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BIOFORTIFICATION OF VEGETABLES FOR NEW CONSUMER DEMAND

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Research highlights

- Micronutrient malnutrition affects 2 billion people worldwide.
- Biofortification is the process of increasing micronutrient content in crops to improve human nutrition.
- This thesis is focused on the agronomic biofortification of lettuce, carrot and tomato.
- Tomato plants treated with Fe through foliar spray and nutrient solution showed higher Fe concentration in fruits.
- Fe supply in the nutrient solution can increase Fe concentration in the leaves of lettuce plants.
- Foliar sprays of Fe, Zn, Se and Si improved the mineral profile of carrots roots and tomatoes.
- The results suggest that biofortification is a promising strategy to face malnutrition that should be further investigated.

Abstract

Over two-thirds of the world's population lack one or more essential mineral elements in the diet. Biofortification of vegetables is a promising approach to increase the dietary intake of minerals, by increasing the mineral content of commonly consumed vegetables. Because of their global relevance in the human diet, carrot, tomato and lettuce were submitted to different biofortification protocols aiming to increase the mineral content in the edible part of these crops. Giving iron importance, 3 out of 5 experiments of this thesis are dedicated to the Fe-biofortification. Firstly, cherry tomatoes were submitted to three concentrations of Fe in the nutrient solution (0, 1 and 2 mM), and received foliar applications of Fe (0, 250 and 500 mM). The combined application of 2 mM and 500 mM of Fe successfully increased Fe content of tomato fruits. Secondly, foliar Se-biofortification (8 mM) of cherry tomato was carried out both at fruit set and fruit ripening. Evidence shows applying the fertilizer at fruit ripening increases Se-biofortification efficiency. In the third experiment, two cultivars of lettuce were submitted to three concentrations of Fe in the nutrient solution (0, 1 and 2 mM). The results showed that the best concentration to obtain Fe-biofortified lettuce is 1 mM. After, carrots received four foliar applications of Fe and Zn (6 mM) in different chemical forms. Results show chelate is the best form to obtain bioaccessible Fe and Zn in the diet, when compared to sulfate. Finally, in the fifth experiment, Si-biofortification of carrot was investigated at the concentration of 15 mM. Results indicate that foliar applications of Si successfully improved mineral content in carrots besides improving the quality during post-harvest. In general, these results indicate that foliar sprays are a good biofortification tool both in the open field and in the greenhouse. Also, soilless cultivated crops as tomato and lettuce can be Fe-biofortified through the management of the nutrient solution.

Keywords: hidden hunger; nutrition; minerals; fertilization.

Riassunto

La dieta di oltre i due terzi della popolazione mondiale manca di uno o più elementi minerali essenziali per l'uomo. La biofortificazione degli ortaggi rappresenta un approccio promettente per aumentare l'assunzione di minerali nella dieta, attraverso l'incremento del contenuto di alcuni minerali negli ortaggi comunemente consumati. A causa della loro rilevanza globale nella dieta umana, carota, pomodoro e lattuga sono stati sottoposti a diversi protocolli di biofortificazione con l'obiettivo di aumentare il contenuto di minerali nella parte edibile di queste colture. In considerazione dell'importanza che il ferro assume nella nutrizione umana, tre capitoli su cinque del presente elaborato sono dedicati alla biofortificazione con ferro. In un primo esperimento, piante di pomodoro, tipologia "cherry" sono state sottoposte a tre concentrazioni di Fe nella soluzione nutritiva (0, 1 e 2 mM) e hanno ricevuto applicazioni fogliari di Fe (0, 250 e 500 mM). L'applicazione combinata di 2 mM nella soluzione nutritiva e 500 mM di Fe nella soluzione apportata alla chioma ha aumentato in maniera significativa il contenuto di Fe dei frutti di pomodoro. In un secondo esperimento, è stata effettuata la biofortificazione del pomodoro, sempre appartenente alla tipologia "cherry" con Se (8 mM), applicato sia all'allegagione che alla maturazione dei frutti. I risultati sperimentali hanno mostrato che l'applicazione della soluzione alla maturazione dei frutti ha aumentato l'efficacia della biofortificazione. In un'ulteriore prova, due cultivar di lattuga sono state sottoposte a tre concentrazioni di Fe nella soluzione nutritiva (0, 1 e 2 mM). I risultati hanno mostrato che la migliore concentrazione per ottenere lattuga biofortificata con Fe è 1 mM. Nel quarto capitolo, una coltura di carota 'Dordogne' ha ricevuto quattro applicazioni fogliari di Fe e Zn (6 mM) in diverse forme chimiche. I risultati mostrano che le forme chelate si sono dimostrate migliori per ottenere Fe e Zn bioaccessibili nella dieta, rispetto al solfato. Infine, nel quinto capitolo, è stata studiata la biofortificazione della carota con Si alla concentrazione di 15 mM. I risultati indicano che le

applicazioni fogliari di Si hanno migliorato il contenuto di minerali nei fittoni di carota oltre a migliorare la qualità in post-raccolta. In generale, i risultati riportati nella presente tesi indicano che le applicazioni fogliari sono un buon mezzo agronomico di biofortificazione sia in pieno campo che in serra. Inoltre, alcune colture, come pomodoro e lattuga, allevate con sistemi di coltivazione fuori suolo possono essere biofortificate con Fe attraverso l'incremento della concentrazione dell'elemento nella soluzione nutritiva.

Parole chiave: fame nascosta; nutrizione; minerali; concimazione.

1 Introduction

1.1 Preface

The world population is estimated in almost 8 billion people and projected to reach 10 billion in few decades (United Nations 2019). Adequately feeding such a large population is one of the biggest challenges of our century. In addition, in the last two years, world hunger was exacerbated by the impact of COVID-19 and increased to 720–811 million people. This number is even higher when we consider all cases of malnutrition, as today over 3 billion people are suffering from some kind of malnutrition (FAO 2021). Malnutrition appears because humans require several minerals and vitamins in order to maintain a good health, and the inadequate intake of even one of these micronutrients will result in adverse metabolic disturbances. Agriculture products have always been the primary source of nutrients and micronutrients for humans (Welch and Gabelman 2015). To answer this strenuous challenge, food production will have not only to increase but also to improve. Since malnutrition does not regard only the quantity, but also the quality of the food consumed.

In this context, micronutrient deficiency is defined as the insufficient intake of essential minerals and vitamins, also known as hidden hunger (White and Broadley 2005). In underdeveloped and developing countries it is mostly linked to poverty and the lack of a varied and balanced diet. Meanwhile, in industrialized countries, these micronutrient deficiencies can have multiple causes (O'Hare 2015).

Decades of intensive agricultural practices have affected soils quality and as a consequence, agriculture products cultivated in these areas and consumed by the population can be poor in micronutrients. This is mainly the case of iron (Fe), zinc (Zn), iodine (I) or selenium (Se) deficiencies in advanced economies population (Alloway 2009;

Hu et al. 2022). Furthermore, high-income urban population has developed new dietary habits. On one side, as a result of a busy work schedule, the average population has increased the consumption of readily available food (Van Rongen et al. 2020). These canned meals and other fast foods may be inexpensive and read to eat but are also, usually, high in calories and poor in essential micronutrients (Poelman et al. 2018). On the other side, for ethical or environmental reasons, many people have been replacing traditional food by restricted diets, as in the case of vegetarians and vegans, which are estimated in 1.5 and 0.8 billion of people, respectively (Leahy et al. 2010). Additionally, advances in the medical field have led to the unravel of new cases of allergies and intolerances (Ortolani and Pastorello 2006), also resulting in changes in the diet, that may limit the intake of important micronutrients. In all above cases, the limited consumption of food products that should provide micronutrients to the diet can increase the incidence of hidden hunger in the population.

At the beginning of the 20th century, when the first cases of micronutrient malnutrition were identified in the population, food fortification started to be used as an approach to improve the nutritional status of the population and, therefore, fight the hidden hunger. Fortification is a simple and direct strategy based on adding a nutrient to a product at the time of manufacture (O'Hare 2015). The first fortified food products commercialized were iodized salt, milk enriched with vitamin D and Fe-enriched flour (Rosenberg et al. 2004). Today, the market has expanded and there is a plethora of processed functional foods available to the consumer, as nutritional bars, spreadable fats, instant soups and energy or fruit drinks (Kroker-Lobos et al. 2022). However, this strategy is limited to processed food, and fresh products, such as vegetables, cannot be fortified.

In addition to the hidden hunger situation, the population from advanced economies is living longer and, often, desires to improve health and physical performance through better eating habits, which

includes the consumption of high-quality fresh products. This modern scenario creates a market opportunity related to the creation of special foods (O'Hare 2015).

Biofortification is defined as the practice to increase the concentration of specific micronutrients in the edible part of a plant species, while is still growing (Buturi et al. 2021). This approach is different from the direct fortification because is an indirect intervention carried out completely during preharvest and the only fortification strategy available for fresh consumed products, as vegetables (de Valença et al. 2017). Biofortification of crops are usually effected through agronomic and/or genetic approaches (White and Broadley 2009). Genetic biofortification protocols include genetically modified organisms and traditional breeding, these strategies face limitations related to the high costs, strict laws and high renewal rate of cultivars made by the vegetable seed industry (Gómez-Galera et al. 2010).

Agronomic biofortification involves simple techniques as the application of fertilizers to accumulate or to stimulate the production of micronutrients at plant level (Kyriacou and Rouphael 2018). In precision agriculture, nutrients can be efficiently applied to the above and/or belowground plant parts depending on the crop and targeted minerals (Chan 2006). Open-field crops can benefit especially from foliar spray applications of mineral fertilizers, meanwhile plants grown in protected cultivation systems can easily receive the minerals directly through the nutrient solution too (Buturi et al. 2021). However, attention should be paid to the mobility of the mineral applied and the translocation ability of the plant (Marschner 2011). In all cases, the concentration and chemical form of the fertilizer play a key role on the success of a biofortification program (White and Broadley 2009; O'Hare 2015).

Vegetables make up a substantial part of the human diet and provide a major part of nutrients, fibers and other compounds (such as

vitamins, antioxidants, minerals) necessary to prevent diseases and keep a healthy life (Fan 2016). For example, high vegetable diets have been associated with lower risk of cardiovascular disease in humans (Mullie and Clarys 2011). In addition, vegetables consumption is rising, reflecting the consumers awareness of their nutritional benefits (Simon 2014; Ridoutt et al. 2022).

Tomato (*Solanum lycopersicum* L.) is the most consumed solanaceous vegetable worldwide and its commercially importance throughout the world for the fresh fruit market and the processed food industry is remarkable (WHO 2005). Tomato plants are cultivated in a wide range of climates in the field, under protection in plastic greenhouses and in heated glasshouses (Singh et al. 2017). The consumption of this vegetable is associated with lower risk of developing certain cancers, cardiovascular diseases and osteoporosis (Burton-Freeman and Reimers 2011).

Lettuce (*Lactuca sativa* L.) is another popular product and the most consumed leafy vegetable in the world (WHO 2005). As tomato, lettuce is grown in the field or under protected cultivation systems (Filho et al. 2009). This vegetable is low in calories, fat and sodium and rich in fiber, folate, and vitamin C, as well as essential minerals such as iron (Kim et al. 2016).

Carrot (*Daucus carota* L.) is another important vegetable crop and undoubtedly the most relevant crop of the family Apiaceae (WHO 2005). It presents good amounts of carotenoids, flavonoids, polyacetylenes, vitamins, and minerals, offering numerous nutritional and health benefits (Prasad et al. 2017). It can be considered an anti-diabetic vegetable and it contributes to lowering bad and improving good cholesterol levels, reducing this way the risk of developing cardiovascular diseases. It is also known for its anti-hypertensive, hepatoprotective, renoprotective characteristics, besides acting as anti-bacterial, anti-fungal, anti-inflammatory (da Silva Dias 2014; Fabiyi et al. 2015).

Given that shortage of Fe is the most common micronutrient deficiency worldwide and that Fe is essential for brain development, myelination, growth, and cognitive function, 3 out of 5 experiments were dedicated to the crops biofortification with this mineral.

The first research was focused on the Fe-biofortification of cherry tomato fruits. The aim of this study was to increase the Fe content in cherry tomato fruits cultivated in a soilless system. Plants of cherry tomato 'Creativo' were submitted to three concentrations of Fe (as N,N'-Bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid, HBED) in the nutrient solution (0.022, 1 and 2 mmol L^{-1}) and received foliar applications of Fe (as diethylenetriaminepentaacetic acid, DTPA) at three different concentrations (0, 250 and 500 µmol L^{-1}).

The second research was focused on the Fe-biofortification of two lettuce cultivars. This experiment addressed the effects of different iron (Fe) concentrations in the nutrient solution supplied as Fe-HBED, i.e., 0.02 (Fe0, control), 1.02 (Fe1) and 2.02 mmol L^{-1} (Fe2) on lettuce ('Nauplus' and 'Romana') yield and compositional traits, the tolerance to Fe stress was also evaluated.

In the third experiment, the Fe and Zn biofortification of carrot was investigated. This study aimed to compare the efficiency between the chelate and sulfate forms of Fe and Zn applied through foliar sprays for the biofortification and bioaccessibility of both minerals in the off-season carrot cv. Dordogne.

Due the importance of Se for human health, the fourth experiment was focused on the Se-biofortification of cherry tomatoes. The aim of this study was to increase the Se content in cherry tomato fruits cultivated in a soilless system through a single foliar application of Se, as sodium selenate (Na_2SeO_4), at two different stages of fruit development: fruit set (immature green stage) and ripening (breaker stage).

Lastly, the biofortification potential of silicon (Si) is a

promising strategy that might improve vegetables tolerance to postharvest storage conditions. For this reason, the fifth experiment of this thesis was focused on investigating the effect of Si as foliar spray on carrot morphological and compositional traits, along with postharvest behavior also in relation to the leaf presence.

1.2 State of the art

1.2.1 Mineral biofortification of vegetables as a tool to improve human diet

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1.2.1.1 Introduction

Many nutritional recommendations for human well-being and disease prevention have highlighted dietary styles based on the growing consumption of fresh fruits and vegetables and the reduction of simple carbohydrates, sodium and saturated and trans-fats consumption (Wang et al. 2016). In order to maintain a good health, people require several mineral nutrients that must be included in the diet. The essentiality of minerals can be demonstrated by the fact that vitamins cannot be absorbed solely or work in the absence of specific minerals, which are necessary in many physicochemical processes (Gupta and Gupta 2014).

Deficiencies of specific mineral elements affect, in both underdeveloped areas and industrialized countries, up to two-thirds of the world's population (Bailey et al. 2015; Hefferon 2015; O'Hare 2015) and the insufficient intake can cause severe damage to people's health (Tardy et al. 2019). For instance, in Europe and Central Asia, malnutrition problems related to diets with low micronutrient contents are increasing the number of women and children with anemia. In fact, iron and iodine deficiency disorders are the most common forms of malnutrition (FAO 2018). Besides, a recent study conducted in South Italy, showed that the population has low intake of calcium and

potassium (Castiglione et al. 2018).

Food, mainly plant-based, is the source of all important minerals, therefore it is important to keep on a regular basis a good and balanced diet that can provide the adequate proportion of minerals (Gharibzahedi and Jafari 2017). The enrichment of food with health-related compounds and mineral elements could, however, be considered a strategy to fight undernourishment or to face with specific nutritional need (Vlaic et al. 2019).

In the case of not processed food, such as vegetables, the only option to enhance the nutrient content of products in preharvest using improved genotypes or adopting specific agronomical techniques (Kyriacou and Rouphael 2018).

The increasing interest in the enrichment of fresh consumed vegetables with mineral elements has encouraged intensive research activity focusing on the elaboration of suitable application protocols. This review describes developments in agronomic biofortification of vegetables with reference to some mineral elements often lacking or not adequately present in human diets. i.e. calcium (Ca), magnesium (Mg), iodine (I), zinc (Zn), selenium (Se), iron (Fe), copper (Cu) and silicon (Si). After synthetically considering the role in human nutrition and in plant physiology, this review aims to discuss the most successful agronomic strategies to increase the amount of the considered minerals in the edible portion of vegetables.

1.2.1.2 The role of vegetables for human health and how biofortification can have an impact

Plant foods make up a substantial part of the human diet and they provide most of calories, nutrients and bioactive compounds necessary to keep a healthy status and prevent diseases. Vegetables are one of the pillars of a plant-based good diet, providing in particular dietary fiber, phytochemicals (such as vitamins, antioxidants) and

minerals (Fan 2016; Wang et al. 2017). Minerals are considered essential nutrients: they are not synthesized by humans and must be obtained from the diet. Humankind evolved thanks to the dietary assumption of a significant number of vegetables and their insufficient intake is one of the reasons of many non-communicable diseases, which are spread in Westernized societies. As an example, potassium, calcium, selenium and iodine obtained through a vegetable-rich diet, can contribute to maintain good blood pressure, bone strength, hormonal production, heart and mental health (Fairweather-Tait and Cashman 2015; Schreinemachers et al. 2018). In a recent study carried out in the UK, data analysis from more than 40.000 people showed that changes in fruit and vegetable consumption may not only benefit physical health in the long-run, but also mental well-being in short term (Lalji et al. 2018; Ocean et al. 2019), besides general population these benefits were also observed in cancer survivors (Zhang et al. 2021). On the other hand, vegetables play an important role in the economy, fighting poverty, hunger and undernutrition, since they can be locally cultivated and consumed in a high diversity of shapes, sizes, colors and tastes (Dixon and Aldous 2014; Cicco 2016; Fan 2016).

Non-optimal intake of micronutrients and undernutrition, the so-called hidden hunger, can be particularly severe for people following restricted diet for religious, ethical or medical reasons (Hefferon 2015; O'Hare 2015; Sharma and Verma 2019). Health authorities have established dietary reference intakes (DRI) based on recommended daily allowances (RDA) and tolerable upper levels (UL). As general principle, strategies that address vitamin or mineral deficiency must aim to achieve the DRI for each component without exceeding the UL (Sanahuja et al. 2013).

However, the actual contribution of phytochemicals and minerals to human diet is not only related to their concentration in a certain plant tissue. The micronutrients must be released from the food matrix during the passage in the gastrointestinal track, absorbed into

the blood and transported to their target tissues (Boland et al. 2014). In fact, only the fraction released from the plant tissue become eventually available for absorption. This fraction is indicated as bioaccessible and to increase the bioaccessibility of plant phytochemicals and minerals is a promising target of agronomical strategies to improve the nutritional quality of vegetables (D'Imperio et al. 2016a).

Vegetable consumption should increase in the coming years for sustainability and healthy reasons. To deal with the rise of global population, more sustainable food sources will be needed (Ruini et al. 2015). According to Schreinemachers et al. (2018), the most important vegetables in the current global economy are tomatoes, cucurbits (pumpkins, squashes, cucumbers and gherkins), alliums (onions, shallots and garlic), chilies, spinach, potatoes, carrots and brassicas therefore it makes sense to focus the biofortification efforts on these species.

1.2.1.3 Biofortification of vegetables

The approaches to address micronutrient malnutrition are different; medical supplementation and products fortifications are the most adopted. Fortification is the process of foods enrichment with nutrients, adopting different methods during processing. However, in some contexts, fortification is challenged due to poor investments, infrastructure and delivery system (Govindaraj 2015). Under these conditions, an alternative strategy is to adopt new genotypes, characterized by improved compositional profiles, or to tailor specific agronomic techniques aimed to enhance the content of specific health effective compounds in widespread crops (Govindaraj 2015). While this can be considered an option for products which are transformed before they are used (e.g. staple foods), for fresh consumed products, such as vegetables, biofortification is the only choice to improve the

content of health-related compounds in the edible portion.

Among the different strategies to obtain biofortified vegetables there are agronomic and genetic approaches, the latter can be done either through conventional breeding or transgenic methods (White and Broadley 2009; Siwela et al. 2020). The objectives are to increase in the edible portion the minerals content or other specific health related compounds. Transgenic programs involve biotechnology studies that allow to genetically modify a species, to obtain a plant with targeted characteristics (i.e.: higher content of specific nutrients). Even though this approach could be cost effective in the long run, it is the least employed methodology today because the phase of research and development is still very slow and expensive. In any case, in developed countries the higher prices involved in the production of biofortified vegetables is countered by the achievement of a premium product with a superior nutritional quality, that can satisfy the new consumers' demand willing to pay for a healthier way of eating (Timpanaro et al. 2020). In addition, some countries have restrictive laws, that forbid genetically modified organisms (GMOs). Along the same lines, there is the option to cross different genotypes, with the aim to introduce in new cultivars desirable traits naturally occurring in plants. This genetic approach (traditional breeding) has been performed from decades and can allow to create new varieties with a higher content of certain nutrients. In this case, the limitation is to find the desired characteristics in the available genetic resources (Gómez-Galera et al. 2010). On the other hand, breeding programs, even when effective, may vanish their effect due to the high renewal rate of cultivars coming from the large number of new introductions made by vegetable seed industry (Maynard 2002).

Biofortification programs carried out through the agronomic approach are the best option, since they involve simple techniques to accumulate or to stimulate the production of specific compounds at

plant level. A substantial part of the biofortification research that has been carried out in the last decades focused the attention on specific compounds such as vitamins and amino acids, rather than minerals (Carvalho and Vasconcelos 2013; Davies and Espley 2013; Hefferon 2015; Wang et al. 2017; Scarano et al. 2018). A variety of biofortified products with vitamins or their precursor include banana, mango, sweet potato, wheat and cauliflower (Shwetha et al. 2020). In the same line, biofortification with amino acids proved to be effective in producing high lysine-rice, using the double strategy of maximizing its biosynthesis and minimizing its catabolism (Yang et al. 2016). Besides, evidence shows that sulfur fertilization on wheat, barley and potato can increase the sulfur-containing amino acids (SAAs) methionine and cysteine content in their edible part. In the same way that the application of nitrogen plus potassium is potential in increasing carotenoid content in carrots (Prasad and Shivay 2020).

However, besides the increase of the content of some specific compounds (e.g.: antioxidants) with controlled doses of stressors (Rouphael and Kyriacou 2018), agronomic biofortification consists in increasing or optimizing the application of mineral elements to the crop in order to increase the corresponding content in the edible organs. In this case the focus is on setting up the form of the mineral, the concentration and the application form; indeed, certain mineral forms or quantities can cause indirect effects, damaging or compromising a crop (White and Broadley 2009; O'Hare 2015).

1.2.1.4 Agronomic mineral biofortification

The production of vegetables is carried out in very diversified agronomic contexts as regards crop cycles, soil conditions or growth environments. Agronomic approaches to rise the concentration of minerals in edible organs generally rely on the supply of mineral fertilizers and/or improvement of the mobilization and solubilization

of mineral elements in the rhizosphere (White and Broadley 2009). Vegetable crops are generally grown in agro-systems characterized by a high degree of intensification of the production processes and in which the supply of nutrients is increasingly based on the use of fertigation, soilless cultivation and foliar fertilization. These alternatives offer different opportunities to implement targeted biofortification programs. In the case of the application of mineral elements by fertigation on soil cultivated crops, some interference may derive from element availability for the plant (phytoavailability), therefore selecting mineral forms and concentrations may have a relevant importance (2013White and Broadley 2009; Carvalho and Vasconcelos). One alternative strategy to overcome the low mineral phytoavailability into the soil is the cultivation through hydroponic systems (soilless cultivation). The possibility of optimizing limited water resources and cultivating in the absence of suitable agricultural soils, has led to a considerable spread of soilless cultivation systems. It has been observed, for example, that hydroponic cultures can be among the best options to increase the nutrient content of plant tissues (Li et al. 2017a; Wiesner-Reinhold et al. 2017). In the case of minerals not readily translocated to the edible tissues, such as for crops grown on soil and/or for minerals with scarce mobility, another alternative is the use of foliar fertilization (Niu et al. 2020).

1.2.1.4.1 Calcium

In human health, calcium (Ca) is required in several systems, like musculoskeletal, nervous and cardiac. It is essential to maintain good bones, teeth and mineral homeostasis. It also acts as a cofactor in many enzyme reactions and contributes to the function of the parathyroid gland (Beto 2015). The RDA of Ca ranges between 1000 and 1300 mg day^{-1}. The UL for adults is 2500 mg day^{-1} (Yates et al 1997). Calcium is an important nutrient for plant metabolism, involved in structural functions of cell, acting as a counter-cation for

organic and inorganic anions trafficking across the tonoplast and as an intracellular, cytosolic messenger (Marschner 2011). It is one of most abundant nutrients in the earth's crust, with an average concentration of about 36.4 g kg^{-1} (Lawlor 2004). Ca^{2+} concentration in the soil solution is usually enough (0.1-20 mM) to meet the plants' demand or, in neutral and calcareous soils, to exceed their requirement, thus leading to Ca accumulation in the vicinity or inside the roots (Marschner 2011). However, some Ca-deficient conditions can sometimes be encountered, especially in highly weathered tropical soils or in saline/sodic soils. Calcium is absorbed as divalent cation by the root apex and/or regions of lateral shoot initiation (White and Broadley 2003), where Casparian band between endodermal cells is absent or disrupted, and/or the endodermal cells surrounding the stele are not suberized (Olle and Bender 2009). Once inside the plant, Ca moves primarily through the xylem (White and Broadley 2003) with the water flow driven by transpiration (Kerton et al. 2009; Demidchik et al. 2018), either as Ca^{2+} or complexed with organic acids (Welch 1995). However, Ca^{2+} movement inside the xylem vessels cannot be explained simply in terms of mass flow, as Ca^{2+} ions are also absorbed by adjacent cells and are complexed to non-diffusible anions in the xylem walls (Demidchik et al. 2018). Because of its slow phloematic mobility, this element is present at lower concentrations in mostly phloem-fed organs (e.g. young leaves, fruits and tubers) than in the older leaves (~10-times less). Considering the mineral partitioning inside the plant, leafy vegetables can play a primary role in the dietary intake of Ca, so being possible targets for Ca biofortification (Neeser et al. 2007). This last should be addressed at increasing the Ca content of the edible portions, without adversely impacting both plant growth and production costs (White and Broadley 2009). Most plant species can accumulate high Ca contents in leaf laminae (up to 100 g kg^{-1} DW) without any symptoms of toxicity, because Ca exceeding plant's needs is detoxified by sequestering as insoluble Ca oxalate and deposited

either in the cell wall or stored inside the vacuole (Olle and Bender 2009; Marschner 2011). Depending on the plant species, tissues and growing conditions, Ca concentration in plants varies between 1 and > 50 mg kg^{-1}. However, some species may have insufficient detoxification mechanisms, so their growth can be severely depressed at high Ca tissue content (Marschner 2011). Excessive Ca can cause toxicity symptoms such as the presence of yellow flecks formed by crystals of calcium oxalate and growth inhibition, the latter can be observed even in calcicole species (plants occurring in calcareous soils) when submitted to a soil solution with a concentration higher than 10 mM Ca (White and Broadley 2003). Strategies for Ca biofortification should include: (i) increasing Ca supply to cells; (ii) increasing Ca uptake into cells; (iii) removing compounds making Ca unavailable and/or (iv) increasing Ca storage at the cellular and/or tissue level (Frossard et al. 2000; White and Broadley 2009; Yang et al. 2012). The application of Ca fertilizers can increase its concentration mostly in leafy vegetables (Table 1-1.1), whereas for grain, seeds and fruits, sound indications are still to be reached. In 21-day old *Brassica rapa* plants grown on soil, the increased Ca supply to roots (compost mix supplemented with 0.4 vs. 3.5 g $CaCl_2$ L^{-1}) significantly enhanced its concentration in leaves (0.75 and 25 g kg^{-1} DW, respectively). The result was not influenced by the different supply of Mg fertilizer (Rios et al. 2012). To reduce the effects of different soil characteristics (e.g. minerals concentration, pH) on Ca availability, soilless cultivation on inert substrates or water (e.g. floating system) allows a better control of the ion concentration in the root environment. In some leafy vegetables, D'Imperio et al. (2016b) increasing the Ca concentration adding calcium phosphate and calcium chloride in the nutrient solution (from 100 to 200 mg L^{-1}), determining an increase of Ca concentration in leaves of basil (~15%) and mizuna (~12%), but not in tatsoi or endive (Table 1-2). Moreover, the biofortification process did not influence their oxalate content nor

Ca bioaccessibility. A higher Ca content (up to 5-fold higher than control) in lettuce (*Lactuca sativa* L.) grown in a floating system was obtained by Borghesi et al. (2013) with a nutrient solution containing 800 mg Ca L^{-1} (as $CaCl_2$), compared to the control with no Ca addition. However, the high salt content increased both the Cl concentration and electrical conductivity of the nutrient solution, so reducing the marketable quality and yield (-32%). Foliar applications of soluble Ca fertilizers are commonly made for several horticultural crops, to prevent Ca-deficiency disorders. However, only few experiments refer to Ca biofortification through foliar applications. Moreover, these applications are expected to have limited effects on Ca content of roots, tubers and seeds, because of the typical translocation patterns of the element. In one of few experiments, Yuan et al. (2018) observed a significant increase of Ca concentration in lettuce sprayed three times every 20 days with a 120 mg L^{-1} of $CaCl_2$ compared to 60 and 180 mg L^{-1} (21.4% and 5.2%, respectively), despite this effect was genotype-dependent. Overall, Ca biofortification of vegetables using Ca chloride proved to be effective in the majority of the studied leafy crops even if negative effects on yield cannot be excluded; besides, one of the main challenges is related to the presence of oxalate, which can partially limit Ca bioavailability.

1.2.1.4.2 Magnesium

In human health, magnesium (Mg) is essential in maintaining muscle tone and blood pressure. It participates in the glycemic control, neuromuscular function and myocardial contraction. It is also involved in the energy metabolism, besides being a cofactor of many enzymes (Al Alawi et al. 2018). The RDA for Mg ranges between 320 and 420 mg day^{-1}. The UL for adults is 420 mg day^{-1} (Yates et al. 1997). Magnesium is a divalent cation and it is essential in plants because of its ability to interact with strongly nucleophilic ligands, it participates in the processes of enzyme regulation, pH cellular and

cation-anion balance, besides being a key metal in chlorophyll structure (Shaul 2002). Magnesium is relatively mobile in soils, where its average concentration can vary between 0.5 g kg^{-1} and 40 g kg^{-1} (Gransee and Führs 2013), with a worldwide average of 5 g kg^{-1} . In addition to passive diffusion, as it happens with others divalent cations, Mg is actively absorbed by roots through permeable cation channels (Kuhn et al. 2000). Regarding leaf uptake, younger leaves are more likely to absorb Mg than the aged ones (Oland and Opland 1956). Mg^{2+} transporters in higher plants are thought to be derived from the CorA transport system, acting as a gate locking it when the Mg concentration in the cytosol is increasing or opening otherwise (Marschner 2011; Kobayashi and Tanoi 2015). Concentration of Mg in the metabolic pool of leaves is supposed to be between 2–10 mM, while free Mg concentration is expected to be lower (around 0.4 mM). For an optimal growth, plants demand between 1.5-3.5 g kg^{-1} of Mg in vegetative fractions (Marschner 2011). Even though toxicity with Mg is rare, concentrations above 20 mM proved to be phytotoxic, causing symptoms like coppery color leaves, decrease in starch contents and growth reduction (Guo et al. 2015). In contrast to the translocation difficulties observed for Ca, Mg shows a high phloem mobility and the application of Mg fertilizer can efficiently increase its concentration in leaves, tubers, fruits, seeds and grains (White and Broadley 2009; Marschner 2011), making Mg agronomic biofortification of vegetables a feasible option to fight cases of malnutrition. As indicated in Table 1-1.1, plants of Indian colza (*Brassica rapa* ssp. *trilocularis*) submitted to different Mg biofortification protocols, showed on average a 3.6-fold increase in Mg content of leaves, when compared to untreated plants. In one experiment, after growing Indian colza plants on peat with a low (0.20 g L^{-1}) or high (3.04 g L^{-1}) Mg chloride ($MgCl_2$) concentration, leaf content increased up to 12 mg Mg kg^{-1} DW. However, the increase was 50% lower when plants received simultaneously a high dose of

$CaCl_2$ (3.04 g), showing a possible negative interaction between Mg and Ca (Rios et al. 2012). Similarly, Blasco et al. (2015) submitted *Brassica rapa* plants to different nutrient solutions. When comparing the application of a low (4.86 mg L^{-1}) and a high (486.1 mg L^{-1}) dose of Mg (as $MgCl_2$) in the nutrient solution they noticed a 12-fold increase in the Mg content of shoots, passing from low to high dose. The same authors tested the interaction with other minerals and concluded that the Mg concentration in shoots increased with high Zn (500 µM), and low Ca (0.4 mM) supplies and decreased at high Ca (40 mM) supply. Another biofortification study of Mg was conducted applying doses of 0, 50, 100, 150 and 200 mg Mg dm^{-3} soil (as magnesium sulfate, $MgSO_4 \cdot 7H_2O$) on growth of onion plants (*Allium cepa* L.) (Kleiber et al. 2012). The maximum Mg content in bulbs was obtained at the dose 150 mg dm^{-3}, i.e. almost 2-times higher than the untreated plants. However, this dose negatively affected the crop yield, and also caused a reduction in the uptake of Ca and potassium (K), showing that the antagonism between these minerals should be carefully evaluated. Therefore, the authors suggest using the Mg-100 dose, as it allowed to increase the Mg content of the bulbs (up to 1.4-fold, when compared to control), with a contextual increase in crop yield (up to 38%). There is evidence that fertilization of Mg via foliar spray can act to improve crop yield and quality (Zlámalová et al. 2015; Altarugio et al. 2017). The few studies on Mg biofortification show that both $MgSO_4 \cdot 7H_2O$ and $MgCl_2$ are effective in enhancing the element content in vegetables. However, Mg biofortification should be carefully managed considering its interaction with Ca, since high Ca content can inhibit Mg uptake by plants.

1.2.1.4.3 Iodine

Iodine (I) is essential for humans; it is required in the synthesis of the hormones thyroxine and triiodothyronine that are produced in the thyroid gland and are responsible for regulating growth and

development, besides maintaining the basal metabolic rate (Zicker and Schoenherr 2012). The RDA of I is 150 µg day^{-1}, whereas the UL for adults is 1100 µg day^{-1} (Trumbo et al. 2001). Typical I concentration in soils is between 0.5-20 mg kg^{-1}, and even though not essential to plant growth, it can be absorbed and translocated within the plant tissues. Plant leaves absorb I through stomata (60%) and leaf surface (40%), but I losses can occur too, due precipitation, wind and tissue decay; the remaining can be partially transported via phloem to the other plant organs, including roots (Whitehead 1984). According to Smolèn et al. (2014), leaves absorption occurs due the organophilic nature of I and its interaction with cuticular waxes or oxidation of I- (iodide) to I_2 (iodine), facilitating I penetration into the cuticle. It is known that root absorbs iodide better than elemental I or iodate, especially in plants grown in hydroponic systems. This I is majorly retained into the roots, but when in nutrient solution with concentrations higher than 0.01-10 µM it can also be translocated to the shoots (Whitehead 1984; Dobosy et al. 2020). In fact, I is efficiently transported into the xylem, transport in plants is analogous to chloride movement, I^- uptake being catalyzed by H+/anion symporters and released into the xylem by anion channels (White and Broadley 2009). Concentration of I in plants can be zero or extremely low, about 30-100 µg kg^{-1} FW (Medrano-Macías et al. 2016). Depending on plant species, a nutrient solution with concentrations higher than 10 to 100 µM can already be phytotoxic and inhibit plant growth (Whitehead 1984). In general, the different I chemical forms present the following phytotoxicity order: (I_2) > (I^-) > iodate (IO_3^-) (Mackowiak et al. 2005). Horticultural crops are the best candidates for I biofortification, because of their ability to absorb and accumulate exogenous I into the edible fractions (Caffagni et al. 2012). As reported in Table 1-1.1, once submitted to different biofortification protocols, leaf species such as basil and Chinese cabbage, showed an average I increase higher than 100-fold in their edible tissues, while

cabbage, lettuce, mizuna, mustard and spinach resulted in increases varying from 5 to 91 times. Average accumulation of I in vegetable fruit species was higher than 100-fold in both tomato and cowpea. Tuber species such as potato, showed a 13-fold average increased in I content, while root vegetables such as carrot presented a much higher average increase (greater than 100-fold). Biofortification of I through repeated foliar spray has been successfully performed in carrot and mustard plants (Golubkina et al. 2018; Signore et al. 2018). Higher efficacy of lettuce iodine biofortification was noted after foliar application, rather than adding the element to the nutrient solution (Smoleń et al. 2014). On the contrary, Caffagni et al. (2012) demonstrated that, even though it is possible to enhance the I content of tomato fruits through KIO_3 foliar spray, better results were observed through fertigation with a 5 mM solution of KI; this allowed to achieve a 249-fold I increase in this vegetable. When grown in water culture, lettuce plants grown with 90 µg I L^{-1} as potassium iodide (KI) showed better biofortification results than plants submitted to the same amount of I as potassium iodate (KIO_3), with the result consisting in a 30-times more I in leaves than untreated plants (Voogt et al. 2010). Low doses of I, such as 0.25 mg L^{-1} (KI) or 0.50 mg L^{-1} (KIO_3) in the nutrient solution are enough to achieve around 7 mg kg^{-1} DW of I in strawberry fruits, compared to 0 in the control, improving plant growth too (Li et al. 2017b). Analogous results were observed in several leafy vegetables (e.g. broccoli raab, curly kale, mizuna or red mustard) when submitted to low doses of iodine (0.75 mg L^{-1}, 5.9 µM KIO_3) through fertigation, showing an increase ranging from 390 to 471 µg kg^{-1} FW (Gonnella et al. 2019). However, high I levels (50 mg L^{-1}) in the nutrient solution, proved to increase the I content in carrot up to toxic amounts for humans (9 mg kg^{-1} FW) showing also phytotoxic effects on plants (Signore et al. 2018). In addition, I biofortification should be carefully evaluated, since there is evidence that I can decrease Cu uptake by plants (Medrano-Macías et al. 2016). However,

even though insufficient phloem loading and high volatilization rates could limit I accumulation, both K iodate and K iodide have successfully increased the I content in horticultural products.

1.2.1.4.4 Zinc

In human health, zinc (Zn) is essential for maintaining the structure and activity of many enzymes, besides playing a key role in the synthesis of nucleic acids and proteins. It acts in cell differentiation, glucose use and insulin secretion (Roohani et al. 2013). The RDA of Zn ranges between 9 and 14 mg day^{-1}, whereas the UL for adults is 40 mg day^{-1} (Trumbo et al. 2001). Zinc is essential in plant metabolism, as it plays a key role in chloroplast development and function through the Zn-dependent activity of SPP peptidase and repair of photosystem (PS I) I, besides participating in enzyme activation process such as RNA polymerases and superoxide dismutase, protein synthesis and metabolism of carbohydrate, lipid and nucleic acid (Sharma et al. 2013). Although most of the world's cultivated soils contain enough Zn to sustain its accumulation in plants' edible portions (between 10 and 100 mg kg^{-1}), Zn phytoavailability is a factor often limiting its uptake by roots, so that it has been estimated that about one-fifth of the world's population actually suffers from Zn deficiency (White et al. 2018). Under these conditions, agronomic strategies are aimed to improve the Zn phytoavailability into the soil, e.g. by correcting soil alkalinity, implementing more proper crop rotations, introducing beneficial soil microorganisms or delivering phytoavailable Zn through the application of Zn-fertilizers to soil or foliage (White et al. 2018). Zinc is absorbed by the plants from the soil solution primarily as Zn^{2+} (Strategy I plants) or complexed with organic ligands released by roots (phytometallophores), a mechanism which is restricted to cereals (Strategy II plants) (Broadley et al. 2007). Once inside the plant, xylem loading occurs either via symplast and apoplast, whereas in the

xylem sap Zn is transported in its ionic form or in form of metal complexes with asparagine, histidine, organic acids and nicotianamine (Gupta et al. 2016). Similarly, phloem Zn redistribution to various organs is thought to be effected either as divalent cation or in complexed forms with nicotianamine, malate or histidine (White and Broadley 2009). Because of its low phloematic mobility, Zn-supplied plants through the rhizosphere show a decreasing Zn concentration in the order shoot ≈ root > fruit, seed, tuber, thus showing a penalty on phloem-fed organs (White and Broadley 2011). For this reason, root crops and leafy vegetables are thought to have the greater potential to increase dietary Zn uptake (White et al. 2018). It must be pointed out that despite the low Zn phloematic mobility, Zn translocation through phloem for several plant species after application to foliage has been found to be nutritionally considerable for their growth and development, especially when cultivation occurs on substrates with low Zn phytoavailability (Waters and Sankaran 2011). Plants markedly differ in their ability to accumulate Zn in their tissues, but as a general rule, most crops require a leaf Zn concentration higher than 0.015-0.030 g kg^{-1} DW to reach their maximal yield. However, phytotoxicity symptoms are usually noticed at concentrations greater than 0.1-0.7 g kg^{-1} DM, depending on the species and exposure time (White et al. 2018). When toxicity levels are attained, plants show an array of heavy metal stress responses such as growth and yield inhibition, leaf chlorosis and necrosis, restricted stomatal conductance and CO_2 fixation, changes in chlorophyll structure and concentration (Tsonev and Lidon 2012), so the higher threshold concentration actually represents a physiological limit to the biofortification achievements. Nonetheless Zn hyperaccumulation capacity has been observed in members of Brassicaceae, Caryophyllaceae, Polygonaceae and Dichapetalaceae, whereas a greater Zn susceptibility has been noticed in the Linaceae, Poaceae and Solanaceae (Broadley et al. 2007). Common inorganic Zn-fertilizers

include $ZnSO_4$, ZnO, and synthetic chelates (White and Broadley 2009) such as Zn-EDTA, Zn-DTPA or Zn-HEEDTA. When foliar applications are concerned, the Zn compounds used must be highly soluble and enter rapidly into the leaf apoplast, in order to promote Zn translocation to phloem-fed organs, so avoiding possible interferences with mesophyll metabolism (White and Broadley 2011). Because of their ability to hyperaccumulate Zn, leafy *Brassicas* have been extensively studied in biofortification protocols (Table 1-1.1). So that, in kale leaves (*Brassica oleracea* L. var. *acephala*) de De Sousa Lima et al. (2015) reported up to a 28-fold increase of Zn concentration by providing the crop with 300 mg Zn kg^{-1} soil. After applying 22.7 kg ha^{-1} of Zn (in the form of Zn sulphate, $ZnSO_4 \cdot 7H_2O$) to the soil, Mao et al. (2014) observed a significant increase in the Zn concentration of the edible portions of canola (*Brassica napus* L.) and cabbage (*Brassica rapa* L. Chinensis Group) (by 25 and 200%, respectively). Zinc biofortification through foliar spray has been successfully performed in arugula (*Eruca sativa* L.) using 1.5 kg ha^{-1} of $ZnSO_4 \cdot 7H_2O$, with a resulting +94% increase of leaf Zn concentration (Rugeles-Reyes et al. 2019). Among non-Brassicas leafy vegetables, in a study conducted by Barrameda-Medina et al. (2017) hydroponically cultured plants of lettuce (*Lactuca sativa* L.) supplemented with 100 µM $ZnSO_4 \cdot 7H_2O$ in the nutrient solution showed a 251% increase in leaf Zn concentration. Simultaneously biofortification programs must take into account that high Zn concentration on soil cultivated crops can negatively affect Fe absorption and improve the content of Mn and of amino acids (De Sousa Lima et al. 2015). In conclusion, Zn biofortification, especially in the form of sulphate is promising in increasing the mineral content in vegetable products.

1.2.1.4.5 Selenium

Selenium (Se) is an essential trace mineral, constituent of the

selenoproteins responsible for important enzymatic functions. The function of selenoproteins in the human metabolism is most commonly connected to immunocompetence and cancer prevention, but it is known that Se functions go above that, as this mineral plays an important role on fertility and reproduction, brain functions, mood, thyroid health and cardiovascular diseases (Rayman 2012). The RDA of Se is 55 µg day^{-1}, and the UL for adults is set at 400 µg day^{-1} (Trumbo et al. 2001). Selenium is not considered a micronutrient, but its appropriated use in plant nutrition can increase growth, stimulate seed germination and contribute to protect several crops against pathogens and pests (Pandey 2015). Soil concentration of Se is relatively low and it varies according to the type of rocks, being generally between 0.01 and 7 mg kg^{-1}, with a worldwide average of 0.4 mg kg^{-1} (Lopes et al. 2017). Plants take up Se inorganically both as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) (Sors et al. 2005). Plant absorption and transportation of Se are active processes (Li et al. 2008). Into the roots, due to its chemical similarity to sulfur (S), selenate moves through high-affinity sulphate transporters, while selenite movement is partially mediated by phosphate transporters (White et al. 2004; Li et al. 2008). Translocation of Se from root to the aerial parts of the plant is more likely to happen as selenate, since selenite is more prone to be accumulated in roots. Leaf surface can absorb volatile forms of Se from the atmosphere (Terry et al. 2000). Foliar application of Se at late growth stages seems to optimize the uptake, translocation and distribution of Se into the edible portions of plants, whereas selenate is more efficiently accumulated in plant tissues than selenite (Deng et al. 2017). The tolerable Se content in most plant species is between 10-100 mg kg^{-1} DW (White 2016) and phytotoxic effects due to Se excess can compromise plant growth through damages to photosynthetic apparatus, photosynthesis inhibition and over-production of starch (Garousi et al. 2017). In addition, secondary accumulators, also called Se-indicator, as some

vegetables of the Asteraceae, Brassicaceae and Fabaceae family, when supplied with exogenous Se can accumulate up to 1 g kg^{-1} DW, being a good target for Se biofortification (White 2016). Skrypnik et al. (2019) reported that Se biofortification of basil through foliar application of sodium selenite (Na_2SeO_3) at 10 µM (4 applications starting from the 7º day after transplanting) enhanced the Se concentration in leaves to up 10.74 mg kg^{-1} DW (more than 700-time higher than untreated plants). Moreover, five applications of Na_2SeO_4 (0.633 mM), as foliar spray, from the six-leaf phase, resulted in lettuce leaves enriched with up to 40 mg Se kg^{-1} DW, around 40 times greater than the control (Smoleń et al. 2014). In another study, radish plants sprayed with 5 mg Se per plant 7 days before harvest, as sodium selenate, were able to produce roots with 346.5 mg kg^{-1} DW of Se, meaning that the consumption of 1-10 radishes is enough to fulfill the daily human requirement (Schiavon et al. 2016). Meanwhile, da Silva et al. (2020) found that fertigation of radish plants could be more efficient than foliar spray, after treating plants with a low dose of Na_2SeO_4 (3.6 mg of Se per pot). They obtained roots with approximately 50 mg Se kg^{-1} DW, while the leaf spray of the same chemical (0.36 mg of Se per pot, 93 ml per pot) resulted in plants with approximately 15 mg Se kg^{-1} DW. Lettuce appears to be a good candidate for Se biofortification, as demonstrated by do Nascimento da Silva et al. (2017). In this experiment, plants submitted to fertigation at 25 µM Se L^{-1} (as sodium selenate) resulted in lettuce leaves with as much as 39.4 mg Se kg^{-1} DW, around 40 times greater than the control. While higher application rates of both sodium selenate (Na_2SeO_4) and selenite (Na_2SeO_3) reached numbers that exceeded the RDA of Se. Similarly, tomato plants fertigated with 5 mg L^{-1} of Se as sodium selenate, were enough to obtain a significant increase in Se concentration of fruits (35.8 mg kg^{-1} DW), twice the concentration in the untreated plants. At the same time this dose allowed to achieve good physiological responses on plants, such as

increased enzyme activity of catalase, glutathione peroxidase and superoxide dismutase in fruits (Castillo-Godinaet al. 2016). Selenium biofortification was successfully implemented in many vegetable crops, using Na selenate or Na selenite. Besides, possible antioxidant and anti-senescence effects of Se can improve shelf-life during postharvest storage (Puccinelli et al. 2017). However, because of the high toxicity of Se, especially in the form of selenate, attention must be made regarding agricultural workers and product safety.

1.2.1.4.6 Iron

In human health, iron (Fe) main function is related to the synthesis of hemoglobin and myoglobin besides being essential to many metabolic processes such as oxygen transport, deoxyribonucleic acid (DNA) synthesis, and electron transport, it is also required for energy production (Abbaspour et al. 2014). The RDA of Fe ranges between 8 and 18 mg day^{-1}, whereas the UL for adults is 45 mg day^{-1} (Trumbo et al. 2001). Iron is a versatile, essential element in plant metabolism, whose biological functions are primarily based on the reversible redox reaction of Fe^{2+} (ferrous) and Fe^{3+} (ferric) ions, the ability to form octahedral complexes with various ligands and to change its redox potential in response to different environmental conditions. Due to this, Fe is involved in the transfer reactions at the base of life, since electron transfer chains of photosynthesis and respiration rely on iron–sulfur (S) clusters of the 2Fe–2S or 4Fe–4S type (Hell and Stephan 2003). The concentration of this element in soil often exceeds plant requirements, being present at 20-40 mg kg^{-1} (Cornell and Schwertmann 2003), but usually only a small amount of this is available for plant nutrition. Particularly in alkaline and calcareous soils, once applied through fertilization, Fe quickly becomes unavailable to roots absorption, because of precipitation, adsorption and oxidation phenomena (Rengel et al. 1999; Shuman 1998). Plants have evolved two different strategies to acquire Fe from

the growth substrate, based either on its reduction (Strategy I plants) or chelation with organic ligands (Strategy II plants) (Colombo et al. 2014). In nongraminaceous species (Strategy I plants), such as most of vegetable crops, organic acids and phenolic compounds released by roots chelate ferric Fe on the root surface (Fe^{3+}), which is subsequently reduced to its ferrous form (Fe^{2+}) to transport the element across the plasmalemma of root epidermal cells (White and Broadley 2009). The Fe transportation within the plant occurs in chelated forms, mainly with citrate and malate in the xylem, and nicotianamine and its derivatives in the phloem (Connorton and Balk 2019). This condition derives from the peculiarities of this metal, characterized by low solubility and high reactivity, so its transport inside the plant must be associated to proper chelating molecules controlling its redox states between ferrous and ferric forms (Kobayashi and Nishizawa 2012). The status of Fe into a plant is expressed by its quantity, redox state, speciation with chelating molecules, and its compartmentalization (Briat 2011). Chloroplasts represent the main pool of Fe within the cell, as they gather approximately 80-90% of cellular Fe (Marschner 2011). This flows from the high Fe demand of the photosynthetic apparatus, and Fe-deficiency hampers the electron transfer between PSI and PSII, resulting in photooxidative damages (Kobayashi and Nishizawa 2012). Even though the range of Fe in leaves is between 50-150 mg kg^{-1} DW, Fe requirement is highly variable among species. For example, C4 species are more likely to require higher Fe amounts than C3 species; fast growing meristematic and expanding tissues need more Fe. On the other hand, Fe toxicity is reported in concentrations above 500 mg kg^{-1} DW, which can cause damages associated with formation of ROS, inducing the activity of antioxidative enzymes such as ascorbate peroxidase, besides damages to membrane and irreversible impairment of cellular structure, DNA and proteins (Marschner 2011). To improve Fe uptake agronomical solutions to make Fe available are acidification of soil (Shuman 1998)

and/or use as Fe(III)-chelates synthetic fertilizers. Since the latter are expensive, their use is mainly restricted to soilless crops and to high added-value cash crops (Briat 2011). However, in the case of vegetable crops, the knowledge concerning Fe enrichment, and specifically biofortification, is still poor. One alternative to provide Fe to plants is the foliar spray even if, both adopting the chelated or the sulfate-salt form, a large fixation by cuticle can be observed (Ferrandon and Chamel 1988). Foliar spray of Fe sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) proved to be effective to increase Fe content both in leaves and sink organs of herbaceous crops (Moosavi and Ronaghi 2011; Niyigaba et al. 2019). In tomato, leaf spray with a 9 mM $FeSO_4$ solution increased 3.8 times the Fe content in roots, mediated via phloem transport (Carrasco-Gil et al. 2016). In a study conducted on potato, Kromann et al. (2017) did not observe a positive relationship between Fe foliar spray with EDTA-chelated Fe and its concentration in tubers, thus the Authors hypothesized that the limited effect was related to the Fe form used. As shown in Table 1-1.1, biofortification of vegetables with Fe through fertilization has been tested in few species. The use of EDDHA-chelated Fe up to 2.0 mM (112 mg L^{-1}) proved to be effective in soilless cultivated lettuce in increasing the Fe content of the leaves from 2.31 mg kg^{-1} FW (control) to 4.30 mg kg^{-1} FW (Giordano et al. 2019). In addition, it has been reported that low doses of Fe can enhance the accumulation of secondary metabolites such as chlorogenic acid, beta-carotene, violaxanthin or neoxanthin, thus leading to improved functional profiles of vegetables (Giordano et al. 2019). However, the Authors observed a yield reduction of about 25%, which increased proportionally with the amount of Fe added to the nutrient solution. Overall, Fe biofortification has not been investigated enough to draw a clear picture. Using sulphate or chelate forms only in some cases enhanced mineral content in the edible part of vegetables, however the increase was coupled with a yield reduction. Concluding significant

insolubilization in the soil, limited translocation into the plant and accumulation into edible organs and negative effects on yield are the main constraints in Fe biofortification.

1.2.1.4.7 Copper

In human health, copper (Cu) importance is related primarily to enzymes function, contributing also to maintain cardiovascular integrity, lung elasticity, normal development of connective tissue and nerve coverings, neovascularization; it has also neuroendocrine and immune functions and it is involved in the Fe metabolism too (Bost et al. 2016). The RDA of Cu ranges between 1.0 and 1.6 mg day^{-1}, while the UL for adults is 10 mg day^{-1} (Trumbo et al. 2001). Copper is a redox-active transition metal that under physiological conditions exists as Cu^{2+} and Cu^{+} (Reed and Martens 2018). In plants, it is essential to many physiological processes like photosynthesis, respiration, C and N metabolism and protection against oxidative stress. It acts as cofactor of numerous proteins and in plants it is mainly present in complexed forms, being the concentration of free Cu^{2+} and Cu^{+} in the cytoplasm minimal (Marschner 2011). The worldwide average Cu concentration in soils is 14 mg kg^{-1}, while in Europe the average concentration is 12 mg kg^{-1} (Alloway 2013). Copper is mobile in soils and its absorption is directly related to its concentration in the soil solution (Marschner 2011). Plants can absorb Cu in huge amounts by roots and in minor amounts by shoots and leaves (Fu et al. 2015). Mechanisms involved in Cu uptake are supposedly similar to those of Fe. Copper chelate reductases are encoded by ferric reductase oxidases 4 and 5 and Cu reduction occurs at the roots (Strategy I plants) where Cu is absorbed and transported by proteins of the COPT family. Copper uptake from soil depends almost exclusively on the protein COPT1, while COPT2 could act in the processes of Cu and Fe homeostasis and phosphate metabolism (White and Broadley 2009; Printz et al. 2016). Plants can also absorb Cu through leaves, as

observed by Stepien and Wojtkowiak (2016) that after treating wheat plants with a foliar fertilization of copper sulphate in the amount of 0.2 kg Cu ha^{-1} (1% $CuSO_4$ solution) obtained a 13% increase in the Cu content. On the other hand, the redox-active transition characteristic of Cu that makes it essential also contributes to its toxicity, since the reduction between Cu^{2+} and Cu^{+} catalyzes the production of toxic hydroxyl radicals that can damage DNA, cell membranes and other biomolecules. Besides, damage to cell membranes can be reflected in low uptake of ions and water, so Cu toxicity can be indirectly expressed as growth inhibition and chlorosis caused by the generalized deficiency of nutrients and water (Yruela 2005). Normally, crop species can tolerate a maximum of 20 to 30 mg kg^{-1} DW of Cu in leaves, but Cu-tolerant species can accumulate as much as 1000 mg kg^{-1} DW of Cu in leaves (Marschner 2011). Moreover, foliar fertilization of Cu in maize should not exceed 100 g ha^{-1}, since at higher doses, between 200 and 600 g ha^{-1}, Barbosa et al. (2013) noticed phytotoxic effects that caused growth and yield reduction up to 19 and 75%, respectively. In agriculture, Cu has been used for plant disease control for decades, a number of Cu formulations have been used as biocides to contain pathogens such as bacteria, fungi and in some cases, even invertebrates. In high concentrations, Cu interacts with nucleic acids, disrupting cell membranes of pathogens. In addition, direct application of Cu is used for seed treatment, to prevent seedlings infections (Lamichhane et al. 2018). As shown in Table 1-1.1, among the few experiences in the biofortification of copper, Obrador et al (2013) conducted a study with spinach (*Spinacia oleracea* L.), var. 'Viroflay Esmeralda' applying eight different liquid fertilizers to the soil surface, with the irrigation water in a concentration ranging from 0 to 3 mg Cu kg^{-1} soil. Total Cu concentration in the dry matter of shoots increased by up to 450%, from 9.55 mg kg^{-1} (control treatment) to 52.51 mg kg^{-1} in the treatment where plants were submitted to 3 mg Cu kg^{-1} soil (as Cu-EDTA), a

4.54-fold increase (Table 1-1.1). However, at this dose they also noticed a 10% decrease in the dry matter yield. Instead, the dose 1 mg Cu kg^{-1} soil resulted in an increase in Cu content of 153% allowing also to obtain a yield increase of 71% when compared to the control. Regarding the chemical form, their results showed that the best fertilizers to increase Cu content in the edible part of spinach are Cu-DHE (Cu-diethylenetri-aminepentaacetate-N-2-hydroxyethyl-ethylenediamine-triacetate-ethylenediamine-tetraacetate) and especially Cu-EDTA. Curiously, in this study, even though the total concentration of Cu in spinach shoots were higher than the maximum concentration usually tolerated by plants, no visual phytotoxic symptoms and significant yields reduction were observed. In conclusion, Cu biofortification proved to be effective using different chelate forms and its potential as a biocide could benefit biofortification programs. In addition, when Cu biofortification is concerned attention must be made to the release of Cu in the soil substrate in relation to crop rotations and soil biological properties.

1.2.1.4.8 Silicon

Accumulating evidence from the last 30 years strongly suggests that silicon (Si) plays an essential role in bone formation and maintenance, improving the bone matrix quality and facilitating its mineralization. Increased intake of Si has been associated with increased bone mineral density and decreased osteoporosis (Price et al. 2013). Average daily dietary intake of Si is 20–50 mg for European population, the RDA has not been established; however, safe upper levels for humans have been recommended with a maximum range of 700–1,750 mg day^{-1} (Martin 2007). Silicon is considered not essential for plant nutrition, but its inclusion in fertilization programs has proved to increase the crop tolerance to biotic and abiotic stressors (Kaushik and Saini 2019), crop yield (Epstein 1999), or improve the absorption of macro- and microelements (Laane 2018). Silicon

concentration in soil can vary depending on the type of soil. For example, alkaline soils containing sodium carbonate usually present a higher Si content. On average, the concentration of Si in soil is between 0.09 and 23.4 mg kg^{-1} (Tubaña and Heckman 2015). If compared with other minerals, Si metabolism is still poorly understood. It seems that two main mechanism of Si absorption co-exist in plants, i.e. active and passive, whose relative contributions depend upon both plant species and external Si concentration (Ma and Yamaji 2006). This would explain the strong differences in Si concentration reported within tissues of different plants species (Mvondo-She and Marais 2019). In any case, Si is taken up by the roots as monosilicic acid with the involvement of channels belonging to the aquaporins' group, so the water flow resulting from leaf transpiration seems to play a determinant role in defining the rate of Si absorption and transport within the plant (López-Pérez et al. 2018). Once absorbed, monosilicic acid is subsequently translocated to the shoot through the xylem flow, where Si is concentrated thanks to transpiration and polymerized to silica (SiO_2), then deposited in the different tissues (Raven 2003). It has been reported in the Poaceae leaves that Si can be deposited both in mesophyll and epidermal cells, suggesting the co-existence of negative (transpiration-driven) and positive (though specific carriers) mechanisms controlling the Si accumulation process (Motomura et al. 2004). Plants markedly differ in their ability to accumulate Si in their various organs; concentrations ranging between 5 and 50 g kg^{-1} DW have been reported as critical for some species. The species with low mobilization capacity accumulate it in the roots and stems, while the species with high mobilization capacity accumulate Si in stems, leaves, fruits and seeds (López-Pérez et al. 2018). Gao et al. (2006) noticed that excessive Si supply (>2 mM) caused the formation of Si polymers on roots surfaces, a feature that could affect nutrients uptake. In spite of the scarcity of available information, this aspect would deserve extensive study with reference

to vegetable crops, since their potential role as Si source in the human diet. Indeed, thanks to their usually low silicification capacity, vegetable crops are expected to contain high amounts of soluble Si, which is theoretically more available to be assimilated after ingestion, so potentially being optimal candidates as Si source in the human diet (López-Pérez et al. 2018). As shown in Table 1-1.1, as regards the leafy vegetables, in a study concerning 6 crops grown in a greenhouse floating system, namely *Brassica rapa* L. (tatsoi and mizuna group), *Ocimum basilicum* L., *Portulaca oleracea* L., *Cichorium intybus* L. and *Beta vulgaris* L. ssp. *vulgaris*, D'Imperio et al. (2016a) found an increased Si content in plant tissues by providing them up to 100 mg L^{-1} Si (as potassium metasilicate) in the nutrient solution, with basil reaching the highest content of Si (293 mg kg^{-1} FW, expressed as SiO_2). Moreover, the Authors found that Si became bioaccessible in all the considered species, in a range from 23% (basil) to 64% (chicory). In a different experiment concerning two leafy vegetables, namely chard (*Beta vulgaris* L. var. *cicla*) and kale (*Brassica oleracea* L. var. *acephala*) grown in a hydroponic system, De Souza et al. (2019) compared the effects of two Si sources, namely potassium silicate and stabilized sodium potassium silicate with sorbitol, and four Si concentration in a foliar spray solution (from 0.00 to 2.52 g L^{-1}). They found that in both species, the Si concentration in leaves linearly increased in response to Si concentration in the foliar spray solution, with the best biofortification results obtained by spraying potassium silicate. In a study concerning the green bean (*Phaseolus vulgaris* L.) cultivated in a hydroponic system, Montesano et al. (2016) found that biofortified pods (obtained by adding 3.6 mM of Si as potassium metasilicate to a standard nutrient solution) showed a 310% increase of Si (from 853.8 to 2496.3 mg kg^{-1} DW) when compared to unbiofortified ones. Moreover, they found that the bioaccessibility of Si in biofortified pods was higher than control pods (25.1 vs. 7.6%), even after cooking them by steaming or boiling. The Si biofortification

protocol of strawberry fruits (*Fragaria* × *ananassa* Duchesne ex Rozier) was studied by Valentinuzzi et al. (2018), who cultivated for 16 weeks in a hydroponic system provided with a standard nutrient solution, or with nutrient solutions enriched with 50 or 100 mg L^{-1} of Si (as Na_2SiO_3). The Authors found that providing 100 mg L^{-1} of Si allowed to maximize the metalloid concentration in strawberry fruits (which increased from 6.44 up to 85. g kg^{-1} DW) without compromising crop yield. However, the they observed a decrease in total phenols and an increase in the content of flavanols in response to the highest Si supply. Overall, biofortification with Si using K silicate proved to effectively increase the mineral content in vegetables. In addition, its possible role as plant protector and its ability to improve the mineral status of the plant, both make Si a key element in biofortification programs.

Table 1-1. Response of some vegetable crops to different biofortification protocols [(1)].

Element	Crop	Scopus® papers (n.)	Average concentration [(2)] (mg kg^{-1} FW)		Average increase	Application dose to roots or leaves (mg L^{-1})	
			Min	Max		Min	Max
Ca	Basil	1	950	1100	0.2-fold	100	200
Ca	Endive	1	1020	1080	0.1-fold	100	200
Ca	Indian colza	2	928	3000	2.2-fold	6	1603
Ca	Lettuce	2	695	2683	2.9-fold	0	800
Ca	Mizuna	1	1250	1400	0.1-fold	100	200
Ca	Potato	1	144	245	0.7-fold	350	5200
Ca	Tatsoi	1	1100	1150	1.1-fold	100	200
Mg	Indian colza	2	290	1059	2.7-fold	4	486
Mg	Onion	1	652	1627	1.5-fold	0	150
I	Basil	2	1	287	> 100-fold	0.1	127
I	Cabbage	3	0.1	2.5	34.4-fold	0.1	0.6
I	Carrot	7	0.1	7.8	> 50-fold	1	50
I	Chinese cabbage	3	0.1	48.7	> 100-fold	0.1	50
I	Cowpea	2	4	1566	> 100-fold	0.7	15
I	Lettuce	18	2	42.0	17.9-fold	0.1	50
I	Mizuna	2	0	1.0	> 50-fold	0.7	1.1
I	Mustard	2	0	0.4	41-fold	0.7	1.1
I	Onion	1	0	1.0	> 50-fold	0	5
I	Potato	3	0.1	0.7	11.3-fold	0.6	5
I	Spinach	8	4.5	22.4	4.0-fold	1	5.1
I	Tomato	5	0.1	12.0	> 100-fold	1	634
Zn	Arugula microgreens	1	3.0	70	22.3-fold	0	10
Zn	Broccoli	1	9.4	133	13.2-fold	121	408
Zn	Cabbage	4	4.1	39.1	8.6-fold	5	260
Zn	Carrot	1	42.1	802	18.1-fold	2.8	303
Zn	Indian colza	1	2.5	167	> 50-fold	0	32.7
Zn	Kale	1	5.8	167	27.8-fold	2.8	303
Zn	Lettuce	3	2.2	30.4	12.8-fold	5.2	60
Zn	Okra	1	3.0	5.0	0.7-fold	2.8	303
Zn	Onion	1	2.5	7.8	2.1-fold	0	10
Zn	Potato	3	2.7	4.9	0.8-fold	9.7	250

Table 1.1. Continued.

Element	Crop	Scopus® papers (n.)	Average concentration [(2)] (mg kg^{-1} FW)		Average increase	Application dose to roots or leaves (mg L^{-1})	
			Min	Max		Min	Max
Zn	Red cabbage microgreens	1	2.5	75	29-fold	0	10
Zn	Red mustard microgreens	1	2.1	92	42.8-fold	0	10
Se	Basil	4	0	8.3	> 100-fold	2	12
Se	Broccoli	1	1.1	19.2	15.7-fold	10	100
Se	Carrot	3	0.1	1.8	35-fold	0.3	3.9
Se	Chard	1	0	0.5	45-fold	0	10
Se	Cucumber	1	0	0.2	7.6-fold	0	30
Se	Endive	1	0.1	1.2	23.6-fold	0.3	0.6
Se	Garlic	2	0.1	6.1	> 50-fold	0.1	15
Se	Indian mustard	1	0	0.5	> 50-fold	0	50
Se	Lettuce	12	0.1	6.9	> 100-fold	0.5	20
Se	Onion	3	0.4	17.7	49.5-fold	2.0	20
Se	Potato	4	0.1	1.6	16.6-fold	0.5	0.8
Se	Radish	4	0.3	18.2	> 50-fold	1	23.7
Se	Spinach	2	0.1	2.2	21.1-fold	0.2	0.3
Se	Strawberry	1	0.5	3.0	5.2-fold	0	4
Se	Tomato	3	0.3	3.4	9.1-fold	5	20
Se	Turnip	1	0.4	10.6	24.3-fold	0.2	2
Fe	Arugula microgreens	1	4.9	111	21.6-fold	0	40
Fe	Lettuce	1	2.3	4.3	0.9-fold	0.8	112
Fe	Red cabbage microgreens	1	7.7	448	> 50-fold	0	40
Fe	Red mustard microgreens	1	4.9	323	> 50-fold	0	40
Fe	Sweet potato	1	185	253	0.4-fold	0	100
Cu	Spinach	1	0.5	3.0	4.5-fold	0	3
Si	Basil	1	41.2	293	6.1-fold	0	100
Si	Chard	1	500	1450	1.9-fold	0	2.5
Si	Chicory	2	17.2	95	4.5-fold	0	101
Si	Green bean	1	57	252	3.4-fold	0	101
Si	Kale	1	700	2800	3-fold	0	2.5

Table 1.1. Continued.

Element	Crop	Scopus® papers (n.)	Average concentration [2] (mg kg^{-1} FW)		Average increase	Application dose to roots or leaves (mg L^{-1})	
			Min	Max		Min	Max
Si	Mizuna	1	20	110	4.5-fold	0	100
Si	Purslane	1	14.8	98	5.6-fold	0	100
Si	Strawberry	1	475	8075	16-fold	0	100
Si	Swiss chard	1	18	145	7.1-fold	0	100
Si	Tatsoi	1	18	70	2.9-fold	0	100

[1] The list reports the most representative horticultural crops. In this and in the following tables, data refer to research on Scopus® using "biofortification" and "vegetables" as keywords performed in November 2020. Papers which tested more than one species were counted more than one time. [2] Calculated in the edible portion.

Table 1-2. Chemical forms of each mineral used in the biofortification of some vegetable crops.

	Basil	***Brassica* spp.**	**Carrot**	**Lettuce**	**Onion**	**Potato**	**Radish**	**Spinach**	**Tomato**
Ca	Calcium phosphate, calcium chloride	Calcium chloride		Calcium chloride		Calcium chloride, calcium nitrate			
Cu								Cu-EDTA[1]	
Fe		Iron sulphate		Fe-EDDHA[2]					
I	Potassium iodide, potassium iodate	Potassium iodide, potassium iodate	Potassium iodide, potassium iodate	Potassium iodide, potassium iodate	Potassium iodide	Potassium iodide, potassium iodate		Potassium iodide, potassium iodate	Potassium iodide, potassium iodate
Mg		Magnesium chloride, magnesium sulphate			Magnesium sulphate				
Se	Sodium selenate	Sodium selenate, sodium selenite	Sodium selenate, sodium selenite	Sodium selenate, sodium selenite	Sodium selenate	Sodium selenate, sodium selenite	Sodium selenate, sodium selenite	Sodium selenate, sodium selenite	Sodium selenate, sodium selenite
Si	Potassium meta silicate	Potassium silicate, sodium silicate							

Zn	Zinc nitrate, Zinc sulphate	Zinc oxide, zinc sulphate, Zinc EDTA[1]	Zinc sulphate	Zn-AML[3], Zn-EDDHSA[4], Zn-EDTA[1], Zn-PHP[5], Zn-HEDTA[6], Zn-EDTA[1]-HEDTA[6], Zn-DTPA[7]-HEDTA[6]-EDTA[1], Zn-EDDS[8]	Zinc nitrate, zinc oxide, zinc sulphate, Zn-EDTA[1]

[1]Ethylenediaminetetraacetic acid, [2]Ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid), [3]Aminolignosulfonate, [4]ethylenediamine-di-(2-hydroxy-5-sulfophenylacetate), [5]Polyhydroxyphenylcarboxylate, [6]N-2-hydroxyethyl-ethylenediaminetriacetate, [7]Diethylenetriaminepentaacetate, [8]Ethylenediamine disuccinate. The list includes those papers that reported the initial and final concentration of mineral element in the edible part of vegetables.

1.2.1.5 Discussion and future trends

The evidence discussed above pointed out that biofortification should be contemplated as a promising strategy to face with malnutrition in many circumstances. Biofortification can help to obtain products designed according to the needs of two categories of target consumers (Figure 1-1). The first concerns products enriched with minerals that can fulfil specific functional needs; this is the case of vegetables richer in one or more minerals to counter the deficiencies related to ordinary diet or new consumer habits. (e.g: vegans). Besides vitamins, in fact, vegan diet feature inadequate content of calcium, potassium, iron, iodine, magnesium (Kowalska et al. 2020). A second target concerns products with premium quality or superfood aimed at improving health as a whole. This would satisfy the need of an increasing group of health-conscious consumers who look at plant-based foods, especially vegetables, as a sort of medicine to prevent the insurgence of chronic diseases.

Agronomic biofortification is comparatively simpler than other methods and potentially suitable for immediate results. However, the available studies on agronomic fortification of vegetables are of a considerable number only for few crops (e.g.: lettuce, tomato, spinach and *Brassica* spp.) and for few mineral elements (e.g.: selenium, iodine). For these elements, aspects related to the form, application modality, concentration and timing have been clarified for most important crops. For all the considered elements, and particularly for selenium and iodine, the biofortification adopting soilless crops or on soil fertigated crops have been mostly considered. In some cases the model describing the accumulation in relation to the application has also been described (White et al. 2012). For some other mineral elements considered in this review, important as well in human nutrition (e.g. Fe), information is still lacking.

On the other hand, even when empirical evidence on

biofortification showed a significant increase in the concentration of the mineral elements, the fortification is not economically worth. In addition, an effective biofortification protocol is based on regular and frequent applications and a negative environmental impact cannot be excluded (Carvalho and Vasconcelos 2013). Besides, the step between biofortification and plant toxicity effects can be narrow and applications targeting the accumulation of essential micronutrients must be adjusted to avoid negative effects on plant growth (Rouphael and Kyriacou 2018).

The application of biofortifying elements poses some problems related to the interaction with other factors at soil level (e.g.: phytoavailability) and at plant level (e.g.: competition with other elements) (Tran et al. 2019). In many studies it is adopted the traditional fertigation approach, rather than foliar spray, which can be more cost effective and environmentally friendly. Indeed, foliar fertilization represents the simplest and fastest method for the application of mineral elements used for the biofortification of vegetables; but the effectiveness depends on the used plant organ and the mobility of the element inside the plant. To face some of these problems, technical innovations such as precision agriculture, soilless cultivation, etc. may help in defining more efficient biofortification protocols.

There are only few biofortified vegetable products already present on the market (e.g.: selenium enriched potato, carrot and onion, 'Selenella' from Consorzio Patata Italiana di Qualità Soc. Cons., IT, iodine biofortified potato, 'Iodì' from the Pizzoli group, IT, selenium enriched brussels sprouts from Marks & Spencer, UK, etc.). It is clear that mostly iodine and selenium have been commercially considered as biofortification elements, probably because a more efficient accumulation system and for their lower toxicity at plant level. In the future, besides a broad choice of diversified vegetables, it is expected that the market will have biofortified products richer in

more than one mineral. So, research that comprises simultaneous biofortification is essential. In addition, further elements are being studied and are expected to be object of biofortification in the future (e.g. lithium, vanadium, etc.). In this regard, biofortification using Li-sulfate and Li-hydroxide was effective in increasing Li content in lettuce plants (da Silva et al. 2019).

Based on the results in literature, biofortification is not expected to fully control mineral element deficiencies or eradicate them, but, it complements other interventions to provide micronutrients to the people. To be effective, a biofortification program should be based on very appropriate planning concerning: - health and nutrition investigation, - nutritional habits, - design and validation of sustainable biofortification protocols, - estimation of positive effect on health. Concerning biofortification protocols, the attention should be paid on those crops having an element content high enough to be conveniently targeted, and that prove to significantly benefit by mineral elements application.

In the reviewed literature most attention has been posed on the content of specific elements in plant edible portion but key concept like bioaccessibility and bioavailability were seldomly considered. The first regards the nutrient fraction released from the food and available for absorption by the intestinal cells, while the latter expresses the amount of nutrients actually absorbed and therefore available for utilization in physiological functions (Fernández-García et al. 2009; Rousseau et al. 2020). While macronutrients (proteins, carbohydrate and fats) are degraded and absorbed by specific and well-known biochemical mechanisms, phytochemicals and minerals are absorbable without biotransformation and often without a specific carrier (Basu and Donaldson 2003; Jackson and McLaughlin 2009). The consequence of this poorly developed intestinal transport system is that the actual absorption of phytochemicals and minerals is deeply dependent from the food matrix. To modulate mineral bioavailability,

attention should be devoted to those substances (e.g.: vitamin C, β-carotene, oxalic acid, polyphenols, etc.) stimulating or inhibiting bioavailability (Gupta et al. 2006; White and Broadley 2009). Furthermore, some chemical bonds with other component in the food or the physical entrapping inside intact plant cell walls can dramatically decrease the bioaccessible and bioavailable fractions of phytochemicals and minerals (Platel and Srinivasan 2016).

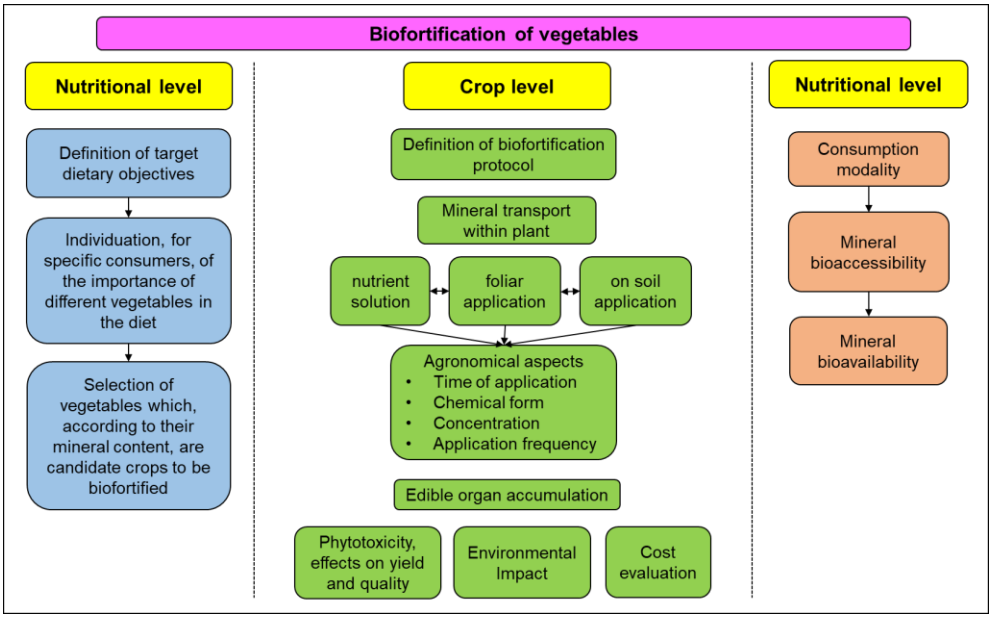

Figure 1-1. Key aspects to be considered in the agronomic mineral biofortification.

1.2.1.6 Conclusion

In conclusion, the production of mineral-dense vegetables will deserve a prominent place in the coming years. Agronomic biofortification, even if involves expensive experimentation activities, represents the only strategy in the case of vegetables, for which genetic improvement programs would be rather complex and not very convenient due to the high rate of varietal turnover. The main challenges for agronomic biofortification in the immediate future will rely on the efficiency of fertilization process and bioavailability of minerals, the high cost of some specific chemical formulations, the possible yield losses due to biofortification-induced alterations of plant metabolism and the potential environmental/health impact deriving from the new agronomic protocols (as in the case, for example of copper and selenium). Deeper knowledge in these areas must be considered indispensable to achieve sound conclusions about the costs/benefits of biofortification.

The papers discussed in this review report promising results for several minerals and pillar vegetables in human diet; however, the results are not entirely consistent and coherent. The future activities, beyond their specific scientific relevance, should be planned in a broader context, adopting an approach involving also farmers, traders, nutritionists, educators. Such an approach, thanks also to the nutritional importance of vegetables, will certainly have a significant impact on improving human diet.

1.3 Aim of the thesis

This thesis is focused on the biofortification of vegetables. For this reason, the main goal was to study and develop the best agronomic biofortification protocols able to increase the concentration of mineral elements in the edible portion of selected vegetables. The main minerals under study were Fe, Zn, Se and Si. In this context, the goal of chapter 2.1 was to study the Fe biofortification of soilless cultivated cherry tomato, by assessing the effects of the application of different levels of Fe chelate in the nutrient solution and in combination with different levels of foliar applications of this metal on the mineral composition and qualitative characteristics of cherry tomato fruits. In chapter 2.2 of this thesis, the aim was to investigate the Se-biofortification of tomatoes. For this we compared the best application time, during fruit development (fruit set vs. ripening), to apply the Na_2SeO_4 foliar spray, in order to obtain Se-biofortified cherry tomato fruits. In chapter 3.1, the goal was to study the Fe biofortification of soilless cultivated lettuce, by improving the concentration of Fe and other health promoting substances in two cultivars of lettuce submitted to high doses of Fe in the nutrient solution. In addition, in this chapter we aimed to compare the tolerance of the two lettuce genotypes to possible stress conditions caused by the presence of high levels of Fe in the nutrient solution. The chapter 4.1 is dedicated to the Fe- and Zn-biofortification of carrot and aimed to compare the efficiency of chelate and sulphate forms of Zn and Fe, applied as foliar sprays, in the biofortification of off-season carrot. In addition, this study aimed to investigate the bioaccessibility of these minerals, by assessing the amount of Fe and Zn actually released from the food matrix during simulated digestion. In chapter 4.2, the aim was to understand the role of preharvest foliar applications of Si in the biofortification of carrots by improving carrot compositional traits and shelf-life performance,

either in presence or not of the leaves. Finally, the general focus of this thesis was to obtain vegetables (carrot, lettuce, tomato) with increased mineral content thanks to the administration of the tested protocols.

2 Experimental activities on tomato

2.1 Iron biofortification of cherry tomato grown in a soilless system

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2.1.1 Introduction

Iron (Fe) deficiency affects almost two billion people worldwide, i.e. around 25% of the global population (McLean et al. 2009; Carrasco-Gil et al. 2016). This mineral is responsible for a variety of metabolic processes, such as DNA synthesis and electron transport (Abbaspour et al. 2014). The recommended daily allowance (RDA) for Fe is from 8 to 18 mg day^{-1} (Trumbo et al. 2001). When the body does not receive an adequate amount of Fe it cannot produce enough of the substances responsible for the transportation of oxygen, leading to a series of complications and diseases such as anemia (Cappellini et al. 2020). The groups at greatest risk of Fe deficiency include women, infants, vegetarians, and frequent blood donors (Tong and Vichinsky 2021). In this framework, biofortification is a promising strategy that allows the delivery of plant foods enriched with one or more nutrients, helping to fight the deficiencies associated with inadequate diets (White and Broadley 2009). Vegetables represent healthy food products that are a natural source of vitamins, minerals, and fibers (Hiel et al. 2019; Distefano et al. 2022a) and are therefore good candidates for biofortification programs. Through biofortification, vegetables can be nutritionally improved through simple agronomic expedients, such as targeted fertilization, helping to respond to the specific dietary needs of consumers (Rouphael and

Kyriacou 2018).

Greenhouse cultivation enables the effective control of the environmental conditions influencing the quality of vegetables, such as air temperature, light, and vapor-pressure deficit (Gruda 2005). Moreover, soilless cultivation systems facilitate the precise control of plant nutrition, allowing improvement of the yield and composition of many vegetables, including their concentrations of minerals and secondary metabolites (Rouphael et al. 2018). In soilless systems, biofortification can be achieved by adding micronutrients to the nutrient solution and/or spraying the leaves of plants with suitable fertilizing solutions (Buturi et al. 2021). When Fe fertilization is concerned, the advantages of soilless systems stem from the poor interaction between the micronutrient and the growing medium, so that the limited mobility of Fe can be managed (Buturi et al. 2022).

The tomato (*Solanum lycopersicum* L.) is the main fruit vegetable in the Mediterranean region, highly appreciated for its functional quality and versatility, which enable it to be consumed either fresh or as a processed product in soups, juices, or sauces (Mauro et al. 2015; Distefano et al. 2022b). Being a pillar of a healthy diet, this product could be effectively used to foster the intake of many important nutraceuticals, including minerals, provided that suitable agronomic protocols are developed and made available at the farm level (Buturi et al. 2021). To this end, the application of fertilizers in nutrient solution or as foliar sprays can affect the yield and product quality of many crops, including tomatoes (Carrasco-Gil et al. 2016; Sellito et al. 2019).

Iron is one of the essential elements for normal plant growth and health, since it participates in a wide range of biochemical and physiological functions. For example, it is involved in the synthesis of chlorophyll and is part of many essential enzymes of the electron transport chain (Marschner 2011). The link between Fe and other minerals is complex and depends on many factors, such as plant

species, chemical form, and concentration applied, but it has been demonstrated that Fe can interact with phosphates, Zn, Cu, and Mn (Rai et al. 2021).

When using Fe fertilizers, attention should be paid to the concentration and chemical form, because of the high potential toxicity of this mineral when excessively present in the crops (Li et al. 2016). In concentrations higher than 500 mg kg^{-1} dry matter, Fe may cause damage related to the formation of reactive oxygen species (ROS), in addition to impairing DNA, cellular structures, and proteins (Marschner 2011).

Consequently, few studies have been conducted with the goal of enhancing the Fe concentrations in vegetables, so there are insufficient operational indications for the biofortification of important vegetables for human nutrition, as in the case of tomatoes (Przybysz et al. 2016; Kromann et al. 2017; Di Gioia et al. 2019; Giordano et al. 2019). For these reasons, the goal of this research was to fine-tune an agronomic protocol of Fe biofortification for cherry tomatoes grown in a soilless system. To maximize the Fe accumulation in tomato fruits, we studied the effects of the application of different concentrations of Fe chelate in the nutrient solution and in combination with foliar applications, and evaluated the subsequent effects on mineral composition, yield, and quality traits of the fruits of the tomato cultivar Creativo.

2.1.2 Materials and Methods

2.1.2.1 *Experimental Site and Plant Material*

The study was conducted from February to May 2021, in the greenhouse of the University of Catania (Sicily, Italy: 37◦24’31.5” N, 15◦03’32.8” E, 6 m a.s.l.). The climate is semi-arid Mediterranean, with dry, warm summers and mild winters. The cold greenhouse used has an area of 810 m^2, and has adjustable windows along the sides and

on the rood, along with a steel tubular structure covered with polycarbonate slabs.

Cherry tomato plants of the cultivar 'Creativo' were transplanted on 1 February 2021 at the stage of four true leaves. The cultivation system adopted was open soilless, where plants were grown in 5 L plastic pots (19 cm width, 20 cm height) and perlite was used as the growing medium (particle size 2–6 mm). Plantlets were selected for healthy appearance and uniform size, before transplanting. Pots were positioned in simple rows, in a 0.30 × 1.00 m rectangular format (center-to-center) with 1 plant per pot (3.33 plants m^{-2}). Seedlings were grown at single stem up to the 5th cluster, while clusters were pruned to 12 fruits. Each net experimental unit contained 8 plants (Figure 2-1A-C). the crop was fertigated with a nutrient solution with the following composition: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 22 µM Fe, 9 µM Mn, 2 µM Cu, 4 µM Zn, 9 µM B, and 1 µM Mo, with pH 6.0 ± 0.2 and an electrical conductivity (EC) of 2600 µS cm^{-1} (Mauro et al. 2020a). A leaching fraction of ~35% was used to mitigate root-zone salinization (Giuffrida et al. 2018).

A split-plot experimental design with three replicates was adopted. On the main plots, the applied treatments consisted of three concentrations of Fe chelate added to the nutrient solution: 0 (only the standard nutrient solution, equal to 0.022 mmol Fe L^{-1}), 1, and 2 mmol Fe L^{-1} (hereafter referred to as R0, R1, and R2, respectively) in the form of Fe-HBED (N,N'-Bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid). In the sub-plots, we carried out five applications of three concentrations of Fe chelate with foliar spray solution: 0, 250, and 500 µmol Fe L^{-1} (hereafter referred to as L0, L250, and L500, respectively, in the form of Fe-DTPA (diethylenetriaminepentaacetic acid). The above concentrations were selected based on primary literature. Foliar treatments were applied on 18 March, 30 March, 13 April, 20 April, and 27 April, around the flowering stage of each

cluster. From 27 April to 11 May, tomatoes belonging to the 1st and 2nd clusters were harvested by hand and subsequently transported to the laboratory. All qualitative determinations were performed on the 2nd cluster, whereas the amount of Fe was measured in both clusters, to check the consistency of the mineral accumulation in the tomato fruits. Once in the laboratory, the fruits were analyzed, flash-frozen with liquid nitrogen, and stored in a freezer at −80 °C for further analytical determinations. Overall, 432 clusters were collected (2 clusters × 3 root concentrations × 3 leaf concentrations × 3 replicates × 8 plants).

2.1.2.2 *Carpometric determinations*

The following measurements were carried out on each sample: Yield and average fruit weight were assessed gravimetrically on 8 fruits per plot detached from their rachis and selected for their uniform appearance and absence of defects. Firmness was measured using a digital texture analyser (model TA-XT2, Stable Micro Systems, Godalming, UK) as described by Distefano et al. (2020). The fruit dry matter (DM) content was calculated by drying fruits in a thermoventilated oven at 70 °C until constant weight. The chromatic coordinates of the fruit were determined as described by McGuire (1992) on the equatorial axis of 12 fruits per plot, using a tristimulus Minolta Chroma meter (model CR-200, Minolta Corp., Ramsey, NJ, USA) calibrated with a standard white tile (UE certificated) with illuminant D65/10°, measuring color in terms of lightness (L*), green-red axis (a*) and blue-yellow axis (b*). Fruit color was expressed as L*, a*, b*, $(a^*/b^*)^2$ and Chroma $[(a^{*2} + b^{*2})^{1/2}]$. Approximately 50 g of cherry tomatoes was homogenized using a home blender (La Moulinette, Groupe SEB, Écully, France) and centrifuged for 15 minutes at 5000 rpm (modell 4235A, ALC centrifuge, Milan, Italy), then samples were then immediately analyzed for soluble solids content and titratable acidity. Soluble solids content (SSC) was

measured using a refractometer (model Abbe 16531, Carl Zeiss, Oberkochen, Germany) and the results were expressed as °Brix. Titratable acidity (TA) was measured by titrating an aliquot of the juice sample with 0.1 M NaOH up to pH 8.1.

2.1.2.3 *Biochemical analyses*

For the biochemical analyses, frozen samples from the 2nd cluster were lyophilized in a freeze-dryer (model Alpha 1-4 LD plus, Martin Christ, Osterode am Harz, Germany) and grounded using liquid nitrogen. All further analyses were performed using plastic cuvettes, and readings were carried out using a UV-Visible spectrophotometer (model 7310, Jeanway, Stone, Staffordshire, UK).

- *Total carotenoids concentration*

Determination of total carotenoids in fruits was conducted as described by Lichtenthaler and Wellburn (1983), with some modifications. For the extraction, 50 mg of lyophilized tomato powder was mixed with 5 mL ethanol (96%) and vortexed for 1 minute; the samples were then left overnight in the dark at 10 °C. After that, samples were sonicated for 10 minutes in an ultrasonic bath (below 10 °C) and centrifuged for 10 minutes (5000 g at 6 °C). The samples were read in 1.5 mL plastic cuvette, using 96% ethanol as the blank. Readings were performed at wavelengths: 470, 649 and 665 and the absorbance values were applied in the following equations:

- $Ca = 13.95 \times A665 - 6.88 \times A649$
- $Cb = 24.96 \times A649 - 7.32 \times A665$
- $Cx+c = (1000 \times A470 - 2.05 \times Ca - 114.8 \times Cb) \div 245$

where Ca stands for Chlorophyll A, Cb for Chlorophyll B and Cx+c for total carotenoids (including xanthophylls).

- *Total phenolic content*

Total polyphenol content (TPC) was quantified through the Folin-Ciocâlteu method (Cicco et al. 2009). To this end, 100 mg of lyophilized tomato powder was mixed with 5 mL of methanol (80%) and vortexed for 1 minute. Samples were then submitted to 10 minutes in an ultrasonic bath (below 10 °C) and centrifuged for 15 minutes at 4000 g and 6 °C. The supernatant was withdrawn and the extraction process was repeated 3 times. The extracts were combined and diluted to 20 mL using methanol (80%). For the reaction, 200 µL of extract solution were mixed with Folin-Ciocâlteu reagent (1000 µL at 10% concentration) and left to react for 2 minutes at room temperature. Next, 800 µL of sodium carbonate (0.7 M) was added to stop the reaction, and the solution was mixed and placed in the dark at room temperature for 60 min. Samples were read at 760 nm and TPC values were obtained from a standard curve prepared by plotting the change in absorbance against different concentrations of gallic acid.

- *DPPH assay*

The DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenging activity of tomato extract was determined via the procedure reported by Brand-Williams et al. (1995). First, 100 mg of lyophilized tomato powder was mixed with of 5 mL methanol (80%) and vortexed for 1 minute. Samples were then submitted to 10 minutes in an ultrasonic bath (below 10 °C) and centrifuged for 15 minutes at 4000 g and 6 °C. For the reaction, 150 µL of supernatant was mixed with 1350 µL of recently prepared DPPH solution (150 µmol), and then samples were vigorously agitated and placed in the dark for 30 minutes. The decrease in absorbance of methanolic solution of DPPH was read at 515 nm and DPPH was calculated from a standard curve prepared by plotting change in absorbance against different Trolox concentrations.

- *FRAP assay*

The ferric-reducing antioxidant power (FRAP) assay of extract was performed as described by Benzie and Strain (1999). For the extraction, 200 mg of lyophilized tomato powder was mixed with 10 mL of methanol (100%), vortexed for 1 minute and placed in the dark for 30 minutes. After that, samples were centrifuged for 10 minutes at 4500 g and 6 °C. Preparation of FRAP reagent consisted of 10 mL of acetate buffer (300 mmol, pH 3.1) mixed with 1 mL of TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution (10 mmol in 40 mmol HCl) and 1mL of ferric chloride (20 mmol). For the reaction, 150 µL of supernatant was mixed with 300 µL of ultrapure water, vortexed and added to 3 mL of FRAP reagent. Samples were placed in the dark at 20 °C for 10 minutes. The FRAP assay, based on the reduction of Fe(III) by the sample extract, was conducted following the shift in absorbance at 593 nm upon the formation of the blue colored Fe(II)-tripyridyltriazine compound from colorless oxidized Fe(III), in the presence of a particular sample concentration. FRAP was calculated from a standard curve prepared by plotting change in absorbance against different Trolox concentrations.

2.1.2.4 *Determination of nitrogen and minerals content*

For nitrogen (N) determination, sulfuric digestion with catalyst salts in a digesting block was employed and distillation was performed in a N distillation unit (model TE-0363, Tecnal, San Juan, PR) according to the Kjeldahl method. Total phosphorus (P) determination was performed using the colorimetric method (AOAC 1995) through spectrophotometry (model DR 2010, Hach, Loveland, Colorado, USA). For the determination of the other minerals, nitric perchloric acid digestion was used (AOAC 1995). Firstly, a pre-digestion of 500 mg of sample was performed with 5 mL of nitric perchloric acid for about 16 hours, then tubes were placed in a

digesting block and the temperature was gradually raised (50 °C hour^{-1}) to 180 °C and maintained for about 4 hours. When samples cooled down, the extracts were filtered with filter paper discs and the volume was adjusted to 50 mL with ultrapure water. The extract was used for the determination of minerals, including macronutrients (i.e., K, Ca, Mg and Na) and micronutrients (i.e., Fe, Cu, Zn, Mn); analyses were performed using an atomic absorption spectrometry (AAS) (model AA-6300, Shimadzu, Kyoto, Japan).

- *Determination of leaf Fe content*

After 72 days of cultivation, the leaves of cherry tomato plants started to show dark spots (Figure 2.1D). In order to assess the Fe content at that time, leaves covering the third cluster of plants submitted to the different treatments were harvested, dried and analyzed to determine their Fe content, following the procedure described above.

2.1.2.5 *Statistical procedures*

The collected data was subjected to a two-way analysis of variance (ANOVA) for split plots, according to the experimental layout adopted in the greenhouse. Means were compared using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$). The calculations were carried out on Excel version 2016 (Microsoft Corporation, Redmond, WA, USA) and Minitab (version 16.1.1, Minitab Inc., State College, PA, USA).

2.1.3 Results

2.1.3.1 *Yield and Carpometric traits*

As shown in Table 2.1, when compared to the control, the

yield and average FW of cherry tomatoes treated with Fe (by the roots) decreased (10 and 13%, for the average of R1 and R2, respectively). However, their DM content at R2 increased significantly (+10.2%), compared to the control. In addition, a significant increase in SSC (+7.7% and +11.8%) was observed in R1 and R2 plants, while TA (+20.5%) was promoted by the R2 treatment. Similarly, fruit firmness was increased in the R2 treatment (+9.3%), compared to the control. Regarding the fruit's chromatic coordinates, only the variable L* showed a reduction in R2 plants, whereas the other chromatic variables showed no significant differences between treatments (Table 2.2). For the plants receiving Fe through leaf spraying, none of the carpometric variables showed significant differences.

2.1.3.1.1 Biochemical analyses

Cherry tomato fruits from the second cluster showed no significant differences in the total carotenoids and total phenolic contents between the compared treatments. The same was observed for the antioxidants DPPH and FRAP (Table 2.3).

2.1.3.1.2 Nutrients concentration

The Fe content in fruits from 1st and 2nd cluster treated with Fe in the nutrient solution showed a significant increase when compared to controls. In this sense, the Fe content increased proportionally with the increase in the Fe concentration in the nutrient solution, being higher at R2. As for the leaf spraying treatment, L500 plants showed significant increases in the Fe content of their fruits (Figure 2.2). In addition, the Fe content of both clusters showed a significant 'nutrient solution × leaf spray' interaction. As shown in Figure 2.1, the R2-L500 plants showed the highest Fe increase (+163%) in cluster 1, when compared to the control plants (Figure 2.2A). This effect was confirmed by the results obtained from the 2nd cluster, where the highest Fe increase was also produced by the R2-L500 plants (+190%)

(Figure 2.2B).

With regard to the other nutrients (Table 2.4), the Fe supplementation through the nutrient solution increased the amounts of K and Na in fruits (by 22 and 35%, on average for R1 and R2, respectively), when compared to the control. Moreover, an increase in the Mg content was noticed in R2 plants (+17%). As for the foliar treatments, fruits from L250 plants showed a decrease in the concentration of Mg (-15%) when compared to the L0 and L500 plants.

In addition, as shown in Table 2.5, the amount of Zn in the fruits of cherry tomato plants increased (+11.3%) with the addition of Fe to the nutrient solution (R2) but decreased after the leaf spray application (L500).

As can be observed in Figure 2.3, the leaves of cherry tomato plants showed a significant and proportional increase in the Fe accumulation according to the concentration of Fe applied. When compared to controls, R2 plants showed the highest Fe concentrations (+132%).

Table 2-1. Effects of Fe application on yield and carpometric parameters of cherry tomato fruits.

	Yield (g plant^{-1})	**Average fruit weight (g)**	**Dry matter content (%)**	**Soluble solid (Brix°)**	**Titratable acidity (g L^{-1})**	**Firmness (N)**
			Fe Nutrient solution			
R0	1067 ± 41 a	14.8 ± 1.0 a	9.69 ± 0.6 b	8.56 ± 0.4 b	6.35 ± 0.5 b	8.87 ± 0.7 b
R1	981 ± 86 b	13.2 ± 1.1 b	10.24 ± 0.5 ab	9.22 ± 0.6 a	6.78 ± 0.6 b	9.37 ± 0.5 ab
R2	932 ± 65 b	12.6 ± 1.1 b	10.69 ± 0.9 a	9.57 ± 0.9 a	7.65 ± 0.9 a	9.70 ± 0.5 a
F-test	*	***	**	**	**	*
			Fe Leaf spray			
L0	958 ± 79	13.2 ± 1.2	10.35 ± 0.6	9.30 ± 0.6	7.01 ± 0.9	9.22 ± 0.7
L250	999 ± 100	13.3 ± 1.8	10.32 ± 1.0	9.27 ± 1.0	7.10 ± 1.0	9.28 ± 0.7
L500	1024 ± 72	14.1 ± 1.0	9.95 ± 0.6	8.78 ± 0.4	6.67 ± 0.7	9.45 ± 0.7
F-test	NS	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (P=0.05). *, **, ***: significance of $P \leq$ 0.05, 0.01, 0.001, respectively. NS: not significant. ± indicates the standard deviation.

Table 2-2. Effects of Fe application on fruit chromatic coordinates of cherry tomato fruits.

	L*	a*	b*	$(a^*/b^*)^2$	Chroma
			Fe Nutrient solution		
R0	42.14 ± 0.8 a	17.52 ± 1.2	24.71 ± 0.6	0.49 ± 0.1	30.30 ± 1.0
R1	41.87 ± 0.9 ab	16.89 ± 1.4	24.37 ± 0.6	0.50 ± 0.1	29.67 ± 0.8
R2	41.30 ± 0.5 b	17.43 ± 1.5	23.84 ± 0.9	0.54 ± 0.1	29.56 ± 1.2
F-test	*	NS	NS	NS	NS
			Fe Leaf spray		
L0	42.01 ± 0.3	17.67 ± 1.1	24.46 ± 0.9	0.54 ± 0.1	30.01 ± 1.1
L250	41.88 ± 0.2	17.24 ± 1.6	24.24 ± 0.3	0.50 ± 0.1	29.95 ± 0.8
L500	41.43 ± 0.3	16.93 ± 1.5	24.22 ± 1.0	0.49 ± 0.1	29.57 ± 1.2
F-test	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (P=0.05). *: significance of $P \leq 0.05$. NS: not significant. ± indicates the standard deviation

Table 2-3. Effects of Fe application on biochemical traits of cherry tomato fruits.

	TPC (GA µmol 100 g^{-1} FW)	**FRAP** (TE µmol 100 g^{-1} FW)	**DPPH** (TE µmol 100 g^{-1} FW)	**Total carotenoids** (µg 100 g^{-1} FW)
		Fe Nutrient solution		
R0	670 ± 58	247 ± 18	266 ± 17	1534 ± 174
R1	665 ± 70	235 ± 19	251 ± 24	1686 ± 160
R2	673 ± 36	232 ± 19	246 ± 24	1587 ± 109
F-test	NS	NS	NS	NS
		Fe Leaf spray		
L0	670 ± 36	233 ± 16	256 ± 20	1576 ± 111
L250	653 ± 61	243 ± 16	259 ± 24	1604 ± 189
L500	684 ± 63	238 ± 25	248 ± 25	1628 ± 178
F-test	NS	NS	NS	NS
Interaction	NS	NS	NS	NS

NS: not significant. ± indicates the standard deviation.

Table 2-4. Effects of Fe application on the macronutrient composition (mg 100 g^{-1} FW) of fruits from 2nd cluster of cherry tomato.

	N	P	K	Mg	Ca	Na
Fe Nutrient solution						
R0	181 ± 3.9	47.8 ± 3.9	272 ± 44 b	16.0 ± 2.6 b	5.2 ± 0.7	9.6 ± 1.8 b
R1	183 ± 5.8	46.6 ± 6.3	323 ± 52 a	15.8 ± 2.2 b	5.3 ± 0.5	12.4 ± 2.4 a
R2	191 ± 11.8	48.7 ± 6.6	342 ± 73 a	18.8 ± 1.9 a	4.9 ± 0.4	13.6 ± 4.4 a
F-test	NS	NS	*	**	NS	*
Fe Leaf spray						
L0	186 ± 7.6	50.5 ± 6.3	326 ± 76	17.8 ± 2.3 a	5.1 ± 0.5	13.1 ± 3.9
L250	188 ± 11.8	47.2 ± 5.0	309 ± 71	15.1 ± 2.5 b	4.9 ± 0.4	11.7 ± 3.6
L500	181 ± 4.3	45.4 ± 4.5	301 ± 42	17.7 ± 2.2 a	5.5 ± 0.6	10.8 ± 2.7
F-test	NS	NS	NS	**	NS	NS
Interaction	NS	NS	NS	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (P=0.05). *, **: significance of $P \leq$ 0.05, 0.01, respectively. NS: not significant. ± indicates the standard deviation.

Table 2-5. Effects of Fe application on the micronutrient composition (μg 100 g^{-1} FW) of fruits from 2nd cluster of cherry tomato.

	Zn	Mn	Cu
Fe Nutrient solution			
R0	239 ± 13 b	70.8 ± 12	120 ± 30
R1	237 ± 12 b	75.5 ± 10	130 ± 30
R2	266 ± 31 a	70.8 ± 6	128 ± 23
F-test	**	NS	NS
Fe Leaf spray			
L0	259 ± 27 a	73.4 ± 9	133 ± 23
L250	250 ± 25 a	71.3 ± 9	129 ± 27
L500	233 ± 8 b	72.3 ± 12	117 ± 31
F-test	**	NS	NS
Interaction	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (P= 0.05). **: significance of P≤ 0.01. NS: not significant. ± indicates the standard deviation.

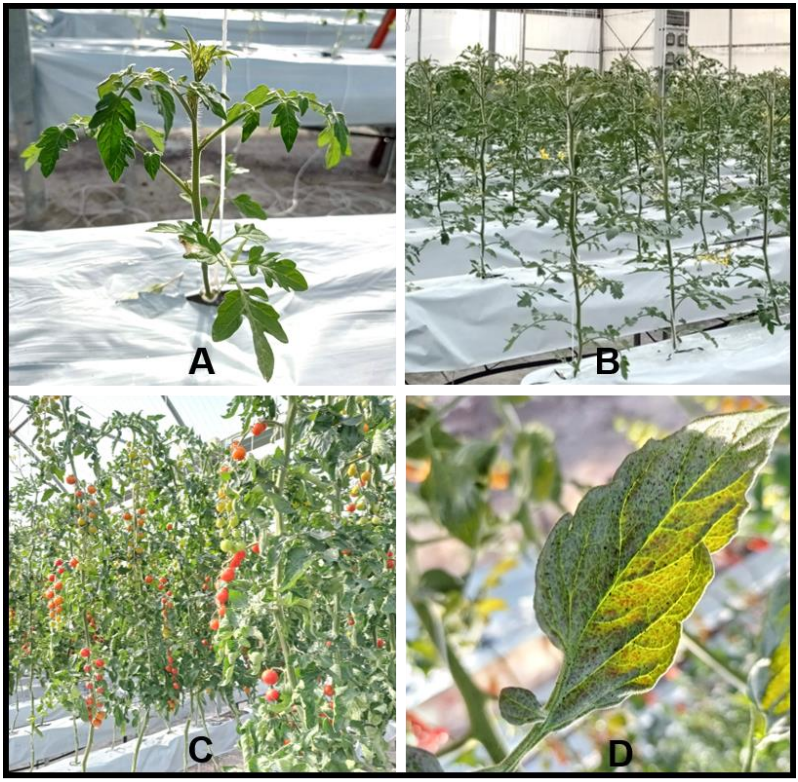

Figure 2-1. Different phases of the crop during experiment. (A) Plant 7 days after transplanting. (B) Flowering of 1st cluster. (C) Maturation of 1st cluster (D) Leaf showing dark spots, 72 days after transplanting.

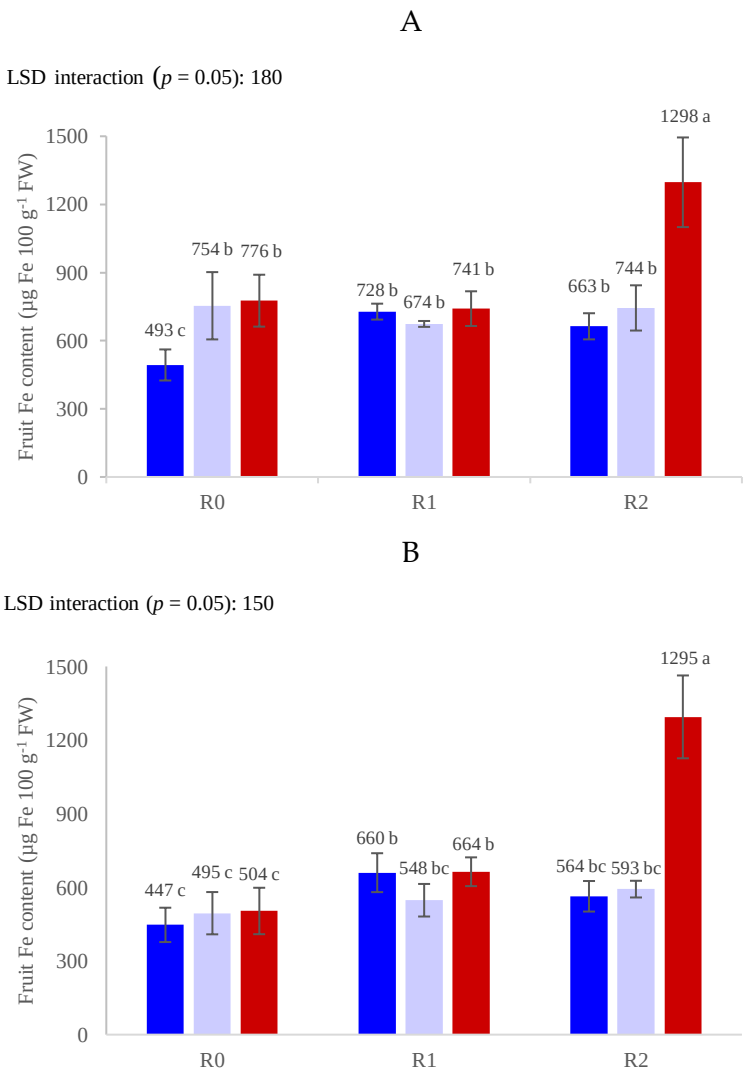

Figure 2-2. Iron content of cherry tomato fruits belonging to 1st (A) and 2nd (B) cluster, as affected by the Fe application (foliar and nutrient solution). Blue bars: L0; lilac bars: L250; red bars: L500. Error bars indicate the standard deviation.

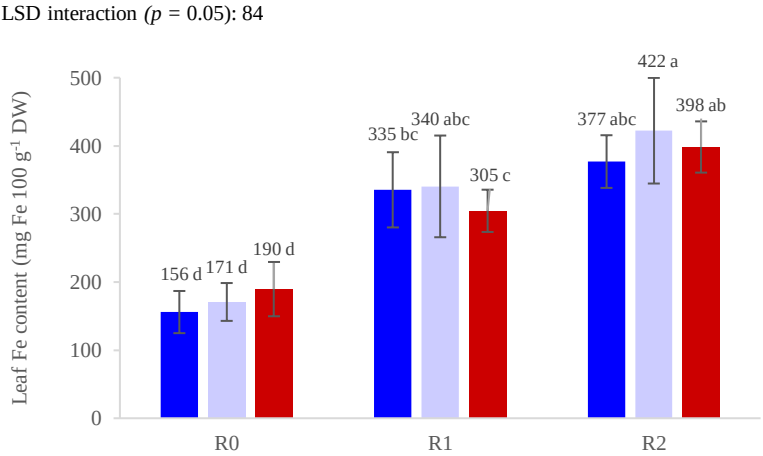

Figure 2-3. Iron content of cherry tomato leaves as affected by the Fe application (foliar and nutrient solution). Blue bars: L0; lilac bars: L250; red bars: L500. Error bars indicate the standard deviation.

2.1.4 Discussion

Cherry tomato plants receiving 2 mmol Fe L^{-1} through the nutrient solution showed a reduction in the average fruit weight and an increase in fruit dry matter. Such phenomenon is probably due a stress condition created by high Fe concentrations and has been observed in other crops, such as lettuce and common chicory (Cecilio Filho et al. 2015; Giordano et al. 2019). At the same time, in a study carried out with rice plants, 0, 2 mmol and 4 mmol L^{-1} of Fe were added to the nutrient solution, and the 2 mmol L^{-1} dose produced the maximum fresh and dry weights, while 4 mmol L^{-1} showed a significant reduction in both fresh and dry weight (De Dorlodot et al. 2005). This reduction in the average fresh weight when plants were submitted to high concentrations of Fe could indicate excessive amounts of Fe that, in turn, can increase ROS generation, causing cell

damage and affecting many biochemical reactions, including reducing the rate of photosynthesis (Nagajyoti et al. 2010). On the other hand, the increase in the dry matter content of cherry tomato fruits obtained in our study can be interpreted as a positive outcome when fruits' postharvest behavior is concerned, as a higher dry matter content at harvest helps increase tolerance to possible mechanical damage during postharvest operations (Valverde-Miranda et al. 2021; Distefano et al. 2022b).

At the same time, titratable acidity and soluble solids content increased in tomatoes biofortified with Fe via the roots, while SS/TA was not affected (data not shown). This suggests that the higher concentrations of Fe in the nutrient solution improved the metabolism of sugars and organic acids, promoting their accumulation in fruits, but without altering the typical balance of the given cultivar (Distefano et al. 2020). This demonstrates that a tailored biofortification approach can also contribute to the production of highly palatable vegetables (Rouphael and Kyriacou 2018).

The present biofortification study showed that the increase in the concentration of Fe provided to the plants through the nutrient solution resulted in Fe being absorbed by the roots and translocated to the fruits. Similarly, a Fe-biofortification experiment conducted with lettuce, showed that a nutrient solution enriched with 1 or 2 mmol L^{-1} Fe as Fe-EDDHA, increased the Fe content in leaves of red and green lettuce, in the range of 41-86% (Giordano et al. 2019). Moreover, Fe's absorption and translocation through the roots in tomato plants has already been demonstrated in a study conducted by Brown and Ambler (1974), who noted how the combined action of protoxylem and metaxylem was able to transport Fe from the lateral roots to the primary ones, before being transported from the roots into the stem exudate, mainly as Fe citrate. This is possible thanks to the reduction of Fe^{3+} to Fe^{2+} in the lateral roots making more Fe available to be transported inside the roots, where it can be chelated into Fe citrate

and transported to the top of the plant. At the same time, foliar applications of Fe (250 and 500 μmol L^{-1}) showed that cherry tomato plants can absorb Fe through the leaves and translocate it to the fruits. This result is consistent with the absorption and translocation pattern of Fe observed by Zhang et al. (2022), after submitting tomato plants to different photoperiods (12h/12h and 16h/8h) and concentrations of Fe-EDTA (100, 150 and 200 μmol L^{-1}), applied through foliar spray. They obtained fruits with 11 and 25% greater Fe concentrations for the 12h/12h and 16h/8h photoperiods, respectively, when the 200 μmol L^{-1} dose was applied, compared to the untreated plants. The same floematic movement was anatomically demonstrated when tomato plants grown using hydroponics received three applications of 3 mmol L^{-1} Fe solution (as $FeSO_4$), where the Fe applied to the leaves was translocated to other parts of the plants (Carrasco-Gil et al. 2016). Finally, the combination of both foliar sprays and nutrient solution enriched with Fe at the higher doses (500 μmol L^{-1} and 2 mmol L^{-1}, respectively) provided the greatest increase in Fe content in cherry tomato fruits. The consistency of the mineral accumulation in tomato fruits subjected to this treatment (R2 L500) can also be confirmed by comparing the first and second clusters, which followed a similar enrichment pattern (Figure 2.2). This indicates that in order to achieve a better biofortification efficacy in all clusters, Fe should be supplied simultaneously through the nutrient solution and as a foliar spray. In addition, the principle of double biofortification efficacy was also demonstrated by Smoleń et al. (2014) when producing Se-biofortified lettuce after the application of the mineral to the roots and leaves of the crop.

The increase we observed in the accumulation of Fe in the leaves of cherry tomato plants (+90 and +132% at R1 and R2, respectively) is consistent with the findings of another study where tomato plants grown in greenhouse received a nutrient solution with 5 mmol of Fe (as Fe-EDTA), and the Fe-enriched nutrient solution

caused a 66% increase in the Fe concentration in tomato shoots (Das et al. 2020).

Plant ionomics indicate that high Fe concentrations in the nutrient solution (2 mmol L^{-1}) synergistically affected the contents of Mg, K, Na and Zn in cherry tomato fruits. A similar result was observed by Olowolaju et al. (2021) when subjecting tomato plants to a nutrient solution 10 times stronger than the standard one (0.053 vs. 0.53 g L^{-1}). They observed that the translocation factor of K, P, Na, Ca, and Mg, along with the bioaccumulation factor of Mg, K, and Na, was higher in the treated plants that received 10 times more Fe. This could have been caused by the increase in the expression of certain proteins responsible for increasing the uptake of Fe, which also causes synergistic increases in the concentrations of other mineral elements, such as Zn, Mn, and Co (Morrissey and Guerinot 2009). This demonstrates that Fe biofortification can not only improve the concentration of the target mineral (Fe), but also increase the concentrations of other elements in the fruits. A better understanding of these synergistic factors could help to improve cherry tomato ionomics and contribute to enhancing the efficiency of biofortification programs.

2.1.5 Conclusion

Since Fe deficiency is among the most relevant types of micronutrient deficiency in both developing and affluent nations, and consumers demand products rich in compounds that can improve health, our study demonstrates that supplementing cherry tomato plants with 2 mmol L^{-1} Fe through the nutrient solution and 500 µmol L^{-1} Fe through foliar spraying can significantly increase the concentration of this mineral in the edible part of the plant (+190%). The application of this mineral also increased the titratable acidity and total soluble solids, potentially improving the taste perception by the consumer. The average fresh weight decreased but, in return, a

significant increase in the dry matter content was noticed, which is a desirable postharvest characteristic. Our results demonstrate that even though more studies are required in order to define an optimal concentration of Fe supplementation to cherry tomatoes, Fe biofortification is facilitated in soilless systems by combining both root and foliar applications, and this strategy could be considered effective to fight malnutrition caused by unbalanced diets, in addition to improving tomato quality.

2.2 Foliar Se-biofortification of greenhouse cherry tomato

2.2.1 Introduction

The 21st century consumer has a growing interest in high quality food, because is commonly knowledge that a proper selection of nutrients intake can substantially impact human health. This knowledge is scientifically supported by the numerous medical studies which demonstrate how healthy eating habits are pivotal for our body condition (Wang et al. 2016; Cho et al. 2021).

Recently, supplemental intake of selenium (Se) is increasing, as this mineral has been associated to prevent aging-related diseases (Cai et al. 2019). As a trace element, Se is required in very small quantities, but it is essential to maintain human metabolism (Ferreira et al. 2021). As constituent of selenoproteins, it is responsible for important enzymatic functions, such as glutathione peroxidase, selenoprotein P, and tetraiodothyronine 5'-deiodinase. The function of selenoproteins in the human metabolism is mainly connected to immunocompetence and cancer prevention, but Se functions go beyond that, as this mineral also plays an important role in fertility and reproduction, brain functions, mood, thyroid health, and cardiovascular diseases (Ip and Lisk 1995; Rayman 2012).

The recommended daily allowance (RDA) of Se is between 55 and 70 μg day^{-1}. In humans, Se deficiency occurs when dietary intake of Se is <40 μg day^{-1}, while tolerable upper level of Se is set at 400 μg day^{-1} (Trumbo et al. 2001). Usually, vegetables contain a low content of Se, caused mostly by its low bioavailability in soils (Dinh et al. 2019). Given this low phytoavailability and the role of plants as the main dietary source of this element, studies aiming to increase the Se content in plants for human consumption are gaining attention. There are already some Se-biofortified vegetables present on the market

(e.g., selenium enriched potato, carrot and onion, 'Selenella' potato from Consorzio Patata Italiana di Qualità Soc. Cons., IT, selenium enriched brussels sprouts from Marks and Spencer, UK), proving that the consumer's interested in these high-quality products.

Tomato (*Solanum lycopersicum* L.) is one of the most consumed and commercially grown vegetable crops, being cultivated in more than four million hectares (FAO 2021). Cherry tomato grown in soilless greenhouse systems can benefit from the control of the environmental conditions such as air temperature, light and vapor pressure deficit, this can positively impact the quality of vegetables (Gruda 2005). Tomato consumption is associated with lower risk of cardiovascular diseases and certain cancer types (Tyssandier et al. 2004). These benefits are attributed to tomato micronutrients, such as lycopene, vitamins, fiber, antioxidants and minerals, and their concentrations are usually determined in ripened fruits (Ali et al. 2021).

Biofortification of Se has been widely studied in many crops, such as lettuce, potato, Brassica, *Allium* (Buturi et al. 2021). However, studies about the Se-biofortification in tomato are scarce and do not provide enough information on the absorption and accumulation of the mineral in this crop. Even though Se is not considered a micronutrient, foliar applications of this mineral have been used to improve crop quality (Mangarotti et al. 2020). In fact, the appropriated use of Se in plant nutrition can increase growth, stimulate seed germination and contribute to protect several crops against pathogens and pests (Hasanuzzaman et al. 2020).

The application time of mineral foliar treatments can affect the biofortification efficiency of fruits, however little information on this factor influence is available. Indeed, there is evidence that the application of Se in different plant developmental stages can influence the Se concentration of cherry tomato (Meucci et al. 2021). Given the importance of the tomato crop in agriculture and the relevance of this

mineral for human health, the aim of the present research was to study the Se biofortification of cherry tomato submitted to foliar applications of Se at different fruit maturation stages. Establish the best application period and evaluate its effects on the development of the plant and on the concentration of other nutrients.

2.2.2 Materials and Methods

2.2.2.1 *Experimental Site and Plant Material*

The experiment was conducted from February to May 2021, in a greenhouse of the experimental farm of the University of Catania (Sicily, Italy: 37◦24'31.5" N, 15◦03'32.8" E, 6 m a.s.l.). The climate of the area is semi-arid Mediterranean, with mild winters and hot, dry summers. An 810 m^2, multi-aisle cold greenhouse was used, having a steel tubular structure with adjustable windows on the roof and along the sides, and covered with polycarbonate slabs.

Plants of *Solanum lycopersicum* L. 'Creativo' were transplanted on 1st February 2021 in the greenhouse at the stage of four true leaves, in an open soilless cultivation system using 5 L plastic pots (20 cm height, 19 cm width) and perlite as growing medium (particle size 2–6 mm). Before transplanting, plantlets were selected for uniform size and health appearance, meanwhile pots were arranged in simple rows, adopting a 0.33 × 1.00 m rectangular format (center to center) and 1 plant per pot (3 plants m^{-2}). Plants were grown at single stem up to the 5th cluster, whereas all clusters were pruned leaving 12 fruits. Each net experimental unit contained 8 plants. During the cycle, the crop was uniformly fertigated with a standard nutrient solution (Mauro et al. 2020a), having the following composition: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 22 µM Fe, 9 µM Mn, 2 µM Cu, 4 µM Zn, 9 µM B, and 1 µM Mo. A leaching fraction of ~25% was adopted, to reduce root zone

salinization (Giuffrida et al. 2018).

2.2.2.2 *Treatments*

The experiment was conducted in a randomized block design with three replicates. The treatments consisted of a single foliar application of Na_2SeO_4 solution, in the concentration of 8 mmol L^{-1}, at two different fruit ripening stages of the second cluster, i.e. (i) P1, when the external fruit coloration of the last 2 fruits of the cluster corresponded to immature green stage and (ii) P2, when the external fruit coloration of the last 2 fruits of the cluster corresponded to breaker stage, according to Gautier et al. (2008). Untreated control (P0) was comparatively considered and didn't receive any foliar application.

The treatments were carried out on 30 March and 20 April, at P1 and P2, respectively. On these days, plants of cherry tomato plants were entirely and uniformly sprayed with ~10 mL of the sodium selenate solution, using a plastic spray bottle (2 L capacity).

Tomatoes belonging to the 2nd cluster were harvested by hand on 11th May; soon after harvest, fruits were transported to the laboratory, analyzed for carpometric variables, flash frozen with liquid nitrogen and stored in a freezer at -80 °C for further biochemical analysis.

Overall, 72 clusters of cherry tomato were collected and analyzed (3 periods of application × 3 replicates × 8 plants).

2.2.2.3 *Carpometric determinations*

The following measurements were made on each sample: Yield and average fruit weight were determined gravimetrically on 8 fruits per plot, detached from their rachis and selected for uniform appearance and absence of defeats; firmness which was determined

through a Digital Texture Analyzer mod. TA-XT2 (Stable Micro Systems, Godalming, UK) and defined as the force (N) needed to impress a 2 mm fruit deformation along the polar axis, between two steel plates. Dry matter (DM) (%), which was obtained by drying the fruits in a thermoventilated oven at 70 °C until constant weight was reached. The fruit chromatic coordinates were measured as described by McGuire (1992) on the equatorial axis of whole fruits, through a tristimulus Minolta Chroma meter (model CR-200, Minolta Corp.) calibrated with a standard white tile (UE certificated) with illuminant D65/10◦, measuring color in terms of lightness (L*), green-red axis (a*) and blue-yellow axis (b*). Fruit color was described as L*, a*, b*, $(a^*/b^*)^2$ and Chroma $[(a^{*2} + b^{*2})^{1/2}]$. For each sample, ~50 g of cherry tomato was homogenized up to a puree in a home blender (La Moulinette, Groupe SEB, Écully, France), this puree was centrifuged for 5 minutes at 1361 g. Then 3 mL of supernatant was immediately analyzed for soluble solids content and reducing sugars. The soluble solids content (SSC) was estimated with an Abbe refractometer 16531 (Carl Zeiss, Oberkochen, Germany) and the results were expressed as ◦Brix. The titratable acidity (TA) was determined by titrating an aliquot of the supernatant with 0.05 N NaOH to pH 8.1, using phenolphthalein as indicator. TA was expressed as g citric acid equivalents (CA) L^{-1}.

2.2.2.4 *Biochemical analyses*

For the biochemical analyses frozen samples from 2nd cluster of cherry tomato, previously conserved in a -80 °C freezer, were lyophilized in a Martin Christ Alpha 1-4 LD plus freeze dryer and grounded using liquid nitrogen. All analyses were performed using plastic cuvettes and readings were carried out using a Jeanway UV/Visible spectrophotometer (Stone, Staffordshire, UK).

- *Total carotenoids*

Determination of total carotenoids was conducted according to Lichtenthaler and Wellburn (1983), with slight modifications. For the extraction, 50 mg of lyophilized tomato powder material was mixed with 5mL of ethanol (96%) and vortexed for one minute; samples were then left overnight in the dark at low temperature (10 °C). After that, samples were submitted to 10 minutes of ultrasonic bath (below 10 °C) and centrifuged for 10 minutes 5000 g at 6 °C. Then samples were read in 1.5 mL plastic cuvette. Ethanol 96% was used as blank. Readings were done in the following wavelengths: 470, 649 and 665 and the absorbance values were applied in the following formula:

- $Ca = 13.95 \times A665 – 6.88 \times A649$
- $Cb = 24.96 \times A649 – 7.32 \times A665$
- $Cx+c = (1000 \times A470 – 2.05 \times Ca – 114.8 \times Cb) \div 245$

Where Ca stands for Chlorophyll A, Cb stands for Chlorophyll B and C x+c stands for total amount of carotenoids, results are expressed in µg every 100 g of fresh weight (FW).

- *Total phenolic content*

Total polyphenol content (TPC) was quantified through the Folin-Ciocâlteu method (Cicco et al. 2009). For the extraction, 100 mg of lyophilized tomato powder material was mixed with 5mL of methanol (80%) and vortexed for 1 minute. Samples were then submitted to 10 minutes of ultrasonic bath (below 10 °C) and centrifuged for 15 minutes at 4000 g and 6 °C. The supernatant was withdrawn and the extraction process was repeated 3 times. Extracts were combined and diluted to 20 mL using methanol. For the reaction, 200 µL of extract solution were mixed with 1000 µL Folin-Ciocâlteu (10%) and left to react at room temperature for 2 minutes Next, 800

µL of sodium carbonate (0.7 M) were added to stop the reaction, mixed and placed in the dark at room temperature for 60 min. Samples were read at 760 nm and TPC values were obtained from a standard curve prepared by plotting change in absorbance against different concentrations of gallic acid, and reported as µmol of gallic acid equivalents (GAE) every 100 g of fresh weight (FW).

- *DPPH assay*

The DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenging activity of extract was determined according to Brand-Williams et al. (1995). For the extraction, 100 mg of lyophilized tomato powder material was mixed with 5mL of methanol (80%) and vortexed for 1 minute. Samples were then submitted to 10 minutes of ultrasonic bath (below 10 °C) and centrifuged for 15 minutes 4000 g at 6 °C. For the reaction, 150 µL of supernatant was mixed to 1350 µL of DPPH solution (150 µM) recently prepared, samples were vigorously agitated and place in the dark for 30 minutes. The decrease in absorbance of methanolic solution of DPPH was read at 515 nm and DPPH was calculated from a standard curve prepared by plotting change in absorbance against different concentrations of trolox and expressed as µmol of trolox equivalents (TE) every 100 g of fresh weight (FW).

- *FRAP assay*

The FRAP (ferric reducing antioxidant power) assay of extract was determined according to Benzie and Strain (1999). For the extraction, 200 mg of lyophilized tomato powder material was mixed with 10 mL of pure methanol, vortexed for 1 minute and placed in the dark for 30 minutes. After that, samples were centrifuged for 10 minutes 4500 g at 6 °C. Preparation of FRAP reagent consisted of 10 mL of acetate buffer (300 mM, pH 3.1) mixed with 1 mL of TPTZ

(2,4,6-Tris(2-pyridyl)-s-triazine) solution (10 mM in 40 mM HCl) and 1mL of ferric chloride (20 mM). For the reaction, 150 µL of supernatant were mixed to 300 µL of ultrapure water, vortexed and added to 3 mL of FRAP reagent. Samples were placed in the dark at 20 °C for 10 minutes. The FRAP, based on the reduction of Fe (III) by the sample extract, was determined following the change in absorbance at 593 nm due to the formation of a blue coloured Fe(II)-tripyridyltriazine compound from colourless oxidized Fe(III) form in presence of a particular concentration of sample. FRAP was calculated from a standard curve prepared by plotting change in absorbance against different concentrations of trolox and expressed as µmol of trolox equivalents (TE) every 100 g of fresh weight (FW).

2.2.2.5 *Determination of minerals content*

The content of Ca, K, Mg, Na and Se was determined after mineralization. For this 2 g of dry and ground sample were placed in the muffle furnace at 550 °C (initially the temperature was gradually raised, 50 °C every 30 minutes) until the resulting ashes were clear and white, this process took approximately 12 hours. The resulting material was further digested in 20 mL of hydrochloric acid (1 M) in water bath for 30 minutes at 100 °C. Finally, samples were diluted to 100 mL using ultrapure water, filtered using filter paper and analyzed by means of ionic chromatography (Dionex IC 25 Ion Chromatograph, 40 EG Eluent Generator) using an Ion Pac CS12A. The determination of Se content in the tomato fruits was performed using an ICP-MS; this analysis was carried out at Mérieux NutriSciences.

The consumer safety of Se-biofortified cherry tomato was evaluated on the basis of the HQ (hazard quotient) values that describe the risk to human health resulting from the intake of Se through the consumption of fresh cherry tomato, according to Smoleń et al. (2019). The calculations of HQ were performed using the following equation: HQ=ADD/RfD, where ADD is the average daily dose of Se

(mg Se per kg body weight per day) and RfD represents the recommended dietary tolerable upper intake level of Se. The average daily dose (ADD) was determined as: ADD = (M × CF × DI)/BW, where M is the Se concentration in cherry tomato fruits (mg kg^{-1} DW), CF is the fresh to dry weight conversion factor for plant samples (dry weight to fresh weight ratio), DI is the daily intake of cherry tomatoes (100 g) and BW is the taken body weight (70 kg). The taken RfD values were 400 μg Se day^{-1} or 5.71 μg Se $kg^{-1}day^{-1}$ for a 70 kg adult (Trumbo et al. 2001). According to the United States Environmental Protection Agency (USEPA) Protocol (IRIS 2020), a hazard quotient less or equal to 1 indicates that adverse effects are not likely to occur, and thus can be considered safe, while when the HQ is ≥ 1, there is a potential health hazard.

2.2.2.6 *Statistical procedures*

Collected and calculated data were firstly subjected to a one-way analysis of variance (ANOVA). Then significant different means were compared using Fisher's protected least significant difference (LSD) test ($p \leq 0.05$). All calculations were performed using Excel and Minitab 19.

2.2.3 Results

When compared to the untreated control, the Se application reduced the DM content (-7% on average) of fruits but did not affect neither yield nor fruit average weight. The Se treatment did not affect any of the other carpometric traits, such as yield, average fruit weight, TSS, titratable acidity, firmness and color parameters (Table 2-6).

Regarding the biochemical traits, total carotenoids and polyphenols were not affected by the applications of Se. As for the antioxidant power, while DPPH showed no significant differences,

FRAP concentration in fruits was reduced in P2 (-24%), when compared to control (Table 2-7).

The Se content of cherry tomato fruits submitted to Se applications (as Na_2SeO_4) was significantly increased both at P1 and P2 (by +1448 and +2567%, respectively), when compared to P0 plants (Table 2-8; Figure 2-4). Regarding Se safety, the hazard quotient (HQ) of fruit of tomatoes did not exceed 1 for none of the treatments. On the other hand, the concentration of K, Ca, Mg and Na was not affected by the none of the Se treatments (Table 2-8).

Table 2-6. Carpometric variables of fruits from the 2nd cluster of cherry tomato as affected by Se treatments

Treatment	Yield (g plant^{-1})	Average fruit weight (g)	DM (%)	TSS (Brix°)	Titratable acidity (g L^{-1})	Firmness (N)	L*	a*	b*	(a*/b*)^2	Chroma
P0	1044	13.5	13.3 a	8.8	6.3	8.7	42.0	17.2	24.1	0.51	29.6
P1	1134	14.5	12.6 b	8.6	6.0	9.1	41.5	17.4	23.9	0.53	29.6
P2	1125	15.1	12.2 b	8.8	6.2	9.7	41.3	17.8	23.9	0.56	29.8
F-test	NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (p = 0.05)

**: significance of $P \leq 0.01$. NS: not significant.

Table 2-7. Biochemical traits of fruits from 2nd cluster of cherry tomato as affected by Se treatments.

Treatment	Total carotenoids (µg 100 g^{-1} FW)	TPC (µmol 100 g^{-1} FW)	FRAP (µmol 100 g^{-1} FW)	DPPH (µmol 100 g^{-1} FW)
P0	1833	965	326 a	284
P1	1666	905	301 a	266
P2	1762	881	248 b	255
F-test	NS	NS	*	NS

Different letters indicate significance fisher's protected LSD Test (p = 0.05)

*: significance of $P \leq 0.05$. NS: not significant.

Table 2-8. Mineral compositional of fruits from 2nd cluster of cherry tomato as affected by Se treatments.

Treatment	Se (µg 100 g^{-1} FW)	HQ	K (mg 100 g^{-1} FW)	Ca (mg 100 g^{-1} FW)	Mg (mg 100 g^{-1} FW)	Na (mg 100 g^{-1} FW)
P0	4.20 c	0.01 b	444	23.8	22.9	19.2
P1	65 b	0.13 ab	433	22.4	21.3	18.8
P2	112 a	0.22 a	428	20.9	20.9	17.4
F-test	***	**	NS	NS	NS	NS

Different letters indicate significance fisher's protected LSD Test (p = 0.05)
, *: significance of $P \leq$ 0.05, 0.01, respectively. NS: not significant.

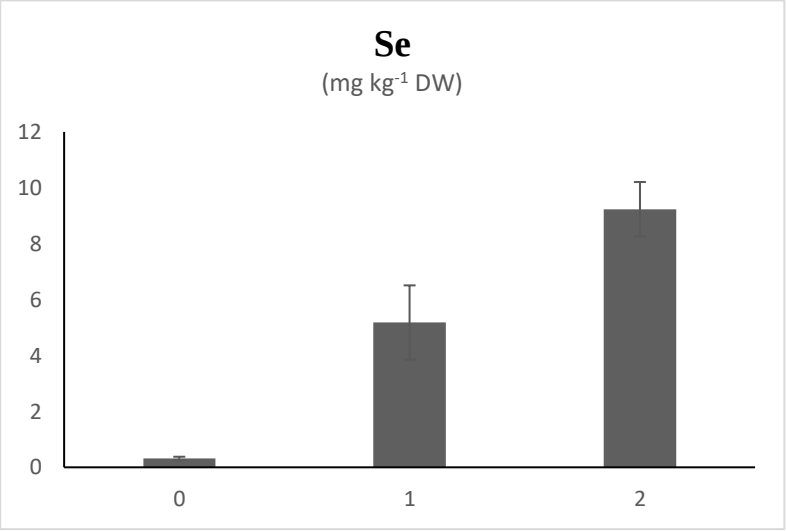

Figure 2-4. Se content of cherry tomato fruits as affected by the Se application. Error bars indicate the standard error.

2.2.4 Discussion

Even though Se application is commonly used in plant nutrition for its beneficial effects (El-Ramady et al. 2016), phytotoxicity of Se has been reported in literature (Hasanuzzaman et al. 2020). After Edelstein et al. (2016) treated tomato plants with Se (selenate) through the nutrient solution (9.5 µmol L^{-1}) they observed a 18% decrease in the fruit DM and this reduction increased linearly with increasing concentrations of Se in the tissues, when compared to the untreated plants. This negative effect is probably caused by an increase in the accumulation of ROS and oxidative stress (Gupta and Gupta 2017). In the present study, the same parameter showed a lower reduction (-7%), when we applied a higher concentration of Se (8 mmol L^{-1}). This indicates that tomato plants are more tolerant to Se stress, when this mineral is applied through foliar sprays. This is highlighted by the fact

that none of the other carpometric parameters evaluated showed significant differences when compared to control plants.

This minor condition of stress can also be the reason why the only biochemical parameter affected by the Se treatment, in this study, was the FRAP assay. Similar studies using high concentrated solutions of Se foliar sprays also didn't show significant differences in the concentration of other antioxidants such as vitamin C and polyphenols (Islam et al. 2018; Andrejiová et al. 2019). However, this is in contrast with the results obtained when small doses of Se were applied in the nutrient solution. Sabatino et al. (2021a), after submitting tomato plants to a Se-enriched nutrient solution (2 and 4 µmol L^{-1}) noticed an increase in the total carotenoids and polyphenols content.

Similarly, the macronutrients content of fruits was not affected by the Se treatments, which suggests that the concentration of Se used in this study did not interfere with the absorption of other elements. Probably because, in tomato, Se does not compete with other minerals, as demonstrated by literature (Castillo-Godina et al. 2016; Edelstein et al. 2016b).

The effectiveness of the foliar spray application of Se in the Se-biofortification of tomato is supported by the results obtained by Schiavon et al. (2013). After a single application of selenate (Na_2SeO_4), in the concentration of 20 mg Se per plant, they obtained tomato fruits with 4 mg kg^{-1} DW of Se, similar to the values obtained in this study at P1. A similar enrichment was obtained when tomato plants received foliar applications of 150 g Se ha^{-1} (Andrejiová et al. 2019).

The abovementioned studies have reported that the Se applications were carried out at the flowering stage. Meucci et al. (2021), compared the Se-enrichment pattern after the application of Se foliar spray at different plant developmental stages (flowering vs. immature green stage). After analyzing cluster 1 and 2, they obtained contrasting results, only cluster 2 showed higher Se concentrations in

fruits, when Se was applied during flowering. They also highlighted that the Se-enrichment obtained in their study was low (100 times lower than ours), which they attributed to the lower concentration used and absence of adhesive substances in the spraying solution. In our study, when comparing the two application stages, P2 showed to be more efficient in increasing Se content in tomato fruits when compared to P1, demonstrating that the best application period to accumulate Se in the cherry tomato fruits is at the breaker stage (ripening). Few studies report the levels of Se during fruit development. Costa et al. (2011) when studying the evolution of mineral contents in cherry tomato fruits, have reported that the concentration of Se slightly decreased from the green stage of fruits to the turning stage but increased five times during the ripening process, suggesting that during ripening there is a greater mobility of minerals. This could be the case of Se, especially in the form of selenate which presents a higher phloematic mobility when compared to the selenite form (Li et al. 2008).

Our results indicate that the consumption of 108 and 63 g FW of Se-biofortified tomato fruits (from plants treated at P1 and P2, respectively) is sufficient to cover the Se RDA. Moreover, based on the calculation of HQ value it can be stated that the consumption of 100 g portion of fresh cherry tomato fruits will pose no risk of Se toxicity as the values were below 1. The HQ would exceed the recommended value after the, unlikely, consumption of around 850 and 485 g tomato day^{-1} (approximately 57 and 33 fruits), from plants treated at P1 and P2, respectively.

Thus, we conclude that Se biofortification of greenhouse soilless grown cherry tomato represents an attractive and easy opportunity for increasing the Se concentration in human diets.

2.2.5 Conclusion

These findings indicate that Se-biofortification of cherry tomato fruits can be successfully carried out after a single foliar application of Na_2SeO_4, both at the stage of fruit set and at ripening, being the second stage more efficient in accumulating Se in the fruits. The consumption of 4-7 enriched tomatoes (108 and 63 g FW) is enough to fulfill the Se daily recommended intake and consuming less than 485 g FW tomato fruits a day poses no risk of toxicity.

3 Experimental activities on lettuce

3.1 Iron biofortification of greenhouse soilless lettuce: an effective agronomic tool to improve the dietary mineral intake

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3.1.1 Introduction

It is well known that iron (Fe) is an essential mineral element for humans, being involved in the synthesis of hemoglobin and myoglobin (Zoroddu et al. 2019). However, the importance of Fe goes beyond the oxygen transport, as it plays a key role in neural systems, immune cell functioning, and homeostasis, it is required for energy metabolism and exercise, being fundamental in the maintenance of human health (Haschka et al. 2021). Moreover, Fe deficiency symptoms usually include weakness, fatigue, difficulty in concentrating, motor and mental impairment and anemia (Camaschella 2017).

The amount of Fe required daily by the human body ranges between 8 and 18 mg, which represents the recommended daily allowance (RDA); in contrast, the tolerable upper intake level (UL) for adults is 40 mg day^{-1} (Wishart 2017). However, in some cases, the minimum intake requirement is not fulfilled with the diet, resulting in cases of micronutrient deficiencies, also called hidden hunger (de Valença et al. 2017; Lillford and Hermansson 2021). This kind of malnutrition is not always easy to detect, and it does not affect only developing countries, but it is also present in the developed world (Biesalski 2017). The causes of the insufficient intake of micronutrients, such as mineral elements, can be attributed to poverty,

but also to the rise of new diets (e.g. veganism) and bad eating habits in developed countries, which include daily intake of high-calorie, low-nutrient-dense foods (Poelman et al. 2018).

Moreover, in the specific case of minerals, not all the elements present in the food matrix are available for the absorption. In fact, only around 14-18% of the Fe present in the diet is bioavailable (Pasricha et al. 2021). This happens because Fe absorption can be limited by many factors such as the presence of inhibiting substances (calcium, phytates, and tannins), age, pregnancy, surgical procedures, and medical conditions (Cappellini et al. 2020; Pasricha et al. 2021).

An alternative to increase the intake of micronutrients is to include, in the diet, foods containing higher concentrations of those elements. Given that, strategies aiming to increase the Fe content in food can be good tools to improve human dietary patterns (Olson et al. 2021). At the same time, vegetables contain a variety of natural health-promoting, such as vitamins, minerals, and antioxidants, being excellent functional food options (Mauro et al. 2020b; Mazzoni et al. 2021).

When mineral micronutrients are concerned, an efficient approach to improve their concentrations in vegetables may be agronomic biofortification, i.e. by growing them with targeted applications of fertilizers (Ierna et al. 2020a; Sabatino et al. 2021a; Sabatino et al. 2021b). In addition, this strategy, when well-managed, can provide more than simply an increase in the target element. Indeed, by using specific elements as eustressors, biofortification can also increase the concentration of many antioxidant compounds, establishing a link between plant nutrition and human nutrition (Rouphael and Kyriacou 2018).

Soilless cultivation systems offer benefits such as the possibility to control water availability, pH and nutrient concentrations in the root zone (Savvas and Gruda 2018). In fact, currently about 3.5% of the total area cultivated under tunnels and greenhouses for vegetable production adopts soilless cultivation systems. This method can increase not only yield but also the quality

and the shelf life of fresh vegetables, meeting the highest demands of modern consumers (Sambo et al. 2019).

Biofortification of vegetables can be carried out in soilless systems by adding higher concentrations of target fertilizers in the nutrient solution (Buturi et al. 2021). Besides, in the specific case of Fe, which presents a low solubility in the soil (Jones 2020), a soilless cultivation system can be a good option to increase micronutrients availability, since it facilitates the pH management in the nutrient solution (Kobayashi et al. 2019).

Another factor that can affect the biofortification effectiveness, is the chemical form of the added micronutrients in the nutrient solution. Considering Fe, chelate forms are highly recommended since they are more easily available for plants and can optimize mineral absorption when compared to inorganic salts (Martens and Westermann 2018).

In addition, it should be taken into consideration that, the introduction of higher amounts of fertilizers in the nutrient solution can also affect vegetable yield and quality (Carrasco-Gil et al. 2016). Since Fe excess can be toxic to the plant, causing damages to the membrane, DNA, and proteins, it is important to understand the activation of the antioxidant enzymes involved in the Fe biofortification (Zahra et al. 2021). So far, few biofortification studies were conducted aiming to improve the Fe and antioxidants content of vegetables and at the same time assess the stress conditions of plants submitted to high Fe levels in the nutrient solution.

Besides being a model plant, lettuce (*Lactuca sativa* L.) is one of the most popular and consumed leafy vegetables in the world (Souza et al. 2022). In this study, we have chosen two different genotypes of lettuce to compare their tolerance to high doses of Fe introduced in the nutrient solution, i.e. *L. sativa* L. var. *capitata* (Looseleaf) and *L. sativa* L. var. *longifolia* (Romaine) as they are among the most commonly consumed (Vargas-Arcila et al. 2017; Giordano et al. 2019; Shatilov et al. 2019).

Given the scarcity of biofortification studies, our investigation

aimed to address the effects of different iron (Fe) concentrations in the nutrient solution supplied as Fe-HBED on yield and compositional traits of two cultivars of greenhouse soilless lettuce and compare the tolerance of these genotypes to the exposure of high levels of this element in the nutrient solution.

Therefore, the hypothesis of this study is that the application of Fe-HBED to lettuce plants will modify the compositional traits of the plants in a genotype-specific manner.

3.1.2 Materials and Methods

3.1.2.1 *Experimental Site and Plant Material*

A greenhouse experiment was carried out from December 2020 to January 2021, at the experimental farm of the University of Catania (Sicily, Italy: 37°24'31.5" N, 15°03'32.8" E, 6 m a.s.l.). The climate of the area is semi-arid Mediterranean, with mild winters and hot, dry summers. An 810 m^2, multi-aisle cold greenhouse was used, having a steel tubular structure with adjustable windows on the roof and along the sides, and covered with polycarbonate slabs. Two lettuce cultivars were selected for the study, i.e., 'Nauplus' (var. *capitata*; Blumen vegetable seeds, Piacenza, Italy) and 'Romana' (var. *longifolia*; Topseed, Sarna, Italy). Plantlets were transplanted on 10th December 2020 in the greenhouse at the stage of four true leaves, in an open soilless cultivation system using 5 L plastic pots (20 cm height, 19 cm width) and perlite as growing medium (particle size 2–6 mm). Before transplanting, plantlets were selected for uniform size and healthy appearance. Pots were arranged in simple rows, adopting a 0.25 × 0.50 m rectangular format (center to center) and 1 plant per pot (8 plants m^{-2}). Plants were harvested on 25th January 2021. Each net experimental unit contained 12 plants.

3.1.2.2 *Treatments*

A split-plot experimental design with three replicates was

adopted. On the main plots we had the treatments, meanwhile the two cultivars were arranged on the subplots. The treatments consisted of three concentrations of Fe chelate added to the nutrient solution: Fe0: 0 mmol L^{-1} Fe (just the standard nutrient solution, equal to 0.022 mmol L^{-1} Fe); Fe1: 1 mmol L^{-1} Fe; Fe2: 2 mmol L^{-1} Fe in the chelate form HBED. Thus, the final concentrations were 0.02, 1.02 and 2.02 mmol L^{-1} Fe. During the cycle, the crop was fertigated with a standard nutrient solution (Mauro et al. 2020a), having the following composition: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 22 µM Fe, 9 µM Mn, 2 µM Cu, 4 µM Zn, 9 µM B, and 1 µM Mo, with an electrical conductivity (EC) of 1400 µS cm^{-1} and a pH of 5.8 ± 0.2. Control plants received only the standard nutrient solution whereas treated plants received the same solution enriched with Fe-HBED. A leaching fraction of ~25% was adopted, to reduce root zone salinization (Giuffrida et al. 2018).

Lettuce harvest was manually carried out on 25th January 2021, avoiding any damage to the leaves. Soon after harvest, plants were transported to the laboratory, characterized for physical variables, flash frozen with liquid nitrogen, and stored at -80 °C for further analysis. Overall, 72 lettuce heads were collected and analyzed (2 cultivars × 3 Fe concentrations × 3 replicates × 4 lettuces).

3.1.2.3 *Lettuce measurements*

In the laboratory, variables such as average fresh weight (FW) and dry matter content (DM) were measured. Average fresh weight was determined using an electronic gage (0.01 g accuracy). For the dry matter content, samples of lettuce leaves were dried at 70 °C in a laboratory oven (Thermo scientific-Herathermoven) with a forced air circulation until constant weight. For biochemical analyses, frozen material was grounded in an IKA A11 analytical mill (Staufen, Germany) using liquid nitrogen. For the mineral content, frozen samples were lyophilized in a Telstar Cryodos-80 freeze dryer (Terrassa, Barcelona, Spain) and grounded in a Taurus aromatic grinder (Oliana, Barcelona, Spain). All biochemical analyses were

performed using fresh frozen material, all mineral analyses were performed using lyophilized plant material. All biochemical analyses as well as the forms of nitrogen were measured through using a spectrophotometer Infinite 200 Nanoquant (Tecan, Switzerland).

3.1.2.4 *Biochemical analyses*

- Leaf chlorophylls and carotenoids concentration

The determination of photosynthetic pigments was performed according to Lichtenthaler and Wellburn (1983), with slight modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of methanol, vortexed, and centrifuged for 5 minutes at 5000 rpm. After that, the absorbance of the supernatant was measured at 3 different wavelengths: 666 nm, 653 nm, and 470 nm. The values obtained were applied in the following equations:

- $\text{Chl a} = 15.65 \times A_{666} - 7.34 \times A_{653}$
- $\text{Chl b} = 27.05 \times A653 - 11.21 \times A_{666}$
- $\text{C x+c} = (1000 \times A_{470} - 2.86 \times \text{Chl a} - 129.2 \times \text{Chl b}) \div 221$

where Chl a stands for Chlorophyll A, Chl b for Chlorophyll B and Cx+c for total carotenoids (including xanthophylls). The results are expressed in µg g^{-1} FW.

- Total phenols and flavonoids concentration

Total phenols and flavonoids concentration were determined according to Rivero et al. (2001), with minor modifications. For the extraction, 100 mg of macerated plant material were mixed with 500 µL of methanol, 500 µL of chloroform, and 250 µL of NaCl (1%), the material was vortexed and centrifuged for 10 minutes at 5000 rpm. For the total phenols, 90 µL of supernatant were mixed with 240 µL of Na_2CO_3 (5%) and 90 µL of Folin-Ciocâlteu reagent (50%). Samples were agitated and incubated at room temperature for 40 minutes. The absorption was measured at 725 nm. The results are expressed in µg caffeic acid (CA) g^{-1} FW. For total flavonoid concentration, 85 µL of

supernatant were mixed with 180 µL of distilled water and 26 µL $NaNO_2$ (5%). Samples were agitated and incubated at room temperature for 5 minutes. Finally, 26 µL of $AlCl_3$ (10%) and 170 µL of NaOH (1 M) were added to the mixture, and samples were incubated as previously. The absorption was measured at 415 nm. The results are expressed in µg rutin g^{-1} FW.

- Anthocyanins concentration

The concentration of anthocyanins was measured according to Giusti and Wrolstad (2001), with minor modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of methanol acidified with 1% HCl, agitated in a vortex, and centrifuged for 5 minutes at 5000 rpm. Then, 250 µL of supernatant were added to react with 1 mL of buffers potassium chloride, pH 1.0 (0.025 M) and sodium acetate, pH 4.5 (0.4 M). The absorption of both solutions was measured at 640 and 710 nm. The values obtained were applied in the following equation:

❖ $[(A640 - A710) - (A640 - A710)] \times 449.2 \div 26900$

The results are expressed as mg cyanidine-3-glucoside per g^{-1} FW.

- Ascorbic acid concentration

Total ascorbic acid (AsA), reduced AsA, and dehydroascorbate (DHA) concentration were determined according to Law et al. (1983), with slight modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of meta-phosphoric acid, agitated in a vortex, and centrifuged for 15 minutes at 13000 rpm. Then, 200 µL of supernatant were mixed with 500 µL of buffer sodium phosphate (150 mM; pH 7.5) and, only, for total ascorbic acid reaction 60 µL of dithiothreitol (DTT) (10 mM) were added. Samples were agitated and incubated at room temperature for 10 minutes. After, 60 µL of N-ethylmaleimide (0.5%), 240 µL of

trifluoroacetic acid, 240 µL of orthophosphoric acid (44%), 240 µL of bipyridyl (4%, in ethanol 70%) and 120 µL of $FeCl_3$ (3%). Finally, samples were incubated at 40 °C for 40 minutes. The absorption of both solutions was measured at 525 nm. The results are expressed in µg g^{-1} FW.

- Antioxidant capacity: FRAP and TEAC assays

The FRAP (ferric reducing antioxidant power) assay was determined according to Benzie and Strain (1999), with minor adaptations. The TEAC (Trolox equivalent antioxidant activity) assay was performed following Cai et al. (2004), with modifications. For both extractions, 100 mg of macerated plant material were mixed with 1 mL of methanol (100%), agitated in a vortex, and centrifuged for 2 minutes at 10200 rpm. Then, for the FRAP reaction, 10 µL of supernatant were mixed with 190 µL of FRAP reagent (acetate sodium, 0.25 M, pH 3.6; TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), 1 mM and $FeCl_3$, 20 mM). The absorption was measured at 593 nm. The results are expressed in µM $FeSO_4$ g^{-1} fresh weight (FW). For the TEAC reaction, 10 µL of supernatant were mixed with 190 µL of TEAC reagent (ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (7 mM) and potassium persulfate (2.45 mM)). The absorption was measured at 734 nm. The results are expressed in mg Trolox g^{-1} FW.

- Superoxide anion

The superoxide anion (O_2^-) detection was performed according to Kubiś (2008), based on the reduction of NBT, with slight modifications. For the extraction, 100 mg of macerated plant material were mixed with 300 µL of buffer potassium phosphate (50 mM, pH 7.8). Then, the material was gently agitated and centrifuged for 15 minutes at 10000 rpm. Subsequently, 250 µL of supernatant were mixed with 225 µL of buffer and 250 µL of hydroxylamine (10 mM). Samples were agitated and incubated for 20 minutes at room temperature. Subsequently, 180 µL of the extract were mixed with 460

µL of sulfanilic acid (17 mM) and 460 µL of 1-Naphthylamine (7 mM). The absorption was measured at 580 nm. The results are expressed in µg g^{-1} FW.

- Proline

The proline concentration was conducted following (Bieleski and Turner (1966), with some adaptations. For the extraction, 100 mg of macerated plant material were mixed with 1.2 mL of ethanol (83%), agitated in a vortex, and centrifuged for 10 minutes a 5500 rpm. Then, 1 mL of supernatant was added to 4 mL of Milli-Q water, 2.5 mL of ninhydrin (140 mM), and 2.5 mL of glacial acetic acid (100%). Samples were agitated and incubated for 45 minutes in a water bath at 100 °C. Subsequently, samples were cooled in ice and 5 mL of benzene (100%) were added and samples were incubated for 10 minutes at room temperature. The absorption of the organic phase was measured at 515 nm. The results are expressed in µg g^{-1} FW.

- MDA

The MDA (malondialdehyde) concentration was carried out according to Fu and Huang (2001), with minor modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of trichloroethanoic acid (TCA; 10%) and thiobarbituric acid (TBA; 0.25%). Samples were agitated and incubated for 30 minutes in a water bath at 95 °C. Subsequently, samples were cooled in ice and centrifuged at 9500 rpm for 10 minutes. The absorption of the organic phase was measured at 532 and 600 nm. The values obtained were applied in the following equation:

❖ $[(A532 – A600)] \div 155$

The results are expressed in µM g^{-1} FW.

- APX

The ascorbate peroxidase (APX) activity was determined

according to Rao et al. (1996), with slight modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of buffer potassium phosphate (100 mM, pH 7.5). Samples were gently agitated and centrifuged for 20 minutes at 12000 rpm. Subsequently, 40 µL of extract were mixed with 80 µL of buffer potassium phosphate, 40 µL of sodium ascorbate (0.5 mM), and 40 µL of H_2O_2 (0.2 mM). The absorption was measured at 290 nm every 30 seconds for 5 minutes. The results are expressed in Δ Abs mg protein^{-1} min^{-1} FW.

- GPX

The glutathione peroxidase (GPX) activity was measured following Elia et al. (2003), with minor modifications. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of buffer tris hydrochloride (100 mM), and added with EDTA (1 mM), and DTT (2 mM). Samples were gently agitated and centrifuged for 20 minutes at 15000 rpm. Subsequently, 30 µL of extract were mixed with 170 µL of buffer potassium phosphate (100 mM). The absorption was measured at 340 nm every 30 seconds for 5 minutes. The results are expressed in Δ Abs mg protein^{-1} min^{-1} FW.

- CAT

The catalase (CAT) activity was performed according to Nakano and Asada (1981), measuring the consumption of H_2O_2, with some adaptations. For the extraction, 100 mg of macerated plant material were mixed with 1 mL of buffer sodium phosphate (25 mM, pH 7). Samples were gently agitated and centrifuged for 20 minutes at 11500 rpm. Subsequently, 40 µL of extract were mixed with 40 µL of buffer HEPES (25 mM), 40 µL of EDTA (0.8 mM), and 80 µL of H_2O_2 (40 mM). The absorption was measured at 240 nm every 30 seconds for 5 minutes. The results are expressed in Δ Abs mg protein^{-1} min^{-1} FW.

3.1.2.5 *Mineral analyses*

Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), Sulphur (S), iron (Fe), manganese (Mn), zinc (Zn), boron (B), and copper (Cu) mineralization was conducted through wet digestion. For the digestion, 150 mg of lyophilized material were mixed with 5 mL of nitric acid (HNO_3) and placed in a sand bath at 100°C for one week, and drops of H_2O_2 at 33% were added daily. Subsequently, the extract was filtered with filter paper and a working solution of 20 mL was prepared with the addition of Milli-Q water. Mineral element concentrations were measured by ICP-OES (Perkin Elmer, Waltham, Massachusetts, USA), according to Martín Peinado et al. (2015). Each measurement was made with three replicates. For calibration, two sets of multi-element standards containing all the analytes of interest at five different levels of concentration were prepared using rhodium as the internal standard. All standards were prepared from ICP single-element standard solutions (Merck, Darmstadt, Germany), after dilution with 10% HNO_3. Internal standards included Sc (50 µg ml^{-1}) and Ir (5 µg ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEX Certi-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V, and Zn, in the range 0-100 µg l^1, and Ca, Mg, K, and Na in the range 0-100 mg. The analytical precision of the analyses was better than ±5% in all cases. The average recoveries ranged between 91% and 105% of the certified reference values. Macronutrients were calculated and expressed as mg 100 g^{-1} FW, while micronutrients as µg 100 g^{-1} FW.

- Forms of nitrogen

The contents of organic nitrogen (N) and ammonium (NH_4^+) were determined according to Krom (1980). For the organic N digestion, 150 mg of lyophilized material were mixed with 5 mL of sulfuric acid (H_2SO_4) and placed in a sand bath at 100 °C for three days, drops of H_2O_2 at 33% were added daily. Subsequently, the extract was filtered with filter paper and a working solution of 20 mL

was prepared with the addition of Milli-Q water.

For the NH_4 extraction, 10 mg of dry plant material were mixed with 1 mL of Milli-Q water. Then, 30 µL of supernatant of both extracts were added to 285 µL of reactive 1 (sodium salicylate, 0.5 M; sodium nitroprusside, 2 mM) and 285 µL of reactive 2 (NaOH, 1 M; sodium dichloroisocyanurate, 28 mM). After, samples were agitated and incubated at 37 °C for 45 minutes. The absorption was measured at 630 nm. Results are expressed in mg g^{-1} dry weight (DW).

The content of NO_3^- (nitrate) was measured according to Cataldo et al. (1975). For the extraction, 10 mg of dry plant material were mixed with 1 mL of Milli-Q water. Samples were agitated in an agitator for 120 minutes. Then, 12 µL of supernatant were added to 24 µL of salicylic acid diluted in H_2SO_4 (10%) plus 565 µL sodium hydroxide (NaOH; 2 N). Samples were agitated and the absorption of the solution was measured at 410 nm. The results are expressed in mg NO_3^- g^{-1} DW).

N total was estimated as the sum of organic N and nitrate. Mineral N was estimated as the sum of NH_4^+ and NO_3^-. N assimilated was assumed as organic N subtracted of NH_4. Results are expressed in mg g^{-1} DW.

3.1.2.6 Statistical procedures

Collected and calculated data were firstly subjected to a two-way analysis of variance (ANOVA), based on a factorial combination (cultivar × Fe concentration in the nutrient solution). Means comparisons were carried out using Fisher's protected least significant difference (LSD) test ($p \leq 0.05$). All statistical analyses were carried out using the Statgraphics Centurion XVI software (The Plains, Virginia, USA).

3.1.3 Results

3.1.3.1 *Lettuce main traits and bioactive compounds concentration*

When compared to untreated control, the Fe application reduced the total plant dry biomass (-18%, on the average of Fe1 and Fe2), but promoted lettuce DM content, total Chls content, and the Chl a/b ratio (by up to 16, 40 and 24%, respectively) (Table 3-1). Excepting the Chl a/b ratio, 'Romana' proved the highest values for all these variables. When compositional traits were concerned, total phenols, anthocyanins, flavonoids, and carotenoids contents peaked at Fe2, with 'Romana' showing the highest carotenoids content, together with the highest rise in anthocyanins and flavonoids content passing from Fe0 to Fe2 (+97 and +210%, respectively) (Table 3-1). Similarly, the Fe application progressively increased both AsA and DHA contents, with 'Romana' proving the sharpest rises passing from Fe0 to Fe2 (+60 and +62% for AsA and DHA, respectively) (Table 3-1). Regarding the antioxidant activity, the highest FRAP values were recorded in Fe2 and 'Romana', while for TEAC, a higher increase was recorded in 'Romana' compared to 'Nauplus' passing from Fe0 to Fe2 (+111%) (Table 3-1).

3.1.3.2 *Oxidative stress indicators*

The Fe supply gradually increased O_2^- concentration, with 'Romana' showing a higher increase passing from Fe0 to Fe2 (+40%) compared to 'Nauplus' (+26.5%) (Table 3-2). When compared to the untreated control, the Fe supplementation promoted proline concentration (by +24% and +61%, at Fe1 and Fe2, respectively) and increased MDA content and APX activity at Fe1 and Fe2, (by up to +47 and +53%, respectively). Meanwhile, when compared to the control, Fe1 and Fe2 plants showed a reduction in the activity of GPX (by -9 and -13%, respectively), and a gradual reduction in the activity of CAT (by +9 and -18%, respectively). Among the tested genotypes, 'Nauplus' proved the highest values of APX and CAT activity, whereas the highest proline content was recorded in 'Romana' (Table

3-2).

3.1.3.3 *Macronutrients and micronutrients content*

Compared to control, the Fe supply generated a progressive increase in N, P, K, and S concentrations of lettuce (by up to 13, 30, 29, and 45% in Fe2), while Mg concentration peaked at Fe1 (+62%) (Table 3-3). Regarding Ca, the response to Fe supply proved to be genotype-dependent, as in 'Nauplus' its concentration increased passing from Fe1 to Fe2 (by 44%), whereas in 'Romana' it raised within the Fe0-Fe1 range (+44%) and declined thereafter (-33%) (Table 3-3). When the genotype per sé was concerned, 'Romana' showed higher concentrations of P, K, Mg and S than 'Nauplus' (Table 3-3).

Regarding the micronutrients content, the supplemental Fe fertilization boosted the accumulation of Fe, Mn, Zn, and B, though in a genotype-dependent way (Table 3-3). Indeed, when compared to the untreated control, 'Romana' showed the highest Fe increase within the Fe1-Fe2 range (+209%, on average), but the Mn, Zn, and B differences were higher at Fe1 (+124, +117 and +96%, respectively), while in 'Nauplus' all these micronutrients were maximized under the Fe2 supply (ranging from +173 to +69% in Fe and B, respectively) (Table 3-3). No differences were found in Cu concentrations.

The amount of Fe accumulated in the dry leaves of 'Romana' ranged from 522 to 520 mg kg^{-1}, at Fe1 and Fe2, respectively. Meanwhile, lower values were observed in 'Nauplus' plants, which varied from 315 to 335 mg kg^{-1} DW, at Fe1 and Fe2, respectively (Figure 3-1).

3.1.3.4 *Nitrogen forms in lettuce leaves*

The analysis of variance revealed that the supplemental Fe application promoted the concentration of NH_4^+ (+40 and +21%, in Fe1 and Fe2, respectively), whereas it decreased the concentration of NO_3^- (-20 and -14%, in Fe1 and Fe2, respectively). When compared to

the control plants, the mineral N content was reduced in Fe1 plants (-15%), while the variable assimilated N was reduced in both Fe1 and Fe2 (-24 and -22%, respectively) (Table 3-4). Regarding the genotype effect, the cultivar Nauplus revealed the highest concentrations of organic N, NO_3^-, total N, mineral N and assimilated N (Table 3-4).

Table 3-1. Lettuce main traits and bioactive compound concentrations as affected by the studied factors.

	Plant Biomass (g DW plant^{-1})	Dry Matter (%)	Total Chls (mg g^{-1} FW)	Chl *a*/*b* Ratio	Total Phenols (µg g^{-1} FW)	Anthocyanins (mg g^{-1} FW)	Flavonoids (µg g^{-1} FW)	Carotenoids (µg g^{-1} FW)	AsA (µg g^{-1} FW)	DHA (µg g^{-1} FW)	FRAP (µM $FeSO_4$ g^{-1} FW)	TEAC (mg trolox g^{-1} FW)
Fe concentration												
Fe0	20.0 a	4.47 b	2.33 c	1.53 c	535 c	1.50 c	542 c	153 c	100 c	61.9 c	6.19 c	0.637 c
Fe1	16.5 b	5.03 a	2.64 b	1.70 b	781 b	2.05 b	901 b	220 b	126 b	79.4 b	9.17 b	0.881 b
Fe2	16.2 b	5.17 a	3.26 a	1.89 a	926 a	2.42 a	1134 a	304 a	143 a	95.9 a	12.3 a	1.173 a
Cultivar												
'Nauplus'	14.6 b	4.44 b	2.67 b	1.69	727	2.11 a	881	203 b	116	70.0 b	8.56 b	0.840 b
'Romana'	20.5 a	5.34 a	2.82 a	1.72	767	1.87 b	838	249 a	130	88.2 a	9.86 a	0.954 a
Fe x Cv												
Fe0 'Nauplus'	15.1	4.09	2.21	1.52	559	1.77 c	686 d	134	97 d	51.2 c	6.07	0.65 d
Fe1 'Nauplus'	15	4.36	2.85	1.76	766	2.11 b	924 bc	234	131 b	83.2 b	9.35	0.84 c
Fe2 'Nauplus'	13.8	4.89	2.96	1.80	856	2.44 a	1032 b	242	121 bc	75.5 b	10.28	1.03 b
Fe0 'Romana'	24.8	4.85	2.46	1.54	511	1.22 d	399 e	173	104 cd	72.7 b	9.00	0.62 d
Fe1 'Romana'	18.0	5.71	2.43	1.65	796	2.00 bc	879 c	207	120 bc	75.7 b	10.28	0.92 bc
Fe2 'Romana'	18.7	5.45	3.56	1.97	995	2.40 a	1236 a	367	166 a	116.4 a	14.28	1.32 a
Significance												
Fe concentration	*	*	***	***	***	**	***	***	**	**	***	***
Cultivar	**	**	**	NS	NS	**	NS	***	NS	**	**	*
Fe x Cv	NS	NS	NS	NS	NS	*	***	NS	*	**	NS	*

Different letters within each column's factor indicate significance at Fisher's protected LSD test (p = 0.05). NS: not significant; *, ** and ***: significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

Table 3-2. Oxidative stress indicators and enzymes activity in lettuce, as affected by the studied factors.

	O_2^- (µg g^{-1} FW)	Proline (µg g^{-1} FW)	MDA (µM g^{-1} FW)	APX (Δ Abs mg $protein^{-1}$ min^{-1} FW)	GPX (Δ Abs mg $protein^{-1}$ min^{-1} FW)	CAT (Δ Abs mg $protein^{-1}$ min^{-1} FW)
Fe concentration						
Fe0	6.91 c	15.3 b	2.92 b	0.055 b	0.171 a	0.011 a
Fe1	8.09 b	18.9 ab	4.16 a	0.084 a	0.149 b	0.010 b
Fe2	9.21 a	24.7 a	4.28 a	0.086 a	0.155 b	0.009 c
Cultivar						
‘Nauplus’	7.86	15.6 b	3.61	0.084 a	0.153	0.011 a
‘Romana’	8.28	23.7 a	3.97	0.066 b	0.163	0.008 b
Fe x Cv						
Fe0 ‘Nauplus’	6.61 d	15.7	2.81	0.064	0.163	0.013
Fe1 ‘Nauplus’	8.61 b	15.2	4.27	0.097	0.146	0.011
Fe2 ‘Nauplus’	8.36 bc	16.0	3.74	0.090	0.150	0.010
Fe0 ‘Romana’	7.21 cd	14.8	3.03	0.046	0.179	0.009
Fe1 ‘Romana’	7.58 bcd	22.7	4.05	0.071	0.151	0.008
Fe2 ‘Romana’	10.06 a	33.5	4.82	0.082	0.160	0.007
Significance						
Fe concentration	**	*	**	*	*	**
Cultivar	NS	*	NS	*	NS	***
Fe x Cv	*	NS	NS	NS	NS	NS

Different letters within each column’s factor indicate significance at Fisher’s protected LSD test ($p = 0.05$). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

Table 3-3. Macronutrients and micronutrients composition of lettuce affected by the studied factors.

	Macronutrients						Micronutrients				
	N	**P**	**K**	**Ca**	**Mg**	**S**	**Fe**	**Mn**	**Zn**	**B**	**Cu**
	(mg g^{-1} FW)						**(µg g^{-1} FW)**				
Fe concentration											
Fe0	4.41 b	3.88 c	2.90 c	0.330 c	0.143 c	0.107 c	7.7 b	3.49 c	3.16 c	1.21 c	0.689
Fe1	4.68 ab	4.60 b	3.07 b	0.385 a	0.231 a	0.135 b	21.8 a	6.28 a	5.74 b	1.93 a	0.607
Fe2	4.99 a	5.06 a	3.75 a	0.371 b	0.215 b	0.155 a	22.4 a	5.64 b	6.04 a	1.80 b	0.695
Cultivar											
‘Nauplus’	4.84 a	3.96 b	3.07 b	0.347 b	0.166 b	0.123 b	12.0 b	4.33 b	4.32 b	1.51 b	0.603
‘Romana’	4.54 b	5.06 a	3.40 a	0.377 a	0.227 a	0.141 a	22.5 a	5.94 a	5.64 a	1.78 a	0.725
Fe x Cv											
Fe0 ‘Nauplus’	4.59	3.75	3.00	0.327 c	0.132	0.109	6.0 d	3.20 d	3.00 d	1.16 d	0.602
Fe1 ‘Nauplus’	4.23	3.64	2.45	0.292 c	0.162	0.117	13.7 b	4.09 c	4.25 c	1.41 cd	0.775
Fe2 ‘Nauplus’	5.70	4.51	3.77	0.421 b	0.204	0.144	16.4 b	5.69 b	5.71 b	1.96 b	0.555
Fe0 ‘Romana’	4.22	4.02	2.80	0.332 c	0.154	0.104	9.4 c	3.78 cd	3.33 cd	1.25 d	0.660
Fe1 ‘Romana’	5.12	5.56	3.69	0.478 a	0.300	0.153	29.8 a	8.46 a	7.23 a	2.45 a	0.651
Fe2 ‘Romana’	4.28	5.62	3.72	0.321 c	0.226	0.166	28.3 a	5.59 b	6.36 ab	1.64 c	0.740
Significance											
Fe concentration	*	***	***	***	***	**	***	***	***	***	NS
Cultivar	*	***	**	*	***	**	***	***	**	**	NS
Fe x Cv	NS	NS	NS	***	NS	NS	***	***	**	***	NS

Different letters within each column’s factor indicate significance at Fisher’s protected LSD test ($p = 0.05$). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

Table 3-4. Forms of N of lettuce as affected by the studied factors.

	Organic N (mg g^{-1} DW)	NH_4^+ (mg g^{-1} DW)	NO_3^- (mg g^{-1} FW)	Total N (mg g^{-1} DW)	Mineral N (mg g^{-1} DW)	Assimilated N (mg g^{-1} DW)
Fe concentration						
Fe0	33.4	6.28 c	1509 a	99.6	72.5 a	27.1 b
Fe1	40.6	8.79 a	1119 b	93.4	61.6 b	31.8 a
Fe2	40.7	7.62 b	1067 b	97.6	64.5 ab	33.1 a
Cultivar						
'Nauplus'	42.2 a	7.48	1503 a	108.7 a	73.9 a	34.7 a
'Romana'	34.3 b	7.65	961 b	85.1 b	58.5 b	26.7 b
Fe x Cv						
Fe0 'Nauplus'	37.1	7.00	1839	112.3	82.2	30.1
Fe1 'Nauplus'	44.5	9.13	1461	97.1	61.8	35.4
Fe2 'Nauplus'	45.2	6.31	1208	116.6	77.7	38.9
Fe0 'Romana'	29.8	5.55	1178	87.0	62.7	24.3
Fe1 'Romana'	36.8	8.46	927	89.7	61.4	28.3
Fe2 'Romana'	36.2	8.94	777	78.6	51.3	27.3
Significance						
Fe concentration	NS	**	*	NS	*	*
Cultivar	**	NS	***	***	***	**
Fe x Cv	NS	NS	NS	NS	NS	NS

Different letters within each column's factor indicate significance at Fisher's protected LSD test (p = 0.05). NS: not significant; *, ** and ***: significant at $p \leq$ 0.05, 0.01 and 0.001, respectively.

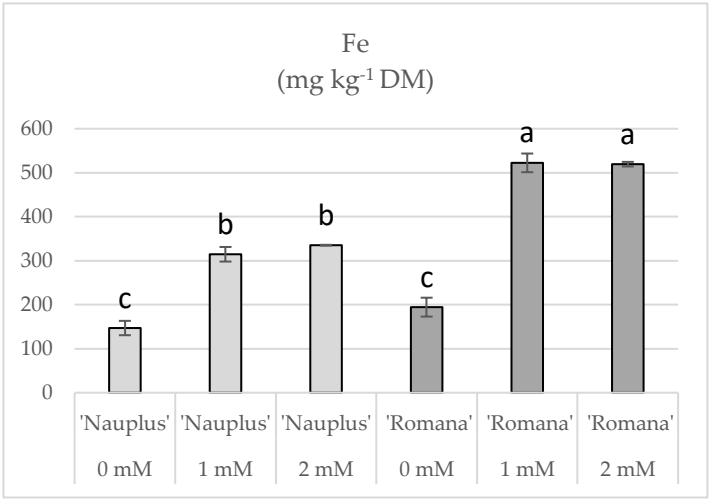

Figure 3-1. Fe content in the leaves of lettuce affected by the studied factors.

3.1.4 Discussion

The plant biomass reduction and the DM content increase observed in the plants of our study were also reported by Giordano et al. (2019), when submitting green and red Salanova cultivars (*Lactuca sativa* L. var. *capitata*) to 1 and 2 mM of Fe-EDDHA in the nutrient solution. The limitation in the growth parameters observed in this and other studies (Broschat and Moore 2004; Cecílio Filho et al. 2015), dealing with Fe supplementation, support the fact that, despite being essential to the plant, Fe excess produce phytotoxic effects (Buturi et al. 2021). Moreover, 'Romana' showed a higher plant biomass and a higher DM content, when compared to 'Nauplus'; this can be attributed to the plant's genetic diversity (Casey Barickman et al. 2018; Hernandez et al. 2020), in fact, the difference of the dry matter between the typologies used in this study is confirmed by Serio and

Elia (2001).

Flavonoids are a group of healthy phenolic compounds found in lettuce plants (Brazaitytė et al. 2022). In our work, the increased concentration of flavonoids in Fe1 and Fe2 plants can be attributed to the plant's defense mechanism, since this antioxidant plays a key role in protecting plants against ROS-related damage and in alleviating oxidative stress caused by Fe excess (Potapovich and Kostyuk 2003; Kejík et al. 2021). This protection ability is a result of the strong chelating properties of flavonoids, capable of forming highly-affinity complexes with transition metals, such as Fe (Kejík et al. 2021). In addition, this antioxidant compound has received considerable attention for its wide spectrum of pharmacological properties. The use of flavonoids has been linked to the prevention of cancer, cardiovascular diseases, gastric and intestine problems, vascular fragility, and infections (Yao et al. 2004). The fact that our lettuce contains such high concentrations of flavonoids contributes to its healthy characteristics.

An important subgroup of flavonoids are anthocyanins, a pigment family responsible for the red color found in some lettuce types (Assefa et al. 2021). In our study, we observed a gradual increase in the anthocyanin content compatible to the increment described by Giordano et al. (2019), when submitting lettuce plants to 2 mM of Fe. The increased concentration of anthocyanins in the presence of Fe could be, also, due its metal chelating properties, as demonstrated by Sigurdson et al. (2017). In addition, Giordano et al. (2019) described differences in anthocyanin concentration among cultivars, being the higher values observed in the red-pigmented cultivar. Similarly, in our study, this parameter was higher in the cultivar Nauplus, as expected, since this is also a red-pigmented cultivar. The same authors highlighted a progressive increase in the profile of other important antioxidants, such as carotenoids. Similarly, we observed a progressive increase in the carotenoid content in Fe1 and Fe2 plants,

probably linked to the high ROS scavenging ability of this antioxidant (Shen et al. 2018).

Fe-biofortification studies can also benefit of the presence of Fe absorption promotors. It is well known that ascorbic acid is the most efficient enhancer of Fe absorption, overcoming the effects of all possible dietary Fe absorption inhibitors (Abbaspour et al. 2014; Ems et al. 2021). Lettuce plants from our study showed a progressive increase in the ascorbic acid content. Ascorbic acid is also a key antioxidant, which have probably been promoted as a protection against Fe excess (Przybysz et al. 2016). Comparing the cultivars, 'Romana' AsA concentration peaked at 2 mM, indicating a more intense stress response. Moreover, the content of the oxidized form of ascorbate (DHA) in our study, followed the same path as AsA (Table 3-1). This oxidized form of vitamin C can be effectively reconverted to AsA in the human body and it is the most common vitamin C form in supplements and cosmetics (Wilson 2002).

Based on our assays, lettuce plants supplied with Fe1 and Fe2 showed a significantly higher antioxidant capacity when compared to control plants (Table 3-1). This increase can be the result of the metal stress caused by the high accumulation of Fe within the plant organs. Similar increases in the antioxidant power of lettuce were observed by Jibril et al. (2017) when plants were subjected to Cd stress.

The increase in the content of all above bioactive compounds suggests, that by enhancing Fe concentration in the nutrient solution, at 1 and 2 mM, we create a condition of metal stress in the lettuce, which produce reactive oxygen species (ROS) (Zahra et al. 2021). In turn, plants increase the production of non-enzymatic antioxidants such as AsA, phenols, flavonoids, carotenoids, whose main role is to scavenge or control ROS generation (Hasanuzzaman et al. 2020).

From a human nutrition perspective, this mechanism favors the production of health promoting substances, making Fe biofortification a simple strategy to produce a healthier lettuce and attend an important

consumer's demand.

As suggested by the biomass reduction, the application of Fe produced a stress response in lettuce plants. This fact is confirmed by the increase of stress indicator parameters such as ROS (O_2^-), lipid peroxidation indicators (MDA), or osmoprotector compounds (proline). Several studies support our results with an increase of these variables in plants subjected to Fe toxicity (Zahra et al. 2021; Szerement et al. 2022). The values obtained for these indicators were higher in 'Romana', highlighting higher proline values, which is consistent with a higher stress response and a greater biomass loss.

Furthermore, plants possess mechanisms to cope with stresses such as those caused by Fe excess. For instance, enzymatic activities such as APX and CAT and antioxidant compounds such as AsA that are key for ROS detoxification are enhanced (Tavanti et al. 2021; Szerement et al. 2022). Thus, several studies observed that adequate Fe fertilization promotes these antioxidant systems because Fe is an enzyme cofactor acting as catalyst for electron transfer reactions necessary for proper antioxidant functioning (Sida-Arreola et al. 2015; Tavanti et al. 2021). Likewise, in our study, a clear increase in antioxidant capacity (antioxidant tests), APX activity, and AsA was observed in biofortified lettuce plants, although no clear response of CAT and GPX enzymes activities were observed. Comparing between the two varieties, the higher activity of APX and CAT enzymes of the 'Nauplus' cultivar could favor ROS detoxification and would support its higher tolerance to Fe and lower biomass loss.

The biofortification treatments progressively stimulated the accumulation of other minerals such as total N, P, K and S. A similar increase was described by Giordano et al. (2019). The authors noticed that lettuce plants submitted to 2 mM of Fe, showed a higher N (in the form of nitrate) and P content. In contrast, the same authors noticed a progressive decrease in Ca and Mg contents, when the Fe concentration in the nutritive solution was enhanced. In our study, as

for the Ca and Mg content, the two cultivars showed different responses when submitted to the different Fe doses. 'Nauplus' presented the higher Ca and Mg concentrations at 1 mM Fe while 'Romana' showed the higher increase at 2 mM Fe. Since this is a Fe-biofortification study, optimizing Fe absorption is a priority, in view of that the Fe doses that do not cause an increase in the Ca content are preferable, because Ca is an inhibitor of Fe absorption (Abioye et al. 2021). When comparing both cultivars, the macronutrient contents (P, K, Ca, Mg and S) were significantly higher in the cultivar Romana, when compared to 'Nauplus'. These results could be explained by the higher DM content accumulated in the former genotype.

The increase in micronutrient content (Mn, Zn, B) observed in this study is consistent with the promotion of Mn and Zn in the leaves of African marigolds (*Tagetes erecta*) and zonal geraniums (*Pelargonium x hortorum*) subjected to high levels of Fe in the nutrient solution (1, 2, 4 and 6 mM) (Broschat and Moore 2004). The genotype responses in our study suggest that for 'Romana', the optimal concentration of Fe in the nutrient solution should not exceed 1 mM, since this concentration allowed to maximize the mineral composition of leaves, mostly in terms of Ca, Mn, and B.

Regarding Fe accumulation, both additional doses of the mineral were able to produce Fe-biofortified lettuce. 'Romana' showed the highest Fe accumulation capacity, when compared to 'Nauplus'. This variability in Fe accumulation among cultivars of lettuce is common (Borghesi et al. 2013; Sularz et al. 2020). In fact, our results are in accordance with Giordano et al. (2019), as they also highlight, in their Fe-biofortification study, a significant difference in the ability to accumulate Fe among the studied cultivars, being 'Red-Salanova' the one with the highest Fe content.

The highest amount of Fe accumulated in the leaves of 'Romana' could also explain its higher decrease in plant biomass and the higher antioxidant accumulation, when compared to 'Nauplus'.

This is supported by the fact that, concentrations above 500 mg kg^{-1} DW are reported as phytotoxic to the plant (Marschner 2011). In fact, 'Romana' exceeded the Fe phytotoxicity limits in the tissues, in Fe1 and Fe2, meanwhile 'Nauplus' did not reach a phytotoxic range, in neither of the treatments. This hypothesis is also supported by the highest proline increase observed in 'Romana', confirming the extreme stress condition of this genotype.

From a nutritional point of view, 100 g of fresh biofortified lettuce (under 1 mM of Fe) can provide 0.94 mg and 2.98 mg of Fe, 'Nauplus' and 'Romana', respectively. These values are comparable to the amount of Fe present in 100 g of prime beef (2.11 mg) and superior to pork loin and chicken breasts (0.68 and 0.62, respectively) (Pretorius et al. 2016). Leaving aside considerations about the bioaccessibility of the element, these data support the hypothesis that Fe-biofortified lettuce can significantly contribute to increase the Fe concentration in the diet, facilitating Fe intake by humans and helping to fight the hidden hunger crisis.

N is a key element in plant growth and plays an important role in plant metabolism. The increase in the organic and assimilated N showed by the plants treated with 1 and 2 mM of Fe, is consistent with the increase in DM, as also observed by Giordano et al. (2019).

In the context of human health, NO_3^- excess is a threat and its consumption should be minimized, because when it encounters the saliva and the bacteria in the gastrointestinal NO_3^- is partially converted to nitrite. Nitrite is associated to diseases such as infantile methemoglobinemia and carcinogenesis (Santamaria 2006). Efforts to reduce NO_3^- can involve different fertilization practices, as the use of organic fertilizers (Jokinen et al. 2022). The European Commission (Anon 2011) has set the maximum nitrate content allowed for the commercialization of fresh lettuce (grown in winter, under cover) as 5000 mg kg^{-1} FW. Both cultivars in this study presented NO_3^-levels within the limit, showing 'Romana' cultivar the lowest NO_3^- content.

In addition, the treated plants (Fe1 and Fe2) showed a reduction in NO_3^-concentration, suggesting that Fe supplementation increases the quality of lettuce, by improving an important food safety parameter. A similar effect was verified when the concentration of another metal mineral (Zn) was increased in the nutrient solution of lettuce, as Barrameda-Medina et al. (2017) found a decrease in the NO_3^- presence.

3.1.5 Conclusion

Our findings indicate that Fe-biofortification of greenhouse-grown soilless lettuce is an effective tool to promote the dietary intake of Fe. We demonstrated that adding 1 mM of Fe (as Fe-HBED) in the nutrient solution not only increased the Fe content in leaves, but also stimulated the plant to produce and accumulate higher concentrations of health promoting compounds, thus adding a possible market value to the product. Regarding the studied genotypes, 'Romana' showed higher concentrations of dry matter, Fe, minerals (N, P, K, Mn and Zn), and a higher antioxidant power. However, high doses of Fe induced plants to stress and from an agronomic perspective the genotype Nauplus proved a higher tolerance to Fe exposure, showing the lowest biomass loss. Moreover, biofortification in soilless systems, through the management of the nutrient solution proved to be simple and effective and should be further investigated. In this sense, studies aiming to mitigate the effects of metal stress on plants and the use of different molecules and concentrations are recommended to optimize the efficiency of lettuce biofortification.

4 Experimental activities on carrot

4.1 Foliar application of zinc and iron effectively achieved carrots biofortification: chelated forms of the minerals are more bioaccessible than corresponding sulfate salts

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4.1.1 Introduction

Zinc (Zn) and iron (Fe) are at the top of the mineral's deficiency in human diet and among the main determinants of the so called "hidden hunger" (Wada, 2004). It affects broad population groups in many countries, including those economically developed (Olivares et al. 1999; Beleggia et al. 2018). Zinc is essential for many biochemical and immunological functions, as it is involved in the activity of more than 100 enzymes, besides playing a key role in the synthesis of nucleic acids and proteins (Roohani et al. 2013). The main function of Fe is related to the synthesis of hemoglobin and myoglobin, so that it is essential in the transfer of oxygen from the lungs to tissues. In addition, there are many Fe-dependent enzymes making this mineral essential to many metabolic processes (Abbaspour et al. 2014). The recommended daily allowance (RDA) of Zn ranges between 9 and 14 mg day^{-1} and the UL (tolerable upper intake level) for adults is 40 mg day^{-1}. The RDA of Fe ranges between 8 and 18 mg day^{-1}, whereas the UL for adults is 45 mg day^{-1} (Trumbo et al. 2001). Wrong dietary patterns or scarce availability of adequate foods, could make difficult to reach these RDA values, causing malnutrition problems (Shridhar

et al. 2015), in particular when regarding micronutrients as is the case of Fe and Zn (Buturi et al. 2021).

Vegetables are consumed worldwide and are good natural sources of minerals, therefore they could be a good vehicle to increase the intake of these elements in the human diet, by implementing targeted biofortification strategies (Buturi et al. 2022). In this view, biofortification of vegetables consists in improving the mineral status of plant tissues (Ierna et al. 2020a). The success of this strategy depends on the market acceptance and consumption of the improved food products. Thus, it is important to choose vegetables commonly present in the human diet. This is the case of carrot (*Daucus carota* L.), one of the most popular vegetables worldwide, which is cultivated on a surface area of approximately 1.13 million hectares and with a production of almost 41 million tons (FAO 2021). The product is represented by the taproot of the plant, which is a versatile product that can be consumed fresh or processed in different ways, alone or as part of many recipes (Ierna et al. 2020b).

The biofortification strategy is based on increasing the concentration of essential mineral elements through the application of specific fertilizers on roots or leaves (White and Broadley 2009). Foliar sprays are commonly used in fertilization and are known for being more targeted than the soil application. Indeed, foliar sprays could be effective on counteracting the low availability of minerals on soil caused by pH anomalies, besides being a simpler, more effective and convenient method, with often faster plant responses to the elements (Fageria et al. 2009; Smoleń et al. 2014; Lawson et al. 2015). Once the nutrient solution is applied directly on leaves, micronutrients can penetrate the cuticle or enter directly through the stomata (Marschner 2011). However, biofortification efficiency varies depending on the chemical form of the fertilizers. Iron and Zn fertilizations are usually based on sulphate or chelated forms and there are controversial debates around which one is more efficient

(Fernández and Ebert 2005). In general, biofortification programs carried out through foliar application can benefit by the enhanced phloematic mobility of the minerals, because of the presence of chelating substances such as sugar or other organic metabolites, which facilitate the translocation from leaf to growing sinks as roots, fruits and grains (Gupta et al. 2016).

Iron biofortification studies shows successful cases of foliar fertilization conducted using FeII ($FeSO_4$), as is the case of tomato and sweet potatoes (Carrasco-Gil et al. 2016; Sun et al. 2019). At the same time, similar studies were performed using FeIII in chelated forms, such as Fe-diethylene-triamine pentaacetic acid (DTPA), Fe-ethylenediamine tetraacetic acid (EDTA) or Fe-ethylenediamine-N,N′-bis(2-hydroxyphenylacetic acid) (EDDHA) (Sida-Arreola et al. 2015; Kromann et al. 2017). Some authors suggest that $FeSO_4$ is the only foliar fertilizer worth it (Rengel et al. 1999), others indicate that chelated forms favor translocation and contribute to improve crop yield and activate antioxidant enzymes (Fernández and Ebert 2005; Sida-Arreola et al. 2015).

Zinc biofortification studies are limited too, and Zn sulphate ($ZnSO_4$) seems to be the most applied inorganic source (Di Gioia et al. 2019). Some studies include the use of Zn nitrate (White et al. 2012; White et al., 2018), while some compare different organic Zn complexes (Almendros et al. 2015). Gupta et al. (2016) suggested that the most effective agronomic fertilizer is Zn-EDTA, but they highlighted the high cost of the molecule.

Looking at this contradictious scenario, the present study compared the efficacy of foliar applications of chelated vs. sulfate forms of Zn or Fe in the biofortification of carrots. In addition, the present study aimed to investigate the amount of Fe and Zn actually released from the food matrix during digestion, so becoming bioaccessible for the absorption through the human intestine.

4.1.2 Materials and methods

4.1.2.1 Experimental site, plant material and crop management

A field trial was carried out during the 2019–2020 growing season at a commercial farm located at Ispica plain (Southeastern Sicily: 36°31'07.2"N 15°04'41.5"E, 42 m a.s.l.), one of the most typical areas for early carrot cultivation in Italy. The climate is semi-arid Mediterranean, with mild winters and hot, dry summers. Frost occurrence is virtually absent in winter. During the experiment (from December 15 to May 6) mean monthly maximum and minimum temperatures progressively decreased from December (16.8 and 14.7 °C, respectively) to January (15.1 and 9.1 °C), then increased up to May (22.0 and 15.2 °C).

The soil is a moderately deep, calcic brown on the basis of the USDA Soil Taxonomy Classification (1999), with a sandy-loam texture, which, at the beginning of the experiment, comprised low N content (0.8 g kg^{-1}) and low organic matter (12.2 g kg^{-1}), P_2O_5 available (57 mg kg^{-1}), K_2O exchangeable (302 mg kg^{-1}), pH 7.4. All soil analyses were carried out according to the procedures approved by the Italian Society of Soil Science (Violante 2000).

The experiment was arranged in a randomized blocks design with three replications, including foliar sprays of Fe and Zn either in an organic or chelated form (see below). The cultivar Dordogne was utilized, a hybrid of the Nantes-type, which is well-adapted to the Mediterranean growing conditions, and it is usually adopted for the production of early carrots.

Seeds were sown at a ≈1 cm depth, through a precision seeder operating in twin rows (0.20 × 0.30 m) on an 0.80 m wide ridges; soon after seeding, the ridges were rolled uniformly. Actual density was 70 plants m^{-2}. Plot size was 3.6 m × 3.6 m, and consisted of 3 ridges, 3.6 m long. Tillage consisted in a preparatory work deep ploughing (~40

cm) and ridges setting with a bed-maker for the formation of raised ridges, ≈2 weeks before sowing. One week before sowing, 70 kg ha^{-1} of P_2O_5 (as mineral superphosphate), 150 kg ha^{-1} of K_2O (as K sulfate) and 60 kg ha^{-1} of N (as ammonium nitrate) were applied. Other 60 kg ha^{-1} N were applied on early March. The crop coefficient of carrot adopted was 1.09 (da Silva et al. 2018). Crop water requirements, starting from early spring, were satisfied by rain irrigation, supplying 100% of crop maximum evapotranspiration, when the accumulated daily evaporation, estimated through the Penman–Monteith equation, reached 25 mm. Over the crop cycle, 170 mm of irrigation water were applied. Weeds and pests' control were performed by applying metribuzin and pirimicarb when needed.

4.1.2.2 *Biofortification treatments*

The biofortification protocols were implemented by leaf spraying aqueous solutions enriched with Fe or Zn, either in the form of inorganic salt ($FeSO_4$ and $ZnSO_4$) or chelated forms (Fe-DTPA (diethylenetriaminepentaacetic acid), and Zn-EDTA (ethylenediaminetetraacetic acid)), at a concentration of 6 mM of these elements.

In total, four applications were effected: the first one was performed on March 4, at the plant stage of full vegetative growth (~30 cm height), while the remaining leaf applications were performed weekly. Leaf sprays were done using a hand pump pressure sprayer. For every treatment, the volume used was 0.25 L m^{-2}. The sprayed solutions contained the non-ionic surfactant Vector® (1 mL L^{-1}; Chimiberg, Caravaggio, BG, Italy) to improve spreading and sticking properties.

4.1.2.3 *Root physical variables*

Roots harvest was manually carried out on May, 6 avoiding any damage to leaves. Within each experimental unit, harvested carrots were selected for uniform size and absence of defects, then arranged by hand in 20 bunches each containing 10 roots. Within 4 h from harvest, all bunched carrots were brought to the laboratory, washed to remove soil particles and dried with paper towels.

In the laboratory, variables such as root average fresh weight (FW), root length, root diameter and root dry matter (DM) (%) were determined. Root average fresh weight was determined by means of an electronic gage (0.01 g accuracy). For the dry matter calculation and mineral content, samples of carrot roots were dried at 70 °C in a laboratory oven (Thermo Scientific-Herathermoven, Waltham, Massachusetts, US), with a forced air circulation until constant weight was reached. After dry weight registration, samples were grounded in a mill and stored at -80 °C for further analyses.

4.1.2.4 *Root chromatic variables*

The external root chromatic coordinates were determined on 3 fresh carrots for each replicate. According to McGuire (1992), measurements were effected on 2 points per root (≈1 cm below the plant collar) through a tristimulus Minolta Chroma meter (model CR-200, Minolta Corp.) calibrated with a standard white tile (UE certificated) with illuminant D65/10◦, measuring color in terms of lightness (L*), green-red axis (a*) and blue-yellow axis (b*). Root color was described as L*, a*, b* and Chroma [as $(a^2 + b^2)^{½}$].

4.1.2.5 *Root compositional variables*

The determination of roots composition included total carotenoids, total phenols and antioxidant assays. All these determinations were effected on lyophilized plant powder by using a

Jeanway UV/Visible spectrophotometer (Stone, Staffordshire, UK).

- *Total carotenoids*

Total carotenoids were determined according to Lichtenthaler and Wellburn (1983), with slight modifications. For the extraction, 50 mg of lyophilized carrot powder were mixed with 5 mL of ethanol 96% and vortexed, then the tubes were placed in the ultrasonic bath for 10 minutes and left overnight in the dark (at 10 °C). After that, tubes were centrifuged for 10 minutes, and samples were read in 1.5 mL plastic cuvette. Ethanol 96% was used as blank. Readings were done in the following wavelengths: 470, 649 and 665 nm and the absorbance values were applied in the following equations:

- $C_a = 13.95\ A_{665} - 6.88\ A_{649}$
- $C_b = 24.96\ A_{649} - 7.32\ A_{665}$
- $C_{x+c} = (1000\ A_{470} - 2.05\ Ca - 114.8\ Cb) / 245$

where C_a stands for chlorophyll A, C_b stands for chlorophyll B and C_{x+c} stands for total amount of carotenoids [xanthophyll (x) plus carotenes (c)]. Results are expressed in µg 100 g^{-1} fresh weight (FW).

- *Total phenolic content*

Total phenolic content (TPC) was quantified using a modified Folin-Ciocâlteu method (Cicco et al., 2009). For the extraction, 100 mg of lyophilized carrot powder were mixed with 1 mL of 70% methanol and agitated for 1 hour at room temperature, then samples were centrifuged at 5000 g for 5 minutes at 25 °C. 100 µL of extract solution were mixed with 100 µL Folin-Ciocâlteu reagent and allowed to react at room temperature for 2 minutes. Next, 800 µL of Na_2CO_3 (5% w/v) were added and tubes were left in a temperature bath at 40 °C for 20 minutes. Samples were read at 760 nm and TPC was reported

as µmol gallic acid equivalents (GAE) 100 g^{-1} FW.

- *DPPH assay*

The DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenging activity of carrot extracts was determined according to Brand-Williams et al. (1995). For the extraction, 100 mg of lyophilized carrot powder were mixed with 5 mL of methanol (80%) and vortexed for 1 minute. Samples were then submitted to 10 minutes of ultrasonic bath (below 10 °C) and centrifuged for 15 minutes at 4000 g (6 °C). For the reaction, 150 µL of supernatant was mixed to 1350 µL of DPPH solution (150 µM) vortexed and placed in the dark for 30 minutes. The decrease in the absorbance of methanolic solution of DPPH was read at 515 nm and DPPH was calculated from a standard curve prepared by plotting change in absorbance against different concentrations of Trolox and expressed as µmol Trolox equivalent (TE) 100 g^{-1} FW.

- *FRAP assay*

The ferric reducing antioxidant power (FRAP) assay of carrot extracts was determined according to Benzie and Strain (1999). For the extraction, 200 mg of lyophilized carrot powder were mixed with 10 mL of methanol 100%, vortexed for 1 minute and placed in the dark for 30 minutes. After that, samples were centrifuged for 10 minutes at 4500 g (6 °C). Preparation of FRAP reagent consisted of 10 mL of acetate buffer (300 mM, pH 3.1) mixed with 1 mL of TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) solution (10 mM in 40 mM HCl) and 1 mL of $FeCl_3$ (20 mM). For the reaction, 150 µL of supernatant were mixed to 300 µL of ultrapure water, vortexed and added to 3 mL of FRAP reagent. Samples were placed in the dark at 20 °C for 10 minutes. The FRAP, based on the reduction of Fe(III) by the sample extract, was determined following the change in absorbance at 593 nm due to the formation of a blue colored Fe(II)-tripyridyltriazine

compound from colorless oxidized Fe(III) form in presence of a particular concentration of the sample. FRAP was calculated from a standard curve prepared by plotting change in absorbance against different concentrations of Trolox and expressed as µmol Trolox equivalent (TE) 100 g^{-1} FW.

- *Mineral analyses*

Dry carrots were grounded and submitted to wet digestion before the ICP-MS-measurement, according to May et al. (2019). The wet digestion was performed with an infrared controlled and power adjusted microwave (Go, Anton Paar, Graz, Austria) up to an end temperature of 180 °C (dwell time of 10 minutes) using approximately 100 mg of the oven-dried sample, 4 mL supra pure HNO_3 and 2 mL ultrapure water. The digested samples were transferred into polypropylene tubes (Greiner bio-one, Kremsmünster, Austria) and made up to 40 mL with water. Subsequently, an aliquot of 2.5 mL of the latter solution was made up to 10 mL with water under addition of 100 µL internal standard (rhodium, 1 mg L^{-1} standard concentration prepared from a 1000 mg L^{-1} stock solution of $Rh(NO_3)_3$, Merck, Darmstadt, Germany), yielding 10 µg L^{-1} rhodium in the final solution. Each sample workup and digestion were done in duplicate.

The ICP-MS measurements were carried out with a NexION 300d (Perkin Elmer, Waltham, Massachusetts, USA) equipped with an S10 autosampler (Perkin Elmer), a Meinhard® concentric nebulizer, a cyclonic spray chamber, a quartz torch, and nickel cones. The following operating conditions and acquisition parameters were used: 1550 W RF (radio frequency) power; 15 L min^{-1} in plasma gas flow; 1.04 L min^{-1} nebulizer gas flow; 1.375 L min^{-1} auxiliary gas flow, and 5.2 L min^{-1} He gas flow in KED (kinetic energy discrimination) mode. Calibration was performed using a custom-made multi-element standard solution (Inorganic Ventures, Christiansburg, VA, USA)

containing the target elements. The calibration standards were matrix-adjusted by adding HNO_3. To avoid possible polyatomic interferences, several elements were quantitated in KED mode (P, K, Ca, Mg, Na, Fe, Mn, Cu, Mo and Ni). The reference material SRM 1570a (Trace Elements in Spinach Leaves, NIST, Gaithersburg, MD, USA) was used to control the accuracy of the method and as a daily quality control standard. LOQ was calculated based on the nine-fold standard deviation of a blank solution prepared and analyzed twelve times. The following LOQs were achieved: Ca: 0.5 mg kg^{-1}, Mg: 0.1 mg kg^{-1}, K: 1.0 mg kg^{-1}, Na: 0.2 mg kg^{-1}, Zn: 30 µg kg^{-1}, Cu: 15 µg kg^{-1} and Mn: 3 µg kg^{-1}. For nitrogen (N) determination, sulfuric digestion with catalyst salts in a digesting block was employed and distillation was performed according to the Kjeldahl method. Nutrients were calculated and expressed either as mg 100 g^{-1} FW or µg 100 g^{-1} FW.

4.1.2.6 *Digestion procedure and bioaccessibility assessment*

Carrot samples were cut into small cubes of 2 mm x 2 mm before being milled with a Retsch Ball Mill MM 400 (Retsch, Haan, Germany) for 90 seconds at a frequency of 30 shakes per second. Five grams of carrot puree was put into a 50 mL Greiner tube. All samples were digested according to the INFOGEST protocol as described by Minekus (2014).

To assess the bioaccessibility of the Zn and Fe from the carrot into the intestine, the mineral amount in the supernatant that was present after digestion was measured. After digestion, 15 mL of the sample was put into a 15 mL Greiner centrifuge tube. All the samples were centrifuged for 15 minutes at a speed of 4000 g at room temperature. Ten mL of the supernatant of the biofortified samples were taken and analyzed as described in 4.1.2.5 for the presence of Fe and Zn minerals. The results were obtained in mg 100 g^{-1} fresh carrot and compared to those present in the carrots before digestion.

4.1.2.7 Statistical procedures

Collected and calculated data were firstly subjected to Shapiro-Wilk's and Levene's test, in order to check for normal distribution and homoscedasticity, respectively. Data were then subjected to a one-way analysis of (ANOVA). For all the variables, the comparison between means was performed by calculating the Fisher's protected least significant difference (LSD, $P = 0.05$). A Pearson's correlation analysis was also performed, to define possible relationships among mineral concentrations. All calculations were performed using Microsoft Excel and Minitab version 19 (Minitab Inc., State College, PA, USA).

4.1.3 Results

- *Carrot quality variables and chromatic coordinates*

As showed in Table 4-1, when compared to control, the average fresh weight of roots was increased in plants treated with $FeSO_4$ (+25%) and with both forms of Zn (+20%, on average), while the dry matter was significantly higher only in the treatment with Zn-EDTA. Root diameter was not affected by any treatment, while roots treated with both Zn forms showed the highest length (+11%). None of the chromatic variables was significantly affected by the biofortification treatments.

- *Biochemical variables*

Total carotenoids content of roots increased (+4%) in plants treated with Zn-EDTA and decreased (-9%) in those treated with Fe-DTPA, in comparison to control roots. A significant difference in plants treated with Fe-DTPA was also noticed for TPC (-14%) DPPH

(-20%) and FRAP (-11%), in comparison to untreated plants (Table 4-2).

- *Total N and mineral composition*

As shown in Figure 4-1, the Fe content of carrots was promoted by the $FeSO_4$ application (+52% compared to control), whereas decreased in plants receiving $ZnSO_4$ (-35%) (Figure 4-1A). On the other hand, Zn content was enhanced by all treatments, showing a 94% increase when submitted to Zn-EDTA and a 57% increase when submitted to $ZnSO_4$, in comparison to control (Figure 4-1B).

When total N was concerned, the strongest differences were recorded among the Zn-EDTA and FeSO4 treatments (103.0 vs. 88.3 mg 100 g^{-1} FW), being the former able to maximize the P and K contents too (Table 4-3).

The Ca content of carrots submitted to both forms of Fe and Zn-EDTA showed a significant increase as compared to control ones, but no difference when compared to the $ZnSO_4$ plants (Table 4-3). The Mg content in Zn-EDTA-treated roots increased only in comparison to those receiving $FeSO_4$ (+28%). On the other hand, a higher root Na content than control was recorded in all biofortification treatments, except for the $FeSO_4$ (Table 4-3). Manganese content was boosted by the two forms of Zn (by 26%, on average) compared to the control. Meanwhile, the Ni content was reduced by Fe-DTPA (-29%) and promoted by $FeSO_4$ (+57%) (Table 4-3).

- *Iron and zinc bioaccessibility*

The amount of Fe obtained in the intestine fluid at the end of the INFOGEST procedure was 530 mg 100 g^{-1} for carrots biofortified with Fe-DTPA, 521 mg 100 g^{-1} for the biofortification with $FeSO_4$. The absolute values were not significantly different respect to the 610 mg 100 g^{-1} found in the non-biofortified control samples. However,

the increased amount present in the $FeSO_4$ biofortified samples was not reflected in the bioaccessibility data (Figure 4-2).

The picture is comparable for Zn biofortified samples: 270 mg 100 g^{-1} in carrot samples that were biofortified with Zn-EDTA and 160 mg 100 g^{-1} in $ZnSO_4$ biofortified carrots were recovered in the digestive fluid. In this case too, while control and chelated fortified samples have bioaccessibility value in line with the actual carrot content, the sulphate biofortified sample lost the advantage gained with the fortification procedure (Figure 4-2).

Table 4-1. Commercial and nutritional traits of carrot as affected by Fe and Zn treatments.

Treatment	Root average fresh weight (g plant^{-1})	Root dry matter (%)	Root diameter (mm)	Root length (cm)	L*	a*	b*	Chroma
Control	88 b	11.0 b	32.0	17.3 b	38.9	32.7	41.0	52.4
Fe-DTPA	98 ab	10.5 b	33.6	17.6 ab	37.6	31.9	40.2	51.4
$FeSO_4$	110 a	10.7 b	34.7	17.1 b	38.1	30.8	38.8	49.5
Zn-EDTA	103 a	11.8 a	33.6	19.2 a	37.9	31.6	39.1	50.2
$ZnSO_4$	108 a	11.0 b	32.5	19.2 a	37.7	32.2	40.7	51.9
LSD	14.4	0.8	3.1	1.6	1.3	1.8	1.7	2.0
F-test	*	*	*NS*	*	*NS*	*NS*	*NS*	*NS*

Different letters indicate significance fisher's protected LSD Test ($p = 0.05$)

*: significance of $P \leq 0.05$. NS: not significant.

Table 4-2. Biochemical traits of carrot as affected by Fe and Zn treatments.

Treatment	Total carotenoids (µg 100 g^{-1} FW)	TPC (µmol 100 g^{-1} FW)	DPPH (µmol 100 g^{-1} FW)	FRAP (µmol 100 g^{-1} FW)
Control	8017 b	56.5 a	49.3 a	49.7 a
Fe-DTPA	7315 c	48.3 b	39.3 b	44.0 b
$FeSO_4$	7981 b	56.3 a	46.5 a	47.8 ab
Zn-EDTA	8337 a	57.3 a	47.0 a	50.7 a
$ZnSO_4$	8318 ab	55.8 a	44.9 a	45.4 ab
LSD	594	6.5	7.0	5.3
F-test	***	*	*	*

Different letters indicate significance fisher's protected LSD Test ($p = 0.05$)
*, ***: significance of $P \leq 0.05$, 0.001, respectively.

Table 4-3. Mineral composition of carrot roots as affected by Fe and Zn treatments.

Treatment	N	P	K	Ca	Mg	Na	Mn	Cu	Ni	Mo
	mg 100 g^{-1} FW						µg 100 g^{-1} FW			
Control	91.3 bc	23.9 d	321 b	30.5 b	10.8 ab	20.6 b	128 b	45.0	3.43 b	1.24
Fe-DTPA	90.9 bc	25.6 cd	322 b	34.7 a	11.9 ab	24.8 a	116 b	44.1	2.43 c	1.26
$FeSO_4$	88.8 c	26.6 bc	322 b	36.4 a	10.2 b	19.4 b	110 b	49.0	5.41 a	1.18
Zn-EDTA	103 a	29.4 a	394 a	36.6 a	13.1 a	24.4 a	157 a	45.7	3.48 ab	1.05
$ZnSO_4$	99.3 ab	27.8 ab	340 b	33.2 ab	12.5 ab	24.6 a	165 a	49.2	3.89 ab	1.09
LSD	9.5	1.7	38.3	3.7	2.4	1.7	221	8.0	1.9	0.2
F-test	**	***	**	***	*	***	**	*NS*	*	*NS*

Different letters indicate significance fisher's protected LSD Test ($p = 0.05$)
*, **, ***: significance of $P \leq 0.05, 0.01, 0.001$, respectively. NS: not significant.

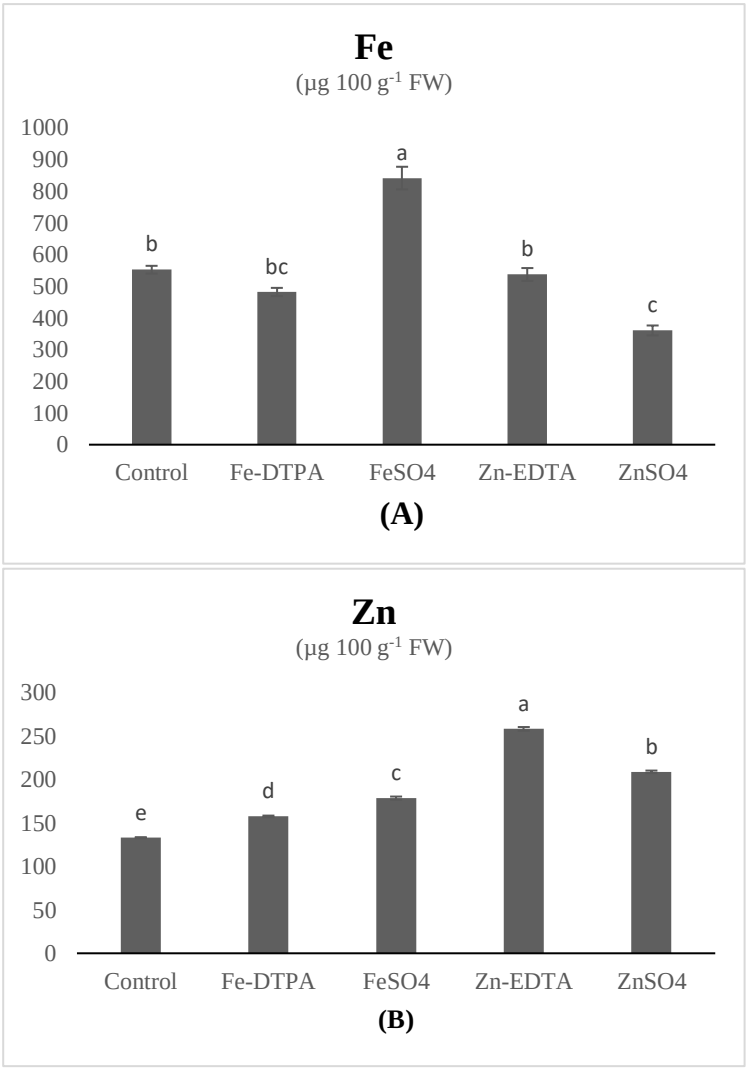

Figure 4-1. (A) Iron content of carrot roots as affected by Fe and Zn treatments. (B) Zn content of carrot roots as affected by Fe and Zn treatments. Different letters indicate significance fisher's protected LSD Test (p = 0.05).

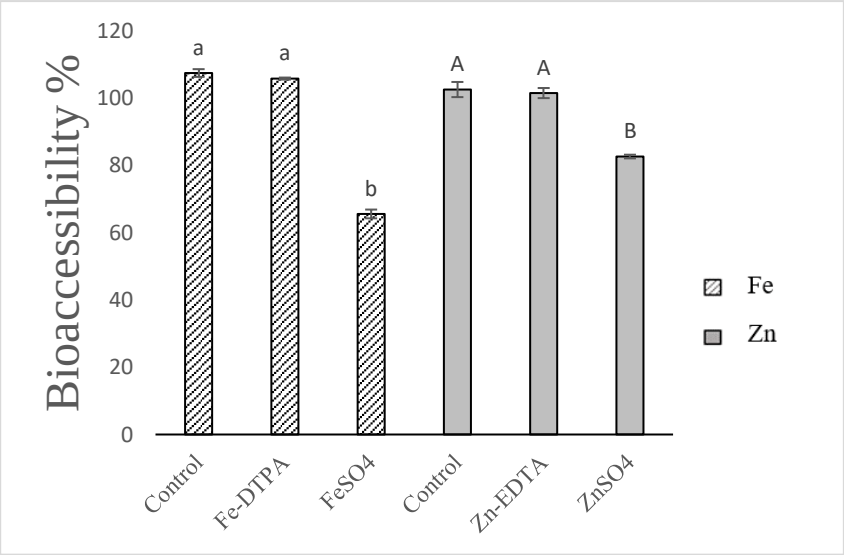

Figure 4-2. Fe and Zn bioaccessibility of carrot roots as affected by Fe and Zn treatments. Different letters indicate significance fisher's protected LSD Test (p = 0.05). Lower cases represent differences among Fe treatments and upper cases represent differences among Zn treatments.

4.1.4 Discussion

4.1.4.1 *Yield and quality traits*

Under the specific conditions of our experiment, the foliar application of both forms of Zn promoted roots' fresh weight and length, while the dry matter content was promoted by Zn-EDTA, suggesting a stimulatory effect of Zn on crop photosynthetic metabolism. This is consistent with the findings of Awad et al. (2021) in an experiment with carrots and foliar sprays of Zn-EDTA. After three applications of a 5.7 mM solution, the authors noticed an increase in the root fresh weight and root dry matter of 39% and 25%,

respectively. Mousavi et al. (2007) also reported an increase in tuber yield and dry matter content after treating potato plants with two foliar applications of $ZnSO_4$, in the concentration of 0.122 mM. The same stimulatory effect was observed by Almendros et al. (2015), after submitting onion plants to different forms of Zn, in the concentration of 0.15 mM through soil fertilization. In their experiment, the most efficient chemical form was Zn-EDTA.

In our study, the application of Zn-EDTA caused an increase in multiple morphometric variables of carrots (mainly in terms of fresh weight, dry matter, root length and total N content). This could be explained by the importance of Zn in maintaining the plant's physiological status, through the stimulation of photosynthesis which increases leaf dry matter production leading to an improvement in the plant growth variables (Rizwan et al. 2019). At the same time Zn-EDTA caused a significant increase in the total carotenoids content but no increase in other biochemical parameters. Rivera-Martin et al. (2021) obtained contrasting results after treating broccoli plants cv. Parthenon with $ZnSO_4$ at 5 mM. They found that the activity of antioxidants (DPPH and ABTS) and TPC significantly increased compared to control. This suggests that, the concentration of Zn-EDTA could be optimized to enhance carrots biochemical traits. Once, these increases in the antioxidant variables demonstrate the potential of Zn biofortification in improving important quality parameters of vegetables (Blasco et al. 2015; Barrameda-Medina et al. 2017).

Regarding Fe, the effect of foliar applications of $FeSO_4$ on carrots stimulated root fresh weight, also improving the content of P and Zn. This positive effect suggests that Fe, in the form of $FeSO_4$, could stimulate plant growth, since Fe is involved in the synthesis of chlorophyll and it is also important to complete the enzyme functions that maintain plant's health (Marschner 2011). In contrast to Fe-DTPA, which did not improve root FW, the stimulatory effect of $FeSO_4$ could be attributed to the presence of sulfur (S), since S

application has been proved to improve carrot yield (Singh et al. 2016). However, it should be highlighted that excess of Fe can be toxic to plants, leading to the formation of ROS (Das et al. 2020). Chelated forms of Fe, as Fe-DTPA, can penetrate leaves more easily than the sulfate form (Ferrandon and Chamel, 1988), being more prone to phytotoxicity effects, a feature that could explain the inhibitory effects of Fe-DTPA on total carotenoids and total polyphenol content and the reduction in the antioxidant activities, looking at the DPPH and FRAP data.

4.1.4.2 *Mineral biofortification*

This stimulatory effect of Zn treatments in our study is also supported by the increase in root concentration of some elements (mainly P, Na, Fe, Zn, Mn), thus suggesting an improved root absorption capacity. This is in accordance with the results showed by Awad et al. (2021) who obtained carrots with a higher content of P, Fe, Mn, Zn and Cu, after foliar application of Zn-EDTA (5.7 mM). This stimulatory effect can be attributed to the fact that Zn plays a key role in increasing membrane function, cell elongation, protein synthesis and positively stimulates plants roots to exchange cations, increasing nutrients absorption (Marschner 2011). On the other hand, White et al. (2017) reported that biofortification of potato with 1.96 g Zn m^{-2} as leaf spray, had little consequence for the concentration of other mineral elements in the tubers.

Regarding micronutrients, foliar sprays of Zn showed to be effective in enhancing the Zn content in carrots, which is the main goal of the present biofortification study. In this sense, our results suggest that biofortification of carrot with Zn can be successfully performed with both forms of Zn (Zn-EDTA or $ZnSO_4$), at the concentration of 6 mM of the element. Moreover, among the two chemical forms, Zn-EDTA treatment proved to be more efficient, as carrots showed

approximatively the double mineral concentration when compared to the control. The reason why Zn-EDTA presented better results could be related to the fact that the chelated form is more soluble and available for the plant when compared to the sulfate form (Gupta et al. 2016).

The positive results obtained in our study concerning Zn biofortification are coherent with those of Awad et al. (2021), as they were able to produce carrots having a Zn concentration 61% higher than those of untreated plants, after spraying with a solution 5.7 mM of Zn-EDTA. Kromann et al. (2017), in a similar experiment with potatoes, using chelated sprays (EDTA) of Zn (3.06 mM) obtained a 2.51-fold increase of the Zn concentration in tubers. Meanwhile, after applying a foliar spray with a lower concentration of Zn (0.122 mM), as $ZnSO_4$, Mousavi et al. (2007) obtained a lower increase (23%) in the tuber Zn concentration, in comparison to the control.

On the other hand, Zn biofortification of carrots through soil applications, contrasting results were obtained by De Sousa Lima et al. (2015) after applying different doses of Zn (0-300 mg kg^{-1}) fertilizer to the soil. They observed no significant increase in the root concentration of Zn, which can be explained by the limited Zn mobility in the xylem. This supports the hypothesis that the foliar spray strategy used in this study, represent the best approach in the Zn biofortification of carrots, probably facilitated by the high solubility and translocation of this mineral in the phloem (Marschner 2011).

In the present study, biofortification of carrots using $FeSO_4$ was successful, as the roots showed a 52% increase in their Fe content. This is consistent with the results of Sun et al. (2019), after applying a Fe solution (6.6 mM) in the leaves of sweet potato, they obtained tubers with 43% more Fe than the untreated ones. In contrast, the inefficient of Fe-DTPA in the Fe biofortification observed in our study was confirmed by the previous author, and Kromann et al. (2017) after applying Fe-EDTA (6.71 mM) on potatoes leaves, they also noticed

no significant Fe increase in the tubers.

A possible explanation why Fe biofortification is more effective using $FeSO_4$ than Fe-DTPA, could be the lower mobility of Fe chelated inside the leaf, when compared to $FeSO_4$; this was demonstrated by Rios et al. (2016), when tracing the uptake pathway of different forms of Fe in the leaves of *Prunus*. In the case of $FeSO_4$, Fe was found in the vascular areas of the leaf, whereas in the case of Fe(III) salts the stain remained in the stomatal areas. Differently, when using a nutrient solution enriched with Fe-DTPA (0.537 mM), carrots leaves showed a 18% increase in the Fe content, when compared to untreated plants. This demonstrates that Fe-DTPA could be more easily absorbed and translocated through the nutrient solution rather than through foliar spray (Gupta and Chipman 1976). In fact, the translocation of Fe chelate from roots to other organs of the plant, has been demonstrated by Sida-Arreola et al. (2015), supporting the hypothesis that biofortification using Fe-chelate could be more efficient when applied via roots using the nutrient solution rather than as foliar sprays.

According to Rengel et al. (1999), the chelated form of Fe limited the Fe biofortification of carrots, because of the high concentration of the solution: it is possible that the chelate competes with ionized groups in the cuticle. Furthermore, possible phytotoxic effects of the application of Fe-DTPA, at 6 mM, could have impaired Fe biofortification. This was not the first case in which $FeSO_4$ demonstrates to be more efficient than Fe-DTPA in translocating Fe from the leaves to the roots. Aciksoz et al. (2011) compared foliar sprays of two chemical forms (Fe-DTPA and $FeSO_4$) and showed that, in the case of biofortification, the sulfate form of Fe was more efficient in increasing Fe content in wheat grains. Another reason for the limited effectiveness of Fe biofortification of carrots could be the limited mobility of the mineral inside the plant (Marschner 2011). Additional Fe biofortification studies in carrots should be performed

in order to better comprehend if lower doses of the chelated form of Fe could be, as effective as the sulfate form, used in the biofortification of carrots through foliar applications.

4.1.4.3 *Bioaccessibility*

Beside studying the fate of the minerals in plant and their accumulation in edible portions, it is extremely important to investigate if they are actually released from the food matrix during the digestion procedure and become bioaccessible for the absorption through the intestine. The data obtained in this study are summarized in Figure 4.2 reporting the percentual increase of the bioaccessible minerals respect to the amount present in the carrot samples. In the control roots, the amount of minerals detected in the intestinal fluid is slightly above the 100% of that present in carrots. This is not statistically significant and it is possibly due the enhanced solubility induced by the enzymatic treatments. Looking at the Fe data, similar results were obtained with Fe-DPTA; however, the bioaccessibility dropped to about 60% for the $FeSO_4$ samples suggesting that this type of biofortification, which was very effective in plant, do not provide an actual nutritional benefit. The figure is similar in Zn-biofortified samples: Zn-EDTA has similar bioaccessibility percentage than the control but in the $ZnSO_4$ fortified samples the Zn bioaccessibility was only about 80% of the expected value.

If the percentage of the bioaccessible minerals would have been similar for all samples the increase in the amount of minerals observed in the carrots would have been actually bioaccessible to the human body. These in vitro data suggest that this is true for chelate forms of Fe and Zn but not for the sulphate ones.

This result is not in line with a previous study showed that biofortification with $ZnSO_4$ led to an increase in bioaccessibility of Zn (Zou et al. 2014). Also, another research showed that high ratios of

EDTA: Zn even led to an inhibitory effect on the absorption of Zn (Hotz et al. 2005). Another factor that could play a role is the amount of Fe that was present in the Zn-biofortified carrots. For both Zn-EDTA as $ZnSO_4$ and control, carrots contained more Fe^{2+} than Zn^{2+}. In itself this might not pose a problem, as it is the goal to obtain more Fe and Zn; nevertheless, literature suggests that non-heme Fe in a ratio of Fe/Zn of 1:1 slightly inhibits Zn absorption, and a ratio of 2:1 substantially inhibits Zn uptake (Solomons and Jacob 1981). As non-heme Fe is found in legumes and crops such as carrot or eggplant (Mauro et al. 2022), this antagonist relation between Zn and Fe should be further analyzed.

4.1.5 Conclusion

Our findings indicate that biofortification of Zn and Fe in carrots through foliar sprays can be potentially successful using four applications of 6 mM of $FeSO_4$ and $ZnSO_4$ or Zn-EDTA, being the Zn-EDTA even more efficient in increasing the Zn content in carrots. However, even though the concentration of minerals was higher with the sulfate form of Fe, bioaccessibility evidence shows that the chelated forms of both minerals are preferable in biofortification programs, since almost all the mineral contents present in the edible part of biofortified carrots with the chelated forms is bioaccessible. This is not true for sulfate forms which showed to be less bioaccessible thus losing most of the benefit potentially achieved with the increased content in the carrots. In general, these results are encouraging and will contribute to defining the utility and application of Fe and Zn biofortification of carrots.

4.2 Quality traits and mineral profile of carrot 'Dordogne' as affected by foliar applications of silicon

This chapter has been submitted to Acta Horticulturae on 15 May 2022

4.2.1 Introduction

Carrot (*Daucus carota* L.) is a valuable crop for worldwide horticulture, which provides important nutrients such as carotenoids, ascorbic acid, phenolics, and minerals (Ahmad et al. 2019). Depending on the region of cultivation, this vegetable is harvested in spring (off-season) or in autumn, this latter being the most appropriate for long-term cold storage (McDonald 2020). In Southern Italy, carrot is an important off-season vegetable for fresh consumption, being harvested in springtime and marketed also as bunches with leaves on (bunched carrots) (Ierna et al. 2020b). This 'early' product is characterized by distinctive organoleptic traits compared to the autumn cold-stored one, but it is more perishable due to the presence of leaves. Indeed, leaves presence drastically increases transpiration rate and deterioration of root tissues, leading to a fast visual decay of the product (Madani et al. 2018). Additionally, the commercialization of bunched carrots limits the possibility to store them under more proper conditions (≈0 °C at 95–98% RH), resulting in higher metabolic activity and fast decay (Ierna et al. 2020b).

The application of silicon (Si) is a promising strategy to extend the shelf-life of vegetables, as well as the crops' agronomic performance (Tripathi et al. 2020). Silicon is one of most abundant elements in soils, being considered non-essential but beneficial for plants, though its role is not completely understood (Coskun et al. 2019). It is known that Si elicits plant antioxidant systems and

modulates plant mineral nutrition by directly interacting with some elements like Mn, or by upregulating the expression of genes involved in minerals translocation, as for P and Fe (Ali et al. 2020). Silicon deposits in cell walls improves plant tissues tolerance to environmental stressors and improves plant canopy and light interception (Weerahewa and Rajapakse 2020).
Silicon has recently been studied also for biofortification purposes. Despite not being essential for humans, adequate Si dietary intake is associated with improved bone formation (Buturi et al. 2021). Besides the beneficial effect of this element on plants and humans, its regulatory role on plant mineral uptake and synthesis of specialized metabolites (e.g. carotenoids and ascorbic acid) justifies the investigation of Si role in improving vegetables quality and nutraceutical traits, which is underexploited for carrots.

For the above-mentioned reasons, in the present study we investigated the role of preharvest application of potassium silicate in regulating early carrot compositional traits and shelf-life performance, either in presence or not of leaves.

4.2.2 Materials and Methods

4.2.2.1 Plant material and experimental design

A field trial was carried out during the 2019–2020 growing season with the carrot cultivar ‘Dordogne’ (Nantes type) at Ispica plain (South-eastern Sicily: 36°47'7"80 N, 14°54'25"56 E, 42 m a.s.l.), a typical area for early carrot cultivation in South Italy. Sowing and crop management practices were performed according to the guidelines for “Carota Novella di Ispica” (a Protected Geographical Indication). The soil was a moderately deep, calcic brown, according to the USDA Soil Taxonomy Classification, with a sandy-loam texture. At the beginning of the experiment, the soil was analyzed for

N content (0.8 g kg^{-1}), organic matter (12.2 g kg^{-1}), available P_2O_5 (57 mg kg^{-1}), exchangeable K_2O (302 mg kg^{-1}) and pH (7.4).

On December 5th seeds were sown at a ≈1 cm depth in three rows on 0.8 m wide ridges. Actual density was 112.5 plants m^{-2} and plot size was 3.6 m × 2.4 m, consisting of 3 ridges. One week before sowing, 100 kg ha^{-1} of P_2O_5 (as mineral superphosphate) and 200 kg ha^{-1} of K_2O (as K_2SO_4) were applied. Nitrogen (120 kg N ha^{-1} as NH_4NO_3) was supplied in four equal amounts, i.e. at sowing, at the stage of 5–6 leaves (71 days after sowing, DAS), at the beginning of roots enlargement (114 DAS) and at the stage of advanced root enlargement (156 DAS). Crop water needs in late spring were satisfied through sprinkler irrigation, supplying the difference between rainfall and crop maximum evapotranspiration. Carrots were manually harvested at commercial maturity (diameter ≈30 mm) on May 6th.

The experiment was arranged as a randomized blocks design with three replications. Plants received four foliar sprays, on a weekly basis (from 42 to 14 days before harvest). These treatments consisted either of Si application (15 mM of Si as K_2SiO_3, hereafter Si+) or untreated control (tap water only, hereafter Si-). Foliar sprays were effected at a dose of 600 L ha^{-1}, whereas a surfactant (isodecyl alcohol ethoxylate) was added to all the spray solutions. At harvest, 40 uniform plants per plot were washed and separated in 4 groups; on 2 groups, measurements were taken the same day of harvest (T0) while the rest were stored at 12 °C for 7 days (T7) either with (L+) or without (L-) leaves. In the second case, leaves were removed soon after harvest with a sharp knife, cutting them at collar level.

4.2.2.2 Physical variables and samples preparation

At T0 the following measurements were performed on roots: diameter (on the collar region), length (from collar region up to root tip), firmness and chromatic coordinates. These latter were taken on

the surface of the root at mid-length with a CR-200 Minolta chroma meter calibrated with a standard white tile (UE certificated) with illuminant D65/10°, measuring color in terms of lightness (L*), green-red axis (a*) and blue-yellow axis (b*). Root color is reported as L* and Hue angle [($\tan^{-1}$(b*/a*)/6.2832)*360]. Firmness was determined in the same region of the root on 3 cm long cylinders, with a TA-XT2 texture analyzer, and reported as force (Newton) needed to apply a 2 mm deformation, by using a cylindrical tip (Ø 2 mm). On both leaves and roots, fresh weight (FW) and dry matter (DM) content were measured (0.01 g accuracy). For DM content, fresh samples were dried at 70 °C in a laboratory oven (Thermo scientific-Herathermoven) with forced air circulation until constant weight was reached. After weight registration, root samples were grounded in a mill and stored for determination of reduced N through the Kjeldahl method.

Another subset of T0 samples was frozen-dried, grounded in a mortar with liquid N and stored at -80 °C. This latter procedure was also applied to T7 samples after they were cut in 3 cm long cylinders and left for 12 hours in distilled water for rehydration, to monitor the DM loss during storage. After that, both sets of samples were used for total carotenoids, phenols and antioxidant activity by DPPH and FRAP assay using a Jeanway UV/Visible spectrophotometer model 7315 (Cole-Parmer, Stone, UK).

4.2.2.3 Roots mineral content

The content of P, K, Ca, Mg, Na, Fe, Zn, Mn, Cu, Mo, Cr, Ni was determined on T0 carrots by means of an ICP-MS. A microwave assisted digestion in HNO_3 was performed on 0.1 g of freeze-dried sample according to May et al. (2019). Each sample's workup and digestion were performed in duplicate. The ICP-MS measurements were carried out with a NexION 300d (Perkin Elmer, Waltham,

Massachusetts, USA) equipped with an S10 autosampler (Perkin Elmer), a MEINHARD® concentric nebulizer, a cyclonic spray chamber, a quartz torch, and nickel cones. The following operating conditions and acquisition parameters were used: 1550 W RF (radio frequency) power; 15 L minutes^{-1} plasma gas flow; 1.04 L minutes^{-1} nebulizer gas flow; 1.375 L minutes^{-1} auxiliary gas flow, and 5.2 mL minutes^{-1} He gas flow in KED (kinetic energy discrimination) mode. Calibration was performed using a custom-made multi-element standard solution (Inorganic Ventures, Christiansburg, VA, USA) containing the target elements. The calibration standards were matrix-adjusted by adding HNO_3. To avoid possible polyatomic interferences, several elements were quantitated in KED mode (Mn, Fe, Ca, Na, Mg, and K). The reference material SRM 1570a (Trace Elements in Spinach Leaves, NIST, Gaithersburg, MD, USA) was used to control the accuracy of the method and as a daily quality control standard. LOQ was calculated based on the nine-fold standard deviation of a blank solution prepared and analyzed twelve times. The following LOQs were achieved: Ca: 0.5 mg kg^{-1}, Mg: 0.1 mg kg^{-1}, K: 1.0 mg kg^{-1}, Na: 0.2 mg kg^{-1}, Zn: 30 µg kg^{-1}, Cu: 15 µg kg^{-1} and Mn: 3 µg kg^{-1}. For nitrogen (N) determination, sulfuric digestion with catalyst salts in a digesting block was employed and distillation was performed according to the Kjeldahl method.

4.2.2.4 Determination of bioactive compounds

- Total carotenoids

Total carotenoids content was determined according to Lichtenthaler and Wellburn (1983). Briefly, 0.05 g of root sample was put in 5 mL of ethanol (96%), vortexed, placed in ultrasonic bath for 10 minutes and left overnight in the dark (at 10 °C). After centrifugation, the absorbance of the supernatant was read at 470, 649

and 665 nm. Ethanol 96% was used as blank. The results are expressed in g kg^{-1} DW. The same procedure was adopted to quantify leaf pigments (chlorophyl a and b, their ratio and total carotenoids).

- Total phenols

Total phenols content (TPC) was quantified in roots using a modified Folin-Ciocâlteu method (Cicco et al. 2009). To this end a 0.1 g sample was mixed with 1 mL of 70% methanol and agitated for 1 hour at room temperature, then centrifuged at 5000 g for 5 minutes at 25 °C. 0.1 mL of extract solution was mixed with 0.1 mL Folin-Ciocâlteu reagent and allowed to react at room temperature for 2 minutes. Next, 0.8 mL of sodium carbonate (5% w/v) were added, and tubes were left in a thermostatic bath at 40 °C for 20 minutes. Samples were read at 760 nm and TPC was reported as µmol gallic acid equivalents (GAE) kg^{-1} DW.

- DPPH essay

Determination of antioxidant activity by inhibition of the free radical DPPH (2,2-diphenyl-1-picrylidrazyl) in roots was adapted from the method reported by Brand-Williams (1995). 0.1 g of sample was placed into plastic microtubes with 1.5 ml methanolic solution (80%), transferred in ultrasonic bath for 14 minutes and centrifuged at 10000 rpm for 5 minutes at 5 °C. Then, 150 µl of supernatant was added to 1350 µl of a previously prepared DPPH solution (150 µM). The solution obtained was allowed to react in the dark for 30 minutes and then used for reading the absorbance at 515 nm, with methanol as blank. The results were compared with a calibration curve prepared the same day using methanolic dilutions of pure grade Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and reported in µmol Trolox equivalent (TE) kg^{-1} DW.

- FRAP essay

FRAP (ferric ion reducing antioxidant power) assay was performed on roots using a modified method from Benzie and Strain (1999). 0.2 g of each sample was incubated in 10 ml of pure methanol in the dark for 30 minutes and then centrifuged at 4500 rpm at 6 °C for 10 minutes. Then, 150 µl of supernatant was transferred into plastic microtubes and added to 300 µl of ultrapure water. The resulting solution was transferred to a plastic cuvette (for visible range) containing 3 ml of FRAP reagent containing TPTZ (2,4,6 tripyridil-S-triazine), iron chloride and an acetate buffer solution at pH 3.1. After 10 minutes, the absorbance was taken at 593 nm and results were then compared to a calibration curve as for DPPH and expressed as µmol TE kg^{-1} DW.

4.2.2.5 Statistical procedures

On the obtained data, normal distribution and homoscedasticity assumptions were checked using Shapiro-Wilk's and Levene's tests, respectively. Results concerning roots physical traits, leaf traits and root mineral content (Tables 4.4, 4.5 and 4.6 and Figure 4.3B) were analyzed by one-way ANOVA with Si as single factor. Root DM% and bioactive composition (Table 4-7) data underwent a three-way ANOVA (Si application × leaf presence × storage time). Means were then separated through Tukey's HSD test ($P \leq 0.05$).

4.2.3 Results and discussion

4.2.3.1 Root physical traits

None of root traits at T0 was responsive to Si application (Table 4-4). Differently, irrespective of leaves presence, DM content was promoted by Si application, both at T0 and at T7 (by 15 and 9%

respectively, when compared to control) (Figure 4-3A). Concomitantly, at T7 Si+ carrots showed no statistical difference in weight loss between L- and L+ storage condition, unlike Si- carrots (Figure 4-3B). The efficacy of Si in mitigating weight loss in postharvest has been reported for other crops such as leeks, which is stored with leaves as well (Weerahewa and Rajapakse, 2020), but not on leafed carrot.

Table 4-4. Physical traits of carrot roots (at T0). Data are expressed as mean ± SEM. Significance to F-test is reported. NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Length (cm)	**Diameter (mm)**	**FW (g)**	**L***	**Hue angle**	**Firmness (N)**
Si-	15.7 ± 0.46	29.7 ± 1.45	80.2 ± 4.89	37.2 ± 0.18	51.15 ± 0.2	35.9 ± 3.23
Si+	16.1 ± 0.6	30.5 ± 1.32	79.1 ± 7.04	38.4 ± 0.39	50.7 ± 0.2	38.2 ± 3.41
F - test	NS	NS	NS	NS	NS	NS

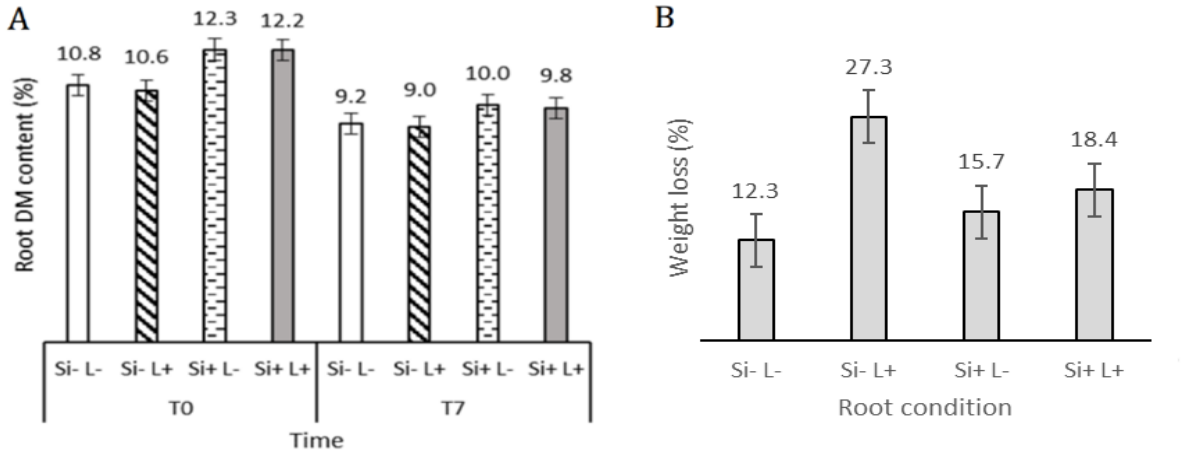

Figure 4-3. (A). Root DM content as affected by Si application, storage time (0 and 7 days, i.e. T0 and T7, respectively) and Leaf presence (L+) or absence (L-). Tukey's HSD (P = 0.05) for Si and T effects is 0.7%. L effect and interactions among factors were not significant. Standard errors bars are reported. **(B).** Root weight loss at T7 in the different Si × L combinations. Tukey's HSD (P = 0.05) is 7.9%. Standard error bars are reported.

4.2.3.2 Leaves composition

At T0 silicon gave increments in terms of DM (+16%), total carotenoids (+89%) and total chlorophyll (a+b) contents (+87%) (Table 4-5). The increased DM content could have resulted from Si deposits in cell wall of the epidermids and guard cells of leaves and petioles, also improving leaf thickness and erectness (Weerahewa and Rajapakse 2020). This is linked to enhanced resistance to abiotic environmental and light interception thus improving photosynthates accumulation (Tripathi et al. 2020) and their translocation to roots, which could explain their higher DM content. Further increments in leaf DM and pigments concentration could be attributed to a modulation of cellular redox homeostasis, resulting from enhanced activity of antioxidant enzymes (Ali et al. 2020). This may have resulted in a slower degradation of chlorophylls and carotenoids and enhanced quantum efficiency of photosystems, as reported for other Si-treated crops (Oliveira et al. 2021).

Table 4-5. Leaves composition at T0. Data are expressed as mean ± SEM. Significance to F-test is reported. NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	DM content (%)	**Total chlorophyll (µg g^{-1} DW)**	**Chlorophyll a/b ratio**	**Total carotenoids (µg g^{-1} DW)**
Si-	11.5 ± 0.17	5.01 ± 0.8	3.42 ± 0.1	811 ± 222
Si+	13.4 ± 0.23	9.37 ± 0.4	3.40± 0.2	1535 ± 91
F - test	*	**	NS	***

4.2.3.3 Root mineral profile

The Si application improved the root content of macro-elements such as P (+20%), Mg (+16%) and Na (+44%) while N, K, and Ca concentrations were not significantly affected (Table 4-6). On the other hand, all the analyzed micro and trace elements were responsive to Si treatment. Zn, Mn, Cu, Cr and Ni concentrations were increased by 19%, 27%, 18% and 93% respectively, while the concentrations of Fe, Mo and Cr decreased in Si+ samples (-32%, -16%, -30% respectively). The increased minerals concentration can partially be ascribed to the higher root DM content (+14% at TO), indicating a strong change in root composition. Moreover, Si is known to play a strong influence on minerals uptake and distribution within the plant (Ali et al. 2020). Our results are in line with those reported by Greger et al. (2018) in carrot and other crops, except for P and Fe. However, a significant increase in P accumulation in response to Si application was observed in rice (Islam and Saha 1969) and wheat (Kostic et al. 2017), this latter resulting from an overexpression of genes involved in P uptake. A strong influence of Si on Fe translocation was reported by Pavlovic et al. (2013) on cucumber (*Cucumis sativus* L.). They observed enhanced xylem-loading of Fe in roots, along with greater translocation towards leaves, due to a higher presence of carboxylase, citric acid and other compounds involved in the reduction and translocation of the metal. Thus, the lower Fe content we observed in roots could have resulted from an increased translocation toward the aerial parts, which did not match the uptake from the soil. On the other hand, the reduction in Fe content we observed could derive from an antagonism with other elements, whose content increased in our samples (e.g. P, Na and Zn), as it has been previously suggested (Mauro et al. 2022). Silicon deposits in root cell walls favor the accumulation of hemicellulose and lignin, leading to a thickening of these cell structures (Khan et al. 2021). This

phenomenon, along with a direct ionic interaction, reduces the translocation of elements such as Mn, Na and heavy metals, leading to their accumulation in root tissues (Ali et al. 2020).

Our results confirm the strong regulatory role of Si in improving crop mineral nutrition, which could lead to better performing crops and to a higher nutrient density of vegetables (Ali et al. 2020). This outcome is important in order to provide the consumer with more nutritious vegetables, thus enhancing their potential in contrasting micro-nutrients deficiencies (Buturi et al. 2021).

Table 4-6. Mineral composition of roots at T0. Data are expressed as mean ± SEM. Significance to F-test is reported. NS: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Macro elements (mg kg^{-1} FW)	**Si-**	**Si+**	**F - test**
Reduced N	1080 ± 94	1180 ± 66	NS
P	270 ± 7	333 ± 11	**
K	3910 ± 135	3620 ± 222	NS
Ca	390 ± 24	395 ± 20	NS
Mg	120 ± 5	142 ± 3	*
Na	201 ± 7	299 ± 13	**
Micro and trace elements (µg kg^{-1} FW)			
Fe	13790 ± 520	9340 ± 420	*
Zn	1800 ± 55	2150 ± 74	*
Mn	1350 ± 29	1710 ± 49	*
Cu	517 ± 40	613 ± 90	*
Mo	14.5 ± 1.1	12.2 ± 1.2	*
Cr	144 ± 5.5	101 ± 6.7	*
Ni	108 ± 12	208 ± 60	**

4.2.3.4 Root bioactive compounds

Root bioactive composition was mostly unaffected by Si application and leaf presence, while a significant variation was observed between T0 and T7 for carotenoids, TPC, DPPH and FRAP (Table 4-7). Total carotenoids content decreased significantly at T7 only in Si- carrots (-22%). Conversely, TPC and DPPH antioxidant activity resulted higher at T7 (by +12% and +31% respectively), regardless of Si treatment and leaf presence. Similarly, the antioxidant activity measured through the FRAP assay increased at T7, more remarkably in Si- L+ (Table 4-7), suggesting that Si might have reduced the oxidative activity due to leaf presence.

The reduction in carotenoids content is likely due to the high oxidative metabolism induced by the non-optimal storage temperature (12 °C instead of 0-2 °C) (McDonald 2020), a condition which may have been attenuated by the Si-induced enhanced antioxidant activity (Oliveira et al. 2021). The concomitant increases in TPC and DPPH during storage contrast with the results reported on carrot (Augspole et al. 2013) though referring to a longer storage time (2 months). However, increments in phenols content and DPPH activity has been reported on lettuce for comparable storage period (Martínez-Ispizua et al. 2022).

Table 4-7. Root bioactive compounds in carrots as affected by Si, L and T. Within each row, different lowercase letters indicate statistical differences for L × T. In Si mean column different uppercase letters indicate statistical difference for Si.

	Si	Root condition				Si mean
		T0 L+	T0 L-	T7 L+	T7 L-	
Carotenoids (g kg^{-1} DW)	**Si-**	11.27 a	11.44 a	7.13 b	7.78 b	9.4 A
	Si+	11.36 a	12.52 a	8.49 b	8.62 b	10.25 A
TPC (GAE µmol kg^{-1} DW)	**Si-**	4.98 c	5.42 bc	8.11 a	7.69 a	6.55 A
	Si+	5.21 bc	5.6 bc	7.95 a	6.67 ab	6.36 A
DPPH (TE µmol kg^{-1} DW)	**Si-**	4.02 c	4.53 bc	8.07 a	6.67 a	5.82 B
	Si+	4.14 b	4.51 b	7.91 a	6.54 a	5.77 A
FRAP (TE µmol kg^{-1} DW)	**Si-**	4.28 c	4.90 abc	5.60 a	5.17 ab	4.99 A
	Si+	4.23 c	4.69 bc	4.85 abc	4.52 bc	4.57 B

4.2.4 Conclusion

The foliar application of Si improved the composition of leaves (DM, chlorophylls and carotenoids contents) and roots traits such as DM content and overall mineral profile, with important possible implications for human nutrition. Moreover, Si slowed down the postharvest quality decay of roots by reducing their weight loss and carotenoids degradation, confirming to be a promising complementary tool to extend the shelf-life of leafed carrots. However, further studies are needed to better understand the interactions between Si and the other minerals in plant nutrition and an eventual synergism with other biostimulant-acting substances in order to maximize the underexploited potential of Si in cropping systems.

5 General discussions and conclusions

This thesis contributes to further insights into the field of the biofortification of vegetables through the study of different agronomic protocols. It highlights the benefits and detriments of implementing this strategy to fight hidden hunger. In detail, the review article provides an overview on the current biofortification scenario discussing key points on this field, as the role of vegetables for human health and how agronomic practices can be used to increase the amount of micronutrients in the edible part of vegetables crops. In addition, the review thoroughly exanimates eight of the most studied minerals elements in the field of the biofortification and summarizes the results obtained from different biofortification protocols found in literature, for each mineral element and vegetable crop. The bibliographic research conducted to write the present review article allowed for the selection of the crops and elements under study in the experimental part of this thesis.

In view of the importance of Fe to the human nutrition, in Chapter 2.1, we demonstrated that supplying cherry tomato plants with 2 mmol L^{-1} Fe through the nutrient solution, and 500 µmol L^{-1} Fe through foliar spray, can significantly increase the concentration of this mineral in tomato fruits, besides improving other compositional traits that increase the consumer's attractiveness for this product.

In Chapter 2.2, the Se-biofortification of cherry tomato fruits through foliar application was successful both at fruit set and at fruit ripening, when compared to control. Among the two periods, the single application of the Se foliar spray at fruit ripening showed to be more efficient in increasing Se content in the tomato fruits, when compared to the fruits from plants sprayed at fruit set.

In Chapter 3.1, lettuce plants were successfully enriched by the administration of Fe in the nutrient solution, demonstrating the effectiveness of the biofortification of greenhouse-grown soilless

lettuce in promoting the dietary intake of Fe. In detail, in both cultivars 1 mM of Fe as HBED added in the nutrient solution was already enough to effectively biofortified the tomato fruits. In addition, even though some biomass reduction was observed, the stress caused by the Fe treatments stimulated the plant to produce and accumulate higher concentrations of health promoting compounds (as minerals and antioxidants). Among the genotypes, from a nutritional point of view, 'Romana' showed higher concentrations of dry matter and micronutrients, but, from an agronomic perspective the genotype Nauplus showed the least biomass loss, proving a higher tolerance to Fe exposure.

In Chapter 4.1, also dedicated to Fe, biofortification of carrots were most effective using foliar sprays of the sulfate forms of Fe and chelate forms of Zn, at the concentration of 6 mM. However, evidence shows that almost all the mineral content present in the carrots biofortified with the chelate forms of both Fe and Zn are bioaccessible, while the sulfate forms showed a decreased bioaccessiblity. This suggests that chelate forms of Fe and Zn are the best choice in carrots biofortification programs.

The last experimental study, Chapter 4.2, shows that carrots submitted to Si treatments were successfully biofortified, in terms of minerals content. The Si treatment improved the content of important micronutrients in carrots roots, such as Mg, Zn, Mn. Moreover, the composition of leaves traits (DM content and pigments) was improved. Finally, Si slowed down the postharvest quality decay of roots by reducing their weight loss and carotenoids degradation, confirming to be a promising complementary tool to extend the shelf-life of leafed carrots.

Finally, it can be concluded that mineral biofortification of carrot, tomato and lettuce was successfully carried out using the protocols tested in this thesis.

The present thesis also confirmed the hypothesis that foliar

sprays are a good strategy to biofortify both plants grown in the field and in the greenhouse (through soilless cultivation systems). At the same time plants grown in soilless systems can also benefit of the management of the nutrient solution, which proved to be simple and effective in the biofortification of the studied vegetables.

In addition, it was highlighted that Fe-biofortification is a promising strategy to fight hidden hunger, that should, however, be further investigated in order to mitigate possible stress effects caused on the plant. The use of new molecules of fertilizers and different concentrations could help, in the future, to optimize the efficiency of the biofortification protocols.

The successful Se-biofortification of cherry tomatoes, demonstrated that foliar sprays with Se can be an alternative to the fertigation strategy (which seems to be the most used). And, that it should be further investigated for other vegetables crops since it is more economically and environmentally sustainable than the constant application and maintenance of Se in the nutrient solution.

The use of foliar sprays of Si in biofortification programs proved to have additional benefits and, further investigations of the potential of this mineral as a biostimulant to improve crop post-harvest traits is recommended.

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Appendix

- **List of papers published on Journals:**

  - **Published:**

Buturi, C.V.; Mauro, R.P.; Fogliano, V.; Leonardi, C.; Giuffrida, F. "Mineral Biofortification of Vegetables as a Tool to Improve Human Diet". *Foods* 2021, 10, 223. https://doi.org/10.3390/foods10020223

Buturi, C.V.; Sabatino, L.; Mauro, R.P.; Navarro-León, E.; Blasco, B.; Leonardi, C.; Giuffrida, F. "Iron Biofortification of Greenhouse Soilless Lettuce: An Effective Agronomic Tool to Improve the Dietary Mineral Intake". *Agronomy* 2022, 12, 1793. https://doi.org/10.3390/agronomy12081793

Buturi, C.V.; Coelho, S.R.M.; Cannata, C.; Basile, F.; Giuffrida, F.; Leonardi, C.; Mauro, R.P. "Iron Biofortification of Greenhouse Cherry Tomatoes Grown in a Soilless System". *Horticulturae* 2022, 8, 858. https://doi.org/10.3390/horticulturae8100858

  - **Submitted:**

"Quality traits and mineral profile of carrot 'Dordogne' as affected by foliar applications of silicon" *Acta Horticulturae*

"Foliar application of zinc and iron effectively achieved carrots biofortification: chelated forms of the minerals are more bioaccessible than corresponding sulfate salts" *Scientia Horticulturae*

- **<u>List of stays in national or international universities:</u>**

  - **The Western Paraná State University – Brazil**

Two months of period abroad were completed at the laboratory of Quality Control of Agricultural products (LACOMP), at The Western Paraná State University. The aim of this internship was to continue the activities of the present PhD project on the topic *Biofortification of vegetables*. Under the guidance of Professor Dr. Silvia Coelho and co-workers, she carried out analyses regarding the mineral characterization of tomato samples.

  - **University of Granada – Spain**

Three months of period abroad were completed at the laboratory of Fisiología y Fitotecnia para el desarrollo de una agricultura sostenible, at the Department of Plant Physiology of the University of Granada. The aim of this internship was to continue the activities of the present PhD project on the topic *Biofortification of vegetables*. Under the guidance of Professor Dr. Begoña Blasco León and co-workers, she performed analyses to assess antioxidants activities, oxidative stress and mineral characterization of lettuce samples.

- **Attendance to congresses, workshops, seminars and courses:**

  - La biofortificazione degli ortaggi: nuove opportunità per un'alimentazione personalizzata – Seminario interattivo tenuto su Internet da Francesco Serio e Massimiliano D'Imperio dell'ISPA-CNR di Bari. 10 December 2020.

  - "Gestione sostenibile delle risorse irrigue nei sistemi ortoflorofrutticoli mediterranei" – Università di Catania. 28 January 2021.

  - 20thAnnual Greenhouse Crop Production and Engineering Design Short Course – The University of Arizona. 03, 10 and 17 March 2021.

  - 2nd International Course "Healthy Food Design" – Wageningen University & Research. 07-10 June 2021.

  - XIII Giornate Scientifiche SOI – Poster:

Buturi V.C., Mauro R.P., Sabatino L., Distefano M., Leonardi C. (2021). Biofortificazione della "Carota Novella di Ispica" mediante applicazioni fogliari di Ferro o Zinco. In XIII Giornate Scientifiche della Società di Ortoflorofrutticoltura Italiana, Catania. 22-23 June 2021.