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"Biofortification with Fe and Zn biochelates and the effects of protein hydrolysate on nutritional quality of *Lactuca sativa* L. in an NFT system"

CANDIDATE:
José Carlos Laban LLiuya
Student ID: 803641

SUPERVISORS:
Prof. Giuseppe Colla
Prof. Agnieszka Lis-Krzyścin

CO-SUPERVISOR:
Iqra Bashir, PhD student

SECOND EXAMINER:
Prof. Roberto Ruggeri

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ABSTRACT:
Iron and zinc deficiencies are major nutritional disorders worldwide. Agronomic biofortification aims to increase micronutrient content in staple crops. Synthetic chelates have been widely used to supply minerals, but their high environmental impact has limited their use. Conversely, peptide based biochelates provide essential minerals while exerting a biostimulant effect. This study evaluated Fe- and Zn-biochelates, as well as vegetal protein hydrolysate, in two lettuce cultivars grown under optimal and heat stress conditions in an NFT system with treatments applied at different days after transplanting. Fe- and Zn-biochelates significantly increased mineral content under both conditions. Treatments also reduced nitrate accumulation, enhancing food safety. Effects on vitamin C, sugars, phenol, and antioxidant activity were cultivar- and condition-dependent. Foliar application of vegetal peptides enhanced root biomass and modulated vitamin C and amino acid levels, confirming their biostimulant potential. These results demonstrate the efficacy of biochelates in biofortifying lettuce and improving nutritional quality.

KEY WORDS: agronomic biofortification, biochelate, micronutrient deficiency, lettuce, heat stress, soilless systems.

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1. INTRODUCTION

Micronutrient deficiency, also known as ‘hidden hunger’, is a major global public health issue that silently affects more than two billion people worldwide, particularly children under the age of five and pregnant women (Muthayya et al., 2013). This condition is primarily caused by inadequate dietary intake of essential vitamins and minerals (Bhardwaj et al., 2022). Among the various micronutrient deficiencies identified globally, iron and zinc are considered the most critical due to their high prevalence and their essential physiological roles in the human body (Caetano-Silva et al., 2021). In particular, insufficient iron intake is the leading cause of anaemia, a condition that impairs oxygen transport throughout the body, while inadequate zinc intake has been associated with the development of autoimmune disorders, cognitive dysfunction, and compromised immune function (Hussain et al., 2022; Kolarš et al., 2024; Baddam et al., 2025).

The causes of malnutrition are closely linked to volatile food prices and climate change, compounded by the aftermath of the COVID-19 pandemic and the conflict in Ukraine. These factors have directly affected global food systems by increasing staple food prices, thereby reducing food availability and affordability, particularly for vulnerable populations (United Nations, 2022; World Bank, 2025). Such populations often rely on cereal- and pulse-based diets that provide sufficient caloric intake but are deficient in essential minerals (Bhardwaj et al., 2022). Consequently, these conditions exacerbate the risk of micronutrient deficiencies, increasing the number of individuals affected by hidden hunger (Muthayya et al., 2013). To address this global challenge, the elimination of micronutrient deficiencies has been identified as a key objective of the United Nations 2030 Agenda, which aims to ensure universal access to essential micronutrients by 2030 (United Nations, 2015). In this context, biofortification has emerged as a promising strategy to enhance the micronutrient content of foods. Agronomic biofortification, in particular, involves the application of mineral-rich fertilizers through seed priming, foliar treatments, or root uptake, with the aim of optimizing nutrient

uptake, translocation, and utilization within plants, ultimately improving their nutritional quality (Shahzad et al., 2021).

Within this framework, *Lactuca sativa* L. represents a strategic crop for biofortification due to its widespread consumption, favourable organoleptic properties, and contribution to dietary micronutrient intake (Giordano et al., 2019). Lettuce is predominantly consumed raw, which allows the preservation of heat-sensitive phytochemicals such as vitamin C, folates, and polyphenols, known to support mineral bioavailability and human health (Kim et al., 2016; Putriani et al., 2020). Moreover, its rapid growth cycle and high capacity for mineral accumulation further enhance its suitability for biofortification strategies (Kicińska and Wikar, 2021). Lettuce is widely cultivated in controlled environment agriculture, including soilless systems (Saavedra Del Real, 2017; Wang et al., 2025), where agronomic biofortification can be efficiently implemented either by enriching the nutrient solution, enabling direct root uptake, or through foliar applications (Giordano et al., 2019; Preciado-Rangel et al., 2022). Several studies have confirmed the effectiveness of these approaches in increasing iron, selenium, and zinc concentrations in lettuce tissues (Giordano et al., 2019; Puccinelli et al., 2022; Ortiz et al., 2024; Marrufo et al., 2025). However, evidence suggests that increasing micronutrient concentrations in the nutrient solution, while enhancing essential mineral accumulation in edible tissues, may partially reduce final yield, highlighting the need to balance mineral enrichment with crop productivity (Giordano et al., 2019). Similar effects have also been reported for foliar applications of zinc and iron in lettuce (Marrufo et al., 2025).

In recent years, innovative agricultural tools such as biochelates have been proposed as alternatives to conventional mineral fertilizers. Biochelates are organic compounds, often peptides or amino acid complexes, capable of chelating micronutrients and exerting a biostimulant effect. In particular, they contain signalling peptides that enhance the uptake and efficient allocation of mineral nutrients within plant tissues (Caetano-Silva et al., 2021; Alzate Zuluaga et al., 2023). For instance, Ortiz et al. (2024) evaluated, in lettuce, the effects of zinc chelated with glycine and citrate (Zn-GLY and Zn-CIT) compared with zinc

sulphate ($ZnSO_4$). The results showed that Zn-GLY led to the highest total zinc accumulation in young leaves and achieved zinc concentrations in mature leaves comparable to those obtained with conventional zinc sources.

Based on these considerations, the present study investigates the potential of foliar-applied iron and zinc biochelates, as well as plant based-protein hydrolysate, to enhance the nutritional quality of lettuce through biofortification in a hydroponic system. This research aims to contribute to the development of nutrient-rich, health-promoting crops as a sustainable strategy to mitigate micronutrient deficiencies worldwide.

2. BIOFORTIFICATION TO REDUCE MICRONUTRIENT DEFICIENCY

2.1. Micronutrients and their biological roles in human health

Micronutrients are a class of nutrients required in small quantities and include individual elements and chemical compounds such as minerals and vitamins (Centers for Disease Control and Prevention, 2025). According to Zhao and Shewry (2011), humans require at least 20 mineral elements, 12 vitamins, 9 amino acids, and 2 fatty acids. Humans cannot synthesize micronutrients, except for vitamin D (Kraemer et al., 2015). Vitamins and mineral elements must therefore be obtained through the daily consumption of fruits and vegetables (Centers for Disease Control and Prevention, 2025). In more detail, micronutrients exert a wide range of biochemical and physiological functions within the human body. Vitamins play various biochemical roles, ranging from hormone-like functions as regulators of mineral metabolism to the regulation of cell and tissue growth and differentiation, while some vitamins also exhibit antioxidant activity. Within the group of mineral elements are included: iron, cobalt, copper, iodine, manganese, selenium, zinc, and molybdenum. The concentration required for human health of the micronutrients described above is less than 100 mg per day (Das and Sen, 2014).

According to Allen et al. (2006), the most widespread micronutrient deficiencies affecting human health, excluding the vitamins, are those related to iron, zinc, iodine, and selenium. The primary physiological roles of these trace elements are presented below.

Iron (Fe) is an essential trace element involved in key physiological and metabolic processes in the human body. It is a key component of haemoglobin, which is responsible for oxygen transport throughout the body, as iron enables the reversible binding of molecular oxygen and thus supports aerobic metabolism (Grzegorzewski et al., 2025). In addition, iron is a fundamental component of the mitochondrial electron transport chain, where it contributes to ATP synthesis, the primary energy currency of cells, thereby supporting cellular energy metabolism (Pal et al., 2026).

Zinc (Zn) is a mineral element required for the catalytic activity of numerous enzymes and is involved in key biological processes such as immune function, protein and DNA synthesis, wound healing, and cell signalling and division (National Institutes of Health, 2023). It contributes to nervous system function, supporting brain health and normal neurological activity. Through its role in enzymatic and metabolic pathways, zinc plays a fundamental role in maintaining proper cellular function and overall physiological homeostasis (Pal et al., 2026).

Iodine (I) is an essential mineral required for the synthesis of thyroxine (T4), a thyroid hormone activated through its conversion to triiodothyronine (T3). Thyroid hormones regulate the metabolic rate of proteins, lipids, carbohydrates, and minerals. Adequate iodine intake is therefore crucial for maintaining normal metabolic function, and iodine deficiency can lead to severe and irreversible developmental disorders (Winder et al., 2022).

Selenium (Se) is mainly involved in immune system function, antioxidant activity, and thyroid hormone regulation. It is a key component of selenoproteins, which protect the organism from oxidative stress. Selenium is also essential for thyroid hormone homeostasis, as it is a component of iodothyronine deiodinases, the enzymes responsible for converting thyroxine (T4) into its biologically active form, triiodothyronine (T3) (Kieliszek and Błażejak, 2015).

2.2. Effects of iron and zinc deficiency in the human health

Inadequate intake of essential micronutrients negatively affects human health. One of the main deficiency pathways is related to the limited intake of iron, zinc, iodine, folate, and vitamin A (Passarelli et al., 2022). Micronutrient deficiencies affect at least one-third of the global population. Iron deficiency is the most prevalent, affecting approximately 2 billion people worldwide and representing the leading cause of anaemia. In addition, iodine deficiency affects nearly 2 billion individuals, while approximately 254 million preschool-aged children are affected by vitamin A inadequacy (Allen et al., 2006).

Iron deficiency anaemia is mainly caused by inadequate dietary intake or impaired absorption during periods of increased iron requirements, such as growth in

children and adolescents and the reproductive years in women, particularly during pregnancy and the postpartum period (Kolarš et al., 2024). It impairs oxygen transport to body tissues, resulting in fatigue, weakness, and reduced concentration. Inadequate iron status during early life can negatively affect cognitive development, while low iron intake during pregnancy is associated with reduced birth weight, increased risk of preterm birth, and higher maternal and neonatal mortality. Finally, iron deficiency compromises immune function, increasing susceptibility to infections (Kolarš et al., 2024).

Zinc deficiency affects multiple physiological processes due to its constitutive role in human physiology. Inadequate zinc intake disrupts cellular and neuronal function, contributing to impaired cognitive development and immune dysfunction (Hussain et al., 2022). Clinically, zinc deficiency manifests as alterations in multiple organs and tissues, including skin, eyes, reproductive and digestive systems, and weakened immune function. Importantly, these effects are largely reversible with adequate zinc supplementation, highlighting the critical role of sufficient zinc intake for maintaining tissue integrity, immune competence, and overall human health (Baddam et al., 2025).

2.3. Iron and zinc in plant metabolism: uptake, transport, storage and toxicity

Iron and zinc are essential elements for plant metabolic and physiological processes (Mir, 2015). Their importance is related to their ability to participate in oxidation-reduction reactions due to their divalent configuration. Iron is involved in the chlorophyll biosynthesis. It plays a key role in nitrogen fixation, DNA replication, scavenging of reactive oxygen species (ROS), and electron transport in both chloroplasts and mitochondria. Iron also acts as a cofactor for numerous proteins and enzymes involved in plant metabolism. Zinc is essential for the metabolism of proteins, nucleic acids, carbohydrates, and lipids. Zn deficiency in plant tissues has been shown to increase the levels of inactive RNAs and starch, indicating that RNA degradation and carbohydrate metabolism are affected by insufficient Zn availability (Mir, 2015).

Iron and zinc availability for the plants depends on the soil physical and chemical characteristics (Khaled and Sayed, 2023).

Plants absorb iron from the rhizosphere through two main strategies: reduction and chelation. The first strategy involves the reduction of Fe^{3+} to Fe^{2+} by the plasma membrane–bound enzyme ferric reductase oxidase, followed by uptake through the iron-regulated transporter 1 (IRT1). In addition, before the reduction occur, plasma membrane H^{+}-ATPases are activated to extrude protons into the rhizosphere, thereby lowering the pH and enhancing the solubilization of soluble Fe^{3+} compounds. The second strategy consists of the release of phytosiderophores into the rhizosphere, which chelate Fe^{3+}. The resulting iron–phytosiderophore complex is subsequently imported into root cells via oligopeptide transporters, such as Yellow Stripe 1 (YS1) (Alzate Zuluaga et al., 2023). With respect to zinc, it is absorbed by plants primarily in the Zn^{2+} form, and its availability in the soil increases as pH decreases. Zinc uptake is mediated by transporters belonging to the ZIP (ZRT–IRT-like Protein) family, which facilitate the entry of Zn^{2+} into root cells. Following uptake, zinc is loaded into the xylem and translocated throughout plant tissues via the transpirational stream (Broadley et al., 2007).

Both elements are transported in the xylem mainly in chelated forms. Iron is primarily translocated as Fe–nicotinamide or Fe–citrate complexes, while nicotinamide can chelate and transport both minerals within the plant. Regarding storage, iron is mainly accumulated in vacuoles and ferritin complexes, with vacuoles representing the principal storage compartment. Zinc is also predominantly stored in vacuoles, as well as bound to protein complexes, contributing to intracellular micronutrient homeostasis (Bhardwat et al., 2022).

While essential for plant metabolism and physiological processes, both iron and zinc can become toxic when present in excessive amounts.

Excessive zinc (Zn) can compromise seed germination, root and stem elongation, reducing overall the plant growth. It alters nitrogen metabolism and enzyme activity, increase the production of antioxidant metabolites, and cause leaf necrosis, potentially leading to plant death (Meng et al., 2023). Similarly, high levels of iron (Fe) can cause toxicity, damaging cell membranes, reducing growth, root

development, photosynthesis, and water and nutrient uptake. It also increases the oxidative stress through the overproduction of reactive oxygen species (ROS). Moreover, iron toxicity is more likely to occur in soils with low pH, high organic matter content, or elevated iron availability (Harish et al., 2023).

2.4. Causes of micronutrient deficiency

Micronutrient deficiency arises from a complex interaction of dietary, socioeconomic, and agronomic factors (United Nations, 2022; Khaled and Sayed, 2023). From a nutritional perspective, insufficient consumption of fruits and vegetables represents primary contributor, as these foods are among the main sources of essential vitamins and minerals (Allen et al., 2006). Diets predominantly based on cereals and pulses are generally energy-dense but nutrient-poor, particularly when the intake of animal-derived products or other micronutrient-rich foods is limited (Pal et al., 2026). Such dietary patterns are especially prevalent among vulnerable populations, who often rely on low-cost staple foods with suboptimal nutritional quality (WHO et al., 2006; FAO et al., 2025).

Socioeconomic factors further exacerbate micronutrient deficiencies. The accessibility and affordability of micronutrient-rich foods are strongly influenced by market prices. Recent increases in extreme weather events and global crises have led to higher prices for staple foods, limiting the ability of vulnerable populations to obtain nutrient-dense foods and prompting a shift toward products with lower nutritional quality (WHO et al., 2006; Kraemer et al., 2015).

From an agronomic perspective, micronutrient deficiency is linked to both historical and contemporary crop production practices.

Since the Green Revolution, plant breeding efforts have primarily focused on increasing yield and optimizing the use of macronutrient fertilizers. While these strategies have successfully enhanced productivity, they have often resulted in a decline in the nutritional quality of crops (Pingali, 2012). In this context, it is well established that maize, wheat, and rice are generally poor sources of essential micronutrients. Rice is characterized by low concentrations of iron, zinc, and provitamin A. Similarly, wheat exhibits limited levels of iron, zinc, selenium, and

provitamin A, and contains high amounts of antinutritional compounds such as phytates, which further reduce micronutrient bioavailability. Likewise, maize is deficient in vitamins A, C, and E and contains antinutritional compounds, contributing to widespread micronutrient deficiencies in populations that rely heavily on these staples (Shahzad et al., 2021).

Only in recent decades, in response to the rising prevalence of mineral-deficiency-related diseases, have breeding programs begun to develop strategies aimed at improving micronutrient uptake by roots and their accumulation in edible plant tissues, while simultaneously reducing the synthesis of antinutritional compounds such as phytates, polyphenols and oxalates, which impair micronutrient absorption in the human digestive tract (Shahzad et al., 2021; Bhardwaj et al., 2022).

From an agronomic perspective, soil chemical and physical properties significantly influence micronutrient bioavailability. High soil pH, calcareous content, and alkaline conditions reduce the availability of essential minerals to plants, resulting in lower micronutrient accumulation and reduced availability for human nutrition and health (Khaled and Sayed, 2023).

Moreover, even when absorbed, micronutrients such as iron and zinc exhibit low phloem mobility, which limits their accumulation in fruits, roots, and grains (Bhardwaj et al., 2022; Sheera et al., 2025).

The interaction between soil-related limitations and the low mobility of micronutrients within the plant represent one of the main agronomic causes of micronutrient deficiencies in edible crops and, together with dietary habits and socioeconomic conditions plays a critical role in determining the prevalence of micronutrient deficiencies at regional and global scales.

2.5. 2030 Agenda and agronomic biofortification

To address micronutrient deficiency the World Health Organization (WHO), in collaboration with other international agencies, has promoted a series of worldwide programs aimed at improving micronutrient intake in both developed and developing countries (Allen et al., 2006; World Health Organization, 2014). In this regard, the 2030 Agenda for Sustainable Development recognizes the eradication

of malnutrition and related micronutrient deficiencies as a central objective. This commitment is embedded within Sustainable Development Goal 2, “Zero Hunger”, which aims to end hunger, ensure food security, and improve nutrition globally (United Nations, 2017).

To achieve these objectives, Allen et al. (2006) describe several strategies to ensure adequate intake of essential vitamins and minerals at the global level, including dietary diversification, food fortification, nutrition education, and supplementation. In this context, biofortification falls within food fortification strategies, as it aims to increase the micronutrient content of the edible parts of plants during growth and development. Biofortification specifically targets crops during their growth phase, whereas conventional food fortification consists of the addition of nutrients to food products after harvest or during processing (Allen et al., 2006; Azeem et al., 2024). Biofortification can be achieved through several approaches including agronomic techniques, conventional breeding methods, and new breeding technologies (Shahzad et al., 2021).

Within agronomic methods, foliar application enables plants to bypass soil-related limitations and enhances the accumulation of micronutrients in edible tissues (Pal et al., 2026). However, not all micronutrients are effectively absorbed through foliar spray. Iron (Fe) and manganese (Mn) are efficiently absorbed, while molybdenum (Mo) and nickel (Ni) are not. Zinc (Zn), boron (B), chlorine (Cl), and copper (Cu) can be applied via both foliar spray and soil application (Sheera et al., 2025).

The direct absorption of foliar-applied fertilizers by leaf tissues reduces nutrient losses and has been successfully used to increase mineral content in cereals such as wheat, maize, and rice, as well as in leafy vegetables (Pal et al., 2026).

Nevertheless, the effectiveness of foliar treatments depends on the plant’s phenological stage, leaf age, and abiotic factors (Bhardwaj et al., 2022). For instance, in wheat, it has been demonstrated that the application of mineral-rich fertilizers during later phenological stages is more beneficial than foliar application at early vegetative stages (Pal et al., 2026).

Environmental factors such as humidity, wind, and temperature have been shown to influence treatment efficacy. High temperature combined with low relative

humidity can reduce foliar absorption due to the rapid water evaporation, micronutrient accumulation on the leaf surface, and reduced mineral permeability (Pal et al., 2026). In addition to these environmental limitations, foliar fertilizers present other drawbacks, including susceptibility to being washed off by rain and higher costs (Sheera et al., 2025).

Despite the potential of agronomic biofortification, practical and economic limitations can constrain its widespread adoption. Bhardwaj et al. (2022) identified these limitations, particularly in terms of economic benefits. While conventional farming practices often rely on fertilizers to enhance yield and productivity, biofortification has minimum effect on crop productivity. Consequently, without any tangible improvement in yield, farmers may be reluctant to invest time and resources in biofortification. Furthermore, the authors highlighted genetic biofortification as the most effective tool for achieving high natural nutrient levels in plants. They also noted that the use of innovative agronomic techniques, such as biochelates, biofertilizers, and nano-fertilizers, can improve nutrient use efficiency and translocation to edible plant parts. In conclusion, the authors emphasized the potential application of agronomic methods to newly developed biofortified crop varieties when soil chemical and physical properties are unfavourable for plant growth.

3. BIOSTIMULANTS AND BIOCHELATES: APPROACHES TO IMPROVE STRESS TOLERANCE AND NUTRITIONAL QUALITY

3.1. Biostimulants: definition, classification and mechanism of action

The earliest scientific interest in biostimulants can be traced back to 1944, when Vladimir Petrovich Filatov introduced the concept of "biogenic stimulants". Filatov proposed that animal and plant tissues, when isolated under certain conditions, accumulate bioactive substances capable of exerting stimulatory effects on metabolic processes (Filatov, 1944). One of the early studies on the mode of action of biostimulants dates to 1991. In that work, Goatley and Schmidt investigated the effects of a freeze-dried extract of *Ascophyllum nodosum* on *Poa pratensis* cv. Plush and determined that biostimulants should be considered materials with no direct fertilizer value but capable of accelerating crop growth when applied at low concentrations (Goatley and Schmidt, 1991). The first definition of biostimulants was introduced by Zhang and Schmidt in 1997. They described biostimulants as substances that, when applied at low concentrations, enhance plant growth (Zhang and Schmidt, 1997). From this early scientific perspective, the concept has progressed toward a legally harmonized definition, now formalized within the EU regulatory framework. According to the current EU Regulation 2019/1009, biostimulants are defined as products that stimulate plant nutritional processes independently of their nutrient content, with the aim of improving at least one of the following aspects: nutrient uptake and use efficiency, tolerance to abiotic stress, crop quality traits, or the availability of nutrients in the soil or rhizosphere.

Biostimulants are natural products derived from organic and inorganic sources that are classified as innovative agricultural tools capable of enhancing plant resilience against biotic and abiotic stresses, which are increasingly associated with climate change, and consist of bioactive compounds whose efficacy depends on the synergistic activity of these molecules on plant physiology (Bulgari et al., 2019).

According to Du Jardin (2015), the major classes of plant biostimulants are:

- humic substances;
- protein hydrolysates;

- seaweed extracts;
- chitosan;
- inorganic compounds;
- beneficial fungi and bacteria.

The following subsections will describe the main biostimulant classes, but it is important to highlight that Juárez-Maldonado et al. (2019) in recent years investigated nanoparticles and nanomaterials with biostimulant effects. These materials should be included in biostimulant classifications to provide a comprehensive overview of biostimulant categories.

3.1.1. Humic substances

Humic substances, derived from the microbial decomposition and chemical transformation of deceased biological matter within the soil, constitute a significant component of soil organic matter. These substances are composed of humic acids, fulvic acids, and humins (Calvo et al., 2014). The biostimulant effect of humic substances include enhanced root growth, increased water and mineral uptake and improved tolerance against abiotic stress factors (Franzoni et al., 2022).

3.1.2. Protein hydrolysates

Protein hydrolysates are mixtures of peptides of various chain lengths and free amino acids obtained through the chemical or enzymatic hydrolysis of proteins derived from animal or plant by-products (Colla et al., 2015). They represent a sustainable strategy for the valorisation of by-products from animal and plant industries (Jolayemi et al., 2022). Proteins in the original biological matrix are present as macromolecules with limited bioavailability for plants. Hydrolysis converts these macromolecules into smaller peptides and free amino acids, increasing their bioavailability and, in many cases, their biological activity (Sun et al., 2020). The molecular weight is an important factor influencing uptake efficiency, as low-molecular-weight peptides and amino acids derived from protein hydrolysates are generally more biologically active and elicit stronger physiological responses in plants compared with larger peptide fractions (Colla et al., 2015).

Regarding the method of protein hydrolysate production, chemical hydrolysis, typically conducted under either alkaline or acidic conditions, is commonly applied to animal-derived residues. Under acidic conditions, hydrolysis is performed using a strong acid, such as hydrochloric acid, at a temperature of 121 °C. In contrast, during alkaline hydrolysis, the protein matrix is solubilized through heating, followed by the addition of alkaline agents, such as calcium, sodium, or potassium hydroxide. Enzymatic hydrolysis, particularly prevalent in the processing of plant-based materials, utilises proteolytic enzymes derived from plants, animals, or microorganisms. This process is characterized by its ability to operate at relatively low temperatures and typically cleaves peptide bonds at specific sites. For instance, papain, an enzyme extracted from *Carica papaya* L. fruit, possesses proteolytic activity that specifically targets the protein chain adjacent to arginine, lysine, and phenylalanine (Colla et al., 2015).

It should be noted that the EU legislation places strict limitations on the use of animal-derived protein hydrolysates on edible plant parts (Jolayemi et al., 2022).

In addition to regulatory constraints, chemical hydrolysis may alter the chemical form of amino acids through racemization, leading to the formation of D-amino acids. While plants can absorb D-amino acids, these forms are generally less efficiently metabolised and exhibit reduced biological activity compared to L-amino acids, which represent the biologically active form in plant metabolism (Colla et al., 2015).

According to Jolayemi et al. (2022), the effectiveness of protein hydrolysate application depends on several factors, including:

- the source of the protein hydrolysate;
- the crop species and cultivar;
- the crop growth stage;
- the application dose and timing;
- leaf permeability and physiological status.

Regarding the application method, biostimulants can be applied via foliar or soil application (Di Sario et al., 2025). Colla et al. (2015) highlighted the limitations of soil application of protein hydrolysates. Plant uptake of protein hydrolysates may

be reduced due to intense competition with soil microorganisms, which rapidly assimilate nitrogen-containing compounds for respiratory metabolism, biomass production, and cellular maintenance. Moreover, plant roots are generally weaker competitors for exogenous amino acids than soil microorganisms. An additional limitation of soil application is that the biological activity of protein hydrolysates may be altered by the secretion of extracellular enzymes by microorganisms, which can further hydrolyse peptide fractions (Colla et al., 2015).

Furthermore, the effectiveness of protein hydrolysate application is strongly influenced by abiotic soil conditions. In particular, abiotic stresses such as mild to moderate water deficit have been shown to reduce root uptake of protein hydrolysates when applied to the soil (Frioni et al., 2022). In contrast, foliar spray application can overcome soil-related limitations by increasing the amount of protein hydrolysates absorbed by leaf tissues, thereby enhancing their biostimulant efficiency. Leaves are able to absorb nutrients and bioactive molecules through the cuticle, stomata, or trichomes, allowing foliar uptake to bypass the soil and root system and ensuring a more rapid and direct effect on plant metabolism (Colla et al., 2015). In this regard, Drobek et al. (2019) suggest applying protein hydrolysates in the early morning, when stomatal conductance is generally higher, thereby favouring nutrient absorption.

Additionally, protein hydrolysates exert both direct and indirect effects on plants. Direct effects include enhanced root and shoot growth, improved photosynthetic performance, and increased crop quality. They can improve plant tolerance to abiotic stresses such as drought, salinity, and the presence of heavy metals in the soil (Colla et al., 2015). Moreover, the enhanced nutrient uptake is accompanied by increased soil microbial and enzymatic activity, as well as improved mobility and solubility of micronutrients, particularly iron, zinc, manganese, and copper. A major effect of protein hydrolysates regards their influence on root architecture by inducing increases in root length, root density, and the number of lateral roots, which ultimately enhance the absorption of nutrients at the root surface (Colla et al., 2015). Indirect effects include an increase in the abundance and activity of telluric microorganisms following biostimulant application. Furthermore, protein

hydrolysates promote the uptake and use efficiency of both micro and macronutrients (Du Jardin, 2015; Bulgari et al., 2019).

In connection with the enhancement of soil and plant enzymatic activity, biostimulant application has been shown to increase the activity of key enzymes involved in nitrogen and iron metabolism, including nitrate reductase, glutamine synthetase, and ferric chelate reductase. These metabolic responses are closely linked to the biochemical composition of protein-based biostimulants, which contain specific peptides and amino acid precursors of phytohormone biosynthesis, such as tryptophan. Through these components, protein hydrolysates can exert hormone-like effects, particularly exhibiting auxin- and gibberellin-like activities, thereby stimulating root development, nutrient assimilation, and overall plant growth (Du Jardin, 2015; Colla et al., 2015).

According to Baltazar et al. (2021), the effects of biostimulants involve both the primary and secondary metabolism of plants. Primary metabolism refers to the synthesis and transformation of carbohydrates derived from photosynthesis, which represent the main source of energy and structural components required for plant growth and development. Secondary metabolism, in contrast, involves the production of bioactive compounds that enable plants to tolerate biotic and abiotic stresses. For instance, the increased synthesis of osmolytes contributes to the alleviation of heat and cold stress, thereby facilitating the maintenance of cellular homeostasis and later enhancing the quality of crop products (Di Sario et al., 2025). Within this latter metabolic pathway, protein hydrolysates have been shown to stimulate the expression of phenylalanine ammoniumyls (PAL), thereby promoting the biosynthesis and accumulation of health-promoting phytocompounds, including ascorbate, tocopherols, carotenoids, and glucosinolates (Colla et al., 2017; Di Sario et al., 2025).

Colla et al., (2017) report several studies demonstrating the effectiveness of protein hydrolysates in improving crop nutritional quality. In *Solanum lycopersicum* L., the application of legume-based biostimulants promoted the accumulation of lycopene, ascorbic acid, and mineral elements. Similarly, in *Vitis vinifera* L., the application of enzymatic plant extracts resulted in increased concentrations of total phenolics

and anthocyanins. Regarding aromatic plants, protein hydrolysates have been shown to enhance the accumulation of citronellal, neral, δ-cadinene, germacrene, and geranial in lemon balm (*Melissa officinalis* L.). Finally, in leafy vegetables such as lettuce (*Lactuca sativa* L.), spinach (*Spinacia oleracea* L.), and rocket (*Eruca sativa* Mill.), protein hydrolysates have been reported to reduce nitrate content, thereby improving nutritional quality and potential benefits for human health (Colla et al., 2017). In line with these findings, several studies have investigated the effects of protein hydrolysates under stress conditions. Di Mola et al. (2019) and El-Nakhel et al. (2023) evaluated lettuce subjected to nitrogen and salinity stress, respectively. In both cases, treatment with protein hydrolysates enhanced plant tolerance and improved antioxidant content, including ascorbic acid, total phenolic acids, total flavonoids, lutein, and β-carotene, without increasing nitrate levels.

3.1.3. Seaweed extracts

Seaweed represents most of the ocean biomass and contains bioactive compounds that can be used in pharmacy, industry and agriculture (Shayen et al., 2023). Most seaweed is commercially harvested in 35 countries, with China, Indonesia, Philippines, Korea, and Japan leading the way. In Europe, macroalgae are collected from natural habitats in France, Ireland, Norway, Portugal, and Spain, with small scale cultivation in France (De Saeger et al., 2020).

Seaweeds are photoautotrophic marine algae, and their seaweed extracts are obtained through alkaline, acidic, or neutral extraction processes and exert multiple beneficial effects on plants such as improvements in growth, yield, and nutritional quality (Stirk et al., 2020). Moreover, they enhance plant tolerance to both biotic and abiotic stresses (Shayen et al., 2023). Seaweeds and seaweed extract can be used as biostimulants, biofertilizers, and soil conditioners (Du Jardin, 2015). According to the current definition of plant biostimulants, seaweed extracts do not act primarily through direct nutrient supply, but by enhancing nutrient use efficiency, stress tolerance, and crop quality via modulation of plant signalling and metabolism (Di Sario et al., 2025).

Brown algae, belonging to the Phaeophyta class, include species such as *Fucus* spp., *Laminaria* spp., *Sargassum* spp., *Ecklonia* spp., *Durvillaea* spp., and *Turbinaria* spp. These species are widely used in agriculture and commercial biostimulant production due to their ability to reach high biomass levels (De Saeger et al., 2020). *Ascophyllum nodosum* (L.) Le Jolis is a widely utilized in the agricultural sector. Polysaccharides constitute the predominant component of brown seaweeds, making it a valuable resource in various agricultural applications. These compounds have been shown to influence plant responses at the transcriptomic, metabolic, and lipidomic levels. Such molecular changes involve multiple pathways and ultimately result in significant phenotypic effects, including enhanced tolerance to oxidative and abiotic stresses, reduced accumulation of reactive oxygen species (ROS), decreased electrolyte leakage, and overall stimulation of plant growth (Baltazar et al., 2021). However, the biological efficacy of seaweed extracts is highly variable and depends on algal species, harvesting period, extraction method, and application rate, which can lead to inconsistent agronomic responses across crops and environments (Baltazar et al., 2021).

Seaweed extracts, depending on the extraction process and the algal species used, are rich in macro and micronutrients, including, potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu) (Shayen et al., 2023). These minerals play essential roles in plant physiological processes. Moreover, seaweed extracts may contain growth-promoting hormone-like compounds that influence plant growth by regulating cell proliferation, elongation, and differentiation. In addition, seaweed extracts contain a wide range of secondary metabolites, such as flavonoids, phenolic compounds, terpenoids, saponins, steroids, and alkaloids, which contribute to improved plant growth and enhanced tolerance to abiotic and biotic stresses. Seaweed extracts also provide proteins, carbohydrates, amino acids, and vitamins that can trigger and support key physiological processes in plants (Shayen et al., 2023).

3.1.4. Chitosan

Chitin is a biopolymer that constitutes crustacean shells, fungal cell walls, and insect exoskeletons. Chitosan is a polysaccharide obtained through the N-deacetylation of chitin. Its structure consists of alternating units of β-(1-4)-linked N-acetylglucosamine (2-acetamido-2-deoxy-β-D-glucopyranose) and glucosamine (2-amino-2-deoxy-β-D-glucopyranose). Currently, the major source of chitin is crustacean waste; however, chitosan derived from *Saccharomyces cerevisiae* has also been evaluated because of its non-allergenic properties in humans (Du Jardin, 2015; Akdaşçi et al., 2025).

According to El Hadrami et al. (2010), chitosan exerts both direct effects on plant pathogens and indirect effects mediated by the activation of plant defence responses. Benhamou et al. (1998) demonstrated that the inhibition of fungal growth is related to the chemical properties of chitosan. Its polycationic nature induces changes in hyphal membrane permeability, resulting in structural alterations of fungal hyphae. In subsequent studies, Amborabé et al. (2008) demonstrated that the primary site of action of chitosan is the plasma membrane H^+-ATPase. This polysaccharide was shown to reduce and inhibit the activity of this membrane protein. Moreover, chitosan affects other H^+-dependent processes, such as the transport of carbohydrates and amino acids into the cell. These combined effects ultimately result in the inhibition of pathogen growth. On the other hand, some fungal populations can counteract the activity of chitosan through the production of chitosanolytic enzymes. Nematophagous and entomopathogenic fungi have been reported to possess such enzymatic activities (Palma-Guerrero et al., 2008).

These direct effects on microorganisms represent only one aspect of chitosan activity, as the compound is also known to act as a biostimulant by triggering defence responses in plants. Plants possess mechanisms to detect pathogenic microorganisms. This recognition relies on transmembrane pattern recognition receptors (PRRs) capable of interacting with pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs). These molecular patterns are conserved structural components of microbial cells. Cell wall

polysaccharides, such as glucans and chitosan, have been reported to function as PAMPs/MAMPs in various plant–pathogen systems (El Hadrami et al., 2010). According to Iriti and Faoro (2009), chitosan behaves as a general elicitor and induces several defence-related physiological responses in plants, including:

- an increase in cytosolic H^+ and Ca^{2+} concentrations;
- activation of mitogen-activated protein kinases (MAPKs);
- callose deposition;
- an oxidative burst;
- induction of the hypersensitive response (HR);
- synthesis of abscisic acid (ABA), jasmonates, phytoalexins, and pathogenesis-related (PR) proteins.

Chitosan activates signal transduction pathways involving hydrogen peroxide (H_2O_2) and nitric oxide (NO), and it can directly influence gene expression through chromatin interactions. Widely used as a biostimulant, chitosan promotes plant growth, enhances abiotic stress tolerance, and induces pathogen resistance. However, these responses depend on chitosan molecular structure, deacetylation degree, concentration, plant species, and developmental stage (Rath et al., 2015).

3.1.5. Inorganic compounds

Within the biostimulant group, inorganic compounds represent a mineral-based class that includes aluminium (Al), cobalt (Co), sodium (Na), selenium (Se), and silicon (Si). These elements are naturally present in soils either as inorganic salts or in insoluble mineral forms, and their availability to plants depends on soil properties and environmental conditions (Du Jardin, 2015).

Silicon (Si) is the mineral with the most promising agricultural application as a biostimulant. Although it has only recently been incorporated into commercial biostimulant formulations and regulatory frameworks, research on silicon has long focused on its ability to enhance crop yield and improve tolerance to abiotic stresses, particularly drought (Henk Maarten, n.d.). Silicon, the second most abundant element in the Earth's crust, is not considered an essential element for plant growth. However, it is present in the soil in the form of aluminium silicate

(Al_2SiO_5) and crystalline silicates. Silicon exhibits a biostimulant effect, enhancing plant growth and photosynthetic efficiency. Additionally, it improves the plant's tolerance to environmental stresses applied in the soil or as foliar treatment (Garcia-Caparros et al., 2025). Silicon exhibits mechanical and metabolic changes in the plant. Mechanical modification on the plant regards "the deposition of silica, leading to the formation of phytoliths within the cell walls of epidermal cells" (Constantinescu-Aruxandei et al., 2020). Silica accumulation in plant cells determines an enhancement of cell thickness that influences the mechanical strength. Moreover, silica influences leaf orientation, enabling the leaf to adopt a position that maximizes photosynthetic efficiency (Garcia-Caparros et al., 2025).
Metabolic changes determined by silica regards its effect on reactive oxygen species (ROS) during stress conditions and improvement of water use efficiency (WUE). In stressful conditions, plants produce reactive oxygen species (ROS). Silicon has been demonstrated to enhance the antioxidant activity of key enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The activity of these antioxidant enzymes safeguards plant cells from ROS-induced damage, preventing the degradation of essential biomolecules such as proteins, lipids, carbohydrates, and DNA. By fortifying antioxidant defence systems, silicon confers enhanced resilience to oxidative stress, thereby supporting plant vigour and adaptability under challenging environmental conditions (Ahmad et al., 2022). In addition to mitigating oxidative damage, silicon has been reported to improve water use efficiency (WUE) under drought conditions by reducing cuticular and stomatal water losses associated with transpiration. This effect is mediated by silicon-induced modifications in both the structural and physiological characteristics of plants (Rea et al., 2022).

3.1.6. Beneficial microorganisms

According to the European Union (2019), plant biostimulant products are classified into microbial and non-microbial biostimulants. Within the microbial category, two main functional groups are recognized: plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF). In accordance with the

Component Material Categories (CMC 7) of the Regulation (EU) 2019/1009, microbial plant biostimulants include microorganisms belonging to *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp., and *Azospirillum* spp.

Plant growth-promoting rhizobacteria, such as *Azotobacter* spp., *Rhizobium* spp., and *Azospirillum* spp., are free-living or endophytic bacteria able to stimulate plant growth through mechanisms including enhanced nutrient uptake, asymbiotic nitrogen fixation, siderophore-mediated iron sequestration, and the production of volatile organic compounds (Pathania et al., 2020). Arbuscular mycorrhizal fungi establish symbiotic associations with plant roots, increasing the effective root surface area and improving nutrient and water uptake (Calvo et al., 2014). These fungi belong to the phylum Glomeromycota, which comprises more than two hundred described species; however, only a limited number are currently exploited in agricultural systems. Consequently, most commercial AMF-based biostimulants include species belonging to the genera *Rhizophagus* and *Funneliformis* (Spatafora et al., 2016; Castiglione et al., 2021). The effectiveness of microbial-based biostimulants depends on several factors, including crop species, stress conditions, soil characteristics, and inoculum formulation. Their main modes of action involve hormonal regulation, modulation of cellular oxidative status, and improvements in water and nutrient use efficiency (Castiglione et al., 2021). Both PGPR and AMF influence the production of key phytohormones and enhance antioxidant defences under stress conditions, thereby mitigating oxidative damage. Furthermore, PGPR can increase the content of photosynthetic pigments, while AMF improve plant water uptake by modulating root hydraulic properties and water transport pathways. AMF-colonized plants can regulate water movement by switching between apo plastic and cell-to-cell pathways, resulting in enhanced water use efficiency under limiting conditions. Ultimately, both PGPR and AMF contribute to improved nutrient use efficiency through mechanisms such as siderophore production, atmospheric nitrogen fixation, and phosphorus solubilization, supporting plant growth and productivity (Castiglione et al., 2021).

3.2. Biochelates: an innovative nutritional strategy for plants

World Health Organization (WHO) and Food and Agriculture Organization (FAO) highlighted the urgent need to mitigate micronutrient malnutrition, which affects populations in both developed and developing countries (Allen et al., 2006). Consequently, research efforts have focused on identifying new strategies to increase micronutrient concentrations in plant tissues or to enhance their bioavailability during digestion (Sun et al., 2020). In this context, biochelates emerge as an innovative solution. They are a peptide-based compounds that effectively chelate divalent micronutrients via coordinate bond, protecting them from precipitation and improving their stability and availability to plants (Caetano-Silva et al., 2021; Leporino et al., 2025).

Biochelates are synthesized from protein matrices derived from industrial and agricultural waste. In their native form, they generally show limited biological activity, whereas enzymatic hydrolysis by specific proteases releases bioactive peptides with enhanced functional properties (Caetano-Silva et al., 2021; Zhang et al., 2023).

The enzymatic hydrolysis, as described in the chapter 3.1.2, simplifies the peptide's complexity, enabling it to acquire physical, chemical, and functional properties. The protease cuts the protein bond in specific target site, breaking it into bioactive peptides and free amino acids. At the end of the enzymatic process, the final mass is composed of peptides of various lengths and molecular masses. To isolate peptides with bioactive properties, the hydrolysate undergoes membrane separation, with ultrafiltration being the most widely applied technique to remove low-molecular-weight analytes and impurities (Zhang et al., 2023). The purified peptide fractions are then further refined using chromatographic methods, such as gel filtration, ion exchange, or reverse-phase chromatography, particularly for metal-binding peptides (Caetano-Silva et al., 2021).

Biochelates enhance micronutrient availability in soils, whose solubility and mobility are influenced by pH. In neutral and acidic soils, availability is generally high, whereas in alkaline soils, iron and zinc precipitate as low soluble hydroxides and carbonates (Wilkinson, 1994; Leporino et al., 2025).

In order to increase the availability of essential elements for plants, synthetic chelates are widely used in agriculture, particularly synthetic chelates such as Ethylenediaminetetraacetic acid (EDTA), Diethylenetriaminepentaacetic acid (DTPA), Ethylenediamine-N,N′-bis 2-hydroxyphenylacetic acid (EDDHA), and Nitrilotriacetic acid (NTA), which complex mineral cations into stable compounds to prevent precipitation in the soil (Leporino et al., 2025).

However, their use presents several drawbacks. Synthetic chelates are poorly biodegradable and can persist in the soil, contributing to environmental pollution. They may also increase the mobility of heavy metals in the presence of non-target minerals and, in some cases accumulate in plant tissues, negatively affecting physiology (Alzate Zuluaga et al., 2023; Leporino et al., 2025). EDTA has been particularly associated with heavy metal mobilization and uptake, toxicity to soil microorganisms and plants, and long-term persistence in the soil, which increases the risk of heavy metal leaching and significant environmental impacts (Evangelou et al., 2007).

In this context, Leporino et al. (2025) conducted a comparative analysis of the effect of a plant-derived biochelate, and a synthetic chelate (EDTA) on *Solanum lycopersicum* L. under alkaline conditions. Micronutrient uptake and metabolic responses were evaluated. Overall plant growth and biomass were similar between the two chelates. However, leaf mineral composition differed: EDTA-based fertilizers showed higher zinc and copper stability at higher rates, while manganese and boron concentrations increased independently of chelate type. Metabolomic analysis revealed a significant effect of biochelates, stimulating pathways involved in phenolic compound synthesis, which are associated with antioxidant defence mechanisms under stress. The results highlight the potential of plant-derived biochelates to modulate micronutrient uptake and enhance stress-related metabolic responses under alkaline conditions with potentially lower environmental impact.

Jie et al. (2008) conducted one of the earliest studies on biochelates, evaluating the effects of foliar application of Fe and Zn-biochelates, synthetic chelates (EDTA), zinc sulphate ($ZnSO_4$), and iron sulphate ($FeSO_4$) on *Oryza sativa* L. The results showed that amino acid–chelated Zn and Fe fertilizers increased growth parameters

by 22–73%, whereas EDTA-chelated Zn and Fe fertilizers resulted in increases of 15–63%. In contrast, $ZnSO_4$ and $FeSO_4$ treatments increased growth parameters by 11–35% compared with the control. In addition, fertilizer application increased chlorophyll content by 11–17%, 3–6%, and 8–12% for amino acid chelates, EDTA chelates, and inorganic salts, respectively, relative to the control. The results showed the potential of biochelates to increase micronutrient content compared with commercial fertilizers and synthetic chelate. Moreover, biochelates have been shown to exert biostimulants effects under abiotic stress condition. In this context, Haghighi et al. (2023) evaluated the foliar application of calcium chelated with tryptophan (Ca–Trp) on *Brassica oleracea* var. *italica* under salt stress. The authors demonstrated that mineral–amino acid complexes act not only as nutrient carriers but also as functional inputs capable of modulating plant physiological and biochemical responses. Consistent with this findings studies on *Cucumis sativus* L. have shown that Fe biochelates derived from spent coffee grounds were more effective in biofortifying cucumbers in greenhouse soil than synthetic bio-chelates (Navajas-Porras et al., 2024).

Together, these studies indicate that protein–based biochelates represent a viable and sustainable alternative to synthetic chelates in horticultural systems, and that their effectiveness in delivering micronutrients to plants can be exploited to produce biofortified crops.

3.3. Biostimulant applications in soilless systems

Sustainable approaches in the agricultural sector are gaining increasing attention, particularly with respect to soilless culture systems, in which biostimulant products can be used to enhance the commercial and nutritional quality of crops. Greenhouses represent the primary setting for soilless systems, as the control of abiotic factors such as temperature, light quality, photoperiod, humidity, and water availability allows precise management of plant growth (Gruda, 2022).

Soilless systems are defined as innovative production methods that eliminate the direct interaction between soil and roots. In these systems, soil is replaced by inert

substrates while essential nutrients are supplied through a nutrient solution which is directly controlled by agrotechnicians (Malorgio et al., 2022).

They are commonly classified according to the type of substrate: liquid substrate cultivation and solid substrate cultivation. Within the first category, the main techniques include the floating system, the nutrient film technique (NFT), and aeroponics (INDIRE, 2017). These production systems allow roots to grow in direct contact with the nutrient solution, thereby facilitating nutrient uptake and supporting plant growth, a condition that also enhances the efficiency of nutrient-based strategies such as biofortification by minimizing losses and soil-related physical, chemical, and biological interactions (Rouphael and Kyriacou, 2018).

The main advantages associated with soilless systems include increased yield per unit area, standardization of the production process, extension of the market supply period, improved water and nutrient use efficiency, and enhanced commercial quality traits (Malorgio et al., 2022).

Biostimulants can be easily applied in soilless systems, either as foliar sprays or directly incorporated into the nutrient solution and represent an agronomic technique to enhance both productivity and nutritional quality of crops grown in these systems. Since hydroponic systems are conducted in protected environments, foliar application of biostimulants can be performed at any time, as the system is completely independent of external abiotic factors (Koukounaras et al., 2020).

Colla et al. (2013) evaluated the effect of different doses of a plant-derived protein hydrolysate in lettuce (*Lactuca sativa* L.) and maize (*Zea mays* L.) cultivated in a floating system. In lettuce, the effect of the biostimulant was also evaluated under induced nutrient stress. The protein hydrolysate was applied as a foliar spray in lettuce and incorporated into the nutrient solution in maize. The results revealed that the protein hydrolysate was effective in increasing the SPAD index in maize, whereas in lettuce under non-stress conditions no significant differences were observed. Conversely, under induced nutrient stress in lettuce, the protein hydrolysate enhanced the SPAD index compared to the control.

Similarly, Vernieri et al. (2005) investigated the effect of nutrient-induced stress in *Eruca sativa* Mill. cultivated in a floating system. In their study, a plant-based

biostimulant, Actiwave® (Valagro S.p.A.), was applied through the nutrient solution to evaluate its impact on crop performance and quality. The results showed that the application of the biostimulant during nutrient stress promoted yield, reaching levels comparable to the control under non-stress conditions.

Overall, soilless systems aim to increase the sustainability of agricultural production, while biostimulant products and biofortification strategies applied in such systems, as evidenced above, can be effectively integrated and have significant positive effects on crop productivity.

4. LETTUCE: FROM NUTRITIONAL PROFILE TO MINERAL BIOFORTIFICATION STRATEGIES

Lettuce (*Lactuca sativa* L.), characterized by its distinctive shape and diverse colours, stands as one of the most widely consumed vegetables globally. Its consumption is associated with a low caloric intake, primarily due its high-water content, which account for approximately 94-96% of its fresh weight (Kim et al., 2016). Despite its core production being confined to mid and subtropical regions, lettuce cultivation encompasses various methods, including open field, greenhouse, soil-based systems, and hydroponics. The latter approach effectively eliminates the growth limitations imposed by climatic factors such as light intensity and soil composition (Saavedra Del Real, 2017).

According to the Food and Agriculture Organization (FAO) in 2024, the area harvested, yield, and production of lettuce and chicory amounted to 1.26 million hectares, 22.27 tons per hectare, and 28.08 million tons, respectively. China emerged as the leading producer in this plant category, with a yield of 14.90 million tonnes destined for internal consumption. Following China, the United States produced 4.71 million tonnes, while India contributed 1.17 million tonnes. As reported by the Food and Agriculture Organization (FAO) for the 2023 dataset, Spain, Italy, and Belgium were the main lettuce producers in Europe, with production levels of 0.86 million tonnes in Spain, 0.66 million tonnes in Italy, and 0.53 million tonnes in Belgium. In Italy, as indicated in the Italian National Institute of Statistics (2024), the production of lettuce reached 0.52 million tons, distributed as follows: 0.33 million tons cultivated in open fields and 0.19 million tons cultivated in greenhouses.

Regarding commercialization, lettuce follows Regulation (EU) 543/2011, which specifies the included cultivars, namely *Lactuca sativa* L. var. *capitata*, *Lactuca sativa* L. var. *longifolia* Lam., and *Lactuca sativa* L. var. *crispa*.

Lettuce is predominantly consumed raw, which preserves heat-sensitive phytochemicals. Depending on the cultivar selected, it is a rich source of folate (vitamin B9), vitamin C, beta-carotene, and phenolic compounds, all known to

support mineral bioavailability and human health (Kim et al., 2016; Putriani et al., 2020). According to the European Food Safety Authority (EFSA), the Average Requirement (AR) for folate and vitamin C in adults is 250 µg dietary folate equivalents (DFE) per day and 90 mg per day, respectively.

Lettuce contains approximately 73 µg of folate per 100 g fresh weight (FW) and about 4 mg of vitamin C per 100 g FW. β-carotene provides provitamin A activity, and β-carotene and lutein are the main carotenoids present in lettuce. The vitamin A content of lettuce is approximately 166 µg retinol equivalents (RE) per 100 g FW. The Average Requirement for vitamin A is 570 µg RE day^{-1} for adult men and 490 µg RE day^{-1} for pre- and post-menopausal women, while pregnant women require 540 µg RE day^{-1} (Mampholo et al., 2016; European Food Safety Authority, n.d.). High β-carotene content in lettuce may reduce the risk of cancer, cataract, cardiovascular diseases and stroke.

In addition, lettuce is also a source of essential elements (potassium, calcium, phosphorus, magnesium, iron, zinc), polyunsaturated fatty acids and low-digestibility carbohydrates (Kim et al. 2016). Its phenolic compounds, particularly flavonoids and phenolic acids, possess strong antioxidant activity, comparable to that of vitamin C, and may protect against cardiovascular and degenerative diseases. Flavonoids additionally exhibit antiviral, anti-inflammatory, cytostatic, cytotoxic, antimutagenic, and anticarcinogenic effects (Mampholo et al., 2016; Boutahiri et al., 2024).

Moreover, lettuce commercial quality is subject to its nitrate content. High consumption of lettuce may be associated with elevated nitrate intake, which may pose potential health risks. The source of nitrate intake is the consumption of leafy vegetables such as lettuce and spinach. Nitrate is transformed into nitrites by microorganisms in the oral cavity and gastrointestinal tract. Nitrites can oxidize myoglobin, determining the formation of methaemoglobin. High concentrations of methaemoglobin affect the erythrocytes' oxygen transport activity. In addition, nitrate intake may contribute to the formation of nitrosamines, some of which are recognized as carcinogenic compounds (European Food Safety Authority, 2017).

For the reason described above, the European Union has established in the regulation (EU) No. 1258/2011 the maximum allowable nitrate content in lettuce:

- 4000-5000 mg kg^{-1} fresh weight (FW) during the winter season;
- 3000-4000 mg kg^{-1} FW during the summer season.

For 'Iceberg' lettuce, the maximum nitrate content is set at 2000 mg kg^{-1} FW for crops grown in the open field and 2500 mg kg^{-1} FW for greenhouse-grown crops.

The regulation doesn't highlight the functional quality aspects that are crucial for consumers. Organoleptic, nutritional, and bioactive compound values are absent. These latter aspects are important to attract the consumer interest (Kyriacou and Rouphael, 2018)

Despite its high nutritional and health-promoting value, lettuce yield and quality are strongly dependent on environmental conditions during cultivation.

Abiotic stresses such as drought, salinity, and extreme temperatures determine a negative effect on the plant physiology, determining mainly a decrease in the production of primary metabolites, producing a reduction in commercial quality and yield (Boutahiri et al., 2024). In particular, exposure to temperature around 30 °C results in reduced yield and chlorophyll content due to the generation of stress in the photosystem II, a sensitive component of the photosynthetic process (Zhou et al. 2022). Moreover, according to Jenni and Yan (2009), drought stress in lettuce causes rib discoloration, tip burn, premature blooming and ribbiness, all of which reduce the commercial value of the final product.

Lactuca sativa L. is an ideal model crop for biofortification due its widespread consumption, rapid growth cycle, and high capacity for mineral accumulation (Kim et al., 2016).

Several scientific publications have documented the supply of iron and zinc via both root uptake and foliar application. Sahin (2021) showed that biofortification of lettuce, cultivated in a soilless system, can be achieved by adding zinc, selenium, and iodine. In particular, the addition in the nutritive solution of 50 mM Zn ($ZnSO_4 \cdot 7H_2O$), 150 mM I ($NaIO_3$), and 20 mM Se (Na_2SeO_4) led to a significant increase in zinc and selenium content in lettuce tissues. Similarly, Giordano et al. (2019) reported comparable results. They investigated the effects of two lettuce

cultivars exposed to three doses of synthetic chelated iron in the nutrient solution (0.5 mM, 1.0 mM, and 2.0 mM). Their findings showed a proportional relationship between the iron content in lettuce and the supplied iron dose. However, increasing iron content led to a decrease in yield. The optimum iron dose was 1.0 mM, which corresponded to a 21% increase in iron content but caused a 13.3% reduction in yield. In addition, the lettuce cultivars showed different levels of iron accumulation. Specifically, red leaf lettuce has shown higher iron content than green leaf lettuce.

With respect to lettuce biofortification through foliar treatments, Marrufo et al. (2025) investigated the effects of different iron and zinc sulphate applications on *Lactuca sativa* L. cv. Parris Island, grown using a nutrient film technique (NFT). The trial consisted of supplying various mixed treatments with different zinc and iron doses. Their findings showed that zinc and iron contents exhibited an inversely proportional relationship when Zn–Fe doses were increased. At a concentration of 200 μM, the iron content in lettuce increased, while the zinc content decreased significantly. The optimal zinc and iron content corresponded to 50 μM Zn-Fe, indicating a possible competition between iron and zinc at the leaf tissue level.

Currently, alternative mineral enrichment approaches are being evaluated. Puccinelli et al. (2022) studied the effect of selenium application at the pre-transplanting phase on lettuce and basil. The results showed that increasing the selenium dose in the seedling substrate promoted selenium accumulation in plant tissues. This technique could potentially be applied for iron and zinc biofortification. Moreover, the authors demonstrated that selenium fertilization also affects zinc and iron contents. In fact, higher doses of selenium corresponded to increased zinc and iron concentrations both at the transplant stage and at harvest.

Another innovative agricultural tool which led to the substitution of mineral fertilization is the biochelates. Ortiz et al. (2024) evaluated the effect the zinc chelate with glycine and citrate (Zn-GLY, Zn-CIT) compared to zinc sulphate ($ZnSO_4$) on lettuce cultivated in an acid soil. The authors observed that Zn-GLY achieved the highest total zinc content in young leaves, reaching similar levels in mature leaves achieved with the traditional commercial zinc source.

Overall, the available evidence indicates that several methods are being applied to *Lactuca sativa* L. to improve its biofortification potential. While the timing of mineral application has been widely investigated in cereal crops, this aspect remains poorly explored in lettuce, likely due to its short life cycle.

5. AIM OF THE RESEARCH

The research evaluated the effects of foliar applications of biochelates and vegetal peptides on two *Lactuca sativa* L. cultivars grown in a nutrient film technique (NFT) system during two growth periods. During the first period (March–June), under optimal growth conditions, iron and zinc biochelates were applied, whereas during the second period (May–August), under heat stress, zinc and iron biochelates as well as a vegetal peptides treatment were applied. Morphophysiological, productivity, and qualitative parameters of lettuce were assessed in both periods.

6. MATERIAL AND METHODS

The research trial was conducted at the University of Agriculture in Kraków (Poland) in collaboration with the University of Tuscia (Viterbo, Italy). Two lettuce (*Lactuca sativa* L.) cultivars belonging to the Salanova® group (Aquino RZ and Barlach RZ), from Rijk Zwaan (Bologna, Italy), were used. The trial was divided into two distinct growth periods: March–June (Experiment 1), under optimal growth condition, and May–August (Experiment 2) characterized by high temperature from transplanting to the harvest. The nutrient film technique (NFT) was used as a cultivation system. During the first cycle, the NFT system was managed inside a tunnel greenhouse covered with PVC plastic film, whereas during the second cycle the NFT system was implemented within a glass greenhouse. The collected samples were analysed at the Plant Nutrition Laboratory of the Faculty of Biotechnology and Horticulture at the University of Agriculture in Kraków.

6.1. EXPERIMENT 1

During the first growth cycle, a nutrient film technique was used with channels composed by aluminium, with dimension of 3.0 m x 0.50 m x 0.15 m each. The lines were covered with a white plastic sheet and connected to a 200 L polyethylene tank, positioned downstream for recirculation of nutrient solution. Mineral composition of the recirculating nutrient solution having pH of 6.0 is shown in the Table 1.

Table 1. Formulation of the nutrient solution.

Fertilizer	**g L^{-1}**
Calcium nitrate (15.5% N; 26.3% CaO)	0.58
Monopotassium phosphate (22.7% P; 28.2% K)	0.18
Potassium sulphate (51% K2O; 45% SO3)	0.29

Magnesium sulphate (16% MgO; 32.5% SO3)	0.36
Sodium tetraborate decahydrate (11.34% B; 12.06% Na)	0.01
	mg L^{-1}
Chelated iron (6% Fe-EDTA)	1.85
Zinc sulphate (21% Zn; 10% S)	0.88
Manganese sulphate (31.04% Mn; 18.79% S)	1.69
Copper sulphate (25.45% Cu; 12.82% S)	0.35
Sodium molybdate (39% Mo; 30% Na)	0.08

Two sizes of polystyrene trays were used to support the crops:

- 6 trays measuring 1 m x 0.17 m x 0.02 m;
- 3 trays measuring 1 m x 0.11 m x 0.02 m.

This arrangement allows the entire surface of the channel to be covered. Each tray had 5 holes at the end plant site, spaced 21 cm apart, allowing each channel to accommodate 42 plants arranged in a quincunx pattern. A closed-loop NFT system was used during the trial. Non-distilled water was used to prepare the nutritive solution, with a volume of 180 L per tank. The nutrient salts were dosed using the following equipment: macronutrients, quantified in grams, were weighed using a PS-50M technical scale (CELY), while micronutrients, quantified in milligrams, were weighed using an Adventurer Pro (OHAUS) analytical scale.

Initially, 40 mL of concentrated nitric acid (65%, POSH-BASIC) was added to the nutrient solution. The pH was then measured using a CP-505 pH meter (ELMETRON). Since the initial pH was not within the optimal range, 10 mL of nitric acid was added at a time until a pH value close to 6 was reached. In total, 75 mL of nitric acid (65%, POSH-BASIC) was added.

For the circulation of the nutrient solution, a submersible pump model HSB-1500 (Hasbao®) was used. The irrigation regime was established using an automatic timer, consisting of irrigation events lasting 3 minutes each and repeated every two hours throughout the day. On 29 May 2024, due to a decrease in the nutrient solution level, 25 L of new solution were added to each tank.

Lettuce seeds were sown on 20 March 2024. Four perforated rock wool panels (160 holes each) were used. One seed per hole is covered by sterilized quartz sand, sub-irrigated in the greenhouse. The sterilization of quartz sand was carried out by washing with running water and heat treatment in SNOL 8.2/1100 LHM01 oven for 60 mins.

Lettuce seedlings were transplanted at the six-leaf stage, 20 days after sowing (DAS), on 9 April 2024. Within each channel the two *Lactuca sativa* L. cultivars under study were arranged as follows:

- Plot I: 4 Barlach RZ plants and 9 Aquino RZ plants;
- Plot II: 5 Barlach RZ plants and 10 Aquino RZ plants;
- Plot III: 5 Barlach RZ plants and 9 Aquino RZ plants.

A randomized complete block design was used to test the factorial combination of two lettuce cultivars with three foliar treatments i.e. control (non-distilled water), zinc and iron biochelate (9% Zn; KeylanZn, 11% Fe; KeylanFe, Hello Nature, Italy) with 5 replicates.

The first foliar treatment was carried out 30 days after transplant (DAT), on 9 May 2024. The others were conducted at weekly intervals at 37, 44, and 51 DAT. The harvest was performed on 3 June 2024 at 55 DAT.

Zinc and iron biochelates were dissolved in non-distilled water at a concentration of 1.5 g L^{-1}. The dosage of biochelates was measured using an analytical balance, model AS 82/220.R2 (RADWAG). The solutions were prepared on the same day of application to ensure their stability and efficacy. The treatments were applied using a manual pressure sprayer Super Venus 2.0 L (Kwazar). During application, special care was taken to evenly spray both sides of the leaves. To limit the risk of cross-contamination between adjacent plots, the surrounding areas were protected with plastic film.

6.2. EXPERIMENT 2

The experimental trial was conducted in a glass greenhouse equipped with five sun-tracking shading nets, two fans for air recirculation, and an opening roof with movable panels at the upper section to ensure proper air circulation and cooling.

The NFT system was provided by NETAFIM IRYGACJA and consisted of three channels, each measuring 5.0 m x 0.3 m in length and width. During the trial, only the lateral channels were used. Each channel contained polystyrene trays measuring 1 m x 0.17 m x 0.02 m, perforated at 21 cm intervals. Four trays were placed in each channel.

Each tray was assigned to a specific treatment: control (non-distilled water), zinc and iron biochelates, or a vegetal peptide treatment. Five plants were placed in each tray. The surface of each channel was completely covered by polystyrene trays to prevent algae formation.

A closed-loop NFT system was used during the trial. Non-distilled water was used to prepare the nutritive solution, with a volume of 400 L per tank. The nutrient salts (Table 1) were dosed using the following equipment: macronutrients, quantified in grams, were weighed using a PS-50M technical scale (CELY), while micronutrients, quantified in milligrams, were weighed using an Adventurer Pro (OHAUS) analytical scale.

Initially, 15 mL of concentrated nitric acid (65%, POSH-BASIC) was added to the nutrient solution. The pH was then measured using a CP-505 pH meter (ELMETRON). Since the initial pH was not within the optimal range, 10 mL of nitric acid was added at a time until a pH value close to 6 was reached. In total, 85 mL of nitric acid (65%, POSH-BASIC) was added.

For the circulation of the nutrient solution, a submersible pump model JETS80 (Malec-pompy) was used. The irrigation regime was established using an automatic timer, consisting of irrigation events lasting 20 second each and repeated every 9 min throughout the day. On 23 July 2024, due to a decrease in the nutrient solution level, 50 L of new solution were added to each tank.

Lettuce seeds were sown on 29 May 2024. Four perforated rock wool panels (160 holes each) were used. One seed per hole is covered by sterilized quartz sand, sub-

irrigated in the greenhouse. The sterilization of quartz sand was carried out by washing with running water and heat treatment in SNOL 8.2/1100 LHM01 oven for 60 min.

Lettuce seedlings were transplanted 28 DAS, on 25 June 2024. Each channel was divided into four plots, each containing 10 plants.

A randomized complete block design was used to test the factorial combination of two lettuce cultivars with four foliar treatments i.e. control (non-distilled water), zinc and iron biochelates (9% Zn; KeylanZn, 11% Fe; KeylanFe, Hello Nature, Italy), and a vegetal peptide (10% Total N, 10% Organic N, 62% vegetal peptides; Aquamin, Hello Nature, Italy) with 8 replicates.

Zinc and iron biochelates were dissolved in non-distilled water at a concentration of 1.5 g L^{-1}, whereas the vegetal peptide was applied at the dose of 0.75 g L^{-1}. The amount of biochelates and the peptide was measured using an analytical balance, model AS 82/220.R2 (RADWAG). The solutions were prepared on the same day of application to ensure their stability and efficacy. The treatments were applied using a manual pressure sprayer Super Venus 2.0 L (Kwazar). During application, special care was taken to evenly spray both sides of the leaves. To limit the risk of cross-contamination between adjacent plots, the surrounding areas were protected with plastic film. In total, five foliar treatments were applied at weekly intervals, starting at 8 DAT on 3 July 2024, and repeated at 15, 22, 29, and 36 DAT. The final treatment was performed on 31 July 2024, one day before harvest on 1 August 2024 (38 DAT).

6.3. QUANTITATIVE ANALYSIS

Lettuce plants in Experiment 1 and Experiment 2 were cut at the collar, and the fresh weight was measured through the AM-15 (EXCELL Computing Scale) technical balance. The butterheads were cut lengthwise, and only one of the two sections obtained was used for the following analysis. The remaining section was discarded. Subsequently, the sections belonging to each cultivar and treatment were mixed separately to homogenize the tissue intended for analysis. The resulting material was then washed with running water, chopped, and gently dried. A portion

of the fresh material was used to determine dry matter content. The remaining fresh material was subjected to three different chemical extraction procedures.

Ethanol extraction was performed to determine sugar and amino acid contents, phenolic profile, and antioxidant activity using the DPPH assay. In a 250 mL Erlenmeyer flask, 20 g of fresh chopped plant tissue and 80 mL of 96% ethanol were added and mixed thoroughly. The containers were then connected to a reflux condenser (WSL POLAND) and heated to boiling (100 °C), maintaining reflux for 15 min from the start of boil. After the reflux phase, the Erlenmeyer flasks were cooled to room temperature. The residual plant material was further shredded directly inside the flask using an Ultra-Turrax model T18 (IKA), then filtered with filter paper and final volume of the extract was adjusted to 100 mL with ethanol.

Acetic acid extraction was used to analyse nitrate and ammonium contents. Oxalic acid extraction was carried out to quantify vitamin C content. The remaining fresh material was subjected to oven desiccation to determine total nitrogen (Kjeldahl method) and macro- and microelement concentrations. Specifically, the fresh material was dried in a UF 110 (Memmert) forced-air oven at 65 °C. The resulting dry material was then ground using a PC-KSW 1021 grinder (PROFICOOK).

6.3.1. Determination of the dry matter content

Glass drying containers were weighed using an AS 82/220.R2 (RADWAG) precision balance. A small aliquot of fresh plant material was placed in each container, and the total weight was recorded using the same balance. The containers were then closed with their lids and placed in a Venticell 55 drying oven (MMM Group) set at 105 °C with 100% ventilation. The following day, the containers were reweighed, and the percentage of dry matter was calculated using the following equation (Eq.1):

$$\%\ Dry\ Matter\ (DM) = \frac{Dry\ weight\ (DW)}{Fresh\ weight\ (FW)} \times 100 \quad \textit{(Eq. 1)}$$

6.3.2. Determination of the phenolic profile

An aliquot of 0.5 mL of plant extract stored in ethanol was transferred into a test tube. Subsequently, 0.5 mL of 1% HCl diluted in ethanol was added, and the mixture was shaken vigorously to ensure proper homogenization. Then, 9 mL of 2% HCl diluted in distilled water was added, and the solution was mixed again for a few seconds using a TK3S agitator (TECHNOKARTELL). The sample was left to stand to allow stabilization prior to spectrophotometric analysis. Measurements were performed using a U-2900 UV–VIS spectrophotometer (HITACHI) equipped with quartz cuvettes (HELLMA ANALYTICS) at four wavelengths: 280, 320, 360, and 520 nm. Ethanol was used as the blank for instrument calibration. Spectrophotometric data were used to quantify different classes of compounds in the samples, expressed as specific standard equivalents using the following equations (Eq. 2,3,4,5):

$$Total\ phenols\ (mg\ CGA \cdot 100\ g^{-1}\ FW) = \left(\frac{Absorbance\ at\ 280\ nm}{0.264} \times 100\right) \div 1.25 \textit{ (Eq. 2)}$$

$$Total\ phenylpropanoids\ (mg\ CAE \cdot 100\ g^{-1}\ FW) = \left(\frac{Absorbance\ at\ 320\ nm}{0.887} \times 100\right) \div 1.25 \textit{ (Eq. 3)}$$

$$Total\ flavonoids\ (mg\ QE \cdot 100\ g^{-1}\ FW) = (\frac{Absorbance\ at\ 360\ nm}{0.513} \times 100) \div 1.25 \textit{ (Eq. 4)}$$

$$Total\ anthocyanins\ (mg\ CyE \cdot 100\ g^{-1}\ FW) = (\frac{Absorbance\ at\ 520\ nm}{0.645} \times 100) \div 1.25 \textit{ (Eq. 5)}$$

Where:

CGA: Chlorogenic Acid Equivalents;

CAE: Caffeic Acid Equivalents;

QE: Quercetin Equivalents;

Cye: Cyanidin Equivalents.

6.3.3. Determination of antioxidant activity by DPPH assay

For the evaluation of the antioxidant activity of plant samples, the DPPH assay based on the reduction of the free radical DPPH was used. The working solution of DPPH was prepared by mixing 100 mL of stock solution of DPPH with 20 mL of 96% ethanol, ensuring complete dissolution by stirring. In each test tube, 5.9 mL of DPPH solution and 0.1 mL of plant material extracted by ethanol were added. The blank test was performed with ethanol as a control reference. Absorbance measurements of each plant sample were taken at 5 and 30 minutes after the addition of the sample. Spectrophotometric analyses were conducted using a spectrophotometer model UNICAM 5675 (ATIUNICAM), operating at a wavelength of 516 nm. The following equation was used to calculate the antioxidant activity (Eq.6):

$$Antioxidant\ activity\ (\%) = \frac{(A_{516}\ blank\ test - A_{516}\ sample)}{A_{516}\ blank\ test} \times 100 \quad \textit{(Eq. 6)}$$

Where:
A516 blank test is the average absorbance of DPPH without extract;
A516 sample is the absorbance of the plant sample.

6.3.4. Determination of sugar profile

The contents of glucose, fructose and sucrose were assessed in ethanol extracts. Their levels (and their sum as total sugars) were determined by the Beckman Coulter PA 800 Plus capillary electrophoresis system with DAD detection (Smoleń et al., 2019).

6.3.5. Determination of amino acids content

The free amino acid content was determined according to the ninhydrin procedure described by Korenman (1973) and Smoleń et al. (2010). An aliquot of 0.5 cm^3 of plant extract obtained by ethanol extraction was transferred into a test tube, and 3.5 cm^3 of isopropanol and 1.0 cm^3 of 0.2% ninhydrin solution were added. Glycine

was used as the standard. The prepared samples, glycine standards, and a blank (ethanol) were heated in a water bath at 80 °C for 10 min. After cooling, absorbance was measured at 570 nm using a UV spectrophotometer. A calibration curve was constructed based on the absorbance values of the glycine standards, and the results were expressed as mg N 100 g^{-1} of fresh plant material.

6.3.6. Determination of vitamin C content

4 g of chopped plant tissue, weighed with an Adventurer Pro (OHAUS) balance, were placed into an Eppendorf tube with 20 mL of a 2% oxalic acid solution measured using an LMP 10000 pipette (LABMATE PRO). The latter solution was prepared by dissolving 28 g of oxalic acid dihydrate ($C_2H_2O_4 \cdot 2H_2O$) in 1 L of distilled water.

The mixture was homogenized and then centrifuged at 4500 rpm for 15 minutes at 5 °C, using an MPW-380R CENTRIFUGE (MPW MED. INSTRUMENTS). The obtained supernatant was stored in the dark under refrigerated conditions. Subsequently, 1.4 mL of the supernatant, collected using an LMP 5000 pipette (LABMATE PRO), was transferred to a new Eppendorf and centrifuged at 10,000 rpm for 5 minutes.

The capillary was conditioned (prepared for operation) using 1.4 mL of 0.1 M hydrochloric acid (HCl), 1.4 mL of buffer, and 1.4 mL of distilled water. The resulting mixture was then loaded into the wells of the PA 800 Plus capillary electrophoresis system (Beckman Coulter). The 0.2 mL of the sample was analysed at a wavelength of 280 nm using a UV detector. The calibration curve of peak area versus vitamin C concentration was prepared, and the results were determined.

6.3.7. Determination of nitrate and ammonium contents

An acetic acid extraction was performed to determine ammonium and nitrate contents. 5 g of fresh plant tissue were placed in a 500 mL plastic container with 100 mL of 2% acetic acid solution, prepared by diluting 25 mL of 80% acetic acid (CH_3COOH) in 1 L of distilled water. After hermetically sealing the containers, they were placed on a rotary shaker set at 40 revolutions per minute for 30 min. At

the end of the extraction, 30 mL of the solution was transferred to plastic tubes (Sarstedt) for subsequent analysis. The contents of nitrate (NO_3^-), and ammonium (NH_4^+) ions in lettuce leaves were analysed in the 2% acetic acid extracts using an AQ2 discrete analyser (Seal Analytical, USA) (Smoleń et al., 2022).

6.3.8. Determination of total nitrogen and macro- and microelement contents

Total nitrogen content was measured by the Kjeldahl method. During the digestion phase, 0.3 g of dried sample, weighed using an Adventurer Pro analytical balance (OHAUS), were placed in glass digestion tubes. Subsequently, 10 mL of concentrated H_2SO_4 (98%), dispensed using a Hirschmann Laborgeräte EM dispenser, and 0.3 g of catalyst (ratio of 3.5 g of K_2SO_4 to 0.4 g of $CuSO_4 \cdot 5H_2O$) were added. The mixture was subjected to digestion in a Digestor 2020 (FOSS) at 430 °C, allowing complete mineralization of the organic material. After digestion, the samples were cooled to room temperature. During the distillation phase, the digestate was transferred to a UDL 139 distiller (VELP Scientifica). A beaker containing 25 mL of 4% boric acid was placed at the condenser outlet to capture the distilled ammonia. During distillation, the instrument automatically added 55 mL of water and 55 mL of 30% NaOH to alkalize the digestate and release NH_3.
In the final titration phase, the distillate collected in boric acid was stirred magnetically. Titration was performed using a Solarus titrant burette (Hirschmann) with 0.1 N HCl, and a visual indicator was used to detect the end point (stable pink colour). The amount of Kjeldahl nitrogen was calculated with the following equation:

$$N\ \% = \frac{ml\ of\ the\ titrant\ solution \times 14.007 \times N}{mg\ of\ sample} \quad \textit{(Eq. 7)}$$

Where:
N = normality of the titrant solution (0.1);
14.007 = conversion factor.

Approximately 0.3 g of dried lettuce leaves were mineralized in 65% super-pure HNO_3 using a MARS-5 Xpress microwave digestion system (CEM, USA). Then, after cooling, the samples were quantitatively transferred into 25 mL volumetric flasks, and the mineral content was subsequently determined using a high-dispersion inductively coupled plasma optical emission spectrometer (ICP-OES; Prodigy Teledyne Leeman Labs, USA) (Kalisz et al., 2019).

7. STATISTIC ANALYSIS

A two-way analysis of variance (ANOVA) was performed to evaluate the significance of the effects of cultivar, treatment and their interaction using SPSS 10 software for Windows (SAS Inc., Cary, NC, USA). Before analysis, the experimental data were checked for normal distribution and homogeneity of variance using Levene's test. All data are presented as mean ± standard error (SE). The Tukey's post hoc test (p=0.05) was adopted for the comparison between the means of each measured variable.

8. RESULTS

8.1. Yield and dry matter content

8.1.1. Experiment 1

Results of iron and zinc biochelate treatments on shoot fresh weight, shoot dry weight, and shoot dry matter are presented in Table 2.

A significant difference was observed in shoot fresh weight and shoot dry matter with respect to the cultivar effect. The cultivar 'Aquino' showed a significantly higher shoot fresh weight compared to the cultivar 'Barlack'. Conversely, 'Barlack' exhibited a significantly higher shoot dry matter content. Biochelate treatments did not induce any significant effect on shoot FW, shoot DW and shoot DM. Similarly, the Cultivar x Treatment interaction did not show any significant effect.

Table 2. Effect of cultivar and fertilizer treatment on shoot fresh weight, shoot dry weight, and dry matter content in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Shoot dry matter (%)
Cultivar			
Aquino	323.02 ± 4.45 a	13.61 ± 0.19	4.22 ± 0.03 b
Barlack	259.21 ± 7.38 b	13.16 ± 0.37	5.08 ± 0.05 a
Treatment			
Control	305.18 ± 7.96	13.60 ± 0.32	4.66 ± 0.16
Fe-biochelate	298.16 ± 6.92	13.41 ± 0.27	4.65 ± 0.12
Zn-biochelate	301.71 ± 7.80	13.37 ± 0.32	4.63 ± 0.17
Cultivar x Treatment			
Aquino x Control	327.51 ± 7.81	13.82 ± 0.34	4.22 ± 0.03
Aquino x Fe-biochelate	317.78 ± 6.90	13.64 ± 0.30	4.30 ± 0.05
Aquino x Zn-biochelate	323.37 ± 8.36	13.37 ± 0.36	4.13 ± 0.05
Barlack x Control	257.61 ± 14.33	13.14 ± 0.72	5.10 ± 0.11
Barlack x Fe-biochelate	259.78 ± 11.99	12.98 ± 0.56	5.01 ± 0.06
Barlack x Zn-biochelate	260.21 ± 12.49	13.37 ± 0.64	5.13 ± 0.08
Significance			
Cultivar	***	ns	***
Treatment	ns	ns	ns
Cultivar x Treatment	ns	ns	ns

*Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.1.2. Experiment 2

Results of peptide and iron and zinc biochelate treatments on shoot and root fresh weight, dry weight, and dry matter are presented in Table 3.

The cultivar effect was shown to have a significant impact on both shoot (fresh and dry weight) and root (fresh weight, dry weight, and dry matter) parameters. In particular, the cultivar 'Aquino' produced the highest values for both shoot (fresh and dry weight) and root (fresh and dry weight) parameters. Conversely, the cultivar 'Barlack' exhibited a significantly higher root dry matter content.

The treatment factor was shown to have a significant impact on both shoot and root fresh weight. The highest shoot fresh weight was observed in the control treatment, whereas the lowest was recorded following Zn-biochelate application. Treatments with Fe-biochelate and Peptide showed intermediate values and were not significantly different from either the control or the Zn-biochelate treatment. In contrast, regarding root fresh weight, the control treatment exhibited the lowest value, whereas Fe-biochelate and Peptide treatments showed the highest value and did not differ statistically from each other. Zn-biochelate showed intermediate values.

The interaction Cultivar x Treatment was shown to significantly affect shoot dry weight, root fresh weight, and root dry weight. All treatments in cv. 'Aquino' resulted in higher shoot dry weight than cv. 'Barlack' with no significant differences among treatments. Moreover, iron and zinc biochelate and peptide treatments, when applied to cv. 'Barlack', showed a reduction in the shoot dry matter compared to the control, with no differences from each other. With regard to root fresh weight, Aquino x Zn-biochelate and Aquino x Peptide treatments produced the highest values. On the contrary, all the treatments related to the cv. 'Barlack' showed the lowest values with no statistically significant differences among them.

Finally, root dry weight significantly varied among treatments. The highest value was observed in the combination of Aquino x Peptide, while Aquino x Zn-biochelate produced comparable values with no statistical difference. The lowest

values were recorded in Barlack across all treatments, whereas intermediate values were found in Aquino x Fe-biochelate.

Table 3. Effect of cultivar and fertilizer treatment on shoot and root fresh weight, dry weight, and dry matter content in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	Shoots			Roots			Root to shoot
	Fresh weight (g plant^{-1})	Dry Matter (%)	Dry weight (g plant^{-1})	Fresh weight (g plant^{-1})	Dry Matter (%)	Dry weight (g plant^{-1})	
Cultivar							
Aquino	191.92 ± 2.53 a	4.50 ± 0.10	8.62 ± 0.12 a	16.74 ± 0.33 a	4.10 ± 0.05 b	0.69 ± 0.01 a	0.08 ± 0.00
Barlack	133.47 ± 2.71 b	4.35 ± 0.06	5.81 ± 0.12 b	9.53 ± 0.27 b	4.66 ± 0.08 a	0.44 ± 0.01 b	0.08 ± 0.00
Treatment							
Control	170.48 ± 4.55 a	4.36 ± 0.10	7.43 ± 0.20	12.26 ± 0.41 b	4.49 ± 0.16	0.53 ± 0.02	0.07 ± 0.00
Fe-biochelate	165.99 ± 5.09 ab	4.37 ± 0.15	7.12 ± 0.23	13.87 ± 0.63 a	4.35 ± 0.14	0.55 ± 0.02	0.08 ± 0.00
Zn-biochelate	157.38 ± 5.51 b	4.55 ± 0.13	7.19 ± 0.25	13.14 ± 0.64 ab	4.41 ± 0.14	0.57 ± 0.02	0.08 ± 0.00
Peptide	160.9 ± 4.72 ab	4.42 ± 0.10	7.28 ± 0.25	13.89 ± 0.66 a	4.35 ± 0.13	0.59 ± 0.03	0.08 ± 0.00
Cultivar x Treatment							
Aquino x Control	196.02 ± 5.66	4.30 ± 0.18	8.43 ± 0.27 a	14.39 ± 0.43 b	4.10 ± 0.06	0.59 ± 0.02 c	0.07 ± 0.00
Aquino x Fe-biochelate	190.41 ± 4.51	4.39 ± 0.28	8.33 ± 0.24 a	16.83 ± 0.71 ab	4.00 ± 0.11	0.65 ± 0.03 bc	0.08 ± 0.00
Aquino x Zn-biochelate	186.40 ± 5.76	4.69 ± 0.24	8.71 ± 0.25 a	17.25 ± 0.71 a	4.11 ± 0.06	0.71 ± 0.03 ab	0.08 ± 0.00
Aquino x Peptide	194.84 ± 4.19	4.63 ± 0.09	9.01 ± 0.19 a	18.51 ± 0.59 a	4.15 ± 0.14	0.77 ± 0.02 a	0.08 ± 0.00
Barlack x Control	144.93 ± 4.26	4.42 ± 0.12	6.43 ± 0.21 b	10.09 ± 0.52 c	4.78 ± 0.18	0.48 ± 0.02 d	0.07 ± 0.00
Barlack x Fe-biochelate	131.10 ± 6.12	4.36 ± 0.15	5.67 ± 0.25 c	10.28 ± 0.68 c	4.61 ± 0.13	0.47 ± 0.03 d	0.08 ± 0.01
Barlack x Zn-biochelate	124.22 ± 6.13	4.41 ± 0.08	5.59 ± 0.26 c	8.82 ± 0.47 c	4.70 ± 0.19	0.41 ± 0.02 d	0.07 ± 0.00
Barlack x Peptide	132.01 ± 5.00	4.20 ± 0.09	5.50 ± 0.22 c	9.03 ± 0.50 c	4.55 ± 0.17	0.41 ± 0.02 d	0.08 ± 0.00
Significance							
Cultivar	***	ns	***	***	***	***	ns
Treatment	*	ns	ns	*	ns	ns	ns
Cultivar x Treatment	ns	ns	*	***	ns	***	ns

*Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.2. Soluble carbohydrates

8.2.1. Experiment 1

Results of iron and zinc biochelate treatments on soluble carbohydrates content are presented in Table 4.

Significant differences were observed between the cultivars 'Aquino' and 'Barlack'. Among the two, 'Aquino' showed the highest content of glucose, fructose, sucrose and total soluble carbohydrates.

The treatment factor resulted to statistically impact the glucose, sucrose and total carbohydrates. In particular, Zn-biochelate resulted in lower contents of glucose, sucrose and total carbohydrates compare with to the control. Fe-biochelate exhibited lower glucose compared to the control and intermediate values for sucrose and total carbohydrates.

The interaction between cultivar and treatment significantly affected the soluble carbohydrate content. In particular, Aquino x Control produced the highest glucose, fructose, sucrose and total carbohydrate content. Fe- and Zn-biochelate treatments on cv. 'Aquino' significantly reduce glucose, sucrose, fructose, and total carbohydrates contents compared to Aquino x Control, with no significant differences between the two treatments. In contrast, Barlack x Fe-biochelate combination showed no differences compared with the Barlack x Control for any of the parameters studied. Notably the combination Barlack x Zn-biochelate resulted in the lowest glucose and total carbohydrates contents, whereas fructose and sucrose values did not differ significantly from the other treatments.

Table 4. Effect of cultivar and fertilizer treatment on soluble carbohydrates in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	Soluble carbohydrates (mg g^{-1} FW)			
	Glucose	Fructose	Sucrose	Total
Cultivar				
Aquino	2.62 ± 0.12 a	2.78 ± 0.10 a	0.42 ± 0.04 a	5.83 ± 0.24 a
Barlack	2.12 ± 0.08 b	1.53 ± 0.03 b	0.28 ± 0.01 b	3.93 ± 0.10 b
Treatment				
Control	2.77 ± 0.14 a	2.45 ± 0.27	0.43 ± 0.06 a	5.65 ± 0.46 a

Fe-biochelate	2.28 ± 0.07 b	1.98 ± 0.18	0.34 ± 0.02 ab	4.60 ± 0.23 ab
Zn-biochelate	2.06 ± 0.13 b	2.04 ± 0.19	0.28 ± 0.01 b	4.39 ± 0.31 b
Cultivar x Treatment				
Aquino x Control	3.16 ± 0.06 a	3.25 ± 0.06 a	0.59 ± 0.04 a	6.99 ± 0.14 a
Aquino x Fe-biochelate	2.33 ± 0.13 b	2.50 ± 0.11 b	0.38 ± 0.02 b	5.21 ± 0.23 b
Aquino x Zn-biochelate	2.36 ± 0.16 b	2.61 ± 0.08 b	0.30 ± 0.03 bc	5.28 ± 0.16 b
Barlack x Control	2.38 ± 0.08 b	1.65 ± 0.04 c	0.27 ± 0.02 bc	4.30 ± 0.10 c
Barlack x Fe-biochelate	2.23 ± 0.05 b	1.46 ± 0.04 c	0.30 ± 0.02 bc	3.99 ± 0.07 cd
Barlack x Zn-biochelate	1.76 ± 0.04 c	1.47 ± 0.04 c	0.26 ± 0.01 c	3.49 ± 0.05 d
Significance				
Cultivar	**	***	***	***
Treatment	***	ns	*	*
Cultivar x Treatment	***	***	***	***

*FW= fresh weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.2.2. Experiment 2

The effects of peptide treatment and of the iron and zinc biochelate treatments on soluble carbohydrates are presented in Table 5.

Significant differences were observed between the cv. 'Aquino' and 'Barlack' with 'Aquino' exhibiting the highest content of glucose, fructose, sucrose, and total carbohydrates.

Treatments significantly affected the sucrose content, with Fe-biochelate, Zn-biochelate and Peptide treatments resulting in lower values compared to the control, and no statistically significant differences observed among them.

The interaction between cultivar and treatments significantly affected the soluble carbohydrate content. In 'Aquino', both Aquino x Control and Aquino x Peptide treatments resulted in the highest levels of glucose, fructose, and total carbohydrates, while Aquino x Zn-biochelate and Aquino x Fe-biochelate treatments showed no significant differences among them for glucose, fructose, sucrose, or total carbohydrate content.

In Barlack, fructose content under Barlack x Fe-biochelate, Barlack x Zn-biochelate, and Barlack x Peptide treatments was similar to Barlack x Control. In contrast, sucrose levels were lower in all biochelate, and peptide treatments

compared with Barlack x Control. Finally, for glucose and total carbohydrates, Barlack x Peptide exhibited the lowest values, although these did not differ statistically from Barlack x Fe-biochelate.

Table 5. Effect of cultivar and fertilizer treatment soluble carbohydrates in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	Soluble carbohydrates (mg g^{-1} FW)			
	Glucose	**Fructose**	**Sucrose**	**Total**
Cultivar				
Aquino	1.75 ± 0.08 a	1.84 ± 0.09 a	0.46 ± 0.04 a	4.05 ± 0.17 a
Barlack	0.86 ± 0.05 b	0.36 ± 0.03 b	0.27 ± 0.03 b	1.49 ± 0.09 b
Treatment				
Control	1.56 ± 0.18	1.35 ± 0.32	0.54 ± 0.04 a	3.45 ± 0.54
Fe-biochelate	1.12 ± 0.13	0.94 ± 0.25	0.32 ± 0.04 b	2.38 ± 0.40
Zn-biochelate	1.21 ± 0.11	0.90 ± 0.20	0.35 ± 0.05 b	2.46 ± 0.36
Peptide	1.34 ± 0.27	1.21 ± 0.36	0.23 ± 0.03 b	2.78 ± 0.65
Cultivar x Treatment				
Aquino x Control	2.04 ± 0.08 a	2.19 ± 0.03 a	0.64 ± 0.03 a	4.87 ± 0.07 a
Aquino x Fe-biochelate	1.45 ± 0.04 b	1.59 ± 0.12 b	0.41 ± 0.02 bc	3.45 ± 0.10 b
Aquino x Zn-biochelate	1.48 ± 0.06 b	1.43 ± 0.08 b	0.47 ± 0.04 b	3.39 ± 0.14 b
Aquino x Peptide	2.05 ± 0.09 a	2.16 ± 0.05 a	0.30 ± 0.05 cd	4.50 ± 0.12 a
Barlack x Control	1.09 ± 0.03 c	0.50 ± 0.01 c	0.45 ± 0.03 b	2.03 ± 0.04 c
Barlack x Fe-biochelate	0.79 ± 0.02 de	0.30 ± 0.02 c	0.24 ± 0.02 d	1.32 ± 0.04 de
Barlack x Zn-biochelate	0.94 ± 0.03 cd	0.37 ± 0.04 c	0.22 ± 0.01 d	1.53 ± 0.04 d
Barlack x Peptide	0.63 ± 0.01 e	0.26 ± 0,00 c	0.17 ± 0.02 d	1.06 ± 0.02 e
Significance				
Cultivar	***	***	***	***
Treatment	ns	ns	***	ns
Cultivar x Treatment	***	***	***	***

*FW= fresh weight. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.3. Mineral content

8.3.1. Experiment 1

Results of iron and zinc biochelate treatments on macro- and micronutrients are presented in Table 6 and 7. The cultivar factor was shown to significantly affect

nitrogen, sulphur, calcium, and magnesium contents. The cultivar 'Aquino' exhibited a higher nitrogen content than 'Barlack', whereas this latter showed higher contents of sulphur, calcium, and magnesium.

Not statistically significant differences were observed for the treatment factor and for the Cultivar x Treatment interaction.

Table 6. Effect of cultivar and fertilizer treatment on macronutrient content in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	Macronutrients (mg g^{-1} DW)					
	N	**P**	**S**	**K**	**Ca**	**Mg**
Cultivar						
Aquino	41.64 ± 0.66 a	8.52 ± 0.17	3.40 ± 0.08 b	66.37 ± 1.65	18.15 ± 0.47 b	6.26 ± 0.13 b
Barlack	39.50 ± 0.39 b	8.51 ± 0.20	3.71 ± 0.08 a	67.31 ± 1.60	20.85 ± 0.51 a	6.92 ± 0.13 a
Treatment						
Control	39.98 ± 0.87	8.55 ± 0.28	3.48 ± 0.13	68.08 ± 2.17	19.38 ± 0.67	6.59 ± 0.19
Fe-biochelate	40.74 ± 0.72	8.36 ± 0.18	3.56 ± 0.09	66.36 ± 1.91	19.46 ± 0.83	6.53 ± 0.23
Zn-biochelate	41.02 ± 0.71	8.64 ± 0.21	3.63 ± 0.10	66.09 ± 1.96	19.67 ± 0.76	6.65 ± 0.17
Cultivar x Treatment						
Aquino x Control	40.63 ± 1.55	8.67 ± 0.28	3.34 ± 0.13	71.13 ± 2.61	18.64 ± 0.49	6.47 ± 0.25
Aquino x Fe-biochelate	42.17 ± 0.51	8.30 ± 0.25	3.39 ± 0.10	63.33 ± 2.17	17.56 ± 0.87	6.04 ± 0.23
Aquino x Zn-biochelate	42.11 ± 1.25	8.60 ± 0.38	3.48 ± 0.19	64.66 ± 2.92	18.26 ± 1.07	6.26 ± 0.21
Barlack x Control	39.17 ± 0.37	8.44 ± 0.52	3.62 ± 0.22	65.04 ± 3.12	20.12 ± 1.22	6.71 ± 0.30
Barlack x Fe-biochelate	39.32 ± 1.05	8.43 ± 0.29	3.73 ± 0.09	69.39 ± 2.66	21.36 ± 0.73	7.01 ± 0.26
Barlack x Zn-biochelate	39.93 ± 0.38	8.67 ± 0.24	3.78 ± 0.05	67.51 ± 2.79	21.08 ± 0.68	7.05 ± 0.12
Significance						
Cultivar	**	ns	**	ns	***	***
Treatment	ns	ns	ns	ns	ns	ns
Cultivar x Treatment	ns	ns	ns	ns	ns	ns

*DW= dry weight. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

Regarding micronutrient content, the cultivar factor significantly affected copper, boron, and molybdenum concentrations, with cv. 'Barlack' having the highest levels of boron and molybdenum, whereas cv. 'Aquino' showed higher copper content. Fe- and Zn-biochelate treatments significantly affected iron and zinc

concentrations in plant tissues. Fe-biochelate increased leaf iron content compared with the Control and Zn-biochelate treatments, while Zn-biochelate significantly enhanced zinc content compared with the Control and Fe-biochelate treatments.

The Cultivar x Treatment interaction significantly affected iron, zinc, boron, and molybdenum content. For iron, Barlack x Fe-biochelate and Aquino x Fe-biochelate exhibited the highest and similar values, with no significant difference between them. Regarding zinc content, Aquino x Zn-biochelate and Barlack x Zn-biochelate showed similar values, which were significantly higher than all other combinations. For boron Aquino x Zn-biochelate resulted in a higher content; however, it did not differ statistically from Aquino x Control and Aquino x Fe-biochelate, which exhibited similar values. In addition, Barlack x Fe-biochelate and Barlack x Zn-biochelate resulted in higher values respect to Barlack x Control, although the differenced were not statistically significant. Finally, for molybdenum Barlack x Zn-biochelate showed the highest value, while Aquino x Control had the lowest. The remaining Cultivar x Treatment combinations exhibited intermediate values and did not differ statistically.

Table 7. . Effect of cultivar and fertilizer treatment on micronutrient content in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	Micronutrients (µg g^{-1} DW)						
	Fe	Zn	Mn	Cu	B	Mo	Ni
Cultivar							
Aquino	211.99 ± 42.15	203.82 ± 30.20	226.12 ± 8.14	10.01 ± 0.52 a	77.65 ± 3.06 b	1.11 ± 0.08 b	5.74 ± 2.73
Barlack	240.93 ± 64.37	189.12 ± 27.82	206.61 ± 6.45	8.70 ± 0.33 b	117.47 ± 4.95 a	1.69 ± 0.08 a	0.67 ± 0.11
Treatment							
Control	138.14 ± 43.76 b	121.26 ± 3.88 b	217.79 ± 13.12	9.23 ± 0.63	88.66 ± 5.57	1.37 ± 0.16	1.19 ± 0.27
Fe-biochelate	444.67 ± 61.99 a	123.16 ± 6.60 b	212.18 ± 5.50	9.22 ± 0.42	97.17 ± 10.38	1.40 ± 0.13	7.05 ± 4.11
Zn-biochelate	96.56 ± 9.63 b	344.98 ± 14.75 a	219.13 ± 8.83	9.62 ± 0.67	106.84 ± 7.35	1.43 ± 0.13	1.37 ± 0.42
Cultivar x Treatment							
Aquino x Control	174.82 ± 88.12 bc	128.36 ± 3.94 b	241.38 ± 17.25	9.89 ± 1.02	74.34 ± 2.63 c	1.05 ± 0.18 b	1.73 ± 0.37
Aquino x Fe-biochelate	359.77 ± 40.50 ab	123.50 ± 5.34 b	209.75 ± 6.80	9.54 ± 0.45	72.08 ± 5.90 c	1.18 ± 0.16 ab	13.27 ± 7.52
Aquino x Zn-biochelate	101.36 ± 19.64 bc	359.61 ± 20.67 a	227.24 ± 15.06	10.62 ± 1.22	86.53 ± 5.14 bc	1.10 ± 0.10 ab	2.21 ± 0.66

Barlack x Control	101.47 ± 13.38 bc	114.17 ± 5.19 b	194.2 ± 14.10	8.57 ± 0.72	102.99 ± 5.48 ab	1.69 ± 0.18 ab	0.64 ± 0.21
Barlack x Fe-biochelate	529.58 ± 109.76 a	122.83 ± 12.93 b	214.6 ± 9.32	8.91 ± 0.73	122.26 ± 11.63 a	1.62 ± 0.15 ab	0.83 ± 0.25
Barlack x Zn-biochelate	91.76 ± 4.52 c	330.35 ± 21.08 a	211.03 ± 9.57	8.61 ± 0.21	127.15 ± 3.21 a	1.75 ± 0.12 a	0.52 ± 0.09
Significance							
Cultivar	ns	ns	ns	*	***	***	ns
Treatment	***	***	ns	ns	ns	ns	ns
Cultivar x Treatment	***	***	ns	ns	***	**	ns

*DW= dry weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.3.2. Experiment 2

Results of peptide, iron and zinc biochelate treatments on macro- and micronutrients are presented in Table 8 and 9.

The outcomes highlight significant differences in macronutrient composition between the two cultivars under study. The cultivar 'Aquino' showed higher levels of sulphur, potassium, calcium, and magnesium, whereas 'Barlack' exhibited higher nitrogen and potassium contents.

The treatments factor significantly affected phosphorus content, with the Control and Fe-biochelate treatments exhibiting similar values and were statistically higher than Zn-biochelate and Peptide treatments.

The Cultivar x Treatment interaction showed a significant effect on macronutrient content in plant tissue. For phosphorus, Aquino x Fe-biochelate resulted in the highest content, whereas Aquino x Zn-biochelate significantly reduced phosphorus content compared with the other combinations. Aquino x Peptide showed intermediate values. In 'Barlack', none of the treatments differed significantly.

For sulphur, Aquino x Fe-biochelate resulted in the highest content, whereas Aquino x Zn-biochelate and Aquino x Peptide showed intermediate values, not significantly different from Aquino x Control. In 'Barlack' none of the treatments differed significantly.

For potassium, Aquino x Fe-biochelate and Aquino x Peptide resulted in the highest potassium contents, which did not differ significantly from each other, whereas Aquino x Control and Aquino x Zn-biochelate showed lower values. In 'Barlack',

the lowest value was observed in Barlack x Fe-biochelate, which did not differ significantly from all the other 'Barlack' treatment combinations.

For calcium, Aquino x Peptide resulted in the highest calcium content, while Aquino x Zn-biochelate, Aquino x Control, and Aquino x Fe-biochelate showed lower values. Barlack x Fe-biochelate exhibited the lowest calcium content, whereas the other Barlack treatment combinations showed intermediate values, not significantly different from each other.

Finally, for magnesium the highest magnesium contents were observed in Aquino x Control, Aquino x Zn-biochelate, and Aquino x Peptide, which did not differ significantly from each other, whereas Aquino x Fe-biochelate showed slightly lower values. Barlack x Fe-biochelate resulted in the lowest content while the other treatment combinations showed intermediate values, not significantly different from each other.

Table 8. Effect of cultivar and fertilizer treatment on macronutrient content in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	Macronutrients (mg g^{-1} DW)					
	N	P	S	K	Ca	Mg
Cultivar						
Aquino	44.96 ± 0.54 b	6.32 ± 0.11 b	3.62 ± 0.04 a	93.13 ± 0.95 a	19.24 ± 0.37 a	4.87 ± 0.10 a
Barlack	47.41 ± 0.69 a	6.61 ± 0.07 a	3.30 ± 0.04 b	83.79 ± 0.73 b	15.94 ± 0.37 b	4.35 ± 0.09 b
Treatment						
Control	45.77 ± 0.35	6.73 ± 0.12 a	3.46 ± 0.08	87.99 ± 1.50	17.80 ± 0.84	4.69 ± 0.20
Fe-biochelate	47.04 ± 0.64	6.71 ± 0.08 a	3.50 ± 0.09	89.28 ± 2.80	16.15 ± 0.62	4.28 ± 0.12
Zn-biochelate	45.25 ± 0.90	6.15 ± 0.11 b	3.48 ± 0.09	87.16 ± 1.82	18.12 ± 0.69	4.67 ± 0.12
Peptide	46.69 ± 1.60	6.26 ± 0.09 b	3.39 ± 0.07	89.40 ± 2.19	18.30 ± 0.86	4.78 ± 0.15
Cultivar x Treatment						
Aquino x Control	46.16 ± 0.58	6.57 ± 0.18 ab	3.62 ± 0.09 ab	91.31 ± 1.12 ab	19.49 ± 0.80 abc	5.06 ± 0.22 a
Aquino x Fe-biochelate	46.73 ± 1.05	6.70 ± 0.15 a	3.69 ± 0.08 a	95.98 ± 1.81 a	17.70 ± 0.19 abcd	4.48 ± 0.16 ab
Aquino x Zn-biochelate	44.04 ± 0.48	5.89 ± 0.11 c	3.61 ± 0.11 ab	90.99 ± 1.59 abc	19.65 ± 0.42 ab	4.88 ± 0.11 a
Aquino x Peptide	42.92 ± 1.05	6.11 ± 0.15 bc	3.56 ± 0.02 abc	94.24 ± 2.32 a	20.12 ± 0.85 a	5.04 ± 0.16 a
Barlack x Control	45.37 ± 0.37	6.89 ± 0.13 a	3.30 ± 0.07 bc	84.67 ± 1.36 bcd	16.10 ± 0.84 de	4.32 ± 0.19 ab
Barlack x Fe-biochelate	47.36 ± 0.86	6.71 ± 0.10 a	3.31 ± 0.07 abc	82.59 ± 1.86 d	14.59 ± 0.39 e	4.08 ± 0.11 b
Barlack x Zn-biochelate	46.45 ± 1.61	6.42 ± 0.04 abc	3.36 ± 0.12 abc	83.34 ± 1.76 cd	16.60 ± 0.71 bcde	4.46 ± 0.17 ab
Barlack x Peptide	50.45 ± 1.18	6.41 ± 0.05 abc	3.22 ± 0.04 c	84.56 ± 1.14 bcd	16.47 ± 0.74 cde	4.52 ± 0.17 ab
Significance						

Cultivar	*	*	***	***	***	***
Treatment	ns	***	ns	ns	ns	ns
Cultivar x Treatment	ns	***	***	***	***	**

*DW=dry weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

Regarding micronutrients, the cultivar 'Barlack' resulted in significantly higher copper content than 'Aquino', whereas the latter exhibited higher boron content.

Fe-biochelate significantly increased iron content, whereas Zn-biochelate significantly enhanced zinc content compared to all other treatments. For boron, the highest level was observed following the Zn-biochelate treatment.

A significant interaction between cultivar and treatment was observed for iron, zinc, copper and boron. The highest copper values were recorded in the Barlack x Fe-biochelate, Barlack x Zn-biochelate, and Aquino x Fe-biochelate combinations, which were significantly higher than Aquino x Zn-biochelate and Aquino x Peptide. Intermediate values were observed in Aquino x Control, Barlack x Control, and Barlack x Peptide, which did not differ significantly from one another. With regard to iron content, Fe-biochelate treatment, in combination with both cultivars, resulted in higher values compared to all other treatments. The same occurred for Zn-biochelate treatment. Finally, regarding boron, Aquino x Zn-biochelate resulted in the highest content, whereas Aquino x Control and Aquino x Fe-biochelate showed lower values. Aquino x Peptide exhibited intermediate content.

Barlack x Zn-biochelate increased the boron content, while Barlack x Control, Barlack x Fe-biochelate, and Barlack x Peptide showed lower and similar values, not significantly different from each other.

Table 9. Effect of cultivar and fertilizer treatment on micronutrients content in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	Microelements (µg g^{-1} DW)						
	Fe	Zn	Mn	Cu	B	Mo	Ni
Cultivar							
Aquino	176.13 ± 49.16	226.22 ± 24.06	155.93 ± 10.47	8.66 ± 0.21 b	49.79 ± 1.66 a	1.24 ± 0.09	0.48 ± 0.07
Barlack	171.12 ± 41.20	215.11 ± 26.03	170.69 ± 11.16	9.44 ± 0.10 a	44.65 ± 1.81 b	1.18 ± 0.07	0.59 ± 0.09

Treatment							
Control	67.83 ± 2.02 b	171.15 ± 8.44 b	173.76 ± 10.29	9.17 ± 0.22	42.57 ± 1.49 b	1.25 ± 0.07	0.60 ± 0.09
Fe-biochelate	468.15 ± 20.54 a	158.55 ± 15.14 b	138.04 ± 9.70	9.53 ± 0.18	44.03 ± 1.72 b	1.07 ± 0.15	0.50 ± 0.05
Zn-biochelate	91.81 ± 24.06 b	379.23 ± 14.76 a	176.72 ± 21.23	8.93 ± 0.34	57.47 ± 1.46 a	1.16 ± 0.14	0.56 ± 0.20
Peptide	66.69 ± 6.98 b	173.72 ± 8.77 b	164.72 ± 15.86	8.56 ± 0.22	44.8 ± 1.42 b	1.37 ± 0.08	0.49 ± 0.08
Cultivar x Treatment							
Aquino x Control	63.91 ± 2.65 b	173.04 ± 10.64 b	166.39 ± 13.20	9.08 ± 0.43 abc	44.87 ± 1.84 c	1.42 ± 0.04	0.64 ± 0.18
Aquino x Fe-biochelate	504.22 ± 16.16 a	173.89 ± 25.98 b	133.19 ± 18.61	9.40 ± 0.32 a	46.65 ± 1.43 c	0.97 ± 0.22	0.45 ± 0.08
Aquino x Zn-biochelate	65.08 ± 4.30 b	376.15 ± 25.31 a	166.07 ± 30.10	8.13 ± 0.34 bc	59.55 ± 2.23 a	1.20 ± 0.28	0.28 ± 0.04
Aquino x Peptide	71.29 ± 13.60 b	181.82 ± 11.95 b	158.08 ± 22.87	8.02 ± 0.05 c	48.08 ± 0.94 bc	1.36 ± 0.03	0.56 ± 0.15
Barlack x Control	71.75 ± 1.35 b	169.27 ± 14.73 b	181.13 ± 16.84	9.27 ± 0.22 ab	40.28 ± 1.87 c	1.07 ± 0.05	0.56 ± 0.06
Barlack x Fe-biochelate	432.07 ± 28.97 a	143.22 ± 15.41 b	142.88 ± 8.80	9.66 ± 0.18 a	41.42 ± 2.68 c	1.17 ± 0.22	0.55 ± 0.07
Barlack x Zn-biochelate	118.55 ± 46.97 b	382.31 ± 19.22 a	187.38 ± 33.51	9.73 ± 0.05 a	55.4 ± 1.47 ab	1.13 ± 0.10	0.84 ± 0.37
Barlack x Peptide	62.09 ± 5.32 b	165.63 ± 13.14 b	171.35 ± 24.93	9.1 ± 0.17 abc	41.52 ± 1.17 c	1.37 ± 0.18	0.43 ± 0.04
Significance							
Cultivar	ns	ns	ns	**	*	ns	ns
Treatment	***	***	ns	ns	***	ns	ns
Cultivar x Treatment	***	***	ns	***	***	ns	ns

*DW= dry weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.4. Ammonium and nitrate

8.4.1. Experiment 1

Results iron and zinc biochelate treatments on ammonium and nitrate are presented in Table 10.

The cultivar factor significantly influenced the content of ammonium, with the cv. 'Aquino' showing higher mean values compared to cv. 'Barlack'.

Treatments significantly affected ammonium and nitrate levels. For ammonium, the Control and Fe-biochelate treatments showed similar values, both significantly higher than the Zn-biochelate treatment. Regarding nitrate content, Fe-biochelate and Zn-biochelate treatments resulted in lower values compared to the control, with no statistical difference among them.

A significant interaction between cultivar and treatment was observed for both ammonium and nitrate. For ammonium, Aquino x Control and Aquino x Fe-biochelate showed significantly higher values than all other combinations, whereas

Barlack x Zn-biochelate had the lowest ammonium content. Regarding nitrate, the combination Aquino x Control showed the highest content, while Aquino x Fe-biochelate resulted in the lowest nitrate levels. All other Cultivar x Treatment combinations displayed intermediate values.

Table 10. Effect of cultivar and fertilizer treatment on ammonium and nitrate content in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	mg NH_4 kg^{-1} FW	mg NO_3 kg^{-1} FW
Cultivar		
Aquino	23.91 ± 0.78 a	2172.08 ± 305.81
Barlack	16.01 ± 1.02 b	1679.82 ± 82.39
Treatment		
Control	22.54 ± 0.93 a	2727.71 ± 360.74 a
Fe-biochelate	21.77 ± 1.60 a	1313.61 ± 55.77 b
Zn-biochelate	15.57 ± 1.54 b	1736.55 ± 62.72 b
Cultivar x Treatment		
Aquino x Control	25.28 ± 0.33 a	3762.43 ± 65.35 a
Aquino x Fe-biochelate	26.47 ± 0.45 a	1161.24 ± 26.04 c
Aquino x Zn-biochelate	19.98 ± 0.26 b	1592.59 ± 72.98 bc
Barlack x Control	19.80 ± 0.19 b	1692.99 ± 214.49 b
Barlack x Fe-Biochelate	17.08 ± 0.57 c	1465.98 ± 41.35 bc
Barlack x Zn biochelate	11.17 ± 0.95 d	1880.50 ± 44.90 b
Significance		
Cultivar	***	ns
Treatment	**	***
Cultivar x Treatment	***	***

*FW=fresh weight. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.4.2. Experiment 2

Results of peptide, and iron and zinc biochelate treatments on ammonium and nitrate are presented in Table 11.

A significant difference was observed in ammonium content, with cv. 'Barlack' showing significantly higher values compared to cv. 'Aquino'.

Treatment factor did not significantly affect nitrate content. In contrast, ammonium concentrations were significantly influenced by Zn-biochelate, which resulted in

the lowest value, significantly differ from the control. Fe-biochelate and Peptide treatment showed intermediate values.

Finally, the interaction between cultivar and treatments significantly influenced both ammonium and nitrate content. Regarding ammonium, Barlack x Control showed the highest ammonium content. Aquino x Peptide and Barlack x Peptide exhibited significantly higher ammonium content compared to Aquino x Zn-biochelate, while all the others combination did not differ significantly from one another. For nitrate, Barlack x Control exhibited the highest value, statistically different from Aquino x Zn-biochelate. Within each cultivar, 'Aquino' combined with all treatments resulted in similar nitrate levels, which no statistically significant differences among them; a similar pattern was observed for 'Barlack'.

Table 11. Effect of cultivar and fertilizer treatment on ammonium and nitrate content in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	mg NH_4 kg^{-1} FW	mg NO_3 kg^{-1} FW
Cultivar		
Aquino	16.25 ± 0.53 b	71.51 ± 6.71
Barlack	19.61 ± 1.05 a	84.74 ± 4.50
Treatment		
Control	20.71 ± 1.78 a	91.11 ± 7.72
Fe-biochelate	16.61 ± 1.05 ab	69.51 ± 1.70
Zn-biochelate	15.17 ± 0.72 b	66.75 ± 3.14
Peptide	19.16 ± 0.50 ab	84.29 ± 11.51
Cultivar x Treatment		
Aquino x Control	16.13 ± 0.23 bc	70.99 ± 1.84 ab
Aquino x Fe-biochelate	16.59 ± 0.44 bc	72.97 ± 1.46 ab
Aquino x Zn-biochelate	13.20 ± 0.29 c	58.06 ± 1.68 b
Aquino x Peptide	18.42 ± 0.31 b	81.03 ± 24.53 ab
Barlack x Control	25.28 ± 0.91 a	111.24 ± 2.09 a
Barlack x Fe-Biochelate	16.63 ± 1.93 bc	66.91 ± 1.96 ab
Barlack x Zn biochelate	16.65 ± 0.23 bc	73.26 ± 0.41 ab

Barlack x Peptide	19.90 ± 0.83 b	87.55 ± 3.04 ab
Significance		
Cultivar	*	ns
Treatment	**	ns
Cultivar x Treatment	***	*

*FW= fresh weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.5. Phenol profile

8.5.1. Experiment 1

Results of iron and zinc biochelate treatments on the phenolic profile are presented in Table 12.

The cultivar factor significantly influenced the phenolic profile. In particular, cv. 'Barlack' showed significantly higher levels of total phenols, total phenylpropanoids, total flavonoids, and total anthocyanins compared to cv. 'Aquino'. No treatment significantly affected the phenolic profile.

A significant Cultivar x Treatment interaction was observed for all the parameters analysed. In the cultivar 'Aquino', the Fe-biochelate, Zn-biochelate, and Control treatments resulted in comparable values of total phenols, total phenylpropanoids, total flavonoids, and total anthocyanins. A similar pattern was observed in 'Barlack'.

Table 12. Effect of cultivar and fertilizer treatment on phenol profile in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	Total phenols (mg CGA 100 g^{-1} FW)	Total phenylpropanoids (mg CAE 100 g^{-1} FW)	Total flavonoids (mg QE 100 g^{-1} FW)	Total anthocyanins (mg CyE 100 g^{-1} FW)
Cultivar				
Aquino	111.45 ± 2.60 b	26.59 ± 0.67 b	37.07 ± 0.93 b	17.96 ± 0.54 b
Barlack	199.92 ± 4.75 a	59.63 ± 1.84 a	73.70 ± 1.90 a	29.74 ± 0.73 a
Treatment				
Control	154.76 ± 17.28	42.16 ± 6.15	54.99 ± 7.11	23.68 ± 2.62
Fe-biochelate	157.30 ± 12.83	43.63 ± 5.10	55.61 ± 5.37	23.93 ± 1.62

Zn-biochelate	155.00 ± 16.03	43.54 ± 5.99	55.56 ± 6.55	23.95 ± 2.00
Cultivar x Treatment				
Aquino x Control	104.48 ± 3.44 b	24.42 ± 0.85 b	34.18 ± 1.23 b	16.07 ± 0.74 b
Aquino x Fe-biochelate	121.33 ± 3.03 b	29.17 ± 0.71 b	40.42 ± 1.23 b	19.52 ± 0.84 b
Aquino x Zn-biochelate	108.55 ± 3.40 b	26.17 ± 0.81 b	36.62 ± 1.04 b	18.28 ± 0.54 b
Barlack x Control	205.03 ± 8.28 a	59.91 ± 3.50 a	75.79 ± 3.08 a	31.28 ± 1.19 a
Barlack x Fe-biochelate	193.27 ± 9.19 a	58.08 ± 3.47 a	70.80 ± 3.59 a	28.33 ± 1.16 a
Barlack x Zn-biochelate	201.45 ± 8.13 a	60.92 ± 3.19 a	74.51 ± 3.51 a	29.62 ± 1.30 a
Significance				
Cultivar	***	***	***	***
Treatment	ns	ns	ns	ns
Cultivar x Treatment	***	***	***	***

*FW= fresh weight; CGA=Chlorogenic acid equivalents; CAE=Caffeic acid equivalents; QE=Quercetin acid equivalents; CyE=Cyanidin acid equivalents. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.5.2. Experiment 2

Results of peptide and iron and zinc biochelate treatments on phenol profile are presented in Table 13.

The cultivar factor significantly influenced the phenolic profile. In particular, the cv. 'Barlack' showed significantly higher levels of total flavonoids and total anthocyanins compared to 'Aquino'. No significant differences were observed for treatment factors. A significant interaction between cultivar and treatment was observed for total phenylpropanoids and total anthocyanins. For total phenylpropanoids, Barlack x Control and Barlack x Fe-biochelate exhibited the highest values whereas Aquino x Fe-biochelate showed the lowest value. The remaining combinations resulted in intermediate values and did not differ significantly. Finally, for total anthocyanins, Barlack x Control had the highest value, whereas Aquino x Peptide showed the lowest. The remaining combinations displayed intermediate values and were not significantly different from either Barlack x Control or Aquino x Peptide.

Table 13. Effect of cultivar and fertilizer treatment on phenol profile in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	Total phenols (mg CGA 100 g^{-1} FW)	Total phenylpropanoids (mg CAE 100 g^{-1} FW)	Total flavonoids (mg QE 100 g^{-1} FW)	Total anthocyanins (mg CyE 100 g^{-1} FW)
Cultivar				
Aquino	103.94 ± 5.16	27.62 ± 1.55	34.67 ± 1.68 b	17.50 ± 1.05 b
Barlack	115.98 ± 7.43	33.29 ± 2.32	41.16 ± 2.46 a	21.41 ± 1.33 a
Treatment				
Control	122.16 ± 8.66	34.13 ± 3.01	42.83 ± 2.97	23.81 ± 1.46
Fe-biochelate	103.14 ± 10.36	29.59 ± 3.74	36.10 ± 3.71	18.05 ± 1.62
Zn-biochelate	106.89 ± 6.82	28.49 ± 2.10	36.14 ± 1.58	17.77 ± 0.84
Peptide	107.65 ± 10.60	29.59 ± 2.80	36.59 ± 3.79	18.19 ± 2.33
Cultivar x Treatment				
Aquino x Control	107.80 ± 8.62	28.41 ± 2.84 ab	38.28 ± 3.37	22.14 ± 1.93 ab
Aquino x Fe-biochelate	83.86 ± 8.34	21.49 ± 2.17 b	29.28 ± 3.04	16.25 ± 1.98 ab
Aquino x Zn-biochelate	120.00 ± 9.16	31.86 ± 3.41 ab	38.05 ± 2.57	16.74 ± 0.94 ab
Aquino x Peptide	104.09 ± 8.92	28.70 ± 2.14 ab	33.06 ± 3.19	14.85 ± 1.87 b
Barlack x Control	136.52 ± 11.76	39.84 ± 3.51 a	47.37 ± 4,00	25.49 ± 2.10 a
Barlack x Fe-biochelate	122.42 ± 13.54	37.70 ± 4.09 a	42.92 ± 4.91	19.84 ± 2.50 ab
Barlack x Zn-biochelate	93.79 ± 4.33	25.12 ± 1.22 ab	34.23 ± 1.61	18.79 ± 1.29 ab
Barlack x Peptide	111.21 ± 20.88	30.48 ± 5.61 ab	40.12 ± 6.96	21.52 ± 3.81 ab
Significance				
Cultivar	ns	ns	*	*
Treatment	ns	ns	ns	ns
Cultivar x Treatment	ns	*	ns	*

*FW= fresh weight; CGA=Chlorogenic acid equivalents; CAE=Caffeic acid equivalents; QE=Quercetin acid equivalents; CyE=Cyanidin acid equivalents. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.6. Vitamin C, DPPH assay, and free amino acids content

8.6.1. Experiment 1

Results of iron and zinc biochelate treatments on Vitamin C, DPPH assay, and free amino acids content results are presented in Table 14.

The cultivar factor significantly affected the studied parameters, with significant differences observed in DPPH radical scavenging activity and vitamin C content, where the cv. 'Barlack' showed significantly higher values compared to cv.

‘Aquino’. Conversely, ‘Aquino’ exhibited significantly higher free amino acids content.

Treatment factor did not significantly affect any of the parameters studied.

Finally, the interaction between cultivar and treatments significantly influenced DPPH radical scavenging activity, vitamin C, and free amino acids content.

For DPPH radical scavenging activity, Aquino x Control, Aquino x Fe-biochelate, and Aquino x Zn-biochelate resulted in similar values. A similar pattern was observed in ‘Barlack’. For vitamin C content, Aquino x Fe-biochelate and Aquino x Zn-biochelate exhibited significantly lower values compared to Aquino x Control. Similarly, Barlack x Fe-biochelate and Barlack x Zn-biochelate showed lower values, with Barlack x Fe-biochelate being significantly lower than Barlack x Zn-biochelate. For free amino acids content, Aquino x Fe-biochelate and Aquino x Zn-biochelate displayed similar values, not significantly different from Aquino x Control. A similar pattern was observed in ‘Barlack’.

Table 14. Effect of cultivar and fertilizer treatment on antioxidant activity, vitamin C, and amino acids content in lettuce, with transplant at 20 days after sowing, treatments applied at 30, 37, 44, and 51 days after transplant, and harvest at 55 days after transplant.

Source of variance	DPPH radical scavenging activity (%)	Vitamin C (mg 100 g^{-1} FW)	Amino acids (mg N 100 g^{-1} FW)
Cultivar			
Aquino	2.68 ± 0.25 b	2.12 ± 0.24 b	0.32 ± 0.01 a
Barlack	18.74 ± 0.80 a	3.16 ± 0.28 a	0.18 ± 0.00 b
Treatment			
Control	10.39 ± 1.97	2.64 ± 0.61	0.24 ± 0.02
Fe-biochelate	10.83 ± 1.91	2.23 ± 0.12	0.25 ± 0.02
Zn-biochelate	10.90 ± 2.06	3.03 ± 0.10	0.26 ± 0.02
Cultivar x Treatment			
Aquino x Control	2.52 ± 0.45 b	1.03 ± 0.05 e	0.31 ± 0.01 a
Aquino x Fe-biochelate	3.00 ± 0.42 b	2.46 ± 0.15 cd	0.31 ± 0.00 a
Aquino x Zn-biochelate	2.52 ± 0.48 b	2.85 ± 0.11 bc	0.33 ± 0.01 a
Barlack x Control	18.26 ± 1.55 a	4.25 ± 0.08 a	0.17 ± 0.01 b
Barlack x Fe-biochelate	18.66 ± 1.27 a	2.01 ± 0.12 d	0.18 ± 0.01 b
Barlack x Zn-biochelate	19.28 ± 1.44 a	3.21 ± 0.11 b	0.20 ± 0.01 b
Significance			
Cultivar	***	*	***
Treatment	ns	ns	ns

Cultivar x Treatment	***	***	***

*FW= fresh weight. Level of significance (p): * (p < 0.05); ** (p < 0.01); *** (p < 0.001); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

8.6.2. Experiment 2

Results of peptide and iron and zinc biochelate treatments on vitamin C, DPPH radical scavenging activity, and free amino acids content are presented in Table 15. A significant difference was observed in amino acids and vitamin C content, with cv 'Aquino' showing significantly higher values compared to 'Barlack'. Conversely, 'Barlack' exhibited significantly higher DPPH radical scavenging activity. Treatment factor did not significantly affect any of the parameters studied. Finally, the interaction between cultivar and treatment significantly influenced vitamin C, DPPH activity, and free amino acids content. For the DPPH activity, Barlack x Fe-biochelate resulted in the highest value, significantly different from Barlack x Zn-biochelate and Barlack x Peptide, but not significantly different from Barlack x Control. For vitamin C, Aquino x Fe-biochelate and Aquino x Zn-biochelate exhibited significantly lower values compared to Aquino x Control. Aquino x Peptide displayed higher values than Aquino x Fe-biochelate, but lower than Aquino x Control.

For free amino acids content Aquino x Peptide resulted in the highest value, significantly different from Aquino x Control, Aquino x Fe-biochelate, and Aquino x Zn-biochelate. Conversely, Barlack x Peptide exhibited the lowest value among Barlack x Control, Barlack x Fe-biochelate, and Barlack x Zn-biochelate.

Table 15. Effect of cultivar and fertilizer treatment on antioxidant activity, vitamin C, and amino acids content in lettuce, with transplant at 28 days after sowing, treatments applied at 8, 15, 22, 29 and 36 days after transplant, and harvest at 38 days after transplant.

Source of variance	DPPH radical scavenging activity (%)	Vitamin C (mg 100 g^{-1} FW)	Amino acids (mg N 100 g^{-1} FW)
Cultivar			
Aquino	6.89 ± 0.43 b	8.70 ± 0.50 a	0.30 ± 0.01 a
Barlack	10.82 ± 0.78 a	4.55 ± 0,14 b	0.20 ± 0.01 b
Treatment			
Control	9.16 ± 0.94	8.14 ± 1.43	0.26 ± 0.01

Fe-biochelate	10.05 ± 1.16	5.68 ± 0.55	0.23 ± 0.02
Zn-biochelate	7.52 ± 0.61	5.92 ± 0.66	0.23 ± 0.02
Peptide	8.69 ± 0.50	6.75 ± 0.55	0.27 ± 0.04
Cultivar x Treatment			
Aquino x Control	5.87 ± 0.52 de	11.90 ± 0.13 a	0.29 ± 0.01 b
Aquino x Fe-biochelate	4.44 ± 0.28 e	7.11 ± 0.17 c	0.27 ± 0.02 b
Aquino x Zn-biochelate	9.16 ± 0.86 bcd	7.61 ± 0.33 bc	0.28 ± 0.01 b
Aquino x Peptide	8.09 ± 0.42 cd	8.17 ± 0.25 b	0.38 ± 0.02 a
Barlack x Control	12.45 ± 0.66 ab	4.37 ± 0.18 e	0.24 ± 0.01 bc
Barlack x Fe-biochelate	15.65 ± 1.42 a	4.25 ± 0.19 e	0.20 ± 0.01 cd
Barlack x Zn-biochelate	5.87 ± 0.26 de	4.24 ± 0.17 e	0.19 ± 0.00 cd
Barlack x Peptide	9.29 ± 0.90 bc	5.33 ± 0.08 d	0.17 ± 0.01 d
Significance			
Cultivar	***	***	***
Treatment	ns	ns	ns
Cultivar x Treatment	***	***	***

*FW=fresh weight. Level of significance (p): * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); ns = non-significant. Different letters indicate significant differences among treatments according to Tukey's HSD test.*

9. DISCUSSION

The present study aimed to evaluate the effects of foliar applications of Fe and Zn biochelates, as well as vegetal peptides, on yield parameters and nutritional quality traits of *Lactuca sativa* L. var. *capitata* cultivars Aquino (green leaf) and Barlack (red leaf) under both optimal and heat stress conditions.

With regard to the shoot fresh weight, the results indicate that zinc and iron biochelates influenced plant growth differently under optimal and heat stress conditions. In Experiment 1, neither treatment resulted in significant effects on shoot fresh weight. In contrast in Experiment 2, Zn-biochelate resulted in a 22% reduction in shoot fresh weight compared with the control. The significant shoot biomass reduction under heat stress with early Zn-biochelate application likely results from a phenological mismatch (Poorter et al., 2012). Applied during the vulnerable transplant establishment phase, the treatment may have disrupted the plant optimal stress-response strategy (Reynolds and Thornley, 1982) forcing a costly reallocation of resources ultimately compromised overall biomass accumulation compared to the non-treated control. Consistently, under the same stress conditions, Zn-biochelate and peptide treatments increased root fresh and dry weight in the cultivar Aquino (green leaf) compared to the control. This phenomenon is the direct effect of foliar application of protein hydrolysate, which stimulates root growth by promoting specific metabolic pathways (Colla et al., 2015). These results are consistent with previous studies demonstrating the biostimulant effect of the protein hydrolysate on root parameters in *Solanum lycopersicum* L. (Colla et al., 2014) and *Lactuca sativa* L. (Rouphael et al., 2017). Moreover, the root dry weight means values obtained for the control plants of both cultivars in Experiment 2 were in line with the results reported by Li et al. (2018).

Beyond growth and biomass responses, the application of Fe- and Zn-biochelates increased mineral concentrations in lettuce leaves. For macronutrient content, both Zn-biochelate and vegetal peptides treatments and their interaction with cv. Aquino, reduced phosphorus content in the leaf tissues. Regarding the Zn-biochelate treatment, the reduction in phosphorus may be related to a Zn-P antagonism within

the plant, as described by Xie et al. (2019). In accordance with these authors, zinc accumulation in leaves through Zn-biochelate application may create a condition in which excessive zinc levels induce the plant to reduce phosphorus content in order to maintain cellular homeostasis. For peptide treatment, the results indicate a highly significant reduction in phosphorus content in the lettuce leaves, approximately 7.0% compared to the untreated plant. These results contrast with those of Carillo et al. (2022), where foliar application of a legume-derived biostimulant increased phosphorus content in lettuce, and with the general effects described by Colla et al. (2015), who reported improved mineral uptake following protein hydrolysate treatment. With regard to iron and zinc content in lettuce, the results indicate the effectiveness of foliar biochelate treatments in enhancing the levels of these minerals under both optimal and heat stress conditions. In both Experiment 1 and Experiment 2, Aquino exhibited iron contents of 360 and 504 μg g^{-1} DW and zinc contents of 360 and 376 μg g^{-1} DW, whereas Barlack showed iron contents of 432 and 530 μg g^{-1} DW and zinc contents of 330 and 382 μg g^{-1} DW. These values exceeded the reference levels for iron (172 and 240 μg g^{-1} DW for green and red lettuce, respectively) and for zinc (60 μg g^{-1} DW) (USDA, 2015, 2022). Notably, biochelate treatments resulted in similar values whether applied one month after transplanting with four applications or starting one week after transplanting with five applications. The effects of cumulative applications should be further evaluated in future research. During optimal conditions, Fe-biochelate increased iron content by 105% in Aquino and 422% in Barlack, whereas under heat stress the increase was 689% in Aquino and 502% in Barlack, compared to their respective untreated controls. These latter results suggest that the efficacy of Fe-biochelate under heat stress conditions may be higher than under optimal conditions. It is important to note that in the present study the number of days from transplanting to harvest differed between first and second growth cycles, which likely contributed to the observed differences in iron and zinc accumulation. Moreover, heat stress induced a lower yield compared to cultivation under optimal conditions; therefore, this trade-off between yield and mineral content must be considered.

Regarding Zn-biochelate, it increased the zinc content by 180% in Aquino and 189% in Barlack under optimal conditions, and by 119% in Aquino and by 126% in Barlack under stress conditions, relative to their respective untreated controls. The present study demonstrated that innovative techniques, such as mineral biochelation through peptides, can effectively increase the content of essential minerals when supplied as foliar sprays in lettuce, potentially enhancing their dietary intake. For instance, in the first growth cycle, the red-leaf cv. Barlack, following biochelate treatments, provided 2.69 mg 100 g^{-1} FW of iron and 1.68 mg 100 g^{-1} FW of zinc. For a vulnerable group such as women aged 19–30 years, the estimated average requirements are 8.1 mg day^{-1} for iron and 6.8 mg day^{-1} for zinc (Institute of Medicine, US Panel on Micronutrients, 2001). Therefore, a 100 g serving of this biofortified lettuce supplies approximately 33% and 25% of the estimated average requirements for iron and zinc, respectively, highlighting the potential of foliar biochelate treatments to contribute to the dietary intake of essential trace elements in vulnerable groups.

Moreover, the results indicate a higher boron content in lettuce tissues following Zn-biochelate treatments compared to the untreated plant under heat stress. These findings are not consistent with those reported by Behtash et al. (2023), who observed an inverse relationship between zinc and boron in *Lactuca sativa* L. cv. Parris Island, although both minerals were supplied by the nutrient solution. In contrast, Ahmad et al. (2025) suggested that zinc application may enhance boron accumulation in plant tissues through Zn–B interactions, as evidenced by their trial on *Solanum tuberosum* L., where zinc was supplied by foliar application. These contrasting results suggest that the increased boron accumulation observed in the present study may be modulated by heat stress and foliar Zn application, highlighting further investigation into Zn–B interactions under abiotic stress conditions. Regarding commercial and nutritional quality, nitrate and vitamin C contents were evaluated. Under optimal growth conditions, both Fe-biochelate and Zn-biochelate treatments led to a reduction in nitrate content compared with the control. The application of these biochelates had a pronounced effect on nitrate accumulation in cv. ‘Aquino’, where nitrate levels were reduced by 69% with Fe-

biochelate and by 58% with Zn-biochelate relative to the untreated control. In contrast, no significant differences were observed in cv. 'Barlack' between the biochelate treatments and the control. Conversely, under heat stress conditions, no significant differences in nitrate content were observed among treatments in either cultivar. All the results were consistent with Giordano et al. (2019) and El-Nakhel et al. (2023) and all recorded nitrate contents among the Experiment 1 and Experiment 2 were below the nitrate limits set by the European Union for safe lettuce marketing, which range from 3000 to 5000 mg kg^{-1} FW depending on the cultivation season (Commission regulation (EU) No. 1258/2011). According to Liang et al. (2023), differences in nitrogen content between the two lettuce cultivars can be largely explained by genetic and physiological traits. Specifically, red lettuce cultivars are able to activate metabolic pathways involved in the synthesis of phenylpropanoids and anthocyanins. This process has a metabolic cost that may negatively affect the activity of enzymes responsible for nitrate uptake and reduction, ultimately resulting in lower nitrate accumulation in plant tissues. Conversely, under heat stress conditions, nitrate levels in both lettuce cultivars were markedly lower than those recorded during the first growth cycle, and no significant differences were observed between the cultivars. This plant response, according to Klimenko et al. (2006), is likely due to the inactivation of nitrate reductase caused by high temperatures, which reduces nitrate accumulation in plant tissues. The decrease in nitrate accumulation under heat stress was accompanied by the inability of cv. Barlack to develop its characteristic intense red coloration. Temperature regulates anthocyanin biosynthesis, and in this context, high temperatures suppress this process by downregulating anthocyanin-related genes, destabilizing biosynthetic enzymes, and inhibiting the transport of anthocyanins into the vacuole (Li and Ahammed, 2023), while also reducing nitrate reductase activity, ultimately lowering nitrate content in plant tissues (Klimenko et al., 2006). Regarding to the peptide treatments, the results of the present study are not consistent with those described by Colla et al. (2015), who showed that protein hydrolysates, through high phloem loading of amino acids, may repress nitrate uptake at the root level. The lack of response to peptide treatment observed in the present study may be

related to differences in amino acid composition or to the high temperature conditions that characterized the plant growth cycle. Finally, in cv. Aquino under optimal growth conditions, following Zn-biochelate treatment, and in cv. Barlack following both Fe- and Zn-biochelate treatments under optimal and heat stress conditions, a reduction in ammonium content was observed. This decrease in ammonium is likely a direct consequence of the observed reduction in nitrate levels. Lower nitrate availability in plant tissue can limit the activity of nitrate reductase, the primary enzyme responsible for the reduction of nitrate to ammonium (Zayed et al., 2023). The effects of biochelates on vitamin C were both cultivar- and condition-dependent. In Aquino, Zn- and Fe-biochelates increased vitamin C under optimal conditions but reduced it under heat stress, as did the protein hydrolysate. In Barlack, biochelate treatments decreased vitamin C under optimal conditions and had no effect under heat stress, whereas peptide treatment increased vitamin C compared to the control under heat stress. The reduction in vitamin C in cv. Aquino following peptide treatment is consistent with the findings of Toscano et al. (2023), who reported a decrease in ascorbic acid content in radish microgreens after application of plant-based protein hydrolysates. Regarding the effect of Fe-biochelate, the increase in vitamin C observed in Aquino under optimal conditions aligns with the direct relationship between tissue iron accumulation and total ascorbic acid content reported by Buturi et al. (2022) in lettuce. In contrast, the significant effect of Zn-biochelate on vitamin C content in the present study differs from the results of Brito et al. (2026), who found that zinc application did not significantly affect vitamin C in lettuce. This discrepancy may be related to the different chemical forms used (biochelate and sulphate). However, a promoting effect of zinc on vitamin C synthesis has been documented in other species, such as tomato (Uddin et al., 2023). With regard the soluble sugar content, several studies have shown that higher soluble sugar proportion and lower crude fiber proportion in total carbohydrate resulted in better taste of lettuce (Fillion and Kilcast, 2002; Lin et al., 2013). The present study provides evidence that both Zn- and Fe-biochelates, as well as peptides, reduce total sugar content in lettuce leaves and influence overall lettuce quality. The lowest sugar content was observed in Barlack

treated with Zn-biochelate under optimal conditions, whereas under heat stress, the lowest levels were found in Barlack treated with both biochelates (Fe and Zn) and peptide. This decrease in sugar following biochelate and peptide applications, as these compounds play a crucial role in plant metabolism (Zhao et al., 2020). The reduction in soluble carbohydrates may be linked to the application of biochelates and peptides, which could enhance nitrogen metabolism and increase amino acid synthesis at the expense of carbohydrate accumulation in lettuce. The findings of the study are in accordance with Rouphael et al. (2021), who tested a vegetal-derived protein hydrolysate and observed a decrease in glucose and fructose content in sweet basil. Similarly, Colla et al. (2015) reported that protein hydrolysate application stimulated enzymes involved in carbon metabolism and nitrogen reduction and assimilation, thereby affecting key metabolic pathways such as the Krebs cycle and glycolysis. Furthermore, the effect of the peptide was similar to that reported by Toscano et al. (2023), where its efficacy depended on the genotype. In their research, total sugar content was significantly reduced in radish, whereas no effect was observed in turnip greens. Consistent with these findings, peptide treatment in Barlack led to a decrease in total sugar content, while in Aquino, total sugar content remained comparable to that of the untreated control. Overall, the stimulated metabolic activity following biochelate and peptide treatments may have increased energy demand, resulting in reduced carbohydrates levels.

In this study, neither treatment significantly affected the phenol profile or antioxidant activity under optimal conditions. Under heat stress, however, Aquino treated with protein hydrolysate exhibited higher DPPH activity compared to the untreated control, whereas Zn-biochelate application on Barlack reduced DPPH values. Moreover, the cultivar factor significantly affected both parameters, with Barlack showed higher phenolic values than Aquino. These cultivar differences are consistent with El-Nakhel et al. (2023), who reported 8.90 mg GAE 100 g^{-1} for Aquino and 18.58 mg GAE 100 g^{-1} for Barlack 19 days after transplant. Red leaf lettuce generally contains higher levels of flavones, flavonols, and anthocyanins than green leaf lettuce (Llorach et al., 2008; Mampholo et al., 2016), which contributes to its stronger antioxidant activity, as also confirmed by Liu et al.

(2007), who identified anthocyanins as the main phenolic compounds in red lettuce. Moreover, the peptide treatment did not show significant efficacy, which, according to Colla et al. (2015), would normally be expected to enhance phytochemicals such as polyphenols, carotenoids, and flavonoids. This discrepancy suggests that under heat stress and with early foliar application, the protein hydrolysate may be ineffective in promoting the accumulation of these bioactive compounds. Zn-biofortification led to a reduction in DPPH activity in Barlack. This effect may be attributed to a shift in phenolic metabolism, as Zn can indirectly stabilize plant antioxidant systems, reducing the accumulation of free radical scavenging compounds detectable by DPPH. In line with this, foliar application of Zinc-Glycinate, a chelated form of zinc bound to the amino acid glycine, has been shown to enhance the activity of key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which work together to scavenge reactive oxygen species (ROS) and protect the plant from oxidative stress (Peptech Biosciences, n.d.; U et al., 2025).

Ultimately, both Fe-biochelate and Zn-biochelate had similar effects on free amino acids content and were not statistically different from the control. In addition, peptide application, under heat stress, on Aquino resulted in higher amino acid content, whereas on Barlack it reduced amino acid levels compared to the control. These contrasting results indicate a cultivar-dependent response to foliar protein hydrolysate treatment. The results observed in Aquino are in line with those described by Colla et al. (2015), who reported that protein hydrolysate foliar application increases their availability within plant tissues.

10. CONCLUSION

The current study demonstrates that foliar applications of Fe- and Zn-biochelates, as well as vegetal protein hydrolysates, can modulate lettuce growth, mineral content, and nutritional quality in a cultivar and environment dependent manner. Foliar application of iron and zinc biochelates resulted in enrichment of lettuce leaves under both optimal and heat stress conditions, increasing their nutritional value and exceeding reference dietary levels for iron and zinc. Protein hydrolysate treatments promoted root growth and, in some cases, antioxidant activity, while biochelates reduced nitrate accumulation and influenced carbohydrate metabolism. These findings highlight the potential of agronomic biofortification through foliar biochelate applications as an efficient strategy to enhance micronutrient levels in crops, contributing to the 2030 Agenda for Sustainable Development goals, including “Zero Hunger,” and supporting the reduction of diseases associated with micronutrient deficiencies.

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