with higher B_{12} concentrations, such as 100 mg / L (0.083 ± 0.021 mg / g) and 1000 mg / L treatment (0.089 ± 0.021 mg / g). No statistically significant differences were observed across B_{12} and Fe_3O_4 NPs treatments. However, at the same B_{12} concentrations, relatively higher iron contents were measured in the Fe_3O_4 NPs 5 mg / L (0.125 and 0.127 mg / g, respectively), suggesting that Fe_3O_4 NPs concentration had a greater influence on iron uptake and accumulation than B_{12} supplementation.

The lower part of Figure 5.12 presents the iron content in root tissues. On average, iron content in root tissues was approximately 2.7 times higher in the Fe_3O_4 NPs 5 mg /L group (13.959 mg / g) compared to the 2.5 mg / L group (5.112 mg / g). The highest iron content was observed in lettuces treated with Fe_3O_4 NPs 5 mg / L with B_{12} 100 mg / L treatment (21.345 ± 2.893 mg / g, 'bd'), which was significantly higher than the corresponding treatment in the Fe_3O_4 NPs 2.5 mg / L group ('ac'). However, no statistically significant differences were observed across most treatments, as indicated by overlapping group labels ($p > 0.05$). This suggest that B_{12} levels had minimal influence on iron uptake in root tissues, whereas Fe3O4 NPs concentrations exerted a more pronounced effect on iron. Within the Fe_3O_4 NPs 2.5 mg / L group, the highest Fe content was recorded in lettuces treated with B_{12} 10 mg / L (8.134 ± 3.702 mg / g, 'abcd') and no statistically significant differences were found across B_{12} treatments ($p > 0.05$).

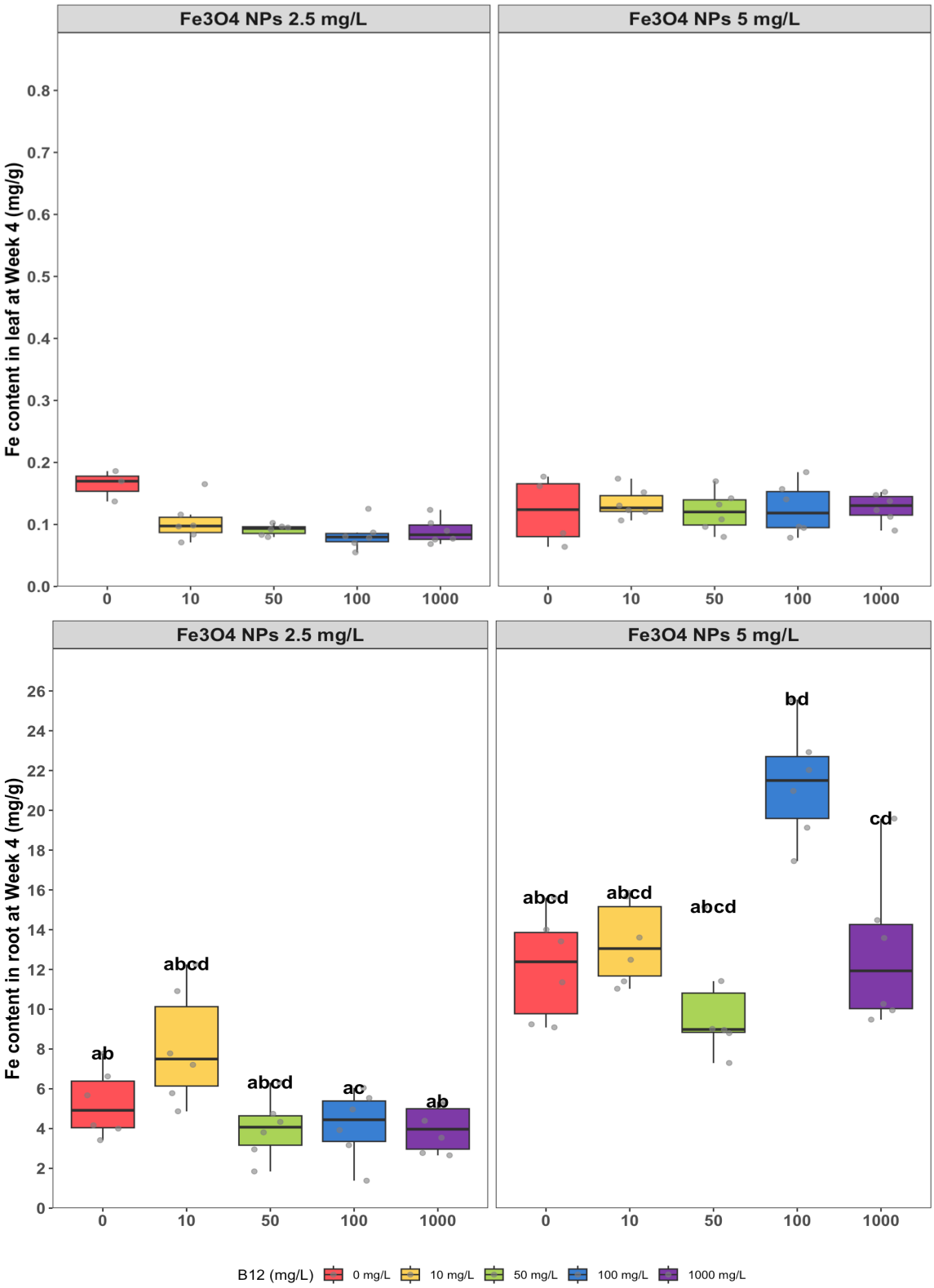


Figure 5.12 Iron quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Potassium (K) quantification

Figure 5.13 presents potassium (K) quantification in both leaf and root tissues across the Fe_3O_4 NPs and B_{12} treatments. On average, the Fe_3O_4 NPs 5 mg / L group exhibited higher K content in leaf tissues (234 mg / g) compared to the Fe_3O_4 NPs 2.5 mg / L (195 mg / g). The highest K concentration was recorded in Fe_3O_4 NPs 2.5 mg / L treatment with B_{12} 0 mg / L (247 ± 59 mg / g, 'abcd'), closely followed by Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L (242 ± 19 mg / g, 'bd'). No statistically significant differences were observed across B_{12} concentrations overall, as indicated by overlapping group labels ($p > 0.05$). However, pairwise comparisons based on 9,999 permutations revealed significant differences between Fe_3O_4 NPs 2.5 mg / L with B_{12} 50 mg / L and the Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L ('cd'), Additionally, Fe_3O_4 NPs 2.5 mg / L with B_{12} 100 mg / L ('ac') showed significant differences compared to Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L ('bd').

The K content in root tissues is depicted in the bottom section of Figure 5.13. On average, the Fe_3O_4 NPs 5 mg / L group exhibited higher K content in root tissues (170 mg / g) compared to the Fe_3O_4 NPs 2.5 mg / L (140 mg / g). The Fe_3O_4 NPs 5 mg / L and B12 1000 mg / L group exhibited the highest K content in root tissues (196 ± 25 mg / g), followed by B12 10 mg / L (169 ± 28 mg / g). Among Fe_3O_4 NPs 2.5 mg / L group, the highest K content was recorded in lettuces treated with B_{12} 100 mg / L (144 ± 37 mg / g). Overall, no statistically significant differences were observed across Fe_3O_4 NPs or B_{12} treatments ($p > 0.05$), indicating that neither treatment had a notable effect on K accumulation in root tissues.

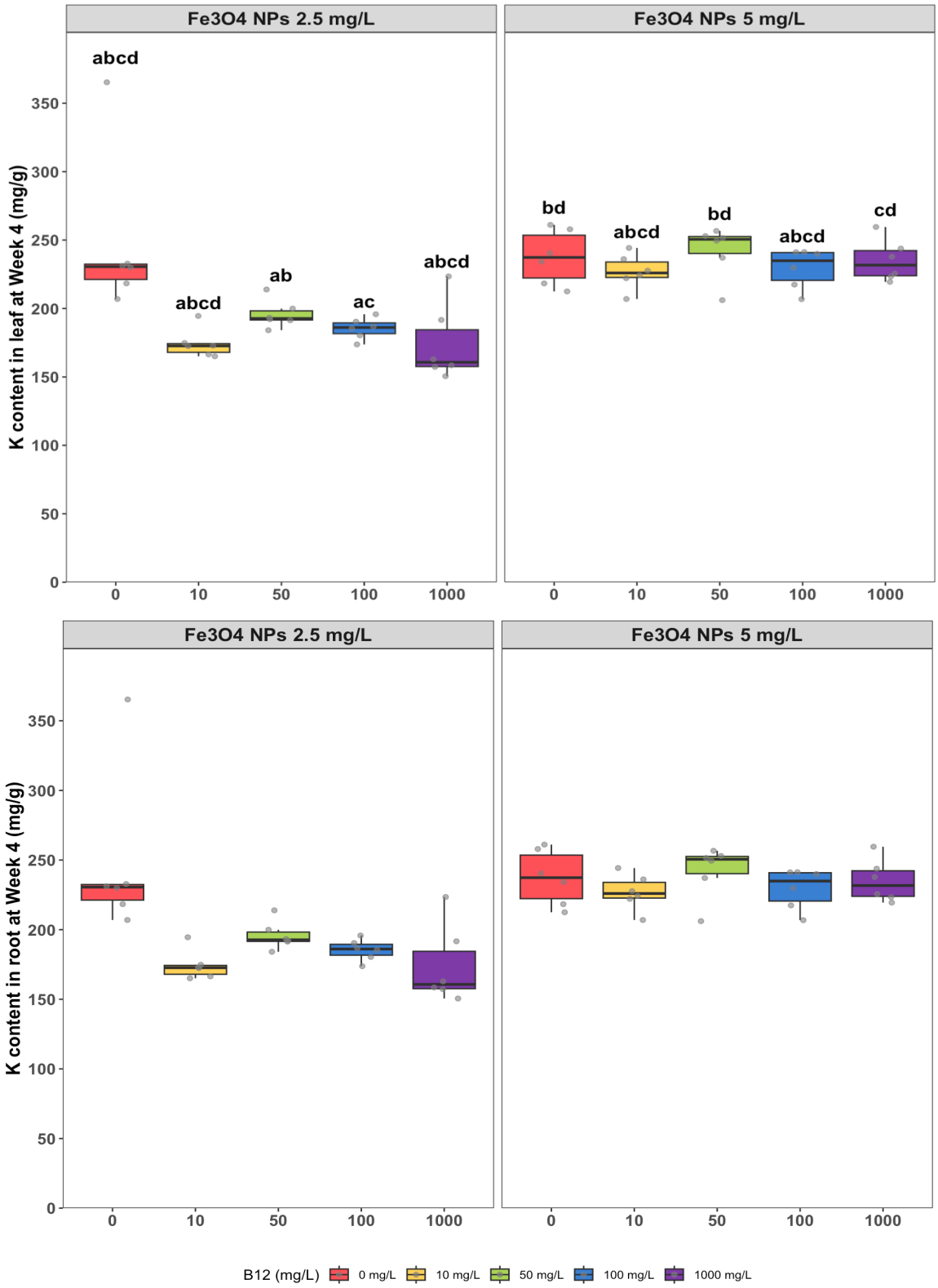


Figure 5.13 Potassium quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Magnesium (Mg) quantification

Figure 5.14 presents magnesium (Mg) quantification in both leaf and root tissues across Fe_3O_4 NPs and B_{12} treatments. Overall, a slightly higher Mg content was recorded in leaf tissues compared to root tissues across all treatments. The upper graph presents that, regardless of B_{12} treatment, the Fe_3O_4 NPs 5 mg / L treatment (15.620 mg / g) resulted in higher Mg content in leaf tissues compared to the Fe_3O_4 NPs 2.5 mg / L treatment (13.784 mg / g). Among the Fe_3O_4 NPs 5 mg / L, lettuces treated with B_{12} 10 mg / L exhibited the highest leaf Mg content (16.729 ± 1.195), closely followed by the B_{12} 0 mg / L treatment (16.484 ± 0.985 mg / g). Within the Fe_3O_4 NPs 2.5 mg / L group, the control without B_{12} treatment contained the highest Mg content (15.923 mg / g). Overall, in Fe_3O_4 NPs 2.5 mg / L group, a negative correlation was observed between increasing B_{12} concentration and leaf Mg content, suggesting a potential antagonistic interaction or physiological trade-off at higher B_{12} levels. This negative correlation was less pronounced in the 5 mg / L Fe_3O_4 NPs group, where Mg content remained relatively stable across lower B_{12} concentrations but decreased at the highest B_{12} level. However, 9,999 times permutation tests revealed no statistically significant differences across all treatments, indicating that neither of Fe_3O_4 NPs nor B_{12} treatments had a significant effect on Mg accumulation in leaf tissues.

The lower section of Figure 5.14 presents Mg content in root tissues. On average, lettuces treated with Fe_3O_4 NPs 5 mg / L exhibited higher Mg accumulation in roots compared to those treated with 2.5 mg / L, regardless of B_{12} concentration. The highest Mg content was observed in the Fe_3O_4 NPs 5 mg / L with B_{12} 100 mg / L treatment (9.578 ± 1.553 mg / g, ‘abd’), followed by B12 0 mg / L (8.846 ± 0.476, ‘abcd’). Among the Fe_3O_4 NPs 2.5 mg / L group, the control group without B_{12} showed the highest Mg content in root tissues (8.239 ± 0.954 mg / g). Similar to the leaf tissue trend, a negative correlating was observed between increasing B_{12} concentration and root Mg content under the Fe_3O_4 NP 2.5 treatment, suggesting a potential antagonistic interaction or physiological trade-off at higher B_{12} levels. Despite these trends, no statistically significant differences were observed across B_{12} treatments with each Fe_3O_4 NPs group ($p > 0.05$), as indicated by group labels. However, pairwise comparisons based on 9,999 permutations revealed statistically significant differences between the lowest Mg content in each Fe_3O_4 NPs group: Fe_3O_4 NPs 2.5 mg / L with B_{12} 1000 mg / L (‘ac’) and Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L (‘bd’).

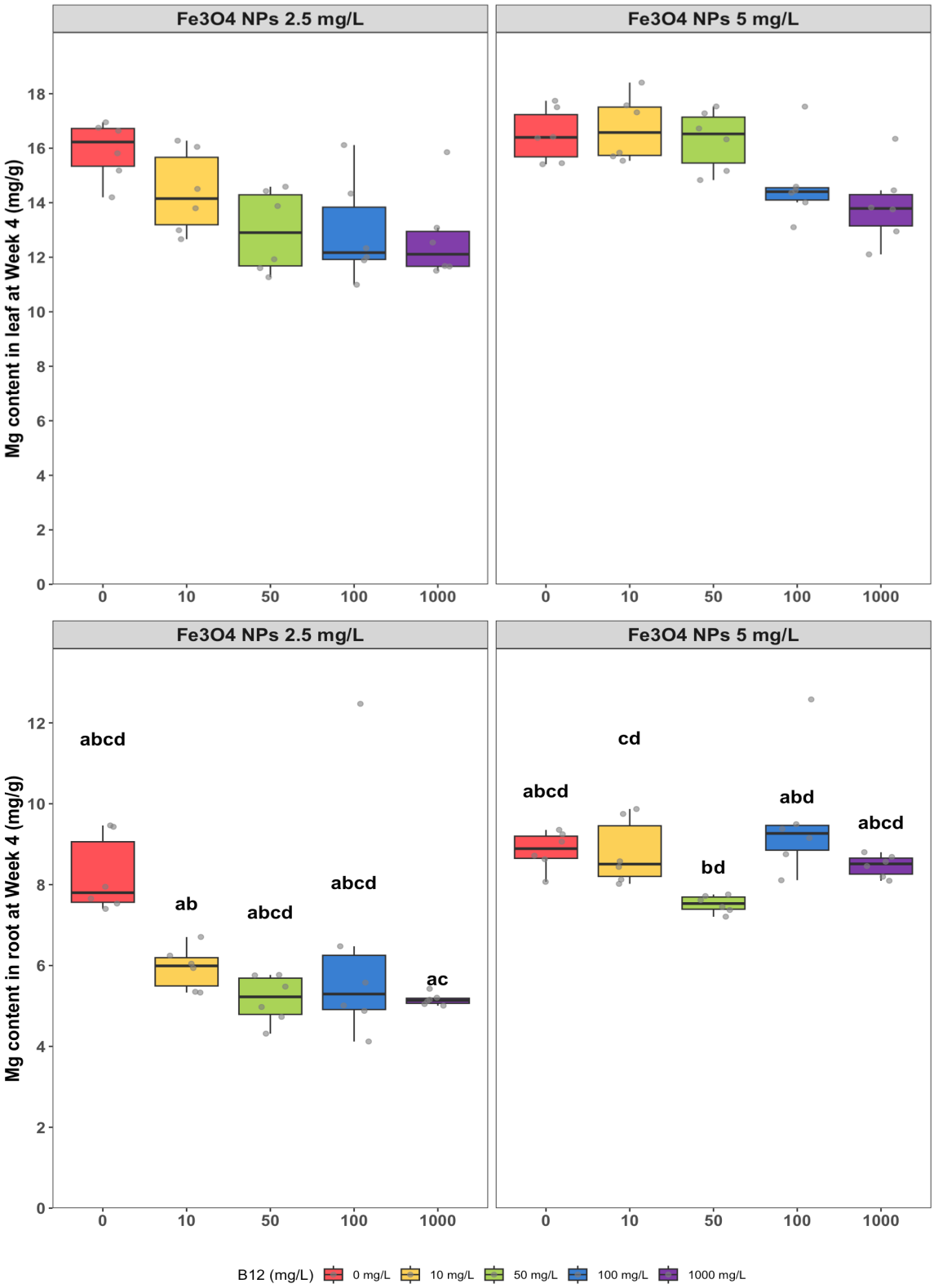


Figure 5.14 Magnesium quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Calcium (Ca) quantification

According to the calcium (Ca) quantification results shown in Figure 5.15, lettuces treated with Fe_3O_4 NPs 5 mg / L generally exhibited higher Ca content in leaf tissues compared to 2.5 mg / L group. Within the Fe_3O_4 NPs 5 mg / L group, the highest Ca content was recorded in the B_{12} 10 mg / L treatment (44.422 ± 2.215 mg / g, 'cdf'), which was significantly higher than the B_{12} 0 mg / L group ('abe', $p < 0.05$). In the Fe_3O_4 NPs 2.5 mg / L group, the control (B_{12} 0 mg / L) showed the highest leaf Ca content (32. 613 ± 2.218, 'abcdef'). A negative correlation between increasing B_{12} concentration and Ca accumulation was evident in the Fe_3O_4 NPs 2.5 mg / L group, suggesting a potential antagonistic interaction or physiological trade-off at higher B_{12} levels. A similar trend of decreasing Ca content with increasing B_{12} concentration was observed in the Fe_3O_4 NPs 5 mg / L group, except for the control (B_{12} 0 mg / L), which exhibited comparatively lower Ca levels than the B_{12} 10 and 50 mg / L treatments. However, most differences across B_{12} concentrations were not statistically significant, as indicated by overlapping group labels ($p > 0.05$).

The lower section of Figure 5.15 presents the Ca content in root tissues. Overall, the Fe_3O_4 NPs 5 mg / L group exhibited higher root Ca content compared to the Fe_3O_4 NPs 2.5 mg / L treatments. The highest Ca content was recorded in the Fe_3O_4 NPs 2.5 mg / L group with B_{12} 100 mg / L (22.984 ± 1.330 mg / g, 'ab'). Notably, a statistically significant difference was found between the Fe_3O_4 NPs 5 mg / L with B12 10 mg / L ('b') treatment and the Fe_3O_4 NPs 2.5 mg / L with B12 1000 mg / L ('a') treatment, based on pairwise permutations ($p < 0.05$). Within the Fe_3O_4 NPs 2.5 mg / L group, the control (B12 0 mg / L, 'ab') exhibited relatively high Ca content in root tissues. Interestingly, a general trend of decreasing root Ca content with increasing B_{12} concentration was observed in the Fe_3O_4 NPs 2.5 mg / L group, whereas no clear pattern was evident in the 5 mg / L group.

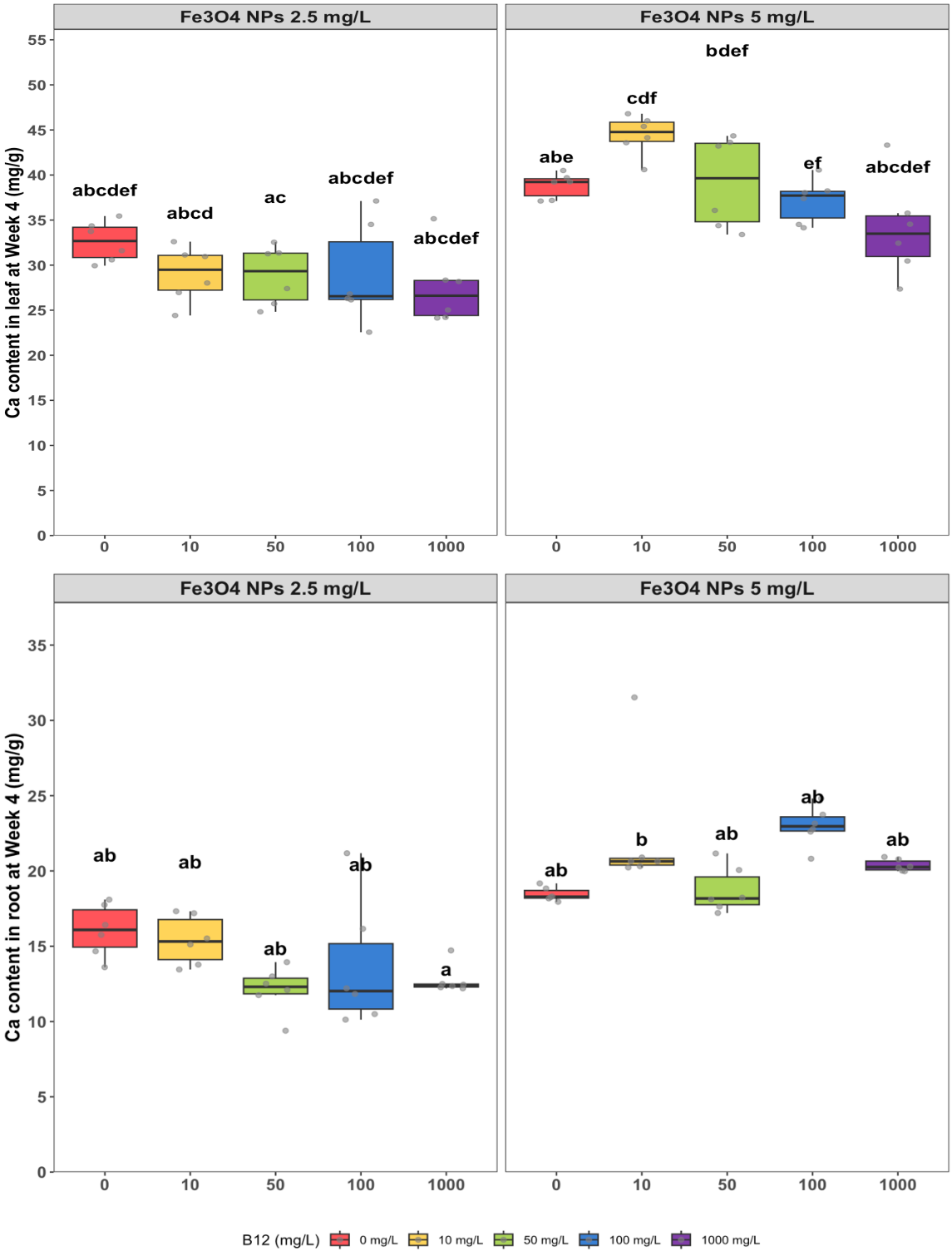


Figure 5.15 Calcium quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Phosphorous (P) quantification

Figure 5.16 presents phosphorous (P) quantification in both leaf and root tissues across Fe_3O_4 NPs and B_{12} treatments. Overall, the Fe_3O_4 NPs 5 mg / L group (29.454 mg / g) exhibited higher P content in leaf tissues compared to Fe_3O_4 NPs 2.5 mg / L treatments (21.188 mg / g). Lettuces treated with the Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L exhibited the highest P content in leaf tissues (31.840 ± 1.065 mg / g, 'a'), which was significantly greater than that observed in the B_{12} 100 mg / L group (27.671 ± 1.532 mg / g, 'b', $p < 0.05$). In the Fe_3O_4 NPs 2.5 mg / L treatments, the highest P content was found in the B_{12} 100 mg / L (22.024 ± 0.960 mg / g, 'ab'). However, no statistically significant differences were observed among B_{12} treatments, as indicated by overlapping group label ('a', and 'ab'). Overall, a clear negative correlation between increasing B_{12} concentration and leaf P content was observed in the Fe_3O_4 NPs 2.5 mg / L group, suggesting a possible antagonistic effect at higher B_{12} levels. In contrast, the Fe_3O_4 NPs 5 mg / L group displayed a non-linear trend, with the highest P accumulation at B_{12} 10 mg / L followed by a decline at higher B_{12} concentrations.

The bottom section of Figure 5.16 presents the P content in root tissues. Overall, lettuces treated with Fe_3O_4 NPs 5 mg / L group exhibited approximately 1.8 times higher root P content compared to those treated with Fe_3O_4 NPs 2.5 mg / L. The highest P accumulation was observed in the Fe_3O_4 NPs 5 mg / L with B_{12} 1000 mg / L treatment (52.240 ± 4.538 mg / g, 'ab'), which was significantly higher than Fe3O4 NPs 2.5 mg / L with B_{12} 10 mg / L ('a'), as determined by pairwise permutation tests ($p < 0.05$). Additionally, Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L treatment also showed a statistically significant difference from the Fe_3O_4 NPs 2.5 mg / L with B_{12} 10 mg / L group ($p < 0.05$).

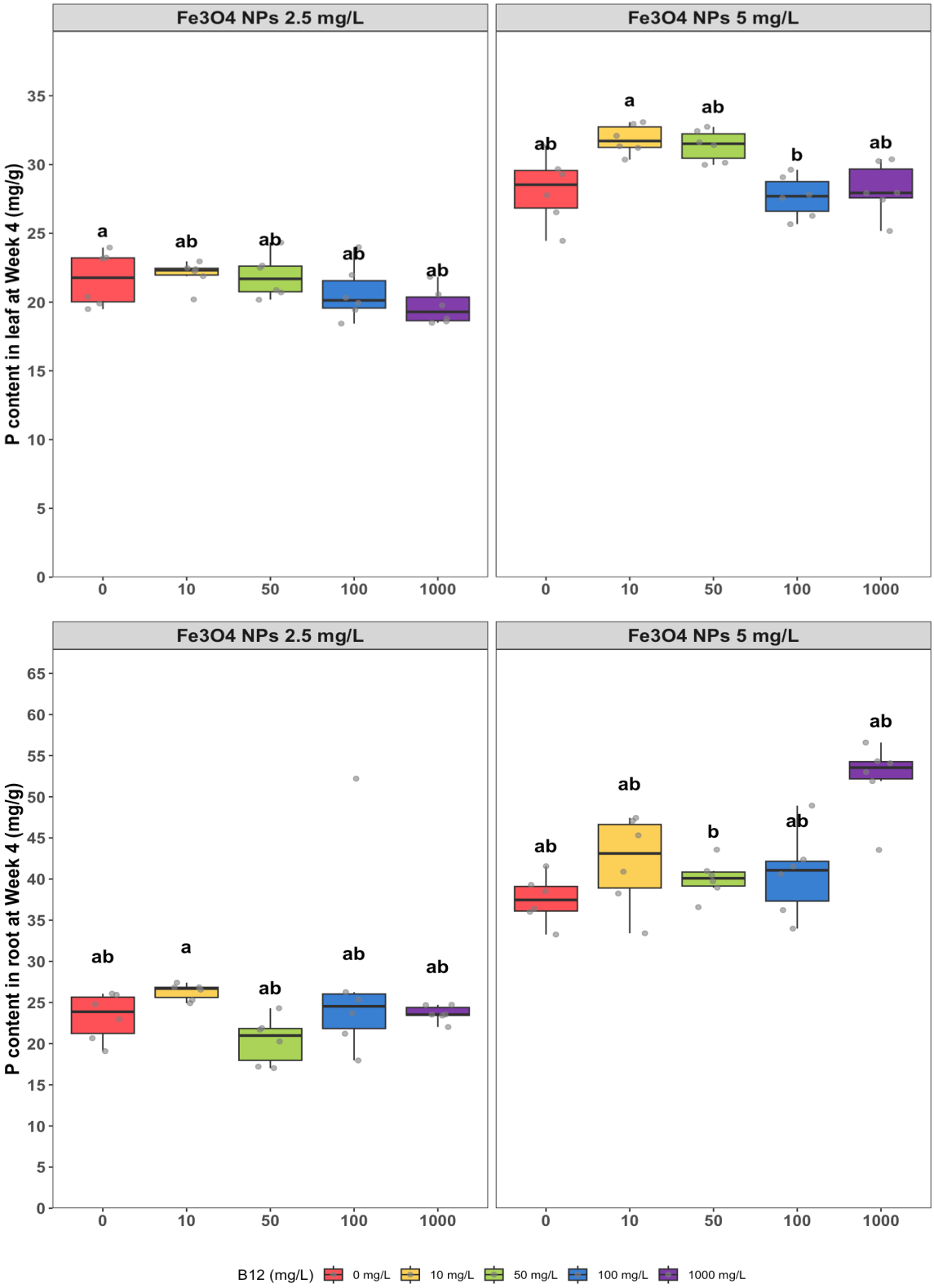


Figure 5.16 Phosphorous quantification in lettuce leaf & root tissues (mg / g) using ICP-MS at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Summary of multielement analysis

In summary, higher concentration of B_{12} and Fe_3O_4 NPs in the treatment corresponded with increased B_{12} and iron content in both leaf and root tissues. Notably, B_{12} and iron accumulation was greater in the tissues directly targeted by the application method, with elevated B_{12} levels observed in leaves following foliar application and increased iron content in root after root application. These findings indicate a strong correlation between the method of application and nutrient uptake efficiency. Interestingly, the Fe_3O_4 NPs 5 mg / L treatment generally resulted in higher B_{12}, Fe, K, Mg, Ca, and P contents compared to the 2.5 mg / L group, except for B_{12} content in root tissues, where the Fe_3O_4 NPs 2.5 mg / L treatment exhibited a higher averaged B_{12} root content.

Notably, Fe_3O_4 NPs 2.5 mg / L group showed a negative correlation between increasing B_{12} concentrations and Mg and Ca content in both leaf and root tissues. In contrast, the Fe_3O_4 NPs 5 mg / L treatment displayed non-linear trends for these nutrients. No significant differences were observed among the B_{12} treatments in K and P accumulation in either leaf or root tissues. Overall, the Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L treatment resulted in the highest accumulation of multiple elements, including the highest Mg content in leaf tissues, the highest Ca content in both leaf and root tissues, and highest P content in leaf tissues. Relatively high amount of B_{12} treatments (100 and 1000 mg / L) showed the highest Fe, K, Mg, and P content in root tissues within Fe_3O_4 NPs 5 mg / L.

5.3.5 Secondary metabolites

The secondary metabolites profiles were evaluated by measuring total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) in lettuce leaf tissues harvested at week 4 after transplanting. The methodology for these analyses followed the procedures described in **Chapter 2.4**. Each parameter was measured using eight biological replicates, each with three technical replicates. Statistical analysis was performed using a PERMANOVA test followed by multiple pairwise comparison test, with significance assessed based on 9,999 permutations.

Total Phenolic Content (TPC)

Phenolic compounds play a key role in plant defence by enhancing stress resistance and prolonging post-harvest shelf life[168]. Figure 5.17 shows the total phenolic content in fresh lettuce leaves, measured using gallic acid equivalence method and expressed as milligrams of gallic acid equivalents per gram of fresh leaf tissue (mg GAE / g). On average, the Fe_3O_4 NPs 5 mg / L group exhibited a 46 % higher TPC than the 2.5 mg / L treatment. The highest TPC was observed in the Fe_3O_4 NPs 5 mg / L with B_{12} 100 mg / L treatment (0.563 ± 0.141 mg GA / g, 'c'), which was significantly higher than the Fe_3O_4 NPs 2.5 mg / L treatments with B_{12} 0 mg / L ('ab'), 50 mg / L ('ab'), and 1000 mg / L ('a') ($p < 0.05$). However, no statistically significant differences were observed among the B_{12} treatment within the Fe_3O_4 NPs 5 mg / L group, as indicated by overlapping statistical groupings ($p > 0.05$). Within the Fe_3O_4 NPs 2.5 mg / L group, the highest TPC was found in lettuce treated with B_{12} 100 mg / L (0.339 ± 0.070 mg GAE / g), although no significance differences were detected among B_{12} concentration within this group ($p > 0.05$).

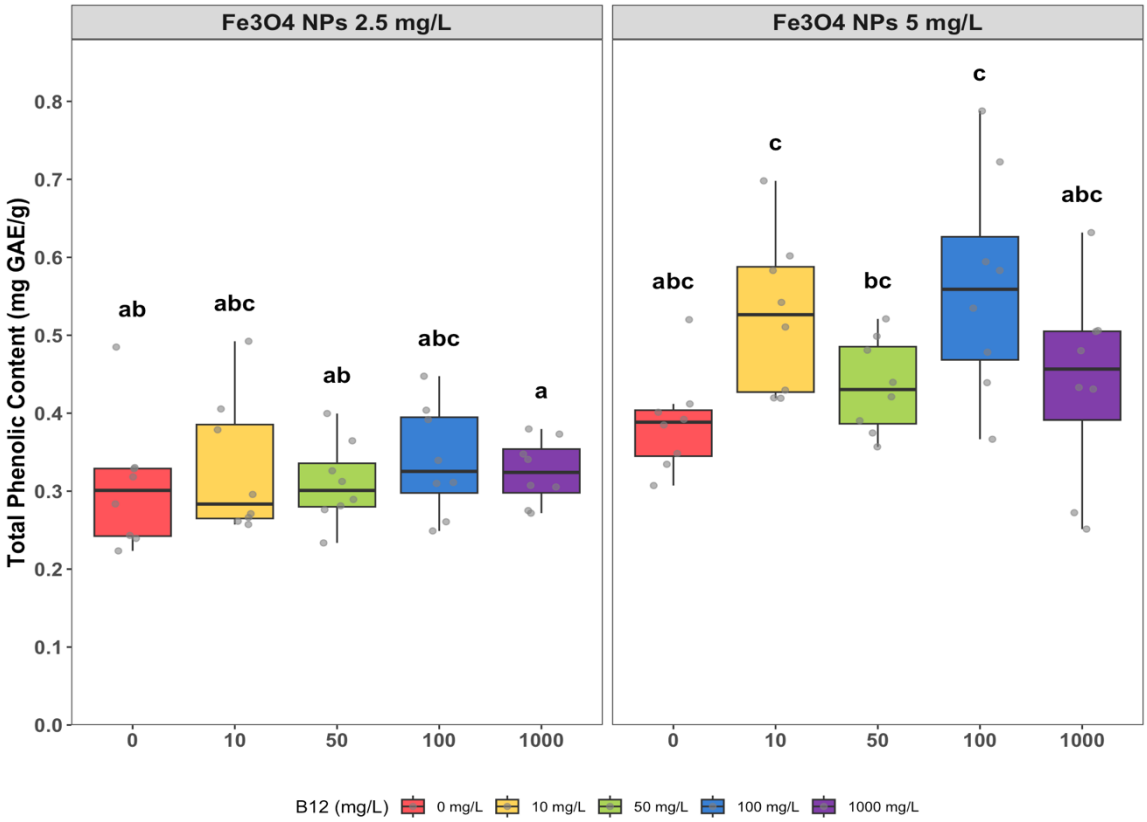


Figure 5.17 Total Phenolic Content (mg GAE / g) in lettuce leaf tissues at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Total Flavonoid Content (TFC)

Flavonoids play an essential role in plant by regulating cell growth, attracting pollinators, providing pigmentation such as chlorophyll and carotenoids, and enhancing stress tolerance against both biotic and abiotic factors [170]. Total flavonoid content (TFC) in fresh lettuce leaves were quantified using the rutin equivalent (RE) method and expressed in milligram of RE per gram of fresh weight (mg RE / g).

As shown in Figure 5.18, on average, the Fe_3O_4 NPs 5 mg / L group exhibited a 73 % higher TFC than Fe_3O_4 NPs 2.5 mg / L group, regardless of B_{12} concentration. The highest TFC was observed in the Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L treatment (0.192 ± 0.041 mg RE / g, 'c'), which was significantly higher than the B_{12} 1000 mg / L treatment within the same Fe_3O_4 NPs group ('bd', $p < 0.05$). This value was also significantly higher than the Fe_3O_4 NPs 2.5 mg / L with B_{12} at 50 mg / L ('ab'), 100 mg/ L ('a'), and 1000 mg / L ('a') ($p < 0.05$). Within the Fe_3O_4 NPs 2.5 mg / L group, showed the highest TFC was recorded in the B_{12} 10 mg / L treatment (0.107 ± 0.056 mg RE / g). However, no significant differences were observed among B_{12} concentrations within the Fe_3O_4 NPs 2.5 mg / L group, as indicated by overlapping statistical groupings ($p > 0.05$)

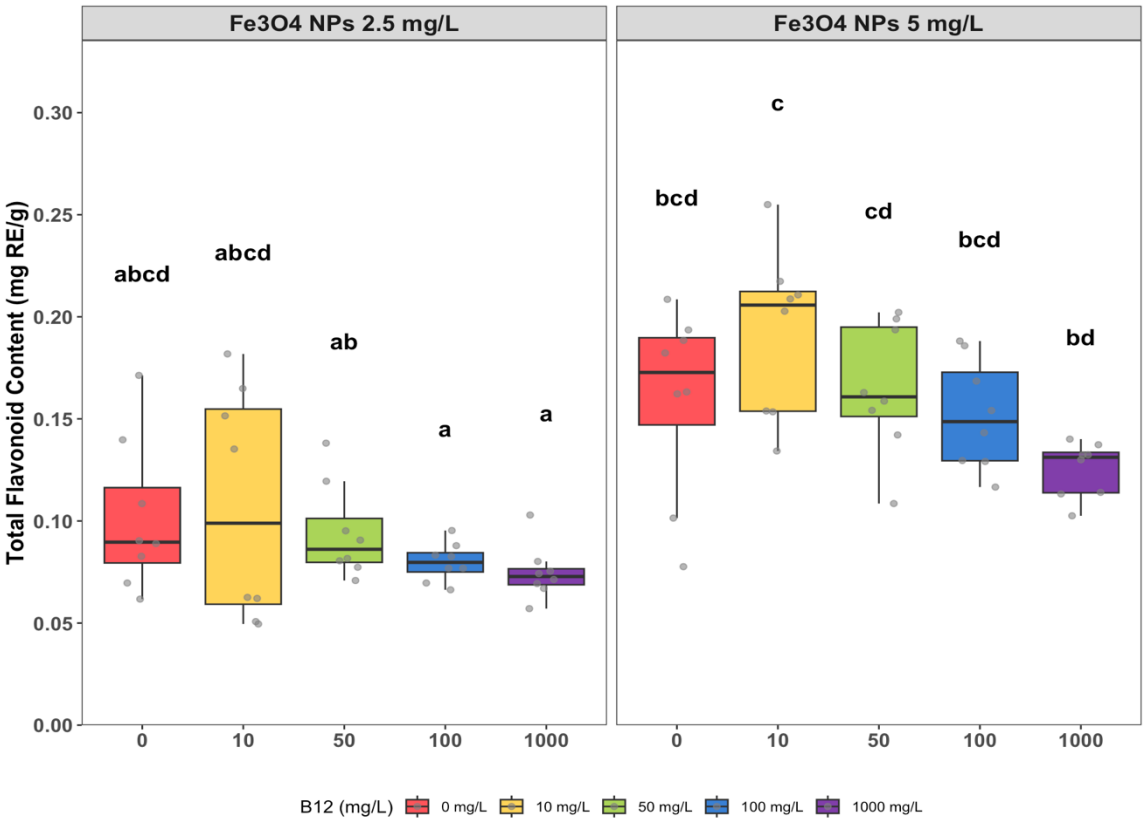


Figure 5.18 Total Flavonoid Content (mg RE / g) in lettuce leaf tissues at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

Total Antioxidant Activity (TAA)

Figure 5.19 presents the results of total antioxidant activity, measured using the Trolox® equivalent antioxidant capacity (TEAC) method. The results are expressed as milligram of TEAC per gram of a gram of fresh lettuce tissue (mg TEAC / g). On average, the Fe_3O_4 NPs 2.5 mg / L treatment exhibited a 3 % higher TAA compared to the 5 mg / L group. The highest TAA was recorded in the of Fe_3O_4 NPs 2.5 mg / L with B_{12} 50 mg / L treatment (5.687 ± 0.176 mg TEAC / g), closely followed by the B_{12} 0 mg / L treatment within the same group. However, no statistically significant differences were observed between any of the Fe_3O_4 NPs or B_{12} treatments, as indicated by the shared statistical groups ('a', $p > 0.05$).

In summary, a positive correlation was observed between total phenolic content and total antioxidant activity, particularly under the combined treatment of Fe_3O_4 NPs 5 with B_{12} 100 mg / L. This suggests that this treatment may stimulate stress defence mechanisms in lettuce by promoting the synthesis of phenolic compounds, potentially due to nutrient imbalance or mild stress induced by the treatment. In contrast, the Fe_3O_4 NPs 2.5 mg / L across various B_{12} concentration consistently exhibited the lowest levels of phenolic and antioxidant activity, which may be due to due to relatively lower iron concentration may trigger stress responses in lettuce.

No significant correlation was found between flavonoid content and pigment or greenness measurements. However, the Fe_3O_4 NPs 5 mg / L treatment resulted in higher flavonoid content in lettuces, which corresponded well with increased relative chlorophyll content measured by SPAD meter. These combined results indicate that higher Fe_3O_4 NPs concentration, especially when paired with optimal B_{12} levels, may enhance secondary metabolite production linked to stress responses and pigment accumulation. Nonetheless, total antioxidant activity did not differ significantly between Fe_3O_4 NPs or B_{12} treatments, indicating that these metabolic changes may not have translated into measurable differences in overall antioxidant capacity.

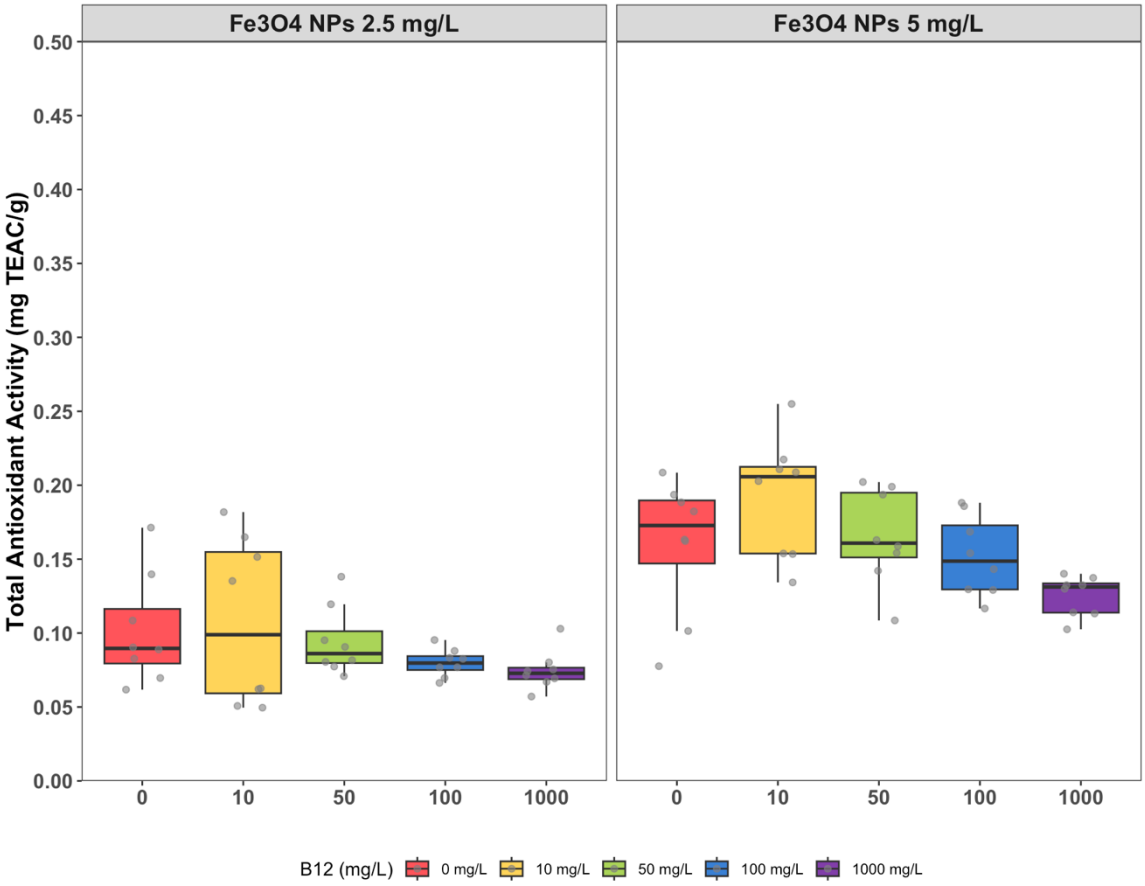


Figure 5.19 Total Antioxidant Activity (mg TEAC / g) in lettuce leaf tissues at Week 4.

Coloured box plots indicate Fe_3O_4 NPs (2.5 and 5mg / L) and B_{12} (0, 10, 50, 100, and 1000 mg / L), as described in the legend at bottom. Letters indicate statistical significance, according to PERMANOVA and multiple permutational pairwise test ($p \leq 0.05$), where different letter indicates a statistic significance difference between groups.

5.4 Discussion: B_{12} and iron fortification

5.4.1 Practical and economic implication of B_{12} and Fe_3O_4 NPs fortification strategies

As described in the result section, B_{12} concentrations were estimated from cobalt content measured by ICP-MS, assuming that all detected cobalt originated from the supplemented B_{12} due to the absence of other cobalt sources in the hydroponic solution. This estimation was based on the molecular ratio of vitamin B_{12} to cobalt. This chapter examines the effects of different application methods and concentrations on B_{12} and iron fortification in lettuce, with additional focus on evaluation the uptake threshold of B_{12} via foliar application at high concentrations (100 and 1000 mg / L). Multielement analysis conducted using ICP-MS enabled investigation of how B_{12} and Fe_3O_4 NPs treatments influence both micro- and macronutrient content in lettuce. These result not only reflect the outcomes of the B_{12} and iron fortification but also contribute to a better understanding of the uptake, translocation, and accumulation of individual elements within plant tissues. B_{12} fortification was successfully achieved in both leaf and root tissues, demonstrating a positive correlation between increasing B_{12} concentrations and elevated B_{12} content in the respective tissues.

To assess the practical implications of the B_{12} fortification strategy, particular attention was given to the B_{12} content in leaf tissues, as they represent the edible part of the plant. The B_{12} contents obtained from ICP-MS analysis were converted from milligram per gram of dry weight (D.W) of lettuce tissues to microgram per gram and further adjusted to reflect micrograms per gram of fresh weight (F.W) of lettuce, based on the fresh to dry weight ratio of the final harvested samples. Given that the recommended daily intake (RDI) of B_{12} for adults is 2.4 µg[1], the overall B_{12} treatment successfully met this daily requirement. Particularly, the Fe_3O_4 NPs 5 mg / L treatment with relatively low B_{12} concentrations, specifically 10 mg / L (2.09 µg / g F.W) and 50 mg /L (3.93 µg / g F.W), are expected to meet or exceed the RDI of B_{12}. These values are notably higher than those observed in the corresponding Fe_3O_4 NPs 2.5 mg / L treatments. Except for the B_{12} 100 mg / L treatment, the Fe_3O_4 NPs 5 mg / L group showed higher B_{12} content in leaf tissues compared to the 2.5 mg / L group across all B_{12} treatment groups. Higher B_{12} concentrations (100 and 1000 mg / L) were investigated to evaluate B_{12} uptake and accumulation in lettuce tissues, as well as to assess any potential toxic effects

on the plants. A dose-dependent effect was observed in B_{12} fortification, with significantly high B_{12} content detected in the B_{12} 1000 mg / L treatment combined with both Fe_3O_4 NPs 2.5 mg / L and 5 mg / L treatment.

Table 5.2 Elemental analysis of vitamin B_{12} in biofortified lettuce tissues: dry and fresh weight (µg / g)

Vitamin B_{12} Leaf application		
	B_{12} content in D.W (µg / g D.W)	B_{12} content in F.W (µg / g F.W)
mg / L	Leaf (µg / g)	Leaf (µg / g)
Fe_3O_4 NPs 2.5 + B_{12} 0	-0.11	-0.01
Fe_3O_4 NPs 2.5 + B_{12} 10	8.86	**0.44**
Fe_3O_4 NPs 2.5 + B_{12} 50	34.12	**1.71**
Fe_3O_4 NPs 2.5 + B_{12} 100	238.38	**11.92**
Fe_3O_4 NPs 2.5 + B_{12} 1000	292.11	**14.61**
Fe_3O_4 NPs 5+ B_{12} 0	-0.62	-0.03
Fe_3O_4 NPs 5+ B_{12} 10	41.88	**2.09**
Fe_3O_4 NPs 5+ B_{12} 50	78.66	**3.93**
Fe_3O_4 NPs 5+ B_{12} 100	121.34	**6.07**
Fe_3O_4 NPs 5+ B_{12} 1000	536.53	**26.83**

For iron fortification, two concentrations of Fe_3O_4 NPs (2.5 and 5 mg / L) were applied to evaluate the optimal iron levels within B_{12} treatments. According to ICP-MS analysis, on average, lettuces treated with the Fe_3O_4 NPs 5 mg / L group showed higher leaf iron content (0.133 mg / g) compared to those treated with Fe_3O_4 NPs 2.5 mg / L. (0.101 mg / g). Notably, the control group without B_{12} supplementation in both Fe_3O_4 NPs treatments (2.5 and 5 mg / L) exhibited the highest Fe content in leaf tissues. As B_{12} concentration increased, a dose-dependent decline in leaf Fe content was observed across both Fe_3O_4 NPs concentrations, suggesting a potential negative interaction between B_{12} supplementation and iron accumulation in plant tissues. However, no statistically significant differences in leaf iron content were observed between the Fe_3O_4 NPs concentrations or among the B_{12} treatments ($p > 0.05$) (Figure 5.12).

In root tissues, iron fortification using Fe_3O_4 NPs proved effective, as Fe_3O_4 NPs 5 mg / L consistently resulted in higher root iron accumulation than the 2.5 mg / L treatment across all B_{12} concentration. This indicates that within the tested range, higher Fe_3O_4 NPs (5 mg / L) more effectively enhanced root Fe accumulation, supporting its potential as a fortification strategy. This positive dose-dependent trend supports previous findings that higher Fe_3O_4 NPs concentration can enhance iron bioavailability and uptake by plant roots [141].

However, the interaction between B_{12} and iron uptake revealed a non-linear interaction. In the Fe_3O_4 NPs 2.5 mg / L group, iron content in root peaked at the

lowest B_{12} concentration (10 mg / L) but declined with increasing B_{12} levels. This pattern suggests that while low B_{12} dose may synergistically support iron uptake or root retention under limited iron availability, higher B_{12} concentrations may exert an inhibitory effect, possible by altering root physiology or transporter activity. Conversely, Fe_3O_4 NPs 5 mg / L group with the B_{12} 100 mg / L treatment yielding the highest iron content in root tissues. These findings indicate that when iron supply is sufficient, high-dose B_{12} treatments may promote iron retention in roots, possibly by reducing translocation to shoot. Collectively, these results points to a complex interaction between iron and B_{12} treatments. While Fe_3O_4 NPs clearly enhanced root Fe accumulation, the B_{12} supplementation modulated this response in a dose-dependent manner. Further investigation is required to determine the relationship between B_{12} and Fe as majority of the research quantified B_{12} [68,69,71] and identified their bioavailability [31].

Compared to the Fe_3O_4 NPs 2.5 mg / L group, the Fe_3O_4 NPs 5 mg / L treatment group showed the higher contents of multielement such as Fe, K, Mg, Ca and P in both root and leaf tissues. Notably, the combined treatment of Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L yielded the highest Mg content in leaf tissues, the highest Ca content in both root and leaf tissues, and the highest P content in leaf tissues. No significant differences were observed between B_{12} treatment, suggesting Fe_3O_4 NPs had a greater influence on the accumulation of the tested micro- and macronutrients. According to Yang et al. (2020), micro- and macronutrient, such as Ca, Mg, K, B, Fe, Mn, and Zn, were increased by the elevated concentrations of the Fe NPs treatment (15, 30, and 60 mg Fe / pot). These results are in line with the present study where that Fe_3O_4 NPs 5 mg / L treatment group obtained higher contents of Fe, K, Mg, Ca, and P compared to Fe_3O_4 NPs 2.5 mg / L treatment group [197]. In contrast, previous studies using foliar Fe_3O_4 NPs applications have reported differing outcomes. For example, Cantu et al. (2022) found that leaf application of Fe_3O_4 NPs 250 mg / L increased iron content but did not significantly improved Cu, Zn, or Mn levels [198]. Similarly, Tombluoglu et al. (2024) reported that leaf application of Fe_3O_4 NPs at 500 mg / L in tomato plants reduced the concentrations of Mn, Co, Mo, B, Cu, Zn, Ca, K, Mg, and P compared to untreated control [140]. These contrasting results suggest that the efficacy of Fe_3O_4 NPs treatment may vary depending on application method, dosage, and plant species. Further research is needed to clarify the mechanisms underlying nutrient uptake, translocation and possible antagonistic or synergistic effects.

Leaf marginal necrosis (leaf blight) was observed in lettuce treated with higher B_{12} concentrations (100 and 1000 mg / L) in both Fe_3O_4 NPs concentrations (2.5 and

5 mg / L), as shown in Figure 5.20. This phenomenon may be attributed to nutrient imbalance or phytotoxic effects resulting from excessive B_{12}, potentially impairing nutrient uptake. Interestingly, these groups treated with B_{12} 100, and 1000 mg / L contained relatively high contents of multielement (e.g., Fe, K, Mg, and P), with no significant differences between the control and B_{12} high concentration groups in multielement profiles. Therefore, the necrosis may have been caused by localised chemical toxicity on the leaf surface rather than systemic nutrient deficiency.

5.4.2 Effects of B_{12} and Fe_3O_4 NPs on lettuce growth and development

This study aimed to fortify lettuce with vitamin B_{12} and iron and assess their effect on morphological, physiological, phytochemical and nutritional traits. Two concentrations of Fe_3O_4 NPs (2.5 and 5 mg / L) were applied via root application, while five concentrations of B_{12} (0, 10, 50, 100, and 1000 mg / L) were administered through leaf application. The results demonstrated that the Fe_3O_4 NPs 5 mg / L treatment generally promoted better growth and physiological development compared to the Fe_3O_4 NPs 2.5 mg / L treatment. These findings align with those of Azizkhani et al (2023), who reported that Fe_3O_4 NPs 6 mg / L significantly enhanced the strawberry growth. Their study also reported strong positive correlations between biomass, leaf area, chlorophyll content, and leaf iron levels, suggesting that improved Fe uptake and bioavailability mediated by Fe_3O_4 NPs contribute to improved plant development. Similarly, in the present study, the higher Fe_3O_4 NPs concentration (5 mg / L) likely promoted greater iron accumulation, resulting in enhanced lettuce growth and performance compared to the lower concentration (2.5 mg / L).

Lettuces treated with the highest B_{12} concentration (1000 mg / L) exhibited poor morphological and physiological development, regardless of Fe_3O_4 NPs concentration. In contrast, the B_{12} 50 mg / L treatment yielded the best final harvest quality in both Fe_3O_4 NPs 2.5 and 5 mg / L groups. Overall, no significant contribution of B_{12} treatment across concentrations was observed in terms of growth and development, which aligned with previous studies [68,69]. These studies reported negligible effects of B_{12} supplementation on final yield (fresh weight) in both lettuce [68] and tomato [69]. However, consistent with the current findings, excessively high B_{12} levels (1000 mg / L) appeared to impair growth and physiological performance in lettuce. A prior experiment conducted as part of this study also found no significant effect of B_{12} treatments on lettuce morphology and

physiology via leaf and root application (**Chapter 3**). Nonetheless, the foliar B_{12} application was associated with increased chlorophyll content.

The relative chlorophyll measurements revealed that the Fe_3O_4 NPs 5 mg / L treatment without B_{12} application recorded the highest SPAD values, while this group also showed the lowest NPCI readings based on PlantEye analysis. However, it is important to note that SPAD measurements were taken from a limited area of the leaf and did not yield statistically significant differences, which may not fully reflect the overall physiological response. Nonetheless, weekly SPAD readings consistently indicated that Fe_3O_4 NPs 5 mg / L group exhibited higher relatively chlorophyll content compared to the 2.5 mg / L group. This trend suggests that the higher Fe_3O_4 NPs concentration (5 mg / L) may enhance photosynthetic efficiency and potentially contribute to improved growth and final yield. These findings are consistent with Azizkhani et al. (2023), who reported that the Fe_3O_4 NPs 6 mg / L treatment significantly enhanced chlorophyll content in strawberry leaves compared to lower concentration (0.06 mg / L) [141]. In contrast, no statistically significant differences observed among the various B_{12} concentrations in the present study, indicating that the effect of B_{12} on chlorophyll content is relatively minor compared to the influence of Fe_3O_4 NPs.

5.4.3 Effect of B_{12} and Fe_3O_4 NPs application on nutrient fortification

As described in the result section, B_{12} concentrations were estimated from cobalt content measured by ICP-MS, assuming that all detected cobalt originated from the supplemented B_{12} due to the absence of other cobalt sources in the hydroponic solution. This estimation was based on the molecular ratio of vitamin B_{12} to cobalt. This chapter examines the effects of different application methods and concentrations on B_{12} and iron fortification in lettuce, with additional focus on evaluation the uptake threshold of B_{12} via foliar application at high concentrations (100 and 1000 mg / L). Multielement analysis conducted using ICP-MS enabled investigation of how B_{12} and Fe_3O_4 NPs treatments influence both micro- and macronutrient content in lettuce. These result not only reflect the outcomes of the B_{12} and iron fortification but also contribute to a better understanding of the uptake, translocation, and accumulation of individual elements within plant tissues. B_{12} fortification was successfully achieved in both leaf and root tissues, demonstrating a positive correlation between increasing B_{12} concentrations and elevated B_{12} content in the respective tissues.

To assess the practical implications of the B_{12} fortification strategy, particular attention was given to the B_{12} content in leaf tissues, as they represent the edible part of the plant. The B_{12} contents obtained from ICP-MS analysis were converted from milligram per gram of dry weight (D.W) of lettuce tissues to microgram per gram and further adjusted to reflect micrograms per gram of fresh weight (F.W) of lettuce, based on the fresh to dry weight ratio of the final harvested samples. Given that the recommended daily intake (RDI) of B_{12} for adults is 2.4 µg[1], the overall B_{12} treatment successfully met this daily requirement. Particularly, the Fe_3O_4 NPs 5 mg / L treatment with relatively low B_{12} concentrations, specifically 10 mg / L (2.09 µg / g F.W) and 50 mg /L (3.93 µg / g F.W), are expected to meet or exceed the RDI of B_{12}. These values are notably higher than those observed in the corresponding Fe_3O_4 NPs 2.5 mg / L treatments. Except for the B_{12} 100 mg / L treatment, the Fe_3O_4 NPs 5 mg / L group showed higher B_{12} content in leaf tissues compared to the 2.5 mg / L group across all B_{12} treatment groups. Higher B_{12} concentrations (100 and 1000 mg / L) were investigated to evaluate B_{12} uptake and accumulation in lettuce tissues, as well as to assess any potential toxic effects on the plants. A dose-dependent effect was observed in B_{12} fortification, with significantly high B_{12} content detected in the B_{12} 1000 mg / L treatment combined with both Fe_3O_4 NPs 2.5 mg / L and 5 mg / L treatment.

Table 5.3 Elemental analysis of vitamin B_{12} in biofortified lettuce tissues: dry and fresh weight (µg / g)

Vitamin B_{12} Leaf application		
	B_{12} content in D.W (µg / g D.W)	**B_{12} content in F.W (µg / g F.W)**
mg / L	**Leaf (µg / g)**	**Leaf (µg / g)**
Fe_3O_4 NPs 2.5 + B_{12} 0	-0.11	-0.01
Fe_3O_4 NPs 2.5 + B_{12} 10	8.86	**0.44**
Fe_3O_4 NPs 2.5 + B_{12} 50	34.12	**1.71**
Fe_3O_4 NPs 2.5 + B_{12} 100	238.38	**11.92**
Fe_3O_4 NPs 2.5 + B_{12} 1000	292.11	**14.61**
Fe_3O_4 NPs 5+ B_{12} 0	-0.62	-0.03
Fe_3O_4 NPs 5+ B_{12} 10	41.88	**2.09**
Fe_3O_4 NPs 5+ B_{12} 50	78.66	**3.93**
Fe_3O_4 NPs 5+ B_{12} 100	121.34	**6.07**
Fe_3O_4 NPs 5+ B_{12} 1000	536.53	**26.83**

For iron fortification, two concentrations of Fe_3O_4 NPs (2.5 and 5 mg / L) were applied to evaluate the optimal iron levels within B_{12} treatments. According to ICP-MS analysis, on average, lettuces treated with the Fe_3O_4 NPs 5 mg / L group showed higher leaf iron content (0.133 mg / g) compared to those treated with Fe_3O_4 NPs 2.5 mg / L. (0.101 mg / g). Notably, the control group without B_{12} supplementation in both Fe_3O_4 NPs treatments (2.5 and 5 mg / L) exhibited the

highest Fe content in leaf tissues. As B_{12} concentration increased, a dose-dependent decline in leaf Fe content was observed across both Fe_3O_4 NPs concentrations, suggesting a potential negative interaction between B_{12} supplementation and iron accumulation in plant tissues. However, no statistically significant differences in leaf iron content were observed between the Fe_3O_4 NPs concentrations or among the B_{12} treatments ($p > 0.05$) (Figure 5.12).

In root tissues, iron fortification using Fe_3O_4 NPs proved effective, as Fe_3O_4 NPs 5 mg / L consistently resulted in higher root iron accumulation than the 2.5 mg / L treatment across all B_{12} concentration. This indicates that within the tested range, higher Fe_3O_4 NPs (5 mg / L) more effectively enhanced root Fe accumulation, supporting its potential as a fortification strategy. This positive dose-dependent trend supports previous findings that higher Fe_3O_4 NPs concentration can enhance iron bioavailability and uptake by plant roots [141].

However, the interaction between B_{12} and iron uptake revealed a non-linear interaction. In the Fe_3O_4 NPs 2.5 mg / L group, iron content in root peaked at the lowest B_{12} concentration (10 mg / L) but declined with increasing B_{12} levels. This pattern suggests that while low B_{12} dose may synergistically support iron uptake or root retention under limited iron availability, higher B_{12} concentrations may exert an inhibitory effect, possible by altering root physiology or transporter activity. Conversely, Fe_3O_4 NPs 5 mg / L group with the B_{12} 100 mg / L treatment yielding the highest iron content in root tissues. These findings indicate that when iron supply is sufficient, high-dose B_{12} treatments may promote iron retention in roots, possibly by reducing translocation to shoot. Collectively, these results points to a complex interaction between iron and B_{12} treatments. While Fe_3O_4 NPs clearly enhanced root Fe accumulation, the B_{12} supplementation modulated this response in a dose-dependent manner. Further investigation is required to determine the relationship between B_{12} and Fe as majority of the research quantified B_{12} [68,69,71] and identified their bioavailability [31].

Compared to the Fe_3O_4 NPs 2.5 mg / L group, the Fe_3O_4 NPs 5 mg / L treatment group showed the higher contents of multielement such as Fe, K, Mg, Ca and P in both root and leaf tissues. Notably, the combined treatment of Fe_3O_4 NPs 5 mg / L with B_{12} 10 mg / L yielded the highest Mg content in leaf tissues, the highest Ca content in both root and leaf tissues, and the highest P content in leaf tissues. No significant differences were observed between B_{12} treatment, suggesting Fe_3O_4 NPs had a greater influence on the accumulation of the tested micro- and macronutrients. According to Yang et al. (2020), micro- and macronutrient, such

as Ca, Mg, K, B, Fe, Mn, and Zn, were increased by the elevated concentrations of the Fe NPs treatment (15, 30, and 60 mg Fe / pot). These results are in line with the present study where that Fe_3O_4 NPs 5 mg / L treatment group obtained higher contents of Fe, K, Mg, Ca, and P compared to Fe_3O_4 NPs 2.5 mg / L treatment group [197]. In contrast, previous studies using foliar Fe_3O_4 NPs applications have reported differing outcomes. For example, Cantu et al. (2022) found that leaf application of Fe_3O_4 NPs 250 mg / L increased iron content but did not significantly improved Cu, Zn, or Mn levels [198]. Similarly, Tombluoglu et al. (2024) reported that leaf application of Fe_3O_4 NPs at 500 mg / L in tomato plants reduced the concentrations of Mn, Co, Mo, B, Cu, Zn, Ca, K, Mg, and P compared to untreated control [140]. These contrasting results suggest that the efficacy of Fe_3O_4 NPs treatment may vary depending on application method, dosage, and plant species. Further research is needed to clarify the mechanisms underlying nutrient uptake, translocation and possible antagonistic or synergistic effects.

Leaf marginal necrosis (leaf blight) was observed in lettuce treated with higher B_{12} concentrations (100 and 1000 mg / L) in both Fe_3O_4 NPs concentrations (2.5 and 5 mg / L), as shown in Figure 5.20. This phenomenon may be attributed to nutrient imbalance or phytotoxic effects resulting from excessive B_{12}, potentially impairing nutrient uptake. Interestingly, these groups treated with B_{12} 100, and 1000 mg / L contained relatively high contents of multielement (e.g., Fe, K, Mg, and P), with no significant differences between the control and B_{12} high concentration groups in multielement profiles. Therefore, the necrosis may have been caused by localised chemical toxicity on the leaf surface rather than systemic nutrient deficiency.

Figure 5.20 Digital photograph of harvest quality.

A. Fe_3O_4 NPs 2.5 mg / L group B. Fe_3O_4 NPs 5 mg / L group within two replicates of the B_{12} treatment (0, 10, 50, 100, and 1000 mg / L).

5.4.4 Effect of B_{12} and Fe_3O_4 NPs on lettuce secondary metabolites

In **Chapter 3.1**, the effect of B_{12} fortification on total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant activity (TAA) were evaluated. A positive correlation between TPC and TAA was observed in lettuces treated with B_{12} 5 mg / L treatment via leaf application. In contrast, the root application experiment described in **Chapter 3.2** showed that control group without B_{12} treatment exhibited the lowest TPC and TAA values. These findings are consistent with the previous studies[72,141,145,199], further supporting the hypothesis that B_{12} fortification enhances the accumulation of secondary metabolites in plants.

Additionally, the effect of Fe_3O_4 NPs application on secondary metabolites was investigate in a prior experiment, described in **Chapter 4.3**. The result showed that the Fe_3O_4 NPs treatment group exhibited higher levels of TPC, TFC and TAA compared to the Fe-EDTA treatment group. Notably, the Fe-EDTA 2.5 mg / L resulted in the lowest TPC and TAA values, likely due to the bioavailable iron supplied by Fe-EDTA providing favourable nutrient conditions that reduced the plant's need to synthesise secondary metabolites. These findings are supported by Holghoomi and Colagar (2024), who reported that Fe NPs treatment significantly increased phenolic and flavonoid content in garden cress (*Lepidum sativum* L.) [195], suggesting a stimulatory effect of FeNPs on secondary metabolite synthesis.

Based on the key findings from this study, the effect of Fe_3O_4 NPs and B_{12} treatments on the secondary metabolites of lettuce were examined. Overall, Fe_3O_4 NPs 5 mg / L treatment resulted in higher average values of TPC and TAA compared to the lower concentration of 2.5 mg / L. Notably, lettuces treated with B_{12} 100 mg / L exhibited the highest TPC levels, followed by those treated with B_{12} 10 mg / L, within Fe_3O_4 NPs 5 mg / L treatment group. A similar pattern was observed in the Fe_3O_4 NPs 2.5 mg / L group. These findings suggest that a higher concentration of B_{12} (100 mg / L) may enhance the synthesis of phenolic compounds in lettuces, potentially stimulating the plants' self-defence mechanisms against environmental stresses during growth.

The TFC results indicated that lettuces treated with the Fe_3O_4 NPs 5 mg / L and B_{12} 10 mg / L treatment exhibited the highest TFC. However, TFC decreased as the B_{12} concentration increased to 50, 100, and 1000 mg / L, regardless of the Fe_3O_4 NPs concentration (2.5 and 5 mg / L). Interestingly, a previously conducted

experiment found that B_{12} 10 mg / L resulted in the lowest TFC values in lettuce, which contrasts with the current findings. This suggests that the addition of the Fe_3O_4 NPs 5 mg / L may have played a role in enhancing flavonoid synthesis. Overall, both TPC and TFC levels were significantly higher in lettuces treated with Fe_3O_4 NPs 5 mg / L compared to those treated with 2.5 mg / L, indicating that secondary metabolites in lettuce were responsive to the nanoparticle treatment. In contrast, the influence of B_{12} appeared to be less pronounced. These findings algin with previous studies, such as one demonstrating that Fe_3O_4 NPs 6 mg / L coated with cysteine significantly increased secondary metabolites, such as phenols, vitamin C, flavonoids, anthocyanin, and antioxidant capacity in strawberry fruits, compared to conventional iron fertilisers [141]. Interestingly, in the present study, no significant differences in TAA were observed across the various Fe_3O_4 NPs and B_{12} treatments concentrations.

General overview

This study conducted a comprehensive investigation into the effects of B_{12} and iron fortification on lettuce growth and development. In the experiment design, B_{12} was applied via foliar spray at five concentrations (0, 10, 50, 100, and1000 mg / L), while iron was supplied through root application using Fe_3O_4 NPs at two concentrations (2.5 and 5 mg / L). Overall, Fe_3O_4 NPs 5 mg / L significantly enhanced lettuce growth and development, compared to the Fe_3O_4 NPs 2.5 mg / L treatment, resulting in increased plant height, greater biomass accumulation, improved vegetation index, higher chlorophyll content, and increased final fresh weight. These improvements were associated with the significantly higher iron content quantified in the leaf tissues of plants treated with Fe_3O_4 NPs 5 mg / L. Notably, the combined treatment of Fe_3O_4 NPs 5 mg / L with B_{12} 50 mg / L produced the most substantial improvements in harvest quality, indicating greater fresh and dry biomass in both leaf and root tissues, which corresponded with digital biomass data obtained using the PlantEye scanner. In contrast, within the Fe_3O_4 NPs 2.5 mg / L group, lettuces treated with B_{12} at either 10 mg / L or 1000 mg / L showed suboptimal final harvest performance. These treatments were also associated with lower chlorophyll and flavonoid content, suggesting possible stress responses or nutrient imbalances at these concentrations.

The Fe_3O_4 NPs 5 mg / L treatment resulted in higher levels of phenolic and flavonoid compounds compared to the Fe_3O_4 NPs 2.5 mg / L treatment. These results suggest that Fe_3O_4 NPs 5 mg / L may stimulate the production of phytochemicals involved in the plant's self-defence mechanisms. Secondary

metabolite analysis showed that various B_{12} concentration led to elevated levels of phenolics, flavonoids, and antioxidant activity within the Fe_3O_4 NPs 5 mg / L treatment group. These results suggest that B_{12} treatments alone may have limited influence on secondary metabolite production, and their effectiveness may depend on the presence of Fe_3O_4 NPs. Interestingly, the combination of Fe_3O_4 NPs 2.5 mg / L with B_{12} 1000 mg / L resulted in the lowest flavonoids content, which corresponded with reduced greenness, vegetation index, and chlorophyll content.

The results of the multielement analysis by ICP-MS suggest that a clear dose-dependent effect of B_{12} was observed in both leaf and root tissues, regardless of Fe_3O_4 NPs treatments. Notably, Fe_3O_4 NPs 5 mg / L group displayed higher contents of B_{12}, Fe, K, Mg, Ca, and P contents in both leaf and root tissues, except for B_{12} root content, where the Fe_3O_4 NPs 2.5 mg / L treatment showed a higher average concentration than the 5 mg / L treatment. In summary, the Fe_3O_4 NPs plays critical role in morphological, physiological, and phytochemical development of lettuces compared to B_{12} concentration.

5.5 Conclusion

Comprehensive experimental tests on B_{12} and Fe fortification have been useful to determine the pathway of both B_{12} and Fe and allowed analysis of the effects of combined application of B_{12} and Fe_3O_4 NPs on lettuce growth, development, and quality. Through this, the following conclusions can be made:

- The combined treatment of Fe_3O_4 NPs and B_{12} effectively fortified lettuce with both B_{12} and iron. Notably, the Fe_3O_4 NPs 5 mg / L treatment with relatively low B_{12} concentrations (10 and 50 mg / L) resulted in B_{12} contents of 2.09 and 3.93 µg / g F.W, respectively. These levels translate to approximately 63 and 118 µg / g of B_{12} per 30 g severing of fresh lettuce, which exceeded the adult recommended daily intake of 2.4 µg. Similarly, the Fe_3O_4 NPs 2.5 mg / L group also achieved B_{12} concentrations (0.44 and 1.71 µg / g F.W) sufficient to meet or exceed RDI when consumed in standard portions. These findings demonstrate that low B_{12} concentrations, when combined with Fe_3O_4 NPs, can achieve effective B_{12} fortification in lettuce.

- A clear dose-dependent increase in B_{12} content was observed in both leaf and root tissues across all treatments, indicating no restriction in uptake or accumulation even at the high concentrations (100 and 1000 mg / L). In contrast, Fe content exhibited non-linear trends across B_{12} concentrations, although the Fe_3O_4 NPs 5 mg / L consistently showed higher Fe accumulation than the 2.5 mg / L group. These variations suggest that iron uptake was primarily influenced by Fe_3O_4 NPs concentration, while B_{12} level may have modulated the translocation or interaction of iron within plant tissues.

- The Fe_3O_4 NPs 5 mg / L treatment group exhibited significantly enhanced morphological and physiological development compared to the 2.5 mg / L group, including increased accumulation of key nutrients (e.g., Fe, K, Mg, Ca, and P) in lettuce tissues. Notably, the combined treatment of Fe_3O_4 NPs 5 mg / L and B_{12} 50 mg / L resulted in the highest fresh and dry biomass at final harvest, highlighting the potential synergistic effect of optimised Fe and B_{12} application. These results support the use of Fe_3O_4 NPs at 5 mg / L as a more effective iron source for enhancing lettuce productivity and nutritional quality under combined micronutrient fortification.

- The highest B_{12} concentration (1000 mg / L) resulted in reduced growth and development in lettuce, with the Fe_3O_4 NPs 2.5 mg / L treatment combined with this B_{12} level showing notably lower fresh and dry biomass at final harvest. Furthermore, B_{12} 100 and 1000 mg / L treatment induced leaf marginal necrosis (browning), indicating potential phytotoxicity at elevated B_{12} doses. These findings indicate that while uptake and accumulation were dose-dependent and not limited even at high concentrations, B_{12} fortification should be applied at lower levels to avoid adverse morphological and physiological effects.
- Significantly higher micro- and macronutrient contents were recorded in the Fe_3O_4 NPs 5 mg / L treatment, accompanied by elevated total phenolic and total flavonoid content at B_{12} 100 and B_{12} 10 mg / L, respectively. This suggests the Fe_3O_4 NPs 5 mg / L may enhance iron bioavailability, potentially promoting synergistic nutrient interactions and improving plant stress resilience.
- The combined application Fe_3O_4 NPs and B_{12} in lettuce demonstrates a promising biofortification strategy to simultaneously enhance essential micronutrient content in food corps. This approach can improve dietary intake of both B_{12} and iron, addressing widespread deficiencies especially in population with limited access to B_{12} or iron supplementation. Moreover, nanoparticle-mediated delivery offers efficient nutrient uptake and potential cost-effective fortification, making it suitable for diverse agricultural system.

Chapter 6. Summary and Conclusion

In summary, the present study fortified vitamin B_{12} using cyanocobalamin and iron using iron oxide nanoparticles (Fe_3O_4 NPs) in lettuces grown in hydroponic/ aeroponic cultivation. This study determined the uptake, translocation, and accumulation of B_{12} and Fe, respectively in lettuce tissues and identified the effects of B_{12} and Fe_3O_4 NPs treatments individually and the combined treatments of B_{12} and Fe_3O_4 NPs in lettuce which were determined by analysing morphological, physiological, quality, and elemental traits from the lettuce tissues.

The results from **Chapter 3** demonstrated that B_{12} was successfully fortified into lettuce via both leaf and root application methods under hydroponic / aeroponic system. B_{12} treatments at varying concentrations (0, 2.5, 5, 7.5, and 10 mg / L) enhanced plant morphological and physiological traits compared to the untreated control. B_{12} treated lettuces showed increased chlorophyll content, improved Water Use Efficiency (*WUE*), and more efficient gas exchange. Specifically, leaf application at B_{12} 5 mg / L resulted in the improved growth and development, while root application at B_{12} 2.5 mg / L led to enhancements in morphological and physiological responses. However, no statistically significant differences were observed between the B_{12} treatment groups in terms of growth-related parameters. Elemental analysis revealed a dose-dependent increase in B_{12} content in both leaf and root tissues. Notably, lettuces treated with B_{12} 7.5 mg / L via root application accumulated the highest B_{12} content, followed by treatment of B_{12} 10 mg / L treatment in both leaf and root tissues. To assess cost efficiency, the total B_{12} used for both application methods was calculated. Given the high cost of research-grade cyanocobalamin (B_{12}), root application required approximately 100 times more B_{12} than leaf application. In practical agricultural settings, cost-effective B_{12} fortification for growers will likely depend on approaches such as leaf application (foliar spray) or microbial biofortification with optimised formulations [136], rather than direct application of pure B_{12} powder via root application.

Chapter 4 investigated the applicability of Fe_3O_4 NPs compared to the conventional iron fertiliser (Fe-EDTA). Initial results suggested that Fe_3O_4 NPs capped with potato starch impeded iron uptake in hydroponically grown lettuce, likely due to the formation of starch cluster from unbound potato starch, or potentially due to microbial contamination or biofilm formation on the nanoparticle surface under hydroponic conditions. To improve nanoparticle stability and uptake,

Fe_3O_4 NPs capped with L-lysine were used in subsequent experiments. Comparative analysis showed that lettuces treated with Fe_3O_4 NPs exhibited improved morphological and physiological development, increased biomass, and enhanced relative chlorophyll content, along with more efficient control regulation of stomatal activities. Iron accumulation was also significantly higher in both leaf and root tissues compared to the conventional Fe-EDTA treatment over the growth period, despite Fe-EDTA showing higher accumulation at the early stage (week 2). This suggests effective uptake and sustained translocation of Fe_3O_4 NPs through the xylem pathway as the plants developed. In particular, iron content in the leaves was markedly higher in the Fe_3O_4 NPs-treated group, supporting the hypothesis that their nanoscale size facilitates active root-to-shoot translocation via the xylem pathway. In addition to these nutritional and physiological benefits, the use of L-lysine as a capping agent may further contribute to enhanced plant performance, offering added value beyond nanoparticle stability. This study presents that the treatments of Fe_3O_4 NPs capped with L-lysine could offer a more cost-effective iron fortification strategy for growers, potentially reducing input costs while enhancing nutrient uptake and crop performance compared to the conventional iron fertiliser, Fe-EDTA.

Chapter 5. provides a contribution to the ongoing discourse in literature regarding the role of B_{12} and Fe_3O_4 NPs applications on lettuce growth and development. This has been achieved by investigating the effects of the combined applications of B_{12} and Fe_3O_4 NPs at different concentrations on lettuce morphology, physiology, biochemistry, and harvest quality. The current study compared the lettuce growth and development by the applications of Fe_3O_4 NPs treatments at 2.5 and 5 mg / L concentrations via root application along with the B_{12} treatments (0, 10, 50, 100, and 1000 mg / L) by leaf application. The results showed that Fe_3O_4 NPs 5 mg / L treatment enhanced the growth performance and nutrient uptake, resulting in the highest harvest yield compared to the 2.5 mg / L group. Notably, the combination of Fe_3O_4 NPs 5 mg / L and B_{12} 50 mg / L treatment showed significant increases in both fresh and dry biomass. For the B_{12} application, the study found that the highest concentration (100 to 1000 mg / L) impedes the lettuce growth and development, although these treatments resulted in relatively high contents of Fe, K, Mg, and P in root tissues. Overall, the Fe_3O_4 NPs treatment had a more pronounced influence on the uptake of micro- and macronutrients, as well as the accumulation of secondary metabolites (TPC, TFC, and TAA), compared to the effects observed from varying B_{12} concentrations. To conclude, the combined treatment of Fe_3O_4 NPs and B_{12} effectively fortify lettuce with micro- and

macronutrients as well as beneficial phytochemicals, promoting growth development with potential synergistic effect. However, since a B_{12} concentration of 10 mg / L meets or exceed the recommended daily intake for adults (2.4 µg per day) in both Fe_3O_4 NPs treatments (2.5 and 5 mg / L), using lower B_{12} treatments level is recommended for efficient fortification.

This research demonstrates that leaf application of B_{12} and root supplementation with Fe_3O_4 NPs are effective strategies for enhancing the nutritional quality and physiological performance of lettuce in controlled hydroponic systems. Foliar B12 application was more efficient and cost-effective than root application, requiring significantly less input to achieve meaningful fortification levels. Meanwhile, Fe_3O_4 NPs capped with L-lysine not only improved iron uptake and translocation due to their high bioavailability but also, support better overall plant growth and micronutrient accumulation compared to conventional Fe-EDTA. Furthermore, to achieve equivalent Fe concentrations, Fe_3O_4 NPs required significantly lower input amounts than Fe-EDTA highlighting their potential cost-efficiency.

For practical application, growers should consider that using foliar B_{12} spray at low concentrations (2.5 to 5 mg / L) to achieve effective fortification while avoiding excess input costs and growth inhibition observed at higher concentrations (100 and 1000 mg / L). The Fe_3O_4 NPs-mediated fertilisation offers promising potentials for sustainable and efficient micronutrient fortification in food crops, with the flexibility to tailor delivery strategies using various capping agents and nanoparticle formulations. The Fe_3O_4 NPs 5 mg / L treatment is recommended for hydroponically cultivated lettuce to promote enhanced growth and superior harvest quality.

Chapter 7. Future Work

In this thesis, vitamin B_{12} and iron has been fortified using cyanocobalamin (B_{12}) and iron oxide nanoparticles (Fe_3O_4 NPs) and determine their effect on lettuces morphology, physiology, and biochemistry traits. This has highlighted areas where additional studies will be beneficial to gain further insights and understanding of the working mechanisms of B_{12} and Fe_3O_4 NPs application on food crops.

The present study conducted multielement analysis using ICP-MS analysis to quantify the fortified elements such as B_{12} and Fe. This provided quantitative analysis of B_{12} and Fe by analysing different growth stage (week 2 and 4) within various tissues of the lettuce samples such as root and leaf tissues. However, additional microscopic analysis such SEM/EDS or TEM, along with isotope tracing techniques, would provide a more comprehensive understanding for the uptake, translocation, and accumulation of the B_{12} and Fe_3O_4 NPs within the plant structure [142,174,200,201].

Further study would be beneficial to evaluate active analogue of B_{12} form in food crops and to provide bioavailability and stability of B_{12} in lettuce for further human intake. Previously, the B_{12} bioavailability has been investigated via HPLC [31] or via bioassay using *E.coli* 215 bioautogram by LC/ESI-MS/MS analysis [69]. Additionally, further efforts should be made to determined Fe bioavailability and stability after plant uptake. By an in vitro digestion/Caco-2 cell model, iron bio-accessibility and bioavailability in plant could be investigated. This approach would provide further comparison between Fe_3O_4 NPs and conventional iron source such as Fe-EDTA. Additionally, further investigation is required to determine the relationship between B_{12} and iron as majority of the research quantified B_{12} [68,69,71] and identify their bioavailability [31].

This study is limited to the use of only two capping agents, potato starch and L-lysine, for Fe_3O_4 NPs. To address this limitation, further research should explore a broader range of capping agents to improve nanoparticle stability, enhance bioavailability, and ensure better plant compatibility. Potential alternatives include polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA), ethylene diamine tetra acetic acid (ETDA), chitosan, and plant-based extracts [202]. These capping agents are widely used in nanoparticle fortification due to their beneficial traits, such as improved stability and additional nutritional benefits. Additionally, it would be beneficial to explore coating technique for Fe_3O_4 NPs with

minerals or vitamin (e.g., B_{12}). Although B_{12} -coated Fe_3O_4 NPs were prepared in this study, their effectiveness was not fully evaluated during root application, possibly due to the low concentration of B_{12} coated on the nanoparticles. Future research should investigate optimising the coating concentration and application method to better assess the potential synergistic effects of combined nutrient fortification.

In the present study, the application was conducted via root and leaf application, respectively. It would be promising to investigate the effect of high concentration (e.g., 100, 500, and 1000 mg / L) via root application on lettuce. Additionally, the current study is mainly focusing on hydroponic/aeroponic cultivation. It would be also useful to investigate the optimal droplet size, flow rate, and nutrient solution conditions such as temperature and nanoparticle application in aeroponic systems [157]. It is required to identify optimal nanoparticle application to fortify micronutrients in closed soilless cultivation such as in hydroponic and aeroponic systems, especially in ultrasonic atomisation aeroponics. Also, efforts should be made for identifying adequate growing systems for food crops based on understanding of their genotypes and physiological characteristics.

Given that the present study was conducted in a controlled environment, it would be beneficial to evaluate the efficacy and safety of Fe_3O_4 NPs fortification under open field conditions and across multiple crop cycles to better assess its practical applicability in agriculture. Additionally, investigating the interactions between Fe_3O_4 NPs and soil microorganisms, as well as the potential environmental impacts of Fe_3O_4 NPs application, would provide valuable insights for sustainable nanoparticle-mediated fertiliser use. Future studies should explore the optimised foliar application of Fe_3O_4 NPs, focusing on parameters such as spray timing, particle stability, and compatibility with nutrient solutions for improved micronutrient delivery.

Lastly, the present study evaluated the effect of B_{12} and Fe_3O_4 NPs treatment on lettuce growth and development, with Fe_3O_4 NPs showing significant improvement in plant health and quality. To gain a deeper understanding of the underlying mechanisms, future studies would beneficial to focus on molecular level research. For instance, Tombuloglu et al. (2024) investigated the effect of Fe_3O_4 NPs on barley by analysing photosynthetic marker genes using RT-qPCR along with the pigment measurements (e.g., chlorophyll a, b, and carotenoids) [140,142]. These gene expression studies provide insights into the ways Fe_3O_4 NPs regulate key processes such as photosynthesis and nutrient uptake. This helps clarify the

molecular pathways of nanoparticle uptake, translocation and accumulation, as well as their regulatory impact on photosynthetic efficiecny and overall plant performance. Understanding these mechanisms can guide the optimisation of nanoparticle-based fertilisers for enhanced crop productivity and nurtional quality.

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Appendices

Appendix A - Chapter 3. Vitamin B_{12} Fortification

3.1.3.1 B_{12} leaf application: growth and morphological measurement by Planteye

Height (cm)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	6.92	0.83	8.58	0.90	10.44	1.20	15.26	1.71
2.5 mg / L	7.39	1.45	9.04	1.48	10.28	0.76	15.11	0.70
5 mg / L	5.76	1.05	7.68	1.27	8.00	1.01	13.13	1.69
7.5 mg / L	6.76	1.03	9.28	1.15	9.80	0.79	14.86	1.65
10 mg / L	5.96	1.00	8.39	1.40	9.49	1.61	14.49	1.41

	Week 1	Week 2	Week 3	Week 4
Min.	4.00	5.92	6.47	10.28
Max.	9.78	11.74	12.77	17.50

Leaf area (cm^2)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	99	34	345	90	677	71	768	95
2.5 mg / L	111	29	356	110	603	42	619	137
5 mg / L	105	27	356	77	658	79	771	90
7.5 mg / L	102	31	339	75	613	36	708	88
10 mg / L	114	33	356	100	561	95	577	141

	Week 1	Week 2	Week 3	Week 4
Min.	55	201	453	388
Max.	179	565	765	945

Digital biomass (cm^3)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	698	301	2968	836	7093	1270	11637	1353
2.5 mg / L	842	375	3262	1151	6208	778	9357	2111
5 mg / L	619	231	2736	710	5288	1040	10063	1232
7.5 mg / L	709	271	3105	595	6010	585	10584	2060
10 mg / L	680	231	2965	879	5245	715	8312	2027

	Week 1	Week 2	Week 3	Week 4
Min.	302	1289	3221	6099
Max.	1678	4760	9313	13896

Hue								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	97.18	2.70	99.39	1.95	100.30	2.64	101.74	1.91
2.5 mg / L	97.02	3.11	99.48	1.70	101.67	2.37	101.38	1.75
5 mg / L	97.25	3.36	100.68	2.11	101.73	2.67	103.16	2.44

7.5 mg / L	97.98	3.16	100.92	2.08	102.70	2.25	102.51	2.06
10 mg / L	98.34	3.60	101.07	2.50	101.33	2.80	101.45	3.46

	Week 1	Week 2	Week 3	Week 4
Min.	93.18	96.77	96.67	96.53
Max.	102.54	104.65	105.63	106.29

Greenness

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.42	0.02	0.56	0.01	0.46	0.01	0.51	0.01
2.5 mg / L	0.41	0.01	0.54	0.02	0.47	0.01	0.50	0.02
5 mg / L	0.41	0.01	0.55	0.01	0.46	0.01	0.49	0.03
7.5 mg / L	0.41	0.01	0.56	0.01	0.46	0.01	0.47	0.01
10 mg / L	0.41	0.01	0.56	0.01	0.46	0.01	0.48	0.02

	Week 1	Week 2	Week 3	Week 4
Min.	0.38	0.50	0.43	0.45
Max.	0.44	0.58	0.48	0.53

NDVI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.54	0.03	0.70	0.03	0.66	0.02	0.71	0.02
2.5 mg / L	0.52	0.02	0.68	0.04	0.67	0.02	0.70	0.03
5 mg / L	0.53	0.02	0.71	0.01	0.68	0.01	0.72	0.03
7.5 mg / L	0.53	0.02	0.71	0.03	0.67	0.00	0.70	0.02
10 mg / L	0.55	0.03	0.72	0.02	0.67	0.02	0.70	0.03

	Week 1	Week 2	Week 3	Week 4
Min.	0.48	0.59	0.63	0.64
Max.	0.58	0.75	0.70	0.75

NPCI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.36	0.03	0.57	0.05	0.37	0.05	0.40	0.04
2.5 mg / L	0.35	0.05	0.53	0.05	0.35	0.04	0.41	0.04
5 mg / L	0.33	0.05	0.51	0.06	0.34	0.05	0.35	0.05
7.5 mg / L	0.32	0.04	0.53	0.05	0.32	0.04	0.34	0.04
10 mg / L	0.32	0.05	0.52	0.06	0.34	0.05	0.37	0.06

	1st week	2nd week	3rd week	4th week
Min.	0.25	0.44	0.26	0.26
Max.	0.40	0.66	0.44	0.46

PSRI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.15	0.02	0.12	0.01	0.10	0.01	0.09	0.01
2.5 mg / L	0.16	0.02	0.12	0.02	0.09	0.01	0.09	0.01
5 mg / L	0.15	0.03	0.11	0.01	0.09	0.01	0.08	0.01
7.5 mg / L	0.15	0.02	0.11	0.02	0.09	0.01	0.09	0.01
10 mg / L	0.14	0.03	0.10	0.01	0.09	0.01	0.09	0.02

	Week 1	Week 2	Week 3	Week 4
Min.	0.11	0.08	0.07	0.06
Max.	0.19	0.16	0.12	0.12

3.1.3.2 B_{12} leaf application: water management and gas exchange including WUE, iWUE and Ls

Water use efficiency (WUE, A/E) at week 2						
Vitamin B_{12} (mg / L)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	1456.34	168.85	2376.23	248.46	2362.08	280.90
2.5 mg / L	1891.44	254.95	3245.72	450.00	3124.48	487.22
5 mg / L	2262.90	43.22	4313.65	271.64	5086.01	567.12
7.5 mg / L	2593.37	322.16	4487.83	529.72	5369.89	632.67
10 mg / L	2373.85	317.31	4607.56	562.76	5661.99	883.45
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	1232.013		1930.195		1796.606	
Max.	3289.918		5510.276		7222.885	
Water use efficiency (WUE, A/E) at week 4						
Vitamin B_{12} (mg / L)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	2474.46	497.17	4089.25	492.40	4198.78	797.88
2.5 mg / L	3040.88	470.64	4615.63	396.00	4363.35	663.12
5 mg / L	3286.49	603.93	5354.19	417.41	5970.53	509.84
7.5 mg / L	2664.45	858.28	4373.13	1071.56	5080.20	1059.29
10 mg / L	2923.14	806.48	4443.55	1282.95	4403.86	1390.55
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	1256.166		2267.241		2678.752	
Max.	4621.546		5982.543		6828.501	
Intrinsic water use efficiency (iWUE, A/gsw) at week 2						
Vitamin B_{12} (mg / L)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	17.81	4.88	32.38	7.28	43.16	8.54
2.5 mg / L	12.96	2.82	26.17	4.49	39.22	5.55
5 mg / L	11.73	1.30	27.00	2.32	58.65	6.91
7.5 mg / L	14.60	4.83	31.97	8.76	72.00	11.43
10 mg / L	11.16	3.63	29.06	8.05	71.74	20.39
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	5.28		17.73		32.31	
Max.	25.87		52.48		108.12	
Intrinsic water use efficiency (iWUE, A/gsw) at week 4						
Vitamin B_{12} (mg / L)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	24.38	13.40	45.17	18.45	67.01	16.93
2.5 mg / L	22.77	7.20	39.88	10.48	58.28	7.95
5 mg / L	23.99	13.33	45.63	19.67	83.57	24.23
7.5 mg / L	18.51	10.58	40.34	19.28	82.65	37.39
10 mg / L	22.04	8.01	40.92	14.66	67.04	15.83
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	4.29		13.08		44.66	
Max.	53.07		83.38		157.94	
Stomatal limitation (L_s, Ci/Ca) at week 2						
Vitamin B_{12} (mg / L)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	0.11	0.02	0.19	0.03	0.25	0.04
2.5 mg / L	0.09	0.01	0.16	0.02	0.23	0.02
5 mg / L	0.08	0.01	0.16	0.01	0.32	0.03
7.5 mg / L	0.09	0.02	0.18	0.04	0.36	0.05

10 mg / L	0.07	0.02	0.17	0.04	0.37	0.09
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	0.05		0.12		0.20	
Max.	0.15		0.26		0.52	
Stomatal limitation (L_s, Ci/Ca) at week 4						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.13	0.06	0.23	0.07	0.34	0.06
2.5 mg / L	0.12	0.03	0.21	0.04	0.30	0.03
5 mg / L	0.13	0.05	0.24	0.07	0.41	0.09
7.5 mg / L	0.10	0.05	0.20	0.08	0.39	0.15
10 mg / L	0.12	0.04	0.21	0.06	0.33	0.07
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	0.04		0.10		0.24	
Max.	0.25		0.37		0.70	

3.1.3.3 B_{12} leaf application: relative chlorophyll measurement by SPAD meter

Vitamin B_{12} (mg / L)	SPAD at Week 2		SPAD at Week 4	
	mean	SD	mean	SD
0 mg / L	21.1	1.5	23.4	1.7
2.5 mg / L	20.3	1.4	22.7	2.3
5 mg / L	22.4	2.3	25.8	2.0
7.5 mg / L	21.1	1.1	24.5	1.3
10 mg / L	21.7	2.1	26.4	2.0
	Week 2		Week 4	
Min.	18.3		18.4	
Max.	27.4		30.3	

3.1.3.4 B_{12} leaf application: Harvest quality (fresh weight)

Vitamin B_{12} (mg / L)	F.W (g) at week 2				F.W (g) at week 4			
	Leaf		Root		Leaf		Root	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	35.12	14.53	6.32	2.66	208.24	43.20	21.65	4.35
2.5 mg / L	39.05	9.52	6.60	1.95	198.57	32.25	19.53	2.93
5 mg / L	45.98	9.17	9.00	1.91	240.14	25.59	26.26	3.28
7.5 mg / L	42.18	10.19	8.06	2.50	223.65	26.12	24.72	2.53
10 mg / L	61.83	15.80	11.77	3.18	229.08	34.04	26.65	4.18
	F.W Leaf at week 2		F.W Root at week 2		F.W Leaf at week 4		F.W Root at week 4	
Min.	20.54		2.79		145.13		14.54	
Max.	83.42		16.38		291.44		35.14	

3.1.3.5 B_{12} leaf application: Vitamin B_{12} content in lettuce tissues

Vitamin B_{12} (mg / L)	B_{12} content at week 2				B_{12} content at week 4			
	Leaf (mg / g)		Root (mg / g)		Leaf (mg / g)		Root (mg / g)	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.004	0.001	0.007	0.002	0.004	0.004	0.003	0.002
2.5 mg / L	0.005	0.002	0.008	0.002	0.005	0.002	0.005	0.001
5 mg / L	0.005	0.002	0.007	0.001	0.007	0.005	0.005	0.003
7.5 mg / L	0.007	0.002	0.007	0.003	0.007	0.002	0.005	0.002
10 mg / L	0.008	0.003	0.012	0.003	0.010	0.003	0.009	0.003
	B_{12} in leaf at week 2		B_{12} in root at week 2		B_{12} in leaf at week 4		B_{12} in root at week 4	
Min.	0.002		0.004		0.0000		0.001	
Max.	0.011		0.017		0.019		0.014	

3.2 Vitamin B12 fortification - Root application

3.2.3.1 B_{12} root application: growth and morphological measurement

Height (cm)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	6.50	0.56	9.56	0.92	13.29	1.24	16.00	0.81
2.5 mg / L	6.61	0.82	10.06	1.28	13.74	1.37	17.45	1.23
5 mg / L	6.59	0.63	10.63	1.50	13.19	1.38	17.65	0.66
7.5 mg / L	7.45	0.44	10.82	1.05	13.10	0.41	16.10	1.29
10 mg / L	5.89	0.96	8.89	0.69	13.21	1.11	16.26	1.04
	Week 1		Week 2		Week 3		Week 4	
Min.	4.73		7.92		11.20		14.20	
Max.	8.05		13.06		15.43		19.74	

Leaf area (cm^2)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	170	26	454	67	790	39	905	86
2.5 mg / L	191	45	495	61	885	110	984	112
5 mg / L	160	51	372	63	794	90	898	89
7.5 mg / L	168	42	416	84	829	114	891	43
10 mg / L	158	27	421	79	771	75	800	100
	Week 1		Week 2		Week 3		Week 4	
Min.	108		262		657		693	
Max.	267		585		1014		1210	

Digital biomass (cm^3)								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	1117	235	4343	786	10515	1198	14496	1701
2.5 mg / L	1269	346	4983	872	12137	1711	17158	2066
5 mg / L	1071	427	3986	1080	10573	2192	15871	1985
7.5 mg / L	1249	309	4500	948	10855	1503	14371	1709
10 mg / L	941	240	3747	770	10142	905	12973	1446
	Week 1		Week 2		Week 3		Week 4	
Min.	512		2234		7654		10918	
Max.	1886		6200		13618		20565	

Hue								
Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	97.62	1.59	100.86	1.19	102.72	1.86	101.00	1.31
2.5 mg / L	99.59	1.11	101.77	0.90	102.27	1.60	101.48	1.86
5 mg / L	99.24	1.54	101.75	1.33	102.21	1.80	101.04	0.93
7.5 mg / L	99.85	1.84	101.38	1.96	101.56	1.76	100.65	1.34
10 mg / L	99.18	1.83	102.30	1.80	102.03	2.28	100.33	1.61
	Week 1		Week 2		Week 3		Week 4	
Min.	95.45		98.72		99.09		98.46	
Max.	102.10		104.15		105.19		104.55	

Greenness				
Vitamin B_{12}	Week 1	Week 2	Week 3	Week 4

(mg / L)	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.44	0.01	0.42	0.01	0.46	0.01	0.42	0.01
2.5 mg / L	0.44	0.01	0.42	0.01	0.46	0.01	0.46	0.00
5 mg / L	0.44	0.01	0.44	0.01	0.45	0.01	0.45	0.01
7.5 mg / L	0.45	0.02	0.44	0.02	0.43	0.01	0.43	0.01
10 mg / L	0.44	0.01	0.43	0.01	0.45	0.01	0.44	0.01

	Week 1	Week 2	Week 3	Week 4
Min.	0.42	0.40	0.42	0.42
Max.	0.47	0.46	0.48	0.46

NDVI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.59	0.02	0.62	0.01	0.66	0.02	0.64	0.01
2.5 mg / L	0.60	0.01	0.63	0.01	0.66	0.01	0.66	0.01
5 mg / L	0.60	0.02	0.65	0.01	0.65	0.02	0.66	0.01
7.5 mg / L	0.60	0.03	0.64	0.03	0.63	0.02	0.63	0.02
10 mg / L	0.60	0.02	0.65	0.02	0.65	0.01	0.64	0.01

	Week 1	Week 2	Week 3	Week 4
Min.	0.56	0.58	0.61	0.60
Max.	0.64	0.67	0.69	0.67

NPCI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.38	0.02	0.29	0.02	0.31	0.03	0.30	0.02
2.5 mg / L	0.34	0.01	0.28	0.01	0.32	0.03	0.33	0.03
5 mg / L	0.36	0.03	0.30	0.03	0.31	0.03	0.33	0.02
7.5 mg / L	0.36	0.04	0.31	0.03	0.30	0.04	0.32	0.03
10 mg / L	0.36	0.03	0.28	0.03	0.30	0.04	0.33	0.03

	Week 1	Week 2	Week 3	Week 4
Min.	0.32	0.25	0.25	0.27
Max.	0.42	0.34	0.36	0.38

PSRI

Vitamin B_{12} (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.14	0.01	0.10	0.01	0.09	0.01	0.10	0.01
2.5 mg / L	0.12	0.01	0.09	0.00	0.09	0.01	0.10	0.01
5 mg / L	0.12	0.02	0.10	0.01	0.09	0.01	0.10	0.01
7.5 mg / L	0.12	0.02	0.10	0.01	0.10	0.01	0.10	0.01
10 mg / L	0.12	0.02	0.09	0.01	0.09	0.01	0.10	0.01

	Week 1	Week 2	Week 3	Week 4
Min.	0.10	0.08	0.08	0.08
Max.	0.15	0.12	0.11	0.12

3.2.3.2 B_{12} root application: Water management and gas exchange including WUE, iWUE and Ls

Water use efficiency (WUE, A/E) at week 2						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	1655.18	268.37	2739.04	190.13	2628.14	59.43
2.5 mg / L	1448.53	196.65	2491.74	272.33	2522.87	194.15
5 mg / L	1598.46	164.10	2618.31	245.96	2564.61	211.96
7.5 mg / L	1369.68	64.72	2247.45	270.70	2307.34	332.01
10 mg / L	1377.07	101.33	2411.94	263.47	2493.92	278.99
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	1216.82		1880.83		1867.74	
Max.	2129.81		3017.30		2890.46	
Water use efficiency (WUE, A/E) at week 4						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	1908.64	438.55	2782.49	339.33	2595.20	213.09
2.5 mg / L	1834.30	346.33	2822.97	459.29	2696.56	333.20
5 mg / L	2362.09	321.84	3175.19	493.13	2778.35	363.59
7.5 mg / L	2211.81	516.78	3150.40	425.75	2798.75	227.27
10 mg / L	1858.41	484.12	2805.18	457.42	2673.23	280.96
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	1373.93		2216.83		2100.05	
Max.	3048.21		3946.45		3214.69	
Intrinsic water use efficiency (iWUE, A/gsw) at week 2						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	24.95	9.99	45.14	12.78	55.39	10.01
2.5 mg / L	19.02	5.65	35.03	9.22	43.97	8.82
5 mg / L	23.10	5.93	41.50	8.83	50.60	7.86
7.5 mg / L	16.18	1.93	28.76	3.89	37.95	6.15
10 mg / L	14.85	2.09	28.27	4.64	37.81	5.05
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	11.42		22.67		31.16	
Max.	43.51		66.88		71.16	
Intrinsic water use efficiency (iWUE, A/gsw) at week 4						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	33.61	14.67	52.83	18.67	62.71	18.73
2.5 mg / L	30.60	9.76	51.37	13.94	60.51	11.06
5 mg / L	49.59	11.56	73.55	17.70	77.42	17.47
7.5 mg / L	44.34	17.42	68.78	18.32	73.60	8.22
10 mg / L	34.94	16.35	55.34	17.94	63.17	12.77
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	19.44		34.55		42.60	
Max.	72.80		100.04		97.91	
Stomatal limitation (L_s, Ci/Ca) at week 2						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.15	0.04	0.25	0.05	0.30	0.04

2.5 mg / L	0.12	0.03	0.21	0.04	0.26	0.04
5 mg / L	0.14	0.03	0.23	0.04	0.28	0.03
7.5 mg / L	0.11	0.01	0.18	0.02	0.23	0.03
10 mg / L	0.10	0.01	0.18	0.02	0.23	0.03
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	0.09		0.15		0.20	
Max.	0.23		0.33		0.36	
Stomatal limitation (L_s, Ci/Ca) at week 4						
Vitamin B_{12} (mg / L)	**200 µmol m⁻² s⁻¹**		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.18	0.06	0.27	0.07	0.33	0.07
2.5 mg / L	0.17	0.04	0.27	0.06	0.32	0.05
5 mg / L	0.25	0.05	0.36	0.07	0.38	0.07
7.5 mg / L	0.23	0.07	0.34	0.07	0.37	0.03
10 mg / L	0.19	0.07	0.28	0.07	0.33	0.05
	200 µmol m⁻² s⁻¹		**500 µmol m⁻² s⁻¹**		**1800 µmol m⁻² s⁻¹**	
Min.	0.12		0.20		0.25	
Max.	0.34		0.46		0.45	

3.2.3.3 B_{12} root application: relative chlorophyll measurement by SPAD meter

Vitamin B_{12} (mg / L)	SPAD at week 2		SPAD at week 4	
	mean	SD	mean	SD
0 mg / L	19.0	1.2	20.7	1.7
2.5 mg / L	18.2	1.7	20.8	1.8
5 mg / L	19.7	1.8	20.4	2.3
7.5 mg / L	19.3	1.6	20.2	2.0
10 mg / L	19.8	1.7	21.3	1.9
	2^{nd} week		4^{th} week	
Min.	14.6		16.5	
Max.	22.4		25.1	

3.2.3.4 B_{12} root application: harvest quality (fresh weight)

Vitamin B_{12} (mg / L)	F.W (g) at week 2				F.W (g) at week 4			
	Leaf		Root		Leaf		Root	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	17.86	2.33	2.38	0.33	162.84	8.32	18.99	1.29
2.5 mg / L	20.05	5.78	2.86	1.00	172.75	7.43	20.90	2.14
5 mg / L	14.40	1.79	1.88	0.28	159.81	12.78	17.83	1.77
7.5 mg / L	14.64	2.99	1.97	0.36	165.91	16.20	19.86	2.78
10 mg / L	16.44	5.28	2.47	1.03	170.49	18.08	20.58	3.47
	F.W Leaf at week 2		F.W Root at week 2		F.W Leaf at week 4		F.W Root at week 4	
Min.	9.47		1.42		142.08		15.06	
Max.	30.76		4.56		202.68		25.75	

3.2.3.5 B_{12} root application: Vitamin B_{12} content in lettuce tissues

Vitamin B_{12} (mg / L)	B_{12} in lettuce at week 2				B_{12} in lettuce at week 4			
	Leaf (mg / g)		Root (mg / g)		Leaf (mg / g)		Root (mg / g)	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.001	0.000	0.002	0.000	0.001	0.000	0.002	0.001
2.5 mg / L	0.006	0.002	0.013	0.002	0.010	0.002	0.060	0.026
5 mg / L	0.013	0.008	0.017	0.012	0.017	0.009	0.100	0.019
7.5 mg / L	0.006	0.004	0.040	0.008	0.028	0.007	0.141	0.025
10 mg / L	0.018	0.012	0.051	0.020	0.023	0.003	0.118	0.032
	B_{12} in leaf at week 2		B_{12} in root at week 2		B_{12} in leaf at week 4		B_{12} in root at week 4	
Min.	0.001		0.002		0.001		0.002	
Max.	0.018		0.051		0.028		0.141	

3.2.3.6 B_{12} root vs. leaf application: secondary metabolites (TPC, TFC, and TAA)

Vitamin B_{12} (method & mg / L)	TPC (mg GAE / g F.W)			TFC (mg RE / g F.W)			TAA (mg TEAC / g F.W)		
	mean	SE	SD	mean	SE	SD	mean	SE	SD
B_{12}_R_0	0.174	0.010	0.043	0.092	0.004	0.017	4.315	0.047	0.200
B_{12}_R_2.5	0.181	0.011	0.045	0.140	0.009	0.038	4.360	0.077	0.328
B_{12}_R_5	0.209	0.012	0.050	0.105	0.002	0.010	4.543	0.023	0.097
B_{12}_R_7.5	0.219	0.012	0.050	0.080	0.003	0.011	4.486	0.054	0.230
B_{12}_R_10	0.228	0.032	0.137	0.080	0.004	0.016	4.349	0.043	0.181
B_{12}_L_0	0.432	0.013	0.055	0.123	0.005	0.022	4.277	0.045	0.193
B_{12}_L_2.5	0.435	0.012	0.050	0.100	0.002	0.008	4.363	0.079	0.334
B_{12}_L_5	0.460	0.018	0.075	0.100	0.004	0.016	4.545	0.042	0.179
B_{12}_L_7.5	0.416	0.012	0.053	0.074	0.003	0.011	4.455	0.058	0.244
B_{12}_L_10	0.431	0.023	0.099	0.070	0.002	0.009	4.340	0.048	0.202

	TPC (mg GAE / g F.W)	TFC (mg RE / g F.W)	TAA (mg TEAC / g F.W)
Min.	0.083	0.054	3.781
Max.	0.654	0.176	4.795

Appendix B - Chapter 4. Iron Fortification Using Fe_3O_4 NPs

4.2.3.1 Growth and development measurement

4.2.3.1 Descriptive statistic - Growth and morphological measurement by Planteye

Height (cm)								
Fe_3O_4 NPs (concentration)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	4.645	0.674	8.944	0.614	9.936	0.842	13.435	0.835
0.625 mg / L	3.569	0.505	8.144	0.713	7.289	1.407	7.974	2.097
1.25 mg / L	4.790	0.533	8.615	1.247	10.009	0.981	12.183	0.684
2.5 mg / L	2.793	0.799	7.076	0.696	8.708	0.551	12.658	1.757
5 mg / L	3.594	1.055	8.947	1.205	9.114	1.610	13.103	0.824
	Week 1		Week 2		Week 3		Week 4	
Min.	1.925		6.187		4.515		5.093	
Max.	5.645		10.719		11.664		14.918	
Leaf area (cm^2)								
Fe_3O_4 NPs (concentration)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	105.85	20.73	301.82	32.82	412.86	61.90	376.20	98.07
0.625 mg / L	55.89	18.95	157.30	30.89	224.26	57.57	311.72	71.12
1.25 mg / L	79.08	15.53	182.70	56.42	405.77	118.80	476.83	133.94
2.5 mg / L	40.50	11.66	158.38	88.12	284.94	81.66	436.10	132.16
5 mg / L	65.19	25.83	244.04	58.71	330.67	94.32	422.58	64.95
	1st week		2nd week		3rd week		4th week	
Min.	2133		7813		16699		19742	
Max.	13401		37814		55927		65456	
Digital biomass (cm^3)								
Fe_3O_4 NPs (concentration)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	483.08	71.46	2701.95	377.59	4083.43	576.18	5053.75	1331.75
0.625 mg / L	204.02	79.32	1295.71	354.64	1688.72	714.63	2555.09	1046.09
1.25 mg / L	377.67	83.35	1553.49	431.97	4106.97	1358.99	5845.97	1828.12
2.5 mg / L	118.09	62.34	1127.26	640.63	2504.18	838.08	5600.71	2229.70
5 mg / L	250.91	164.78	2191.52	640.35	3015.53	967.06	5528.82	824.15
	Week 1		Week 2		Week 3		Week 4	
Min.	50515		522821		764183		1005450	
Max.	612302		3425620		5315440		9416890	
Hue								
Fe_3O_4 NPs (concentration)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	99.948	2.020	102.593	1.225	102.897	1.906	94.406	5.254
0.625 mg / L	86.956	4.954	81.271	6.154	71.115	4.228	67.743	3.600
1.25 mg / L	97.430	1.947	89.390	5.534	87.462	8.449	85.665	7.917
2.5 mg / L	80.858	3.390	85.911	3.084	76.075	2.952	75.089	5.914
5 mg / L	91.936	4.227	95.032	2.661	90.231	3.785	92.331	3.344
	Week 1		Week 2		Week 3		Week 4	

Min.	75.80		71.33		63.98		63.32	
Max.	102.71		105.16		105.50		101.46	
Greenness								
Fe_3O_4 NPs (concentration)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.426	0.017	0.446	0.025	0.449	0.023	0.401	0.031
0.625 mg / L	0.362	0.031	0.336	0.050	0.254	0.041	0.212	0.031
1.25 mg / L	0.433	0.019	0.421	0.067	0.376	0.061	0.353	0.055
2.5 mg / L	0.346	0.025	0.407	0.029	0.318	0.026	0.280	0.041
5 mg / L	0.420	0.035	0.474	0.016	0.428	0.027	0.393	0.022
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.3170		0.2619		0.1796		0.1674	
Max.	0.4638		0.4962		0.4777		0.4288	
NDVI								
Fe_3O_4 NPs (concentration)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.634	0.020	0.702	0.017	0.724	0.014	0.653	0.030
0.625 mg / L	0.418	0.074	0.362	0.091	0.231	0.058	0.182	0.048
1.25 mg / L	0.600	0.017	0.495	0.096	0.457	0.122	0.431	0.112
2.5 mg / L	0.342	0.045	0.448	0.059	0.316	0.043	0.279	0.065
5 mg / L	0.511	0.093	0.607	0.045	0.529	0.063	0.533	0.055
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.2837		0.2168		0.1366		0.1105	
Max.	0.6629		0.7300		0.7420		0.7006	
NPCI								
Fe_3O_4 NPs (concentration)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.314	0.047	0.302	0.048	0.302	0.053	0.357	0.040
0.625 mg / L	0.432	0.038	0.474	0.025	0.461	0.038	0.403	0.031
1.25 mg / L	0.369	0.056	0.501	0.051	0.442	0.050	0.428	0.037
2.5 mg / L	0.510	0.024	0.546	0.028	0.531	0.026	0.450	0.032
5 mg / L	0.446	0.031	0.495	0.046	0.499	0.028	0.390	0.035
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.2581		0.2401		0.2495		0.2844	
Max.	0.5474		0.5866		0.5592		0.4894	
PSRI								
Fe_3O_4 NPs (concentration)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.102	0.010	0.075	0.008	0.068	0.009	0.111	0.021
0.625 mg / L	0.248	0.056	0.304	0.067	0.390	0.029	0.391	0.027
1.25 mg / L	0.130	0.017	0.222	0.052	0.234	0.087	0.243	0.077
2.5 mg / L	0.331	0.038	0.268	0.041	0.360	0.032	0.354	0.053
5 mg / L	0.199	0.057	0.157	0.030	0.205	0.043	0.177	0.038
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.08843		0.06416		0.05573		0.08179	
Max.	0.38897		0.41793		0.41960		0.43973	

4.2.3.2 Water management and gas exchange

4.2.3.2 Water management and gas exchange of WUE, iWUE and Ls

Water use efficiency (WUE, A/E) at week 1						
Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	1360.06	243.00	2719.74	266.98	2957.60	304.65
0.625 mg / L	1225.67	402.94	2502.66	534.70	2665.21	562.88
1.25 mg / L	1409.34	327.05	2849.45	527.21	2968.82	433.63
2.5 mg / L	1479.04	411.01	2880.92	579.41	3132.58	444.10
5 mg / L	1443.35	209.67	2776.52	226.75	3100.76	270.35
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	685.3		1575		1648	
Max.	1973.5		3671		3836	
Water use efficiency (WUE, A/E) at week 2						
Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	1493.82	184.72	3066.06	363.99	3361.73	362.31
0.625 mg / L	1725.05	637.05	3443.90	844.04	3525.63	601.34
1.25 mg / L	2486.09	527.20	4077.47	1509.46	4132.98	851.39
2.5 mg / L	1933.53	676.31	3735.02	1028.99	3662.89	977.82
5 mg / L	3365.71	276.54	5552.55	538.59	4922.50	566.21
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	755.3		726.6		2301	
Max.	3755.4		6099.6		5475	
Water use efficiency (WUE, A/E) at week 3						
Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	1649.04	393.94	2904.72	371.44	2921.54	244.90
0.625 mg / L	543.50	186.48	1172.78	535.07	1265.51	613.31
1.25 mg / L	1572.01	640.45	2877.61	822.67	2942.49	686.24
2.5 mg / L	1083.19	447.40	2010.30	559.58	2066.08	539.07
5 mg / L	1272.77	486.39	2738.05	819.56	2808.75	679.96
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	234		574.9		633.9	
Max.	2685		3960.8		3873.3	
Water use efficiency (WUE, A/E) at week 4						
Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	1434.40	482.16	2421.57	798.63	2747.75	509.42
0.625 mg / L	603.95	252.44	1440.55	428.13	1740.94	465.05
1.25 mg / L	1949.13	741.71	2837.78	616.54	2639.07	887.53
2.5 mg / L	727.89	566.62	1405.61	934.36	1479.05	861.12
5 mg / L	1960.53	440.16	2868.11	682.14	2797.10	729.08
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	126.6		460.8		653.6	
Max.	2936.0		4039.6		3998.1	
Intrinsic water use efficiency (iWUE, A/gsw) at week 1						
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	

Fe_3O_4 NPs (concentration)	mean	SD	mean	SD	mean	SD
0 mg / L	21.35	7.04	48.54	10.39	68.48	10.46
0.625 mg / L	22.52	7.55	51.39	12.31	64.69	15.54
1.25 mg / L	25.81	7.94	60.55	14.00	77.34	13.92
2.5 mg / L	34.26	11.28	72.69	15.97	92.41	13.45
5 mg / L	24.43	3.88	53.09	6.67	73.62	9.82
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	14.228		36.481		46.056	
Max.	51.611		90.491		113.369	

Intrinsic water use efficiency (iWUE, A/gsw) at week 2

Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	21.93	5.17	50.28	12.18	69.27	15.33
0.625 mg / L	25.81	10.13	56.47	13.95	70.10	13.05
1.25 mg / L	29.36	7.44	54.17	22.02	70.91	19.56
2.5 mg / L	21.90	8.15	48.58	15.58	63.29	21.45
5 mg / L	40.58	4.73	75.68	8.10	89.61	11.96
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	9.685		9.509		38.557	
Max.	46.701		88.118		108.645	

Intrinsic water use efficiency (iWUE, A/gsw) at week 3

Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	27.37	10.26	54.26	13.33	71.78	11.88
0.625 mg / L	8.59	4.24	20.99	14.01	28.05	18.37
1.25 mg / L	28.06	14.64	56.70	21.57	71.31	21.11
2.5 mg / L	20.51	10.93	41.53	13.88	50.25	10.52
5 mg / L	18.96	8.30	45.27	15.11	57.78	17.41
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	3.981		10.512		14.228	
Max.	57.189		92.201		103.366	

Intrinsic water use efficiency (iWUE, A/gsw) at week 4

Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	27.06	13.09	49.73	20.63	68.55	19.23
0.625 mg / L	11.50	6.59	29.62	12.14	42.88	14.94
1.25 mg / L	43.13	18.92	67.34	19.12	75.53	28.21
2.5 mg / L	15.74	13.90	32.87	24.90	40.38	26.76
5 mg / L	38.98	10.79	63.28	18.50	76.74	23.42
	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	2.626		10.876		18.330	
Max.	70.023		88.949		111.020	

Stomatal limitation (L_s, Ci/Ca) at week 1

Fe_3O_4 NPs (concentration)	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
	mean	SD	mean	SD	mean	SD
0 mg / L	0.13	0.03	0.26	0.04	0.36	0.04
0.625 mg / L	0.13	0.03	0.26	0.05	0.33	0.07
1.25 mg / L	0.15	0.04	0.31	0.06	0.39	0.06
2.5 mg / L	0.18	0.05	0.35	0.07	0.44	0.05
5 mg / L	0.14	0.02	0.28	0.03	0.38	0.04

	200 µmol m^{-2} s^{-1}		500 µmol m^{-2} s^{-1}		1800 µmol m^{-2} s^{-1}	
Min.	0.096		0.202		0.240	
Max.	0.254		0.418		0.517	
Stomatal limitation (L_s, Ci/Ca) at week 2						
Fe_3O_4 NPs (concentration)	**200 µmol m^{-2} s^{-1}**		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.13	0.02	0.27	0.05	0.36	0.06
0.625 mg / L	0.14	0.04	0.28	0.06	0.35	0.06
1.25 mg / L	0.15	0.03	0.26	0.10	0.35	0.08
2.5 mg / L	0.12	0.04	0.24	0.07	0.31	0.09
5 mg / L	0.20	0.02	0.36	0.03	0.43	0.05
	200 µmol m^{-2} s^{-1}		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
Min.	0.063		0.065		0.198	
Max.	0.227		0.415		0.514	
Stomatal limitation (L_s, Ci/Ca) at week 3						
Fe_3O_4 NPs (concentration)	**200 µmol m^{-2} s^{-1}**		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.15	0.04	0.28	0.05	0.37	0.04
0.625 mg / L	0.07	0.02	0.13	0.06	0.16	0.08
1.25 mg / L	0.16	0.06	0.29	0.09	0.36	0.09
2.5 mg / L	0.12	0.05	0.22	0.06	0.26	0.05
5 mg / L	0.12	0.04	0.24	0.07	0.31	0.08
	200 µmol m^{-2} s^{-1}		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
Min.	0.048		0.079		0.101	
Max.	0.281		0.434		0.491	
Stomatal limitation (L_s, Ci/Ca) at week 4						
Fe_3O_4 NPs (concentration)	**200 µmol m^{-2} s^{-1}**		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**
0 mg / L	0.15	0.06	0.25	0.09	0.34	0.08
0.625 mg / L	0.08	0.03	0.16	0.05	0.22	0.06
1.25 mg / L	0.22	0.08	0.32	0.08	0.36	0.12
2.5 mg / L	0.10	0.06	0.17	0.10	0.21	0.11
5 mg / L	0.20	0.05	0.31	0.08	0.38	0.10
	200 µmol m^{-2} s^{-1}		**500 µmol m^{-2} s^{-1}**		**1800 µmol m^{-2} s^{-1}**	
Min.	0.044		0.083		0.119	
Max.	0.328		0.426		0.526	

4.2.3.3 Harvest quality: Fresh weight

4.2.3.3 Harvest quality (fresh weight) - mean, standard deviation, minimum and maximum values

Fe_3O_4 NPs (concentration)	F.W (g) at week 2				F.W (g) at week 4			
	Leaf		Root		Leaf		Root	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	50.30	5.78	10.27	1.69	166.59	42.71	27.49	4.09
0.625 mg / L	26.27	7.47	6.38	1.80	49.16	19.25	6.63	2.51
1.25 mg / L	32.09	8.92	8.96	2.73	153.23	54.11	25.53	9.97
2.5 mg / L	26.67	16.41	7.89	4.77	110.40	36.89	16.92	3.92
5 mg / L	47.06	5.27	11.22	1.51	153.55	29.68	27.73	6.72
	F.W Leaf		F.W Root		D.W Leaf		D.W Root	
Min.	15.158		3.742		22.497		2.883	
Max.	60.712		18.945		253.609		37.782	

4.2.3.4 Elemental analysis: Fe quantification

4.2.3.4 Iron content in lettuce tissues – mean, standard deviation, minimum and maximum

Fe_3O_4 NPs (concentration)	Fe in leaf at week 2				Fe in leaf at week 4			
	Leaf		Root		Leaf		Root	
	mean	SD	mean	SD	mean	SD	mean	SD
0 mg / L	0.225	0.033	6.534	1.423	0.029	0.012	0.687	0.095
0.625 mg / L	0.120	0.025	0.272	0.160	0.012	0.005	0.020	0.008
1.25 mg / L	0.187	0.160	0.458	0.288	0.018	0.013	0.043	0.040
2.5 mg / L	0.161	0.057	0.864	0.915	0.011	0.004	0.027	0.016
5 mg / L	0.229	0.171	2.084	0.959	0.009	0.001	0.094	0.054
	Fe in leaf at week 2		Fe in root at week 2		Fe in leaf at week 4		Fe in root at week 4	
Min.	0.120		0.272		0.009		0.020	
Max.	0.229		6.534		0.029		0.687	

4.3.3.1 Growth and development measurement

4.3.3.1Growth and morphological measurement by Planteye: mean, standard deviation, minimum and maximum values

Height (cm)								
Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	1.641	0.656	2.323	0.783	3.876	1.196	6.140	3.498
Fe NPs 2.5	1.663	0.538	6.479	0.508	11.365	1.394	13.565	0.765
Fe NPs 5	1.376	0.377	6.020	0.503	10.044	1.589	13.697	0.735
Fe NPs 7.5	1.255	0.389	7.242	0.508	11.363	0.813	14.360	0.209
Fe NPs 10	1.867	0.238	7.309	0.661	10.403	0.686	12.947	0.548
Control	1.641	0.656	2.323	0.783	3.876	1.196	6.140	3.498
Fe EDTA 2.5	0.932	0.401	7.088	0.960	10.404	1.154	15.208	0.873
Fe EDTA 5	1.241	0.221	6.714	0.186	10.460	0.963	15.227	0.467
Fe EDTA 7.5	1.553	0.530	6.960	0.316	10.605	0.381	14.053	0.542
Fe EDTA 10	1.506	0.644	7.117	0.380	10.088	0.839	14.857	1.686
	Week 1		Week 2		Week 3		Week 4	
Min.	5.222		14.278		29.727		26.356	
Max.	23.788		81.702		128.936		165.556	
Leaf area (cm^2)								
Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	34.78	16.12	57.40	20.71	76.81	26.19	107.58	78.96
Fe NPs 2.5	43.10	18.56	134.38	32.10	335.32	70.68	453.51	43.56
Fe NPs 5	29.63	5.38	106.59	8.99	293.82	59.53	426.44	50.22
Fe NPs 7.5	35.77	3.71	164.22	21.59	357.45	55.99	457.20	25.98
Fe NPs 10	39.48	2.02	138.66	14.38	370.87	42.90	488.13	65.00
Control	34.78	16.12	57.40	20.71	76.81	26.19	107.58	78.96
Fe EDTA 2.5	28.40	17.56	158.80	12.78	307.87	62.89	362.03	46.72
Fe EDTA 5	37.10	8.77	132.59	8.27	305.67	56.11	396.79	62.46
Fe EDTA 7.5	29.73	2.01	117.82	13.29	296.54	29.48	389.00	36.23
Fe EDTA 10	36.19	17.76	123.83	25.97	309.19	53.90	401.73	26.05
	Week 1		Week 2		Week 3		Week 4	
Min.	1215.22		3750.41		5211.42		4273.64	
Max.	6215.95		18915.3		40915		56296.6	
Digital biomass (cm^3)								
Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	64.11	53.61	141.43	80.00	314.71	201.05	789.58	947.32
Fe NPs 2.5	78.17	56.49	878.29	267.75	3806.46	890.70	6130.35	269.48
Fe NPs 5	41.74	16.06	642.00	83.43	2914.08	539.94	5842.84	763.39
Fe NPs 7.5	44.96	15.67	1191.18	194.11	4038.63	480.03	6561.83	276.41
Fe NPs 10	74.02	12.88	1019.38	195.81	3858.14	531.62	6333.82	1033.80
Control	64.11	53.61	141.43	80.00	314.71	201.05	789.58	947.32
Fe EDTA 2.5	31.10	28.51	1123.54	156.01	3160.12	416.77	5487.73	575.42
Fe EDTA 5	47.09	17.43	889.23	34.89	3194.06	659.89	6060.08	1143.55
Fe EDTA 7.5	46.37	16.36	822.83	131.17	3137.40	207.37	5463.70	496.56
Fe EDTA 10	62.14	53.47	887.89	234.53	3129.22	702.62	5997.36	1068.10
	Week 1		Week 2		Week 3		Week 4	

Min.	6345.82	37299.8	79867.8	219339
Max.	143801	1397250	4582980	7518810

Hue

Fe (concentration)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	89.621	2.394	79.362	1.218	78.494	6.764	81.836	5.612
Fe NPs 2.5	89.931	3.141	99.262	2.837	100.802	1.981	99.024	1.236
Fe NPs 5	89.531	2.639	99.180	1.643	97.974	1.207	96.548	0.729
Fe NPs 7.5	90.462	3.329	101.059	2.113	102.716	1.813	98.086	2.216
Fe NPs 10	91.615	1.706	100.567	2.120	100.376	1.521	97.616	0.344
Control	89.621	2.394	79.362	1.218	78.494	6.764	81.836	5.612
Fe EDTA 2.5	89.625	2.208	101.87	1.036	103.355	1.7	102.307	1.424
Fe EDTA 5	90.721	3.06	102.005	1.45	103.395	0.988	102.354	1.248
Fe EDTA 7.5	91.078	2.936	101.275	2.126	102.898	1.405	103.354	1.744
Fe EDTA 10	90.129	2.529	101.44	1.851	102.462	1.641	102.114	0.65

	Week 1	Week 2	Week 3	Week 4
Min.	87.1417	78.0024	70.6934	75.3702
Max.	94.4341	103.581	104.364	104.961

Greenness

Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	0.309	0.022	0.269	0.024	0.264	0.070	0.283	0.058
Fe NPs 2.5	0.331	0.004	0.388	0.002	0.524	0.013	0.434	0.002
Fe NPs 5	0.315	0.022	0.385	0.016	0.509	0.005	0.412	0.012
Fe NPs 7.5	0.306	0.026	0.406	0.010	0.537	0.023	0.416	0.011
Fe NPs 10	0.322	0.036	0.404	0.010	0.514	0.004	0.412	0.003
Control	0.309	0.022	0.269	0.024	0.264	0.07	0.283	0.058
Fe EDTA 2.5	0.313	0.014	0.402	0.004	0.531	0.009	0.43	0.014
Fe EDTA 5	0.322	0.016	0.399	0.012	0.548	0.008	0.433	0.012
Fe EDTA 7.5	0.319	0.038	0.395	0.007	0.561	0.014	0.44	0.01
Fe EDTA 10	0.323	0.022	0.399	0.009	0.549	0.016	0.449	0.014

	Week 1	Week 2	Week 3	Week 4
Min.	0.27566	0.21793	0.185	0.22016
Max.	0.3549	0.41802	0.57557	0.46163

NDVI

Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	0.376	0.016	0.292	0.021	0.246	0.093	0.320	0.065
Fe NPs 2.5	0.405	0.021	0.560	0.030	0.692	0.012	0.629	0.011
Fe NPs 5	0.375	0.023	0.559	0.020	0.638	0.031	0.563	0.007
Fe NPs 7.5	0.368	0.034	0.602	0.020	0.724	0.020	0.599	0.027
Fe NPs 10	0.400	0.057	0.589	0.027	0.674	0.011	0.579	0.011
Control	0.376	0.016	0.292	0.021	0.246	0.093	0.32	0.065
Fe EDTA 2.5	0.381	0.035	0.616	0.016	0.739	0.004	0.684	0.011
Fe EDTA 5	0.393	0.023	0.612	0.03	0.746	0.007	0.677	0.009
Fe EDTA 7.5	0.393	0.037	0.603	0.006	0.75	0.005	0.693	0.012
Fe EDTA 10	0.39	0.05	0.609	0.023	0.732	0.013	0.682	0.007

	Week 1	Week 2	Week 3	Week 4
Min.	0.33743	0.22624	0.14088	0.24085
Max.	0.4515	0.64001	0.75551	0.70721

NPCI

Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	0.332	0.027	0.388	0.027	0.373	0.037	0.354	0.048
Fe NPs 2.5	0.355	0.024	0.284	0.024	0.450	0.029	0.338	0.020
Fe NPs 5	0.343	0.025	0.286	0.020	0.491	0.025	0.353	0.028
Fe NPs 7.5	0.322	0.030	0.280	0.020	0.429	0.031	0.332	0.027
Fe NPs 10	0.329	0.028	0.284	0.014	0.447	0.034	0.335	0.006
Control	0.332	0.027	0.388	0.027	0.373	0.037	0.354	0.048
Fe EDTA 2.5	0.336	0.01	0.26	0.006	0.402	0.045	0.28	0.013
Fe EDTA 5	0.336	0.025	0.259	0.021	0.436	0.037	0.283	0.011
Fe EDTA 7.5	0.33	0.037	0.263	0.023	0.469	0.023	0.275	0.036
Fe EDTA 10	0.343	0.023	0.264	0.017	0.456	0.037	0.306	0.015
	Week 1		Week 2		Week 3		Week 4	
Min.	0.28717		0.24379		0.33402		0.23797	
Max.	0.37641		0.41511		0.5197		0.40715	

PSRI

Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	0.211	0.026	0.296	0.030	0.313	0.022	0.263	0.015
Fe NPs 2.5	0.210	0.034	0.118	0.026	0.106	0.011	0.112	0.010
Fe NPs 5	0.216	0.027	0.117	0.013	0.140	0.019	0.142	0.012
Fe NPs 7.5	0.205	0.034	0.100	0.014	0.088	0.011	0.123	0.018
Fe NPs 10	0.194	0.013	0.106	0.015	0.112	0.011	0.130	0.004
Control	0.211	0.026	0.296	0.03	0.313	0.022	0.263	0.015
Fe EDTA 2.5	0.212	0.028	0.092	0.007	0.079	0.007	0.079	0.005
Fe EDTA 5	0.204	0.029	0.09	0.011	0.079	0.003	0.081	0.005
Fe EDTA 7.5	0.199	0.028	0.096	0.013	0.083	0.005	0.074	0.01
Fe EDTA 10	0.21	0.035	0.094	0.012	0.089	0.009	0.085	0.002
	Week 1		Week 2		Week 3		Week 4	
Min.	0.1638		0.08202		0.07391		0.06646	
Max.	0.24987		0.32505		0.36378		0.3081	

4.3.3.2 Water management and gas exchange

4.3.3.2 Water management and gas exchange of WUE, iWUE and Ls

Water use efficiency (WUE, A/E) at week 4						
Fe (mg / L)	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	
	mean	SD	mean	SD	mean	SD
Control	1124.18	289.49	1865.47	535.94	1855.50	562.25
Fe NPs 2.5	1788.45	665.85	2802.75	450.33	2802.35	53.79
Fe NPs 5	1374.17	330.76	2047.87	471.17	2095.94	306.71
Fe NPs 7.5	1558.42	284.98	2336.64	255.43	2327.11	250.49
Fe NPs 10	1570.82	71.83	2457.44	104.56	2286.83	191.91
Control	1124.18	289.49	1865.47	535.94	1855.50	562.25
Fe EDTA 2.5	1920.81	886.24	2505.82	1316.34	2333.61	1172.78
Fe EDTA 5	1370.98	182.82	2229.59	288.31	2263.85	255.16
Fe EDTA 7.5	1839.61	294.66	2859.57	282.05	2736.26	257.58
Fe EDTA 10	1952.16	675.28	3253.54	659.23	3581.48	1094.44
	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	
Min.	555.270		907.668		953.672	
Max.	2796.375		4002.119		4819.587	
Intrinsic water use efficiency (iWUE, A/gsw) at week 4						
Fe (mg / L)	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	
	mean	SD	mean	SD	mean	SD
Control	19.15	7.99	35.30	13.57	43.99	17.21
Fe NPs 2.5	31.86	23.22	51.53	27.29	58.87	20.19
Fe NPs 5	17.83	5.27	28.38	8.41	37.20	8.43
Fe NPs 7.5	24.85	8.63	39.70	7.70	50.31	8.51
Fe NPs 10	19.90	1.55	33.84	3.25	41.43	7.66
Control	19.15	7.99	35.30	13.57	43.99	17.21
Fe EDTA 2.5	35.37	25.38	52.12	38.33	63.20	43.24
Fe EDTA 5	12.33	4.65	21.49	7.44	28.75	8.96
Fe EDTA 7.5	19.86	9.08	33.02	13.34	41.15	16.67
Fe EDTA 10	11.92	4.06	21.70	4.26	34.93	13.24
	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	
Min.	7.690		12.769		17.594	
Max.	62.475		90.576		103.594	
Stomatal limitation (L_s, Ci/Ca) at week 4						
Fe (mg / L)	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	
	mean	SD	mean	SD	mean	SD
Control	0.88	0.04	0.81	0.06	0.77	0.08
Fe NPs 2.5	0.83	0.10	0.73	0.10	0.68	0.07
Fe NPs 5	0.88	0.02	0.83	0.04	0.78	0.04
Fe NPs 7.5	0.86	0.04	0.78	0.02	0.73	0.02
Fe NPs 10	0.88	0.01	0.80	0.01	0.76	0.04
Control	0.88	0.04	0.81	0.06	0.77	0.08
Fe EDTA 2.5	0.82	0.11	0.75	0.16	0.69	0.18
Fe EDTA 5	0.91	0.02	0.86	0.03	0.82	0.04
Fe EDTA 7.5	0.88	0.04	0.82	0.05	0.78	0.06
Fe EDTA 10	0.92	0.02	0.87	0.02	0.80	0.06
	200 µmol m⁻² s⁻¹		500 µmol m⁻² s⁻¹		1800 µmol m⁻² s⁻¹	

Min.	0.701	0.583	0.520
Max.	0.942	0.911	0.884

4.3.3.3 Relative chlorophyll measurement

4.3.3.3 Relative chlorophyll measurement - mean, standard deviation, minimum and maximum values

Fe (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	17.467	1.986	10.800	1.752	6.933	1.986	11.733	1.102
Fe NPs 2.5	15.667	1.185	28.733	1.159	28.100	2.427	28.633	2.401
Fe NPs 5	15.933	0.493	28.100	0.964	32.633	1.124	27.100	0.872
Fe NPs 7.5	17.667	1.644	28.633	1.686	28.067	2.026	27.167	3.075
Fe NPs 10	16.667	1.193	27.733	1.159	27.900	1.735	27.467	2.098
Control	17.467	1.986	10.800	1.752	6.933	1.986	11.733	1.102
Fe EDTA 2.5	16.833	0.153	26.067	0.896	23.900	1.082	24.667	2.902
Fe EDTA 5	15.167	2.013	24.967	0.416	23.633	1.563	21.333	1.168
Fe EDTA 7.5	16.767	0.833	26.900	1.000	27.933	1.815	22.167	1.721
Fe EDTA 10	17.433	2.421	24.600	0.624	24.767	2.050	23.900	1.559

	Week 1	Week 2	Week 3	Week 4
Min.	13.3	8.2	5.2	10.6
Max.	20.2	29.8	33.6	31

4.3.3.4 Harvest quality: Fresh weight

4.3.3.4 Harvest quality (fresh weight) - mean, standard deviation, minimum and maximum values

Fe (mg / L)	F.W (g) at week 2		F.W (g) at week 4	
	mean	SD	mean	SD
Control	5.248	0.692	12.590	10.023
Fe NPs 2.5	13.284	0.919	158.778	141.118
Fe NPs 5	10.279	2.377	132.857	115.696
Fe NPs 7.5	18.426	2.590	151.736	133.798
Fe NPs 10	16.601	3.058	133.484	117.720
Control	5.248	0.692	12.590	10.023
Fe EDTA 2.5	18.233	1.447	134.550	119.179
Fe EDTA 5	18.139	2.268	141.143	126.714
Fe EDTA 7.5	19.974	4.835	150.769	135.234
Fe EDTA 10	14.814	2.541	118.317	104.757

	F.W Leaf (g)	F.W Root (g)
Min.	3.471	12.590
Max.	19.974	158.778

4.3.3.5 Elemental analysis

4.3.3.5 Fe, K and Mg in lettuce tissues – mean, standard deviation, minimum and maximum

Fe (mg / L)	Fe in leaf tissues (mg / g)		Fe in root tissues (mg / g)	
	mean	SD	mean	SD
Control	0.065	0.015	0.098	0.011
Fe NPs 2.5	0.291	0.076	1.512	0.290
Fe NPs 5	0.284	0.117	3.053	0.236
Fe NPs 7.5	0.287	0.106	4.338	0.576
Fe NPs 10	0.191	0.021	4.517	1.004
Control	0.065	0.015	0.098	0.011
Fe EDTA 2.5	0.126	0.072	1.598	0.304
Fe EDTA 5	0.064	0.002	3.057	0.170
Fe EDTA 7.5	0.132	0.057	3.753	1.391
Fe EDTA 10	0.072	0.027	4.410	0.913
	Fe in leaf tissues (mg / g)		Fe in root tissues (mg / g)	
Min.	0.04879		0.08736	
Max.	0.41877		5.67455	
Fe (mg / L)	K in leaf tissues (mg / g)		K in root tissues (mg / g)	
	mean	SD	mean	SD
Control	13.895	2.398	11.959	1.580
Fe NPs 2.5	18.471	0.402	12.841	0.949
Fe NPs 5	21.924	1.677	16.474	0.766
Fe NPs 7.5	18.813	3.530	13.453	1.851
Fe NPs 10	20.191	1.551	12.963	2.086
Control	13.895	2.398	11.959	1.580
Fe EDTA 2.5	20.299	1.426	17.667	5.094
Fe EDTA 5	18.157	1.873	18.839	2.710
Fe EDTA 7.5	21.089	2.965	13.285	1.134
Fe EDTA 10	18.235	2.521	16.687	4.396
	K in leaf tissues (mg / g)		K in root tissues (mg / g)	
Min.	11.13025		8.99639	
Max.	24.50504		23.47206	
Fe (mg / L)	Mg in leaf tissues (mg / g)		Mg in root tissues (mg / g)	
	mean	SD	mean	SD
Control	17.312	1.473	3.805	0.197
Fe NPs 2.5	7.963	1.701	3.663	0.107
Fe NPs 5	8.216	2.123	3.218	0.120
Fe NPs 7.5	7.040	1.056	3.137	0.296
Fe NPs 10	6.970	0.733	3.176	0.102
Control	17.312	1.473	3.805	0.197
Fe EDTA 2.5	6.477	1.200	4.521	1.812
Fe EDTA 5	6.762	1.665	4.466	1.298
Fe EDTA 7.5	7.756	0.694	4.063	0.136
Fe EDTA 10	6.371	2.029	5.464	1.737
	Mg in leaf tissues (mg / g)		Mg in root tissues (mg / g)	
Min.	4.10893		2.79711	
Max.	21.90732		7.35144	

4.3.3.6 Secondary metabolites analysis

4.3.3.6 Secondary metabolites (TPC, TFC, and TAA) - mean, standard deviation, minimum and maximum

Fe (mg / L)	TPC (mg GAE / g F.W)			TFC (mg Rutin/g F.W)			TAA (mg TEAC / g F.W)		
	mean	SE	SD	mean	SE	SD	mean	SE	SD
Control	0.495	0.031	0.054	0.058	0.002	0.003	5.983	0.128	0.222
Fe NPs 2.5	0.447	0.030	0.052	0.106	0.002	0.004	6.132	0.090	0.155
Fe NPs 5	0.284	0.054	0.093	0.091	0.002	0.004	5.865	0.281	0.487
Fe NPs 7.5	0.392	0.043	0.075	0.105	0.008	0.014	6.247	0.242	0.419
Fe NPs 10	0.329	0.021	0.036	0.079	0.003	0.006	5.976	0.272	0.470
Control	0.495	0.031	0.054	0.058	0.002	0.003	5.983	0.128	0.222
Fe EDTA 2.5	0.282	0.020	0.034	0.088	0.006	0.011	5.823	0.179	0.311
Fe EDTA 5	0.338	0.010	0.017	0.119	0.008	0.014	6.138	0.200	0.346
Fe EDTA 7.5	0.379	0.024	0.041	0.098	0.010	0.017	6.156	0.202	0.350
Fe EDTA 10	0.349	0.022	0.038	0.066	0.003	0.005	6.006	0.104	0.180

	TPC (mg GAE / g F.W)	TFC (mg Rutin/g F.W)	TAA (mg TEAC / g F.W)
Min.	0.21722	0.05512	5.49233
Max.	0.67393	0.13289	6.51773

Appendix C - Chapter 5. Vitamin B_{12} and Iron Fortification

5.3.1 Descriptive statistic – Fe_3O_4 NPs + B_{12} application: growth and morphological measurement by Planteye

Height (cm)								
Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	4.376	3.086	4.224	0.907	7.351	1.114	9.915	1.478
Fe 2.5 + B_{12} 10	4.629	2.806	4.368	1.152	5.920	1.050	9.406	0.858
Fe 2.5 + B_{12} 50	4.686	2.824	5.692	0.923	8.590	1.616	10.583	1.172
Fe 2.5 + B_{12} 100	4.165	2.267	6.841	0.667	10.193	0.752	13.180	2.436
Fe 2.5 + B_{12} 1000	4.547	2.642	5.822	0.676	9.442	1.946	13.545	0.701
Fe 5 + B_{12} 0	4.117	3.151	3.629	0.757	7.841	2.414	10.561	3.123
Fe 5 + B_{12} 10	3.966	3.281	5.645	1.271	9.059	1.047	13.044	1.947
Fe 5 + B_{12} 50	4.341	2.991	5.616	0.988	8.472	1.231	12.991	1.865
Fe 5 + B_{12} 100	4.213	3.043	4.962	1.402	8.666	1.025	13.392	2.278
Fe 5 + B_{12} 1000	4.518	2.703	5.644	1.323	11.433	1.618	16.248	2.011
	Week 1		Week 2		Week 3		Week 4	
Min.	3.966		3.629		7.351		9.406	
Max.	4.686		6.841		10.193		16.248	

Leaf area (cm^2)								
Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	49.24	17.14	219.16	57.15	762.17	100.54	1014.33	163.46
Fe 2.5 + B_{12} 10	38.78	12.66	156.93	41.96	431.06	111.87	706.82	223.86
Fe 2.5 + B_{12} 50	44.86	7.04	191.91	41.15	552.43	72.97	866.83	126.24
Fe 2.5 + B_{12} 100	46.89	19.13	221.70	60.36	643.65	101.79	777.88	133.76
Fe 2.5 + B_{12} 1000	49.01	13.43	198.35	40.94	524.92	74.27	550.63	79.67
Fe 5 + B_{12} 0	35.71	8.33	162.03	95.00	633.21	105.27	904.86	110.25
Fe 5 + B_{12} 10	43.25	13.98	228.03	22.41	645.55	88.15	847.95	152.36
Fe 5 + B_{12} 50	50.39	16.38	240.43	61.75	708.95	101.49	889.87	114.86
Fe 5 + B_{12} 100	46.63	15.26	210.07	68.36	686.04	95.29	767.37	178.63
Fe 5 + B_{12} 1000	40.39	14.70	209.21	60.48	492.72	56.75	463.39	108.90
	Week 1		Week 2		Week 3		Week 4	
Min.	35.71		156.93		431.06		463.39	
Max.	50.39		240.43		762.17		1014.33	

Digital biomass (cm^3)								
Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	181.03	77.75	938.09	343.10	5621.50	1148.25	10103.70	2370.85
Fe 2.5 + B_{12} 10	207.84	197.39	671.96	196.35	2627.58	1041.59	6724.34	2326.73
Fe 2.5 + B_{12} 50	218.91	155.52	1101.25	320.82	4778.30	1219.04	9132.47	1395.16
Fe 2.5 + B_{12} 100	218.74	191.86	1530.15	499.89	6599.52	1380.19	10211.77	2376.36
Fe 2.5 + B_{12} 1000	229.10	158.50	1160.61	309.75	4929.68	1057.98	7481.70	1340.72
Fe 5 + B_{12} 0	150.70	123.95	633.25	439.65	5149.79	2189.89	9503.43	2684.92
Fe 5 + B_{12} 10	167.64	130.98	1298.24	358.47	5839.66	1083.78	10999.97	2227.75
Fe 5 + B_{12} 50	216.96	154.38	1347.84	382.18	6002.34	1307.63	11486.60	1864.36

Fe 5 + B_{12} 100	211.37	187.18	1077.51	585.31	5929.84	1009.14	10017.44	1642.08
Fe 5 + B_{12} 1000	193.25	168.07	1185.01	389.33	5683.67	1360.40	7566.87	2309.38

	Week 1	Week 2	Week 3	Week 4
Min.	150.70	671.96	2627.58	6724.34
Max.	229.10	1347.84	6599.52	11486.60

Hue

Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	97.03	3.48	103.89	1.67	104.38	2.52	103.59	1.78
Fe 2.5 + B_{12} 10	94.29	4.10	97.87	3.74	100.83	3.97	104.66	3.56
Fe 2.5 + B_{12} 50	96.15	4.36	100.36	2.52	102.64	1.86	106.25	1.50
Fe 2.5 + B_{12} 100	93.73	2.84	103.01	3.12	103.45	2.86	103.21	3.11
Fe 2.5 + B_{12} 1000	93.34	2.79	99.40	2.06	100.62	2.61	101.00	3.58
Fe 5 + B_{12} 0	95.30	4.08	104.33	2.35	106.49	2.20	106.10	1.70
Fe 5 + B_{12} 10	95.69	3.73	103.20	1.45	105.73	1.67	105.13	1.78
Fe 5 + B_{12} 50	95.35	2.02	105.03	1.51	105.44	1.77	104.76	2.53
Fe 5 + B_{12} 100	93.66	4.26	101.08	3.86	106.04	2.02	103.57	2.61
Fe 5 + B_{12} 1000	92.17	3.05	102.87	2.26	102.45	2.14	99.87	3.15

	Week 1	Week 2	Week 3	Week 4
Min.	86.603	92.311	94.457	92.389
Max.	103.468	107.532	109.522	109.293

Greenness

Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	0.37	0.06	0.42	0.02	0.42	0.01	0.43	0.01
Fe 2.5 + B_{12} 10	0.38	0.02	0.40	0.02	0.41	0.02	0.43	0.02
Fe 2.5 + B_{12} 50	0.38	0.03	0.41	0.01	0.42	0.01	0.42	0.01
Fe 2.5 + B_{12} 100	0.38	0.02	0.40	0.01	0.43	0.01	0.42	0.01
Fe 2.5 + B_{12} 1000	0.36	0.03	0.38	0.02	0.40	0.01	0.38	0.03
Fe 5 + B_{12} 0	0.36	0.02	0.38	0.01	0.39	0.01	0.40	0.01
Fe 5 + B_{12} 10	0.37	0.03	0.39	0.01	0.40	0.01	0.41	0.01
Fe 5 + B_{12} 50	0.37	0.04	0.38	0.02	0.40	0.01	0.42	0.01
Fe 5 + B_{12} 100	0.36	0.04	0.40	0.01	0.39	0.01	0.42	0.02
Fe 5 + B_{12} 1000	0.35	0.03	0.38	0.01	0.38	0.02	0.39	0.03

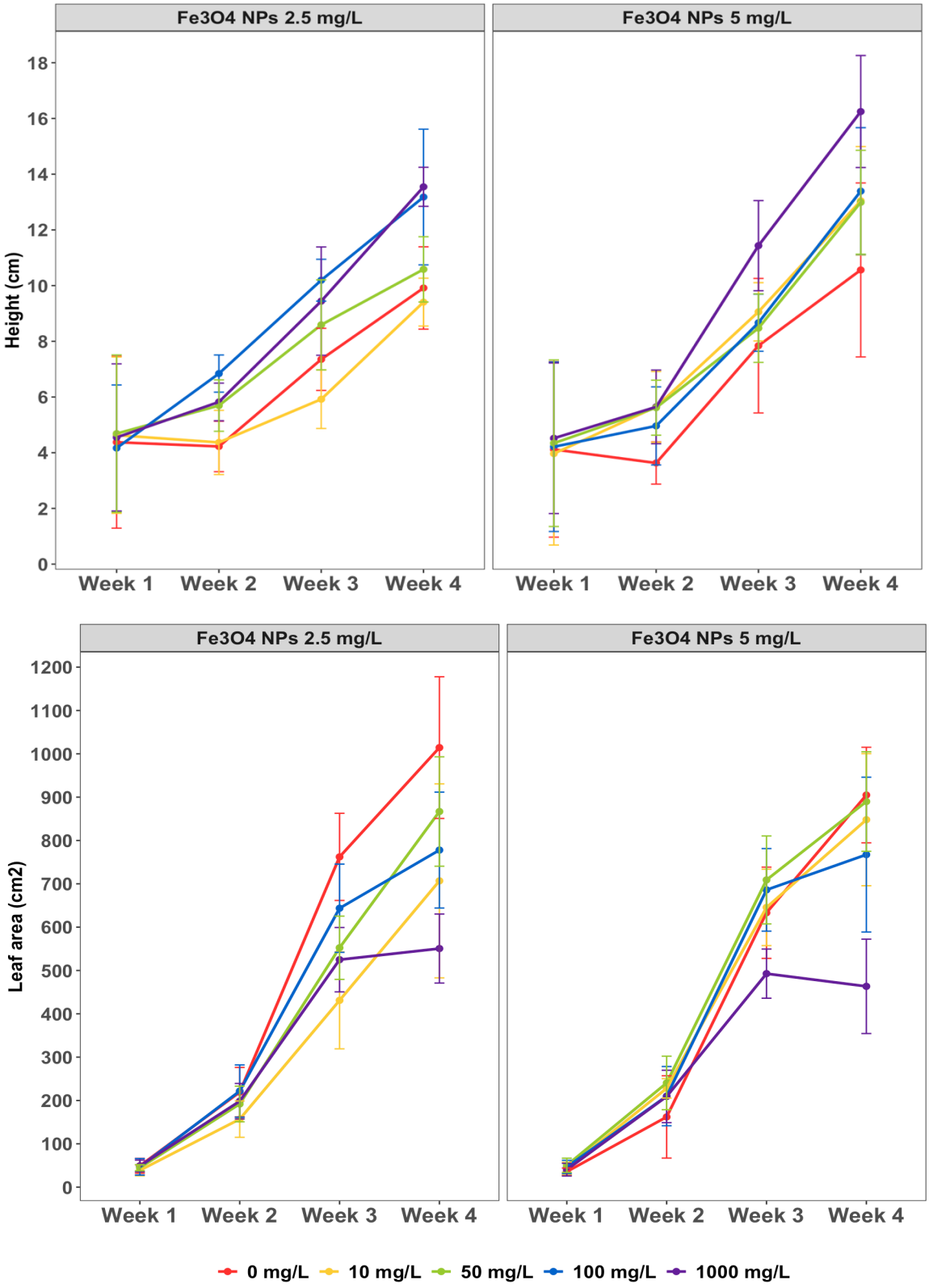
	Week 1	Week 2	Week 3	Week 4
Min.	0.262	0.347	0.352	0.324
Max.	0.437	0.457	0.442	0.446

NDVI

Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	0.48	0.10	0.69	0.04	0.70	0.02	0.70	0.03
Fe 2.5 + B_{12} 10	0.48	0.05	0.58	0.05	0.63	0.06	0.69	0.04
Fe 2.5 + B_{12} 50	0.50	0.06	0.62	0.04	0.67	0.02	0.72	0.01
Fe 2.5 + B_{12} 100	0.47	0.04	0.66	0.05	0.68	0.03	0.69	0.03
Fe 2.5 + B_{12} 1000	0.49	0.05	0.62	0.03	0.66	0.03	0.65	0.03
Fe 5 + B_{12} 0	0.48	0.03	0.67	0.03	0.70	0.01	0.71	0.01
Fe 5 + B_{12} 10	0.47	0.07	0.66	0.02	0.70	0.02	0.71	0.01
Fe 5 + B_{12} 50	0.49	0.09	0.67	0.01	0.70	0.01	0.71	0.02
Fe 5 + B_{12} 100	0.46	0.08	0.63	0.06	0.70	0.01	0.71	0.03
Fe 5 + B_{12} 1000	0.45	0.06	0.67	0.04	0.69	0.01	0.67	0.04

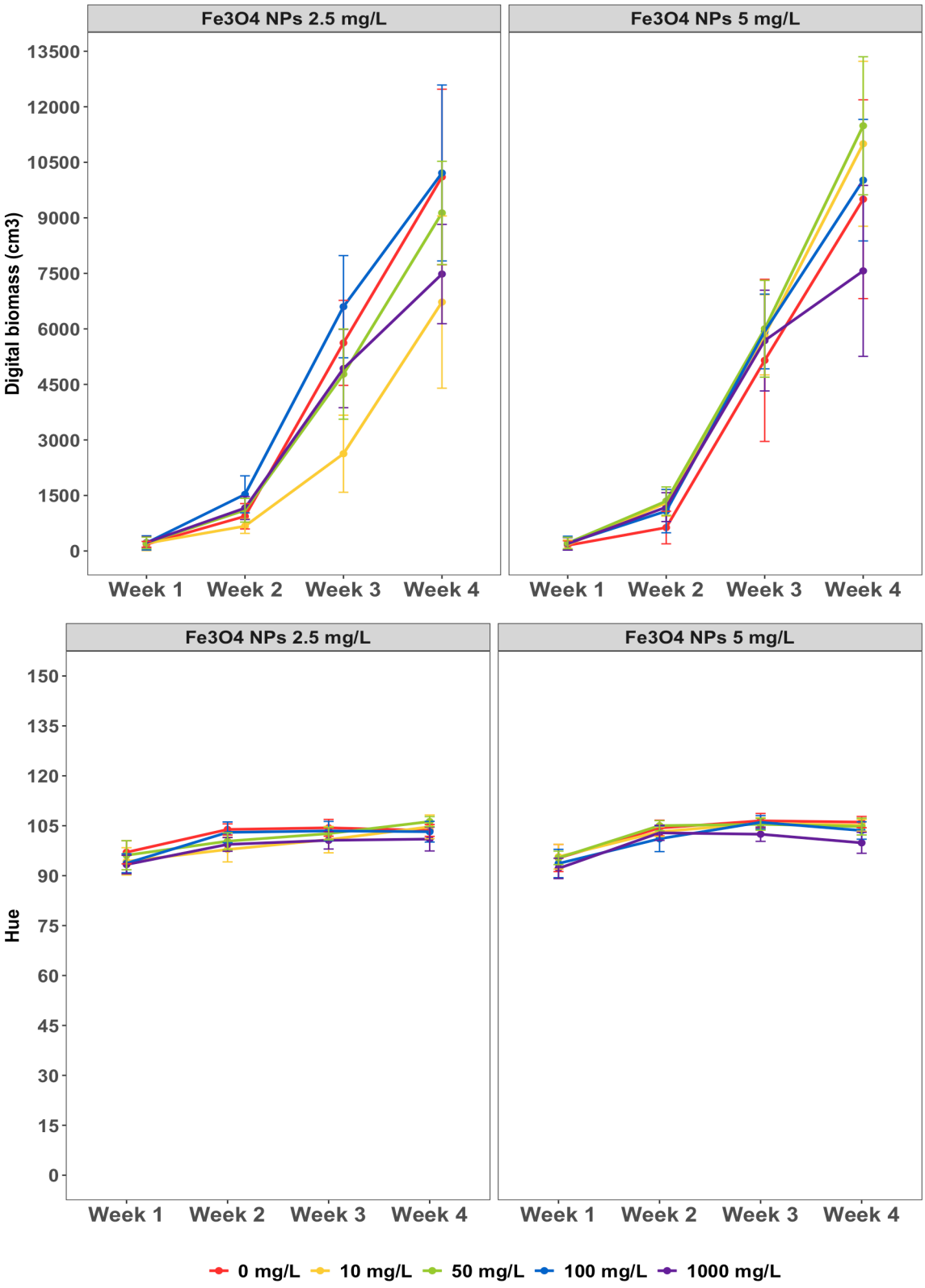
	Week 1	Week 2	Week 3	Week 4

Min.	0.331		0.491		0.524		0.584	
Max.	0.634		0.733		0.732		0.739	
NPCI								
Treatment (mg / L)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Fe 2.5 + B_{12} 0	0.33	0.05	0.26	0.03	0.25	0.04	0.27	0.03
Fe 2.5 + B_{12} 10	0.37	0.04	0.32	0.05	0.29	0.05	0.25	0.06
Fe 2.5 + B_{12} 50	0.34	0.05	0.29	0.03	0.27	0.03	0.22	0.02
Fe 2.5 + B_{12} 100	0.36	0.04	0.24	0.04	0.27	0.04	0.26	0.05
Fe 2.5 + B_{12} 1000	0.35	0.03	0.27	0.03	0.28	0.03	0.24	0.02
Fe 5 + B_{12} 0	0.34	0.04	0.21	0.03	0.19	0.03	0.21	0.02
Fe 5 + B_{12} 10	0.33	0.05	0.23	0.02	0.21	0.02	0.23	0.03
Fe 5 + B_{12} 50	0.33	0.02	0.20	0.03	0.21	0.03	0.24	0.04
Fe 5 + B_{12} 100	0.34	0.04	0.27	0.05	0.20	0.03	0.25	0.02
Fe 5 + B_{12} 1000	0.35	0.04	0.23	0.03	0.23	0.02	0.27	0.03
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.246		0.169		0.150		0.175	
Max.	0.404		0.379		0.340		0.346	
PSRI								
Treatment (mg / L)	**Week 1**		**Week 2**		**Week 3**		**Week 4**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Fe 2.5 + B_{12} 0	0.15	0.03	0.07	0.01	0.07	0.01	0.07	0.01
Fe 2.5 + B_{12} 10	0.18	0.04	0.13	0.04	0.10	0.03	0.07	0.03
Fe 2.5 + B_{12} 50	0.16	0.04	0.10	0.02	0.08	0.01	0.05	0.01
Fe 2.5 + B_{12} 100	0.18	0.03	0.08	0.02	0.07	0.02	0.07	0.02
Fe 2.5 + B_{12} 1000	0.17	0.02	0.09	0.01	0.08	0.02	0.08	0.02
Fe 5 + B_{12} 0	0.16	0.04	0.06	0.02	0.05	0.01	0.05	0.01
Fe 5 + B_{12} 10	0.17	0.04	0.07	0.01	0.06	0.01	0.06	0.01
Fe 5 + B_{12} 50	0.16	0.03	0.06	0.01	0.06	0.01	0.06	0.01
Fe 5 + B_{12} 100	0.18	0.05	0.09	0.04	0.05	0.01	0.06	0.01
Fe 5 + B_{12} 1000	0.18	0.03	0.07	0.01	0.06	0.01	0.08	0.02
	Week 1		**Week 2**		**Week 3**		**Week 4**	
Min.	0.097		0.045		0.041		0.040	
Max.	0.265		0.186		0.156		0.115	



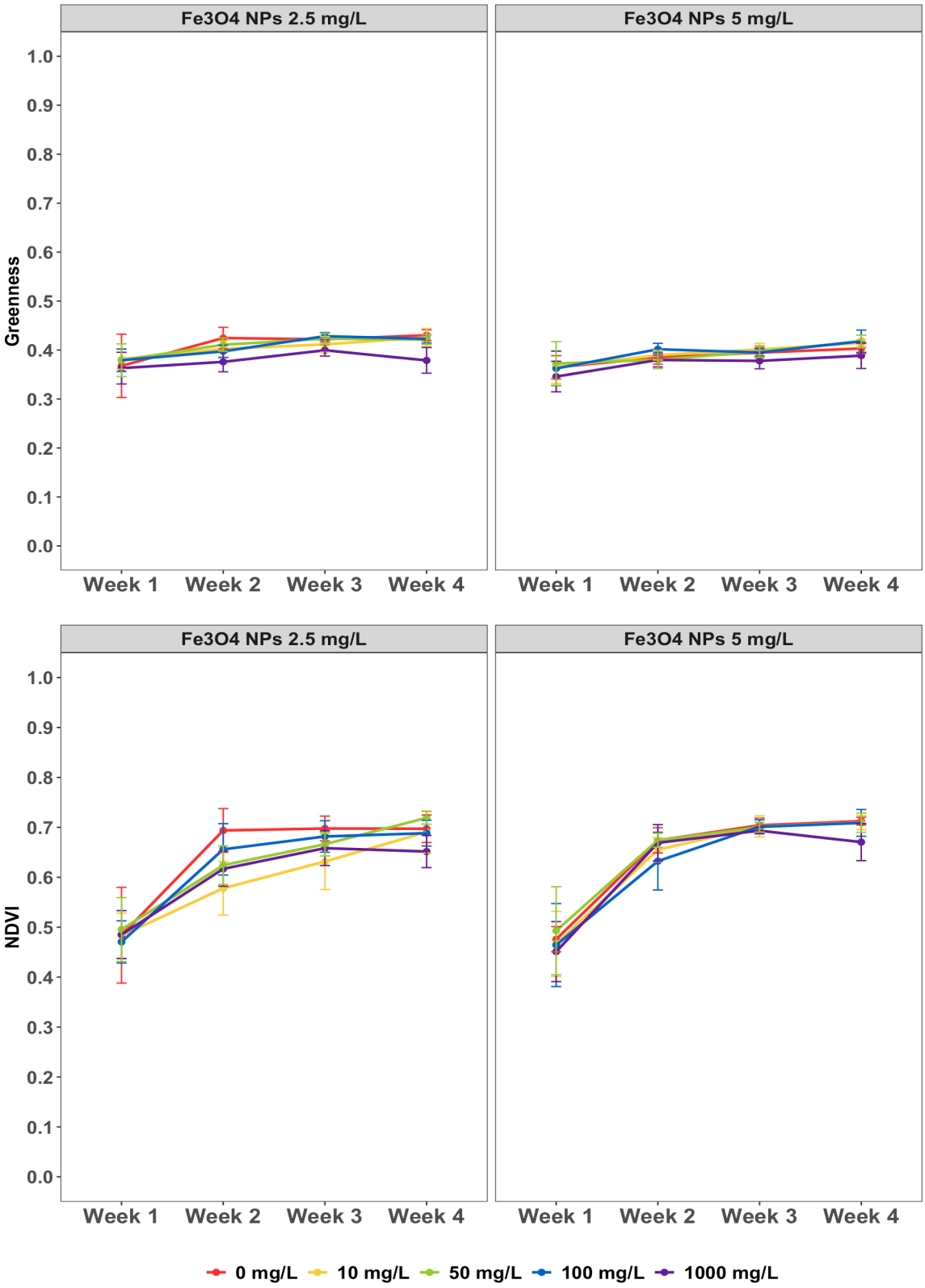
Height (cm) and Leaf area (cm^2) of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.



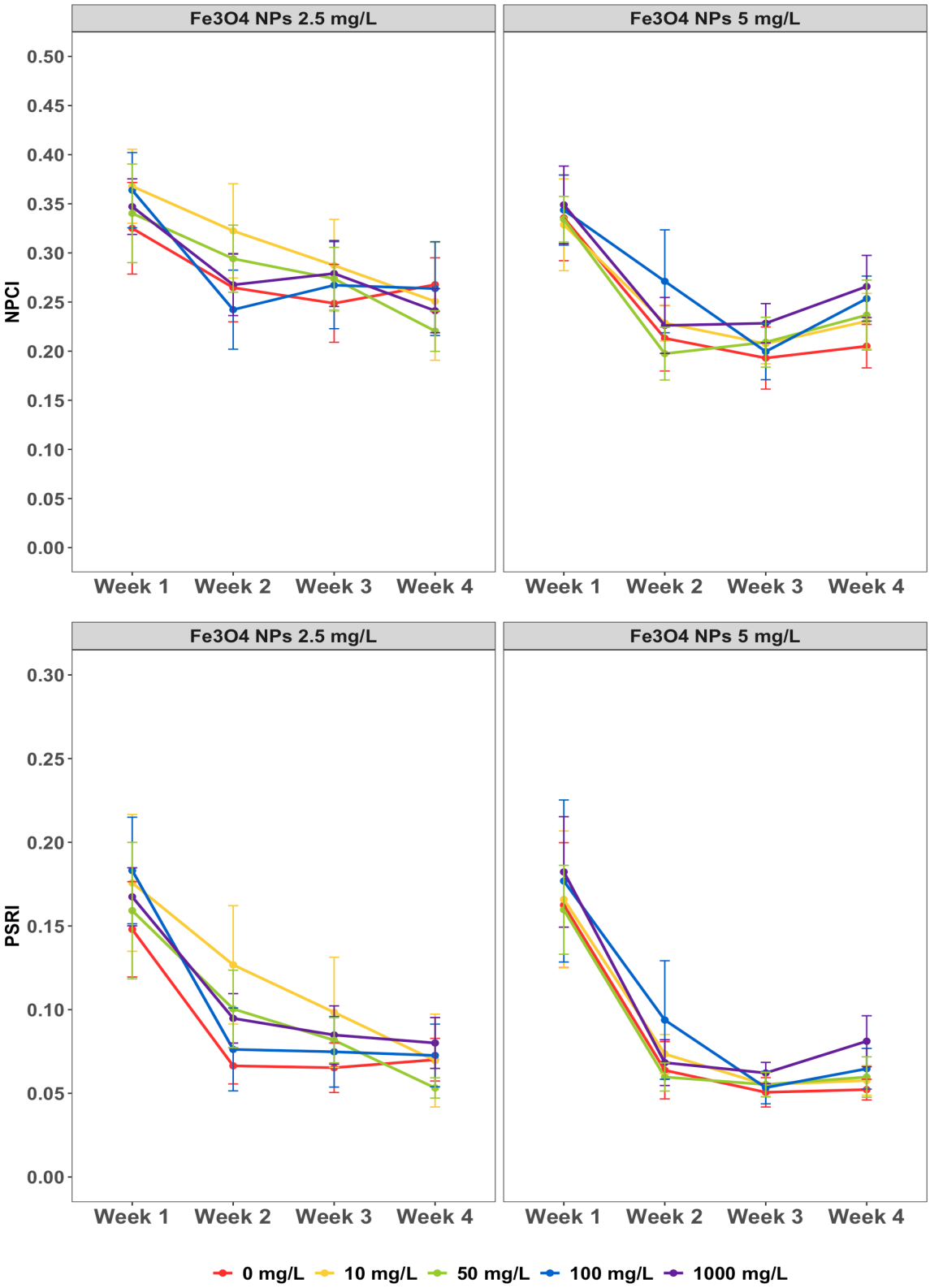
Digital biomass (cm3) and Hue(°) of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.



Greenness and NDVI of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.



NPCI and PSRI of lettuces measured weekly via PlantEye.

Coloured box plots indicate Fe_3O_4 NPs and Fe-EDTA concentrations (0, 2.5, 5, 7.5, and 10 mg / L). Statistical significance was determined using PERMANOVA followed by multiple permutational pairwise test ($p < 0.05$). Different letters denote statistically significant differences between groups.

5.3.2 Relative chlorophyll measurement - mean, standard deviation, minimum and maximum values

Treatment (mg / L)	Week 1		Week 2		Week 3		Week 4	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	23.4	3.439	37.8	3.319	33.0	3.916	32.4	2.305
Fe 2.5 + B_{12} 10	23.3	2.731	25.9	3.375	24.6	3.222	30.8	4.647
Fe 2.5 + B_{12} 50	22.9	3.276	28.6	3.323	29.3	4.104	33.0	1.283
Fe 2.5 + B_{12} 100	22.7	2.366	33.9	3.673	31.0	3.060	33.0	1.782
Fe 2.5 + B_{12} 1000	25.6	3.603	32.4	3.920	29.2	3.960	31.8	2.315
Fe 5 + B_{12} 0	24.1	3.783	34.3	4.925	36.6	3.246	35.3	2.220
Fe 5 + B_{12} 10	24.0	3.760	31.4	5.140	34.6	3.123	33.3	2.873
Fe 5 + B_{12} 50	25.0	4.784	37.3	2.389	37.8	2.839	33.4	2.564
Fe 5 + B_{12} 100	24.5	2.920	32.4	6.522	36.5	2.864	31.3	2.452
Fe 5 + B_{12} 1000	22.6	2.835	36.8	3.442	36.6	3.550	33.8	1.828
	1st week		2nd week		3rd week		4th week	
Min.	16.60		20.90		20.20		24.50	
Max.	33.20		41.60		42.60		38.50	

5.3.3 Harvest quality (fresh/dry weight) - mean, standard deviation, minimum and maximum values

Treatment (mg / L)	Fresh weight (g)				Dry weight (g)			
	Leaf		Root		Leaf		Root	
	mean	SD	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	172.069	31.586	19.368	3.401	5.357	0.947	0.974	0.184
Fe 2.5 + B_{12} 10	89.297	33.476	10.085	3.579	3.144	1.031	0.466	0.159
Fe 2.5 + B_{12} 50	113.495	22.622	14.567	2.052	4.095	0.639	0.609	0.085
Fe 2.5 + B_{12} 100	123.086	28.335	14.348	4.000	4.233	0.987	0.624	0.175
Fe 2.5 + B_{12} 1000	89.940	16.287	8.921	1.387	3.552	0.477	0.431	0.033
Fe 5 + B_{12} 0	167.491	55.625	17.321	3.820	5.885	1.644	0.861	0.295
Fe 5 + B_{12} 10	172.509	27.294	16.250	3.057	5.758	0.925	0.760	0.163
Fe 5 + B_{12} 50	198.188	35.130	21.547	4.964	6.385	1.508	0.975	0.232
Fe 5 + B_{12} 100	187.402	27.050	16.944	3.671	5.891	1.273	0.779	0.173
Fe 5 + B_{12} 1000	114.822	30.779	10.845	2.742	4.578	1.230	0.531	0.128
	F.W Leaf		F.W Root		D.W Leaf		D.W Root	
Min.	40.44		5.70		1.633		0.2649	
Max.	273.35		32.73		9.932		1.4809	

5.3.4 Multielement quantification in lettuce– mean, standard deviation, minimum and maximum

Vitamin B_{12} (B_{12}) quantification				
Treatment (mg / L)	B_{12} in leaf tissues (mg / g)		B_{12} in root tissues (mg / g)	
	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	0.000	0.004	0.001	0.001
Fe 2.5 + B_{12} 10	0.009	0.004	0.108	0.030
Fe 2.5 + B_{12} 50	0.034	0.012	0.159	0.047
Fe 2.5 + B_{12} 100	0.238	0.079	0.275	0.117
Fe 2.5 + B_{12} 1000	0.292	0.110	0.596	0.126
Fe 5 + B_{12} 0	-0.001	0.004	0.001	0.000
Fe 5 + B_{12} 10	0.042	0.010	0.121	0.073
Fe 5 + B_{12} 50	0.079	0.023	0.128	0.057
Fe 5 + B_{12} 100	0.121	0.038	0.239	0.045
Fe 5 + B_{12} 1000	0.537	0.138	0.368	0.094
	B_{12} in leaf tissues (mg / g)		B_{12} in root tissues (mg / g)	
Min.	-0.001		0.000	
Max.	0.537		0.596	
Iron (Fe) quantification				
Treatment (mg / L)	Fe in leaf tissues (mg / g)		Fe in root tissues (mg / g)	
	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	0.137	0.046	5.275	1.704
Fe 2.5 + B_{12} 10	0.105	0.033	8.134	2.893
Fe 2.5 + B_{12} 50	0.092	0.009	3.998	1.534
Fe 2.5 + B_{12} 100	0.083	0.024	4.168	1.723
Fe 2.5 + B_{12} 1000	0.089	0.021	3.986	1.177
Fe 5 + B_{12} 0	0.155	0.090	12.118	2.662
Fe 5 + B_{12} 10	0.134	0.024	13.340	2.080
Fe 5 + B_{12} 50	0.121	0.033	10.102	2.802
Fe 5 + B_{12} 100	0.125	0.042	21.345	2.866
Fe 5 + B_{12} 1000	0.127	0.023	12.890	3.872
	Fe in leaf tissues (mg / g)		Fe in root tissues (mg / g)	
Min.	0.083		21.345	
Max.	0.155		3.986	
Potassium (K) quantification				
Treatment (mg / L)	K in leaf tissues (mg / g)		K in root tissues (mg / g)	
	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	247.412	58.586	143.335	12.320
Fe 2.5 + B_{12} 10	174.371	10.606	142.260	12.689
Fe 2.5 + B_{12} 50	195.791	10.195	130.694	18.899
Fe 2.5 + B_{12} 100	185.419	7.681	144.372	36.681
Fe 2.5 + B_{12} 1000	174.058	28.108	140.787	7.095
Fe 5 + B_{12} 0	237.407	19.933	165.436	18.169
Fe 5 + B_{12} 10	226.892	12.757	169.469	27.548
Fe 5 + B_{12} 50	242.306	18.936	158.725	8.906
Fe 5 + B_{12} 100	229.411	14.449	159.304	28.016
Fe 5 + B_{12} 1000	234.931	15.191	196.201	24.943
	K in leaf tissues (mg / g)		K in root tissues (mg / g)	
Min.	174.058		130.694	
Max.	247.412		196.201	

Magnesium (Mg) quantification				
Treatment (mg / L)	**Mg in leaf tissues (mg / g)**		**Mg in root tissues (mg / g)**	
	mean	**SD**	**mean**	**SD**
Fe 2.5 + B_{12} 0	15.923	1.076	8.239	0.954
Fe 2.5 + B_{12} 10	14.380	1.525	5.935	0.530
Fe 2.5 + B_{12} 50	12.948	1.512	5.170	0.594
Fe 2.5 + B_{12} 100	12.945	1.907	6.422	3.064
Fe 2.5 + B_{12} 1000	12.721	1.653	5.159	0.148
Fe 5 + B_{12} 0	16.484	0.985	8.846	0.476
Fe 5 + B_{12} 10	16.729	1.195	8.797	0.811
Fe 5 + B_{12} 50	16.310	1.104	7.520	0.214
Fe 5 + B_{12} 100	14.673	1.497	9.578	1.553
Fe 5 + B_{12} 1000	13.905	1.445	8.467	0.277
	Mg in leaf tissues (mg / g)		**Mg in root tissues (mg / g)**	
Min.	12.721		5.159	
Max.	16.729		9.578	
Calcium (Ca) quantification				
Treatment (mg / L)	**Ca in leaf tissues (mg / g)**		**Ca in root tissues (mg / g)**	
	mean	**SD**	**mean**	**SD**
Fe 2.5 + B_{12} 0	32.613	2.218	16.051	1.743
Fe 2.5 + B_{12} 10	29.014	3.082	15.398	1.639
Fe 2.5 + B_{12} 50	28.850	3.281	12.115	1.539
Fe 2.5 + B_{12} 100	28.907	5.621	13.667	4.260
Fe 2.5 + B_{12} 1000	27.506	4.193	12.750	0.973
Fe 5 + B_{12} 0	38.825	1.380	18.452	0.459
Fe 5 + B_{12} 10	44.422	2.215	22.374	4.492
Fe 5 + B_{12} 50	39.172	5.074	18.736	1.539
Fe 5 + B_{12} 100	37.147	2.434	22.984	1.330
Fe 5 + B_{12} 1000	33.978	5.465	20.371	0.395
	Ca in leaf tissues (mg / g)		**Ca in root tissues (mg / g)**	
Min.	27.506		12.115	
Max.	44.422		22.984	
Phosphorus (P) quantification				
Treatment (mg / L)	**P in leaf tissues (mg / g)**		**P in root tissues (mg / g)**	
	mean	**SD**	**mean**	**SD**
Fe 2.5 + B_{12} 0	21.686	1.973	23.252	2.887
Fe 2.5 + B_{12} 10	22.024	0.960	26.312	0.979
Fe 2.5 + B_{12} 50	21.874	1.566	20.399	2.854
Fe 2.5 + B_{12} 100	20.681	1.996	27.785	12.333
Fe 2.5 + B_{12} 1000	19.676	1.324	23.650	0.996
Fe 5 + B_{12} 0	28.180	2.467	37.512	2.905
Fe 5 + B_{12} 10	31.840	1.065	42.065	5.574
Fe 5 + B_{12} 50	31.388	1.142	40.048	2.311
Fe 5 + B_{12} 100	27.671	1.532	40.607	5.219
Fe 5 + B_{12} 1000	28.190	1.942	52.240	4.538
	P in leaf tissues (mg / g)		**P in root tissues (mg / g)**	
Min.	19.676		20.399	
Max.	31.840		52.240	

5.3.5 Secondary metabolites (TPC, TFC, and TAA) - mean, standard deviation, minimum and maximum

Treatment	TPC (mg GAE / g F.W)		TFC (mg Rutin/g F.W)		TAA (mg TEAC / g F.W)	
	mean	SD	mean	SD	mean	SD
Fe 2.5 + B_{12} 0	0.306	0.084	0.102	0.037	5.582	0.158
Fe 2.5 + B_{12} 10	0.329	0.087	0.107	0.056	5.462	0.184
Fe 2.5 + B_{12} 50	0.310	0.053	0.094	0.023	5.687	0.176
Fe 2.5 + B_{12} 100	0.339	0.070	0.080	0.009	5.552	0.248
Fe 2.5 + B_{12} 1000	0.325	0.042	0.075	0.013	5.440	0.264
Fe 5 + B_{12} 0	0.388	0.065	0.160	0.046	5.356	0.196
Fe 5 + B_{12} 10	0.526	0.101	0.192	0.041	5.402	0.332
Fe 5 + B_{12} 50	0.435	0.060	0.165	0.032	5.382	0.352
Fe 5 + B_{12} 100	0.563	0.141	0.152	0.027	5.355	0.280
Fe 5 + B_{12} 1000	0.439	0.126	0.125	0.014	5.385	0.323

	TPC (mg GAE / g F.W)	TFC (mg Rutin/g F.W)	TAA (mg TEAC / g F.W)
Min.	0.2234	0.04955	4.816
Max.	0.7878	0.25497	5.942