Università degli Studi di Palermo

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SETTORE STRATEGIA PER LA RICERCA
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BIOFORTIFICATION AND INNOVATIVE SUSTAINABLE AGRONOMIC PRACTICES TO ENHANCE YIELD AND QUALITY OF LEAFY AND FRUITING VEGETABLES

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CICLO XXXV
ANNO CONSEGUIMENTO TITOLO 2022

In memory of my dear friend and colleague

Dr. Raffaele Stassi

1993 – 2020

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1.0. Introduction

Nowadays, climate change is considered one of the most relevant world challenges that need to be addressed as rapidly as possible. Data from the last few decades suggest that the climate macro-changes are the direct effects of intensive human activities (agriculture, industrialization, and urbanization) which have changed the atmosphere composition (IPCC, 2014). Indeed, according to the Intergovernmental Panel on Climate Change (IPCC, 2014), since the year 1750 the concentration in the atmosphere of CH_4, CO_2 and N_2O has significantly increased by 150%, 40% and 20%, respectively. These gases absorb the thermal radiations from the earth and atmosphere, warming the earth and causing a phenomenon called greenhouse effect (Mahli et al., 2021). On the other hand, as stated by Van Dijk et al. (2021), by 2050 the negative effects of climate change on agriculture along with the world increase population will cause an increase in world hunger by 30%. In this scenario, agriculture must face this double challenge and at the same time put special efforts in decreasing the environmental impact on natural ecosystem and on human health. Therefore, agronomic scientists from all over the world have been continuously studying and researching solutions to avoid an environmental disaster. Currently, many conventional and innovative green strategies are largely adopted to increase crop yield and ensure food safety (Bailey-Serres et al., 2019). As reported by Koli et al. (2019), the intensive application of synthetic fertilizers poses environmental problems to ecosystems, however, by 2050 the European Commission wants to substitute 30% of synthetic fertilizers with green options (Li et al., 2022). On this regards, natural product such as plant biostimulants are considered an eco-friendly tool both to overcome plant stresses induced by climate change and to decrease the use of chemical fertilizers (Hunter et al., 2017). The application of plant biostimulants can efficiently achieve a more sustainable agriculture while assuring crop yield under low inputs (Gupta et al., 2020). Concomitantly, another useful tool to increase yield, quality, and tolerance to soilborne diseases is the use of grafting. Grafting is an agamic propagation technique which consists in joining two bionts in such a way that they develop and grow forming a single plant.

Moreover, malnutrition is another critical challenge that modern agriculture must face both in rich and poor country and it is related to the lack of important micro-nutrients that negatively affects human health. Luckily, this kind of malnutrition can be overcome throughout dietary diversification, mineral supplementation, food fortification or via increasing the micronutrients concentration in edible crops (biofortification).

Taking into accounts the above considerations, it emerges that the agricultural sector has a very important role and responsibilities in the modern world. Indeed, it must provide valid answers to solve some of the problems that most afflict humanity. Vegetable crops represent one of the most important sectors of contemporary agriculture. This sector aims to increase quality, yield and sustainability of the production processes of several crops which are crucial for a healthy human diet. In the last decades the application of new sustainable substances and/or microorganisms coupled with valid eco-friendly cultural practices have opened new perspectives to reach successful achievements.

1.1. Plant biostimulants

For years, farmers largely used synthetic pesticides and fertilizers to ensure constant crop yield during the seasons and either under favourable or unfavourable cultivation conditions. However, to reduce the environmental impact of agriculture, in the last decades several green innovations have been proposed to replace synthetic agrochemicals. (Balaban et al., 2016).

The replacement models for these production systems propose the use of technologies that allow efficient management of water and mineral resources and allow higher quality and healthier productions (Colla and Rouphael, 2015). In this regard, thanks to the greater awareness both of people and researchers, greater attention has been paid to the protection of human health and the environment, highlighting the need to reduce the use of synthetic products in agriculture. Continuous research on the subject has led to the introduction of new technical means, characterized by a low environmental impact. Among these means, biostimulants are certainly the most promising and environmentally sustainable

innovation. These products which are not fertilizers, manage to increase the qualitative and quantitative characteristics of the crops. They do not replace existing fertilizers but integrate their action by promoting an increase the absorption and accumulation of macro and micronutrients into the plants and overall enhance their efficiency. Furthermore, the use of biostimulant products favours a rapid emergence of the seedlings and/or a rapid rooting process, anticipates yields, improves growth, fruit set, flowering, and the quality of the production (Colla and Rouphael, 2015).
Biostimulants are real technical means of production and as such they should require in-depth technical knowledge of the professional user. In fact, the result of applying biostimulants is not always the same and depends on the method of administration, the dose applied, genetic, agronomic, and environmental factors. With respect to these considerations, in recent years, several studies have been carried out to understand the real operating mechanisms and the optimal conditions of administration to provide guidelines to farmers who want efficiently to use these innovative tools.

1.1.1. Definition and legislation

The first review regarding biostimulants was published in 1994 by Herve (Herve, 1994) and it reports some characteristics common to all products: low dosage of application and low environmental impact. In 1999 Zhang and Schmidt (Zhang and Schmidt, 1999) of the Virginia Polytechnic Institute and State University wrote an article in the online magazine "Ground Maintenance" describing biostimulants as "*materials that, in minimal quantities, promote plant growth*". In this article the authors emphasize the quantities of product used (“minimal quantities”), underlining the substantial difference between biostimulants (used in low dosages) and fertilizers/soil amendments (applied in high quantities). The plant biostimulants cited in this article were two remarkable group: humic acids and seaweed extracts. Their effect on plants was suggested to be mainly hormonal.
It is only in 2007 that Kauffmann and collaborators (Kauffman et al., 2007) officially introduce the definition of biostimulant products, describing them as "*materials other than fertilizers that promote plant growth when applied in low*

doses", they also distinguish three groups of biostimulants: humic substances, algae extracts and amino acid-based products.

In 2011, a consortium of companies active in the fertilizer sector was established. In 2013 it evolved into the European Biostimulants Industry Council (EBIC). This consortium represents a connecting platform between companies operating in the biostimulants sector and aims to promote the creation of the biostimulants category at European level to overcome the regulatory differences between Member States.

In 2012 du Jardin (du Jardin, 2012) reported that plant biostimulants are heterogeneous materials and he suggested to divide them into 8 categories: humic substances, organic materials, beneficial chemical elements, inorganic salts, seaweed extracts, chitin and chitosan derivated, antitraspirants, free amino acids and N-containing substances. Interestingly, in this first attempt of classification, du Jardin did not incorporate any microbial biostimulants. A few years later, to clarify and order the fertilizers and biostimulants sectors, the European Commission has entrusted Professor Patrick du Jardin with the task of carrying out an in-depth bibliographic study on biostimulants and drawing up a well-defined subdivision. In 2015 du Jardin (du Jardin et al., 2015) published his classification which included the following categories: humic and fulvic acids, protein hydrolysates, algae extracts, chitosan and other biopolymers, inorganic compounds, mycorrhizal fungi, and growth promoting bacteria. Moreover, professor du Jardin defined plant biostimulant as follows; "*A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content*". In the same year, Colla and Rouphael (Colla and Rouphael, 2015) suggested to divide plant biostimulant in 6 non-microbial (chitosan, humic and fulvic acids, protein hydrolysates, phosphites, seaweed extract and silicon) and 3 microbial (arbuscular mycorrhizal fungi, plant growth-promoting bacteria and *Trichoderma* spp.) categories (Canellas et al., 2015; Gómez-Merino and Trejo-Téllez, 2015; Battacharyya et al., 2015; Savvas and Ntatsi, 2015; Ruzzi and Aroca, 2015; López-Bucio et al., 2015).

On 27 March 2019, the European Parliament approved the new Regulation on fertilizers which defines plant biostimulants as a "product that stimulates the

nutritional processes of plants regardless of its nutrient content with the only purpose of improving one or more of the following characteristics of the plant o of the rhizosphere of the plant: a) efficiency of the use of nutrients; b) tolerance to abiotic stress; c) qualitative characteristics; d) availability of nutrients confined in the soil or in the rhizosphere" (EU, 2019). Therefore, plant biostimulants are described based on agronomic effects and not based on their nature or mode of action. Furthermore, the regulation introduces two classes of biostimulants: microbial plant biostimulants and non-microbial plant biostimulants and, as reported by Rouphael and Colla (2020) this needs an advanced classification. A microbial biostimulant is composed of one or more microorganisms (e.g. *Azotobacter* spp, mycorrhizal fungi, *Rhizobium* spp. Or *Azospirillum* spp.). A non-microbial biostimulant includes the remaining part of the products not falling within the previous category (humic substances, animal and vegetable protein hydrolysates, plant extracts, algae extracts or beneficial mineral elements). Nevertheless, the explanation of the effects prompted by microbial or non-microbial biostimulant is judged as an important element to permit plant biostimulants to be placed on the European Union market. For this reason, EBIC members suggested to follows the guidelines of Ricci et al. (2019) "general principles to justify biostimulants claims" for trials and essays on plant biostimulants. As stated by the European Committee for Standardization (CEN), an European legal framework regarding the regulation of sampling, denominations, marking, and test methods are under development and will be published in 2024 (CEN, 2021). Consequently, robust, and reliable experimental data are needed to sustain the plant biostimulants claims and offer useful information for users. Recently, a database on biostimulant products, including plant trials and scientific data on crop performances, was launched (Bio4Safe, 2021).

In Italy, biostimulants are defined in Legislative Decree 75/2010 in the section products with specific action on plants - Biostimulants as "*products that bring to another fertilizer or to the soil or to plants, substances that favour or regulate the absorption of nutrients or correct certain physiological anomalies* ". Annex VI of Legislative Decree 75/2010 recognizes the following categories of biostimulants: inoculum of mycorrhizal fungi (which include mycorrhizae, bacteria, and

Trichoderma spp.), protein hydrolysates of animal or vegetable origin, plant and algae extracts. According to the legislative decree, the beneficial properties of a biostimulating product cannot be claimed when mixed with other fertilizers.

1.1.2. Non-microbial biostimulants

Humic substances

The humic substances together with the organic residues constitute the organic material present in the soil. They are complex macromolecules originating from the decomposition of the organic substance by microorganisms (Nardi et al., 1996). Humic substances are organic polymers with a highly variable composition, and therefore it is difficult to identify and classify them exactly. Traditionally they are classified according to their solubility: fulvic acids are soluble in an acid, neutral or basic environment; humic acids are soluble in an alkaline environment, have a lower acidity and oxygen value; humine is not soluble in water at any pH value and represents the inert component of humic substances (Stevenson, 1994).

The humic substances used to produce biostimulants derived mainly from fossil humus deposits, from agri-food by-products, from compost or vermicompost. Humic substances originating from peat and compost seem to have a greater bio-stimulating activity than those obtained from fossil humus deposits. Recently, synthesis humic substances have also been produced, the lignohumates. These compounds are made in the laboratory from lignin, speeding up a process that would take thousands of years in nature. The lignohumates differ from the humic substances for some characteristics: the material of origin of the lignoumates is young and not subject to long transformations as in the case of the humic substances, the basic material is homogeneous, the lignoumates do not contain polluting substances and the production process does not produce waste. Lignohumates, just like humic substances, possess high biological activity which makes them a good alternative to humic substances of natural origin (Ertani et al., 2011).

Humic substances can modify the chemical-physical properties of the soil, resulting in a structure capable of favouring the exchange of air, water drainage, root penetration, the development of telluric microorganisms, the cation exchange

capacity, the power buffer, the reduced loss of nutrients by leaching. They improve the general fertility of the soil. The humic substances determine different actions on the plant:

• stimulate the growth of hairs and root primordia, making the hypogeal apparatus more efficient (Atiyeh et al., 2002; Nardi et al., 1996; Canellas et al., 2002);

• involve a thickening of the cell walls of xylem vessels (Concheri et al., 1994);

• make nutritional elements more available to the plant that are easily assimilated by it, thanks to the high cation exchange capacity promoted by humic substances (Maggioni et al., 1987);

• thanks to the presence of radical transporters on the roots, humic substances favour the assimilation of inorganic nitrogen (Nardi et al., 2000a);

• promote enzymatic activity in processes such as glycolysis, the Krebs cycle, reductive nitrogen assimilation (Nardi et al., 2000b);

• stimulate ribulose-1,5-bisphosphate carboxylase, an enzyme involved in the Calvin cycle (Merlo et al., 1991);

• stimulate the mobilization of reserve substances in the leaves by-amylase which leads to an increase in the sugar content (Ertani et al., 2011; Muscolo et al., 2005; Nardi et al., 2000a).

More in details, fulvic acids are responsible for the chelation of metals such as iron and aluminium, they can permeate cell membranes and be assimilated by plants (Lobartinin et al., 1998). On the contrary, humic acids are unable to cross the cell plasma membrane and remain indivisibly attached to it. Despite several studies in this regard, the correct structure of humic substances is still not completely clear due to their heterogeneity and the multiplicity of chemical reactions involved in their synthesis. Over the last few years, the researchers' attention has focused on studying the ability of these substances to influence the development and growth of plants both directly (by influencing plant metabolism) and indirectly (by modifying the properties of the soil) (Cannellas et al., 2002; Nardi et al., 1996). As far as indirect influences are concerned, it is known that the characteristics of the soil are strongly influenced by the content of humic substances. In fact, structure, bulk density, water retention capacity and pH of the soil are positively correlated with a good content of SU in the soil (Concheri et al., 1996). The direct effects of humic

substances occur on the plant itself, in fact some studies have shown that they stimulate the emission of root primordia, leading to an increase in the size and complexity of the root system. Furthermore, humic substances play an important role in plant nutrition by making nutrients contained in the soil readily available (Concheri et al., 1994). In this regard, it should be remembered that the solubility of metals such as iron, zinc, nickel, and manganese is influenced by the interactions that occur between metal ions and soluble humic substances (Lombartini and Pape, 1998; Maggioni et al., 1987). The ability of these biostimulants to increase the absorption of nutrients is mainly due to the influence that these substances have on the synthesis and functionality of membrane transport proteins (Colla & Rouphael, 2019). In addition, plant roots through the extrusion of organic acids, can split humic macromolecules into lighter fractions (Nardi et al., 2002, Muscolo et al., 1998). Humic substances also positively affect secondary metabolism, helping the accumulation of antioxidant compounds and the activity of defence enzymes from oxidative stress caused by free radicals that are produced as a result of abiotic stress (Nardi et al., 2002). In addition to the effects mentioned above, humic substances are attributed hormone-like effects that promote growth and regulate the opening and closing of stomata (Nardi et al., 1994; Russel et al., 2006).

From an application point of view, the positive effects of this kind of biostimulant on plants are more visible in poorly fertile soils characterized by a low content of organic matter and with repeated root applications during the crop cycle (Canellas et al., 2015).

Protein hydrolysates

Protein hydrolysates are obtained by chemical or enzymatic hydrolysis of vegetable or animal protein matrix and are made up of a mixture of amino acids, nitrogen compounds and soluble peptides.

The most used matrix to produce animal protein hydrolysates is collagen obtained from the waste of the tanning industry, while to produce plant protein hydrolysates material from legumes is often used (Colla et al., 2015; du Jardin, 2015). However, chemical synthesis could be also employed for single or group of compounds.

Another nitrogen compound are the betaines, in particular glycine-betaine is known for its anti-stress effects on plants (Chen and Murata, 2011).

Plant protein hydrolysates were produced via enzymatic hydrolysis thanks to the action of proteolytic enzymes that break the peptide bonds of proteins. The hydrolysis occurs at temperatures lower than 60 °C so the thermolabile amino acids are not degraded. The final product contains fewer free amino acids and more soluble peptides, moreover, through this process, the thermolabile amino acids are preserved. Whereas, animal protein hydrolysates are made by acid or alkaline chemical hydrolysis, i.e. with the use of acids (such as hydrochloric and sulfuric acids) or alkalis (such as sodium, potassium or of calcium), these substances release chlorides and sodium ions making the hydrolysate more saline than those obtained by enzymatic hydrolysis, the product obtained is rich in free amino acids. The hydrolysis pressures and temperatures are high, specifically the pressures are 220.6 KPa and the temperatures are above 121 °C. This hydrolysis is quite intense and releases many free amino acids, however it determines the degradation of some thermolabile such as tryptophan, tyrosine, and histidine. These biostimulants of animal origin contain hydroxyproline and hydroxylysine which are closely related to collagen (Colla et al., 2015). Compared to animal protein hydrolysates, those of vegetable origin have a higher content of aspartic acid and glutamic acid, essential for the metabolism of inorganic nitrogen, while they contain less glycine and proline involved in the processes of osmoregulation and resistance to stress. All the compounds comprised in the protein hydrolysates showed a biostimulatory action on plants (du Jardin, 2012). A direct effect is the modification of the nitrogen uptake and assimilation via the regulation of key enzymes implicated in nitrogen metabolism. Moreover, hormone-like activities (Colla et al., 2015), chelating effects, and antioxidant action are also described (Rouphael and Colla, 2020). The indirect impact on plant growth and nutrition regards the increase of microbial activity, nutrients availability and overall soil fertility. Applications with protein hydrolysates can be carried out directly in the soil or in nutrient solution through foliar treatments or fertigation. The beneficial effects of these biostimulants concern the improvement of the quality of production with an increase in the content of carotenoids, flavonoids, polyphenols, and proteins. In addition, they improve the

absorption of macro- and microelements and interact with nitrogen metabolism by reducing the nitrate content in the plant (Colla et al., 2015). The biostimulating activity of protein hydrolysates is mainly due to the presence of peptides and amino acids. Amino acids, after being absorbed by the plant, are used for the synthesis of proteins, to produce energy, for the synthesis of molecules with high biological activity or as precursors of compounds that influence the quality of the product (Colla & Rouphael, 2019). The low molecular weight peptides, on the other hand, play the role of signal peptides and have an important action in the exchange of information between cells, in the regulation of growth and development and in the response to plant stress conditions. Furthermore, given that protein hydrolysates are made up of a mixture of molecules with high biological activity, they influence plant metabolism by showing hormone-like activities (Colla and Rouphael, 2019). From an application point of view, the biostimulating effect of protein hydrolysates of animal or vegetable origin is especially evident in crops grown in poorly fertile soils and with repeated applications during the crop cycle. The application of these products can influence plant nutrition, stress tolerance and product quality (Colla et al., 2015). Soil applications with protein hydrolysates are suitable for stimulating rhizogenesis and telluric microflora, while foliar treatments are more suitable for promoting plant growth, especially in conditions of intense metabolic effort.

Both protein hydrolysates, animal or plant derived, can be used in organic farming, the only limitation concerns those of animal origin for which the European Union has banned the use on edible plant parts (Regulation EU no. 354/2014).

Seaweed extracts

Seaweed extracts are one important category of non-microbial biostimulant, the use of them in agriculture became more widespread in the 1950s, thanks to extraction processes that led to the breakdown of algal tissue cells to obtain biostimulating molecules useful for plants (Battacharyya et al., 2015). Algae-based biostimulants have a long tradition of use in coastal agricultural areas, their production takes place in many countries of the world, especially in Asia (China, Indonesia, Philippines, Korea and Japan), while in Europe they are produced by France, Ireland, and

Norway. In the past, algae were used through direct use, it is only since the 1950s that the first commercial extracts were used (Battacharyya et al., 2015).

Seaweed extract are products obtained from the processing of red, green, or brown algae and contain polysaccharides, alcohols, phenols, and compounds with hormone-like activity. The species most frequently used to produce algae extracts are *Ascophyllum nodosum*, *Laminaria* spp, *Ecklonia maxima*, *Fucus* spp, *Himanthalia elongata*, *Durvillaea* spp, *Macrocystis pyrifera*, and *Sargassum* spp.

Despite the high interest in developing new algae extracts as biostimulants, few well-characterized products with reliable performance are on the market. This is mainly due to the high variability of the raw materials (due to the seasons and the time of harvest) (Marinho-Soriano et al., 2006; Marsham et al., 2007). This variability increases the difficulty of standardizing its composition and identifying its mechanisms of action. Therefore, for some years now, the scientific community and private companies/industries have given greater emphasis to the production of microalgae, which can be grown indoors, obtaining harvests with quantitative and qualitative characteristics that are stable over time (Chiaiese et al., 2018; Ronga et al., 2019). The most used microalgae species are the following: *Chlorella vulgaris*, *Acutodesmus dimorphus*, *Spirulina platensis*, *Scenedesmus quadricauda*, *Dunaliella salina*, *Chlorella ellipsoida*, *Spirulina maxima*, and *Calothrix elenkinii*.

To produce the biostimulant, the algae are harvested manually or mechanically along the ocean coasts (Canada, Ireland, South Africa, China) and subjected to washing, cutting and extraction. Today there are several methods of extraction: water, acid, or alkaline, cryo-processing, or high-pressure treatment. In addition, modern techniques which involve microbial fermentation of the basic plant matrix were also developed. However, today the most used method is the cold extraction in high pressure water, which prevents chemical alterations of the highly biological activity molecules.

Algae extracts have been shown to improve crop tolerance to abiotic stress, improve germination rate, growth, root development, fruit set, nutrient absorption, product quality, and determine an increase in chlorophyll of plant tissues (De Pascale et al., 2017; Rouphael et al., 2017).

Among the bioactive substances present in seaweed extracts, there are phytohormones (cytokinins, auxins, gibberellins, and abscisic acid) and other substances with hormone-like activity. In addition, traces of a precursor of the hormone ethylene, responsible for fruit ripening, have been found in some extracts of *Ecklonia maxima* (Nelson & Van Staden, 1985).

Seaweed extracts also contain polysaccharides and derivatives that activate the defence mechanisms of plants (alginates, laminarin, mannitol, carrageenan and fucoidans) (Di Stasio et al., 2018). Polysaccharides play the role of elicitors, favouring the activation of defence responses to stress in the plant (Rouphael et al., 2017a). Due to this mechanism, an increase in resistance to biotic stress is generally observed in plants treated with algae extracts (downy mildew, mites, aphids, nematodes, etc.). Furthermore, it has been proven that the use of algae extracts increases tolerance to abiotic stresses such as drought, salinity, and extreme temperatures (Trivedi et al., 2018). The mechanisms responsible for the increased tolerance to abiotic stress are different and not yet fully known. Among these, the increase in root development, the improvement of the nutritional status of the crop and the presence of abscisic acid which, by regulating the opening and closing of stomata, increases the resistance of plants to drought (Colla and Rouphael, 2019).

From an application point of view, the positive effects of algae extracts are more evident in crops grown in low fertile soils and treated repeatedly during the crop cycle. Foliar applications are preferred due to the rapidity of action and the reduced dosages used.

Chitosan

Chitosan is a linear polysaccharide obtained through the deacetylation of the biopolymer chitin, generally extracted from the exoskeleton of crustaceans (crabs, shrimps, etc.). Chitosan is employed in food, cosmetic, medical, and agricultural sectors (du Jardin, 2015). The effects of chitosan on plants are due to its ability to bind many cellular components such as DNA, cells membrane and specific receptors involved in the activation of defence gene, acting as elicitors (Hadwiger, 2013; Yin et al., 2010). Ferri et al. (2014) and Povero et al. (2011), conducting proteomic and transcriptome analyses of plants treated with chitosan, demonstrated

that the binding of chitosan to cell receptors prompts the hydrogen peroxide accumulation and Ca^{2+} leakage into the cells, which determine physiological changes since they play a fundamental role in signalling and in development regulation. Chitosan act as a stimulator in plant defence responses in case of wounding or pathogen infections (Doares et al., 1995; Yu et al., 2012). When plant tissues are damaged, pectic fragments of cell wall determine an upsurge of reactive oxygen species and pathogenesis-related proteins to defend plant against infections (Ferrari et al., 2013). A similar effect was observed in plants treated with chitosan (Malerba and Cerana, 2015; Mejía-Teniente et al., 2013; Pastor et al., 2013). Moreover, chitosan applications increase the chlorophyll content and is often linked to an enhanced growth and photosynthesis rate (Mondal et al., 2012). Chitosan can also confer resistance to abiotic stresses such as drought, salt, and temperature (Górnik et al., 2008.; Pongprayoon et al., 2013; Mahdavi, 2013).

Field applications of chitosan aimed to obtain several advantages such as plant protection to biotic and abiotic stresses, the increase of quality and secondary metabolites biosynthesis. Agronomic responses of crops depend on the chemical composition of chitosan and on the timing and rate of application. Since chitosan stimulates many plant responses (abiotic and biotic stresses tolerance, increase of plant growth and activation of secondary metabolites production) it could be very useful in horticulture as biostimulant. However, there is still a lot to study to do about chitosan, so further research is needed.

Silicon

After oxygen, silicon (Si) is the most abundant element on the planet (Ma, 2005). Even though most of Si in the soil is insoluble, some water-soluble compounds are also present. Si is considered a non-essential element for plant (Epstein, 1994), however, biostimulatory effects have been documented (Epstein, 1999; Ma and Yamaji, 2006). All elements which are not considered essential for plants but can influence plant physiological processes represent a different category of biostimulants (Savvas and Ntatsi, 2015). Many studies have demonstrated that mono- and dicotyledons plants react positively to a Si supply, especially when subjected to abiotic or biotic stresses (Epstein, 1999; Ma et al., 2007; Ma, 2004;

Fauteux et al., 2005). For this reason, Si is considered a “quasi-essential” element for higher plants since they may be stimulated by Si supply, whereas a shortage may show physical deformities, but it is not lethal for the plants (Rafi and Epstein, 1997; Ma and Yamaji, 2008). Si could be also useful in non-stressed crops (Adatia and Besford, 1986; Savvas et al., 2007), however, the effect of Si become more evident when it is applied to stressed plants (Ma and Yamaji, 2008; Guntzer et al., 2012). Many experiments have revealed that Si can mitigate biotic and abiotic stresses (Van Bockhaven et al., 2013; Zhu and Gong, 2014). The beneficial effects of Si are attributed to the anatomical modifications of plants tissues caused by silica accumulation in cell walls (Raven, 2001; Piperno et al., 2002; Ma, 2004). Further studies revealed that Si beneficial effects are due to its effect on plant metabolism (Epstein,1999; Liang et al., 2003; Zhu et al., 2004). However, the knowledge on the impact of Si on plant metabolism (stress tolerance and growth advantages) is not well understood (Liang et al., 2007; Guntzer et al., 2012; Gonzalo et al., 2013).

Si can be employed as biostimulant via foliar or soil application. As stated by Wang et al. (2015), since Si has a very low mobility in the phloem (Pilon et al., 2013), foliar application might prevent chemical or physical Si immobilization and, consequently, allows a more direct functioning. Foliar treatments were accomplished using highly soluble silicate such as sodium silicate, silicic acid, and potassium silicate. However, application via the soil is more efficient in enhancing the Si concentration in plant tissues and, therefore, stimulating the plant (Haynes, 2014).

Nowadays, the application of Si in horticultural crops is limited. However, since Si is the only element which confers tolerance to multiple stresses and is not harmful for humans and environment, its use as biostimulant is estimated to increase significantly in the future.

Phosphite

During the last 30 years, phosphite ($H_2PO_3^-$) or its conjugate phosphorous acid (H_3PO_3) were used as pesticide, fertilizer, and biostimulant. When used as biostimulant, phosphite increase the nutrient uptake and assimilation, abiotic stress tolerance and crop quality. Furthermore, the application of phosphite is widely used

for controlling pathogens such as Oomycetes and bacteria (Lobato et al., 2008 and 2010; Silva et al., 2011). Recent studies have underlined that phosphite has an important role in enhancing plant metabolic processes (Gómez-Merino and Trejo-Téllez, 2015). In several field and greenhouse experiments, many authors revealed that foliar applications of phosphite increased yield and quality of different horticultural species such as celery, onion, potato, strawberry, and sweet pepper (Rickard, 2000). However, other researches on *Brassica nigra* and *Brassica napus* revealed that phosphite is not suitable as the plants supplied showed a reduction in growth and phytotoxicity effects (Carswell et al., 1996, 1997; Förster et al., 1998; Varadarajan et al., 2002). Thus, more specific trials are needed to improve the knowledge about the role of phosphite in plant metabolism. A deeply understanding of phosphite functioning mechanisms is required to provide new knowledge for the horticultural sector and to promote it as biostimulant. On the other hand, it is also important to find the proper source, rate, method, and stage of application. How phosphite is employed as biostimulant must be discussed with experts to achieve goals related to quality and yield. Furthermore, international requirements and regulations need to be taken into consideration for an appropriate use of this biostimulant in the horticultural sector.

1.1.3. Microbial biostimulants

Arbuscular mycorrhizal fungi

The microorganisms present in the soils play an important role in the fertility, influencing the biochemical and physiological processes of the soils and improving the biological and nutritional properties. The mechanisms of action of microorganisms towards the plant are different, the most important are related to the synthesis of beneficial compounds, the increased absorption of nutrients and the prevention of pathogenic attacks (Azcòn-Aguilar & Barea, 2015).

Plants and fungi have co-developed since the origin of terrestrial plants, among them arbuscular mycorrhizal fungi are probably the most common plant symbionts (Newman and Reddell 1987). Indeed, numerous plant species such as *Solanaceae*, *Alliaceae*, fruit trees, ornamental and herbal plants can form a mutualistic symbiosis

this kind of fungi (Smith and Read, 2008). Arbuscular mycorrhizal fungi take from the soil and transfer mineral elements such as phosphorus, nitrogen, sulphur, potassium, calcium, copper, and zinc to the host plants through a dense network of hyphae that acts as an auxiliary absorption system (Giovannetti et al., 2001; Avio et al., 2006). From host plants, fungi obtain sugars that they would otherwise not be able to synthesize as chemoheterotrophic organisms. It is important to note that arbuscular mycorrhizal fungi can colonize the roots of many plants and are not host-specific. The structure that allows the association between plant and fungus is called arbuscules, it develops within the radical cortical cells and takes the form of a shrub (hence the name) (Colla & Rouphael, 2019).

These fungi are known to be an efficient mean to increase drought tolerance of horticultural crops (Asrar et al., 2012; Wu et al., 2013; Jayne and Quigley, 2014; Baum et al., 2015). The inoculation with arbuscular mycorrhizal fungi prompts modification of the plant root architecture (length, density, and number) (Wu et al., 2013). Thus, a better root system facilitates the uptake of water and nutrients even when plants are cultivated in water-scarce conditions (Smith and Smith, 2011). Another important effect of arbuscular mycorrhizal fungi is the alleviation of salt stress. Even though the salinity negatively influences arbuscular mycorrhizal growth (Juniper and Abbott, 2006), inoculated plants grown under salinity have improved performances (Wu et al., 2010; Khalil, 2013). For example, Navarro et al. (2012) and Gómez-Bellot et al. (2015) reported that arbuscular mycorrhizal fungi can decrease the translocation of toxic ions such as Na^+ and Cl^- and, concomitantly, increase the uptake of useful elements like P, K, Ca, and Mg of plants cultivated under saline stress. Moreover, several authors reported that arbuscular mycorrhizal fungi have a significant role in the mitigation of heavy metal negative effects (Andrade and Silveria, 2008). The protection effects are attributed to a physical immobilization of heavy metal via a binding with the fungi hyphae (Mozafar et al., 2002; Kumar et al., 2015). Evidence revealed that arbuscular mycorrhizal fungi symbiosis can alter host plant primary and secondary metabolism, eliciting the biosynthesis of phytochemicals (Sbrana et al., 2014). These physiological changes might be attributed to the activation of host defence mechanisms in colonized plant roots (Strack and Fester, 2006). Moreover, the

higher content of nutrients can influence the biosynthesis of secondary metabolites (e.g., ascorbic acid and flavonoids) (Schliemann et al., 2008). As stated by Lohse et al. (2005), Krebs cycle and plastid biosynthesis is also influenced by arbuscular mycorrhizal colonisation.

As arbuscular mycorrhizal fungi are symbiont organisms, therefore the availability of highly infectious mycorrhizal fungi represents the most important requirement for their use in agriculture. Infectivity depends on environmental conditions, the germination of fungal spores and the extent of root colonization. It is also necessary to specify that there are dormant spores and others do not, those that do not show dormancy germinate after a few days, while those that show dormancy do not germinate even if in the presence of ideal conditions (Tommerup, 1983). Precisely for this reason, in some cases the inoculum must be subjected to cold treatment to remove dormancy. Other parameters that influence spore germination are the pH and the presence of heavy metals (Giovannetti et al., 2010).

From the application point of view, these biostimulants are used in the transplant phase of crops and, in some cases, a second inoculum in the middle of the production cycle is also provided. To obtain good results it is necessary to have a careful agricultural management of the crop: deep tillage destroys the fungal hyphae compromising their functionality, excessive inorganic phosphate fertilizations reduce mycorrhization, while the use of pesticides has very variable and unpredictable effects (Helgason et al., 1998; Jansa et al., 2002; Hijri et al., 2006; Verbruggen et al., 2010; Oehl et al., 2003; Castillo et al., 2006; Brito et al., 2012). The choice of the crop is also important as some genotypes could present phenomena of incompatibility with the fungi.

The production of these microorganisms takes place by means of host plants as they are obligate biotrophic organisms. Propagation occurs in containers as there is a greater risk of contamination in the open field. The production involves the use of mother cultures in which the fungal strains have been previously isolated; the spores are extracted from the growth substrate of these plants and, before being sold, are analysed under the microscope to determine the absence of contamination (Feldmann and Schneider, 2008; Douds et al., 2012; Berruti et al., 2016; Gerdemann & Nicolson, 1963). The major limitation on the use of arbuscular

mycorrhizal fungi is the propagation on a large scale because of their biotrophic features and the low knowledge about mycorrhizal populations in agroecosystems. Further studies must be focused on considerate the interaction among fungi strains, plant species and environmental conditions, production of high quality inocula which have a high quantity of infective propagules, higher standardization of the inocula production, elucidate the molecular mechanisms behind the positive effects of the arbuscular mycorrhizal inoculation.

Trichoderma spp.

Trichoderma spp. is a very important fungal genus which encloses more than 200 species (Atanasova et al., 2013). Member of this genus are cosmopolitan and can be found in a wide range of ecosystems (Kubicek et al., 2008). *Trichoderma* species often parasitize other fungi that could be found in soil or associated with plants (Jaklitsch, 2009; Chaverri et al., 2011). Nowadays, *Trichoderma* inoculations are widely used as plant biostimulant for several positive effects. Many studies underlined that *Trichoderma* could enhance growth and development of several horticultural crops (Chang et al., 1986; Hermosa et al., 2012; Studholme et al., 2013). Studies from Samolski et al. (2012) and Zhao et al. (2014) revealed the potential use of *Trichoderma* as biofertilizers as it increases the solubility of nutrients and the nutrient uptake efficiency. These beneficial effects could be explained via the modulation of root architecture and the release of compounds that enhance mineral availability such as organic acids (Samolski et al., 2012; Zhao et al., 2014). At this regard, one of the most important roles of *Trichoderma* is the solubilization of phosphate (Rudresh et al., 2005).

Plant inoculated with *Trichoderma* also showed an increase of auxins, ethylene, gibberellins, enzymes, antioxidants, phytoalexins and phenols concentrations that confer environmental stress tolerance (López-Bucio et al., 2015).

Trichoderma application is also useful for overcoming salt (Rawat et al., 2013; Hashem et al., 2014) and drought stresses (Contreras-Cornejo et al., 2009; Shukla et al., 2012). The increase of drought tolerance can be explained by the activation of antioxidant response of plants prompted by the fungal inoculation (Mastouri et al., 2012).

As seen, *Trichoderma* spp. is a promising fungi genus useful for the expansion of sustainable agriculture. Consequently, the is the necessity of speed up its integration in the traditional agricultural practices and to develop efficient production and supply systems.

Plant growth promoting bacteria

Microorganisms with a biostimulant activity are often present in numerous bacteria phyla, comprising strains from genera *Bacillus*, *Pseudomonas*, *Azospirillum*, *Azotobacter, Alcaligens, Arthobacter, Agrobacterium, Burkholderia*, *Comamonas*, *Pantoea*, *Rhizobium*, *Serratia*, and *Variovorax* (Kloepper et al., 1989). The effects and mode of actions of this plant growth promoting bacteria (PGPB) are different (Dey et al., 2004). Among these mechanisms there are changes of the hormonal concentration in host plant, the biosynthesis of volatile compounds, the enhancement of nutrient availability or the increase of abiotic stress tolerance (Choudhary et al., 2019). Indeed, as reported by Bal et al. (2013) and Chen et al. (2013), PGPB can decrease the concentration of ethylene in plant tissues and, consequently, increase plant growth parameters. Another plant hormones involved in the PGPB effects are auxins which are a well-known plant hormone that promote growth (Enders and Strader, 2015). Moreover, abscisic acid, cytokinins, and gibberellins are also involved in the activity of PGPB with beneficial effects for plants in terms of growth and development (Kudoyarova et al., 2014; Pospisilova, 2003; Belimov et al., 2014).

PGPB are also useful to increase plant tolerance to water stress conditions and it was hypothesized that the increase of abscisic acid concentration prompted by the PGPB inoculation decreases water loss (Salomon et al., 2014). However, the real role of abscisic acid in PGBP effects is not well known and further studies are necessary.

It is well documented that PGPB inoculation boost the nutrient content of host plant (Canbolatet al., 2006; Wani et al., 2007; Lai et al., 2008). However, as the abscisic acid, the real mechanisms are not known yet. In addition, each mineral element answers differentially. PGPB are able to increase N and K uptake as well as phosphate solubilization (Lai et al., 2008; Canbolatet al., 2006; Singh et al., 2010).

Another positive effects of PGPB are the increase of abiotic stress tolerance even though the real mechanism is not well understood. However, considering that i) all stresses determine an upsurge of reactive oxygen species production which causes oxidative damage (Gill and Tuteja, 2010); ii) the oxidative damage can be lightened by the dissipation of surplus energy in chloroplasts via carotenoids (Bassi and Caffarri, 2000); iii) PGPB inoculation increase the carotenoid concentration in plants (Chakraborty et al., 2013), the scientific community hypothesized that PGPB can increase the abiotic stress tolerance via a modulation of carotenoids concentration in plant tissues (Ruzzi and Aroca, 2015).

Another important consequence of PGPB application under abiotic stress circumstances is the enhancement of the leaf water status (Naveed et al., 2014). This equilibrium in water status could be attributed to the ability of PGPB to modulate aquaporin genes expression, however, this is only a theory that need to be elucidate with further analyses.

Therefore, it is evident that inoculation with PGPB has considerable advantages for plants, however, the precise mechanisms of operation have yet to be understood.

1.1.4. Use of plant biostimulants in the horticultural crop sector and variable that can affect their effectiveness

In open field operating conditions and in protected environment there are numerous variables that can influence the effects of biostimulant. In fact, it is of fundamental importance to carry out many experimental tests to precisely define the conditions that maximize the effectiveness of the application. The effects of commercial products can vary greatly depending on the genotype, the phenological stage of the plant, the cultivation practices, the growing environment, the doses, and methods of application (Colla & Rouphael, 2019).

The activity of biostimulants depends primarily on the genotype used in cultivation, for example crops treated with arbuscular mycorrhizal fungi show different susceptibility to mycorrhization, therefore their productivity and development vary according to the degree of interaction with the mycetes (Rouphael et al., 2010).

The response of a plant to the biostimulating treatment also depends on the physiological and morphological conditions which can vary according to the

phenological stage. For example, the nutritional status of the plant influences the degree of mycorrhization and the absorption of biostimulants administered through foliar treatments. Similarly, thermal excesses or water stresses cause the stomata closure, reducing the leaf absorption. It is for this reason that it is recommended to administer the biostimulant through the roots when unfavourable environmental conditions are present. In addition, the age of the leaf also affects the absorption capacity, indeed young leaves are more efficient than old ones since they have a greater number of stomata and a less thick cuticle.

Furthermore, agronomic practices such as irrigation, fertilization and phytosanitary defence can influence the activity of biostimulants. In fact, frequent and too deep soil managing reduces the activity of mycorrhizal fungi, while minimal handling allows the development of a large hyphal network. As regards fertilization, in general, the contributions of organic matter with a high degree of humification, together with the use of crop rotations, favours the establishment of a microbial population, reducing the need for exogenous inoculations. Treatments with pesticides can limit the effectiveness of the inocula, although in general insecticides, acaricides, nematicides and herbicides have a mild effect, while negative effects occur with the application of fumigants. As for foliar treatments, care must be taken when a phytosanitary treatment is associated with the biostimulating treatment, in this case it is necessary to check for any incompatibilities between the products to avoid phytotoxicity phenomena. Generally, biostimulants are more effective in managed crops with low agrochemical use.

The cultivation environment plays an important role in the activity of biostimulants, the pedoclimatic characteristics considerably affect the activity of the biostimulants. In general, the response of crops to biostimulant treatments decreases as soil fertility increases. This effect is particularly marked in the case of humic acids. The soil temperature plays a very important role in the case of microbial microorganisms, suboptimal temperatures reduce the effectiveness of the biostimulant. Another important environmental factor is humidity: if high, it allows the stomata to open with a consequent increase in foliar absorption, if low, it inhibits the entry of the biostimulating product due to the closure of the stomata. Light

intensity also plays an important role in the proper functioning of biostimulants, in fact it regulates the stomatal opening. To ensure maximum effectiveness of the treatment, the plants should be treated in the central hours of the day, when the stomata are open due to the high brightness; however, the treatment should only be carried out when the humidity conditions are optimal, otherwise it would be advisable to act in the early hours of the morning.

The variability of plant response to biostimulant treatments is particularly evident in open field crops since the soil and climatic conditions are not always optimal and the treatments are rarely carried out for economic reasons. On the contrary, in protected crops, where repeated treatments are carried out and the environmental parameters are well controlled, it is possible to obtain more marked effects and results that can be reproduced over time.

The choice of the type of biostimulant, the dose, the moment and the method of application depend on the aims to be achieved by the soil and climatic conditions. Increasing doses of biostimulants up to the optimal dose generate an increase in the response of the plant in terms of biomass and production; however, by exceeding the optimal dose, the response of the crop becomes constant or in some cases decreasing due to phytotoxicity phenomena (Colla & Rouphael, 2019). Usually, the repeated foliar administrations are those that ensure the best results on the crop, especially if preceding stress events.

Each class of biostimulant must be applied to the plant according to precise doses and methods. From an application point of view, it is of fundamental importance to consider the recommended doses on the label, since over-dose treatment can cause phytotoxicity phenomena while, on the contrary, too low a dosage could cancel the effect of the biostimulant itself.

As for the methods of administration, the biostimulants can be distributed to the crop via the leaves or via the roots. The best results are achieved when compounds made up of different substances are used that can act synergistically to optimize the biostimulating capacity of the product.

In vegetable crops, biostimulant products are used very frequently both in the nursery, both in open fields and in a protected environment. In nurseries, biostimulants can be used in the production of young seedlings of vegetable species

to increase the quality, growth rates and improve post-transplant performance of the crop. Seed companies often apply biostimulants to seeds using algae extracts, protein hydrolysates and microorganisms to promote rapid and uniform seed germination, high seedling vigour and higher resistance to abiotic stress (Demir et al., 2006; Kanmaz et al., 2018). Biostimulants can also be administered via the growing medium as in the case of inoculations of mycorrhizal fungi, growth promoting bacteria, humic substances, and protein hydrolysates (Tullio et al., 2007). During growth in the nursery, it is also possible to apply biostimulating substances to young plants via foliar and root.

In open field horticulture, the soil administration of inoculations of mycorrhizal fungi to the plant is particularly recommended in soils characterized by physico-chemical anomalies and for long-cycle crops. Biostimulant products such as humic and fulvic acids, protein hydrolysates and algae extracts can be applied at the root level by drip irrigation system to promote root development and nutrient absorption efficiency (Conversa et al., 2013; Colla and Rouphael, 2015).

In protected horticulture, the application of biostimulant products is more widespread thanks to the higher profitability of the crops themselves and the more favourable environmental conditions. Particularly, in the Mediterranean horticulture, the cultivation of vegetables is mainly conducted in greenhouses and in tunnels covered by plastic films and without heating and ventilation systems. In these conditions, plants can suffer stress due to the high temperature range, excessive relative humidity of the air and reduced light intensity. Furthermore, the high cultivation specialization and the use of intensive cultivation techniques lead to a frequent loss of soil fertility. In these contexts, biostimulants can significantly contribute to mitigating environmental stresses. The contribution of biostimulants can also be a valid help to favour fruit set and growth in colder cultivation periods and with less light intensity. For these purposes, algae extracts, plant extracts or protein hydrolysates are preferred.

1.1.5. Perspective and importance

The use of biostimulant products in horticulture optimizes the absorption of nutrients by decreasing the quantities of fertilizers used in agriculture. In addition,

they help to reduce the use of pesticides, to increase the quality of horticultural products, and to respond perfectly to global challenges such as increased production and climate change while ensuring greater tolerance of the plant to abiotic and biotic stresses. Most of the published scientific papers report the effects of the application of biostimulants on plants, but few have evaluated their effects on the physiology and biochemistry of these. However, recent articles have focused their attention on the mechanisms of action of these products. The characterization of a biostimulant should be carried out based on the plant's responses, specifying the physiological and metabolic effects involved. Therefore, it is important to carry out specific studies in different environments and with multiple genotypes to better understand the biochemical characteristics of biostimulant products. This approach would allow greater awareness of biostimulants effects, of the mechanism of action and of the functions of the individual components. In this way, precise guidelines could be issued for their use in the field. Another crucial aspect is the support to farmers, in fact they would need specialized technicians to help them understand when, how and why to use a specific biostimulant.

The interest in the creation and use of these products is confirmed by the constantly growing market. In fact, in 2015 the European market stood at around half a billion euros. The global market in 2018 was worth 2 billion dollars but it is estimated that by 2021 this figure will reach 3 billion and by 2025 to 4 billion dollars (Colla and Rouphael, 2019). The reasons for this rapid rise are mainly related to the need to reduce damage to crops due to abiotic stress and the need to increase the inputs use efficiency. Furthermore, some biostimulant products have been shown to improve the characteristics of agricultural products (size, colour and absence of defects), increase the nutraceutical value and reduce the presence of harmful compounds such as nitrates and heavy metals.

In agriculture, algae extracts are used to a greater extent for their biostimulating action (76%), then protein hydrolysates (61%), humic and fulvic acids (52%) and, finally, microorganisms (20%) are used. (Cittar, 2018).

In conclusion, the application of biostimulants in horticultural crops allows the realization of a model of sustainable intensification of agricultural production which is promoted by FAO and also allows farmers to "produce more with less",

preserving resources and reducing the negative impact of horticultural crops on the environment.

1.2. Biofortification

1.2.1. Human malnutrition and biofortification

Contemporary technologies employed in agriculture are useful for satisfying the world food demand and feed more people. In the last decades, the most efforts were focused on the production increase rather than food quality. Currently, agriculture is going through a transition period from a phase of producing larger quantities of food, to a phase of producing foods enriched in micronutrients. This new approach should help fight micronutrient malnutrition, also called "hidden hunger", especially in poor and underdeveloped countries, where, in many cases, diets are predominantly made up of micronutrient-poor foods. Indeed, around the world, more than 2 billion people suffer from nutritional deficiencies of microelements, with reference to iron, iodine, zinc, and vitamin A (Daher, 2001). It was noted that this condition particularly affects developing countries, where the quality and variability of food is not sufficient to satisfy different needs (Daher, 2001). Indeed, developing countries depends mostly on staple food which has high content of carbohydrates but low content of vitamins and minerals. This situation will lead to the establishment of a micronutrient deficiency situation, especially of iron, iodine, zinc, and vitamin A (Welch, 2005). What emerges as a direct consequence of this nutritional deficit, are the significant growth disturbances that are mainly evident in pregnant women and in developing children. Unfortunately, further compromises occur to the detriment of the immune defences, especially towards certain types of diseases such as HIV, malaria, and tuberculosis, since they are often responsible for metabolic imbalances and physiological debilitations (Initiative, 2006). Deficiencies in micronutrients can be determined either by a single nutrient or by several concomitant deficiencies.

Micronutrient malnutrition was discussed by the international community in the 1980s, highlighting a major public health challenge in 1990 at the United Nations-backed World Summit for Children with UNICEF support. What emerged from

many researches is that humans need at least 22 mineral elements for their well-being (Welch & Graham, 2004; Graham et al., 2007). These elements can be provided by an appropriate diet. However, it is estimated that over 60% of the 6 billion people in the world are iron (Fe) deficient, over 30% are zinc (Zn) deficient, 30% are iodine (I) and 15% are deficient in selenium (Se). Furthermore, deficiencies in calcium (Ca), magnesium (Mg) and copper (Cu) are common in many developed and developing countries (Frossard et al., 2000; Welch & Graham, 2005; Rude & Gruber, 2004; Grusak & Cakmak, 2009). This situation is attributed to crop production in areas of low mineral availability and/or consumption of (basic) crops with inherently low tissue mineral concentrations, aggravated by a lack of fish or animal products in the diet (Welch & Graham, 2004, Graham et al., 2007).

It is necessary to emphasize the importance of mineral substances which are considered essential nutrients since they are not synthesized by the human body and therefore must be consumed through the diet. Consequently, in order to maintain good health and improve human well-being, the intake of all the mineral elements that the human body needs is very important. Among the essential nutrients for the human body, some have a function directly related to energy production and must be taken in the order of grams/day, these are defined as macronutrients. These micronutrients collectively play a fundamental role in the human body, some of these acts as cofactors for the functioning of many enzymes and therefore regulate important functions and metabolic processes. Deficiencies in mineral nutrients affect both underdeveloped areas and industrialized countries, affecting two thirds of the world population (O'hare, 2015).

Mineral malnutrition can be addressed through dietary diversification, mineral supplementation, food fortification and/or increasing mineral concentrations in edible crops (biofortification). Biofortification is the development of micronutrient-dense core crops using traditional best farming practices and modern biotechnology (Nestel, et al., 2006). However, strategies to increase dietary diversification, mineral supplementation and food fortification have not always been successful. For this reason, the biofortification of crops through the application of mineral fertilizers, combined with breeding varieties with a greater capacity to acquire

mineral elements, is recommended as an immediate strategy not only to increase mineral concentrations in edible crops, but also to improve yields on barren land.

The importance of proper nutrition to prevent the onset of diseases has been underlined by many scientific researches that recommend the consumption of fresh fruit and vegetables and the reduction of saturated fat and sodium consumption (Wang, 2016).

Vitamins and micronutrients are fundamental factors involved in the growth and development of plants and humans. All these important compounds have essential roles in physiological processes of cells such as redox reaction and co-factors for several enzymes. In humans, vitamins are assumed via the consumption of plant or animal sources (Grivetti and Ogle 2000; Asensi-Fabado and Munné-Bosch 2010). Vitamins (A, E, K, B and C) can be easily found in fruits and vegetables and have a high antioxidant ability. All these vitamins are produced and accumulated in plant tissues. To satisfy the metabolic demand, humans need at least 49 nutrients, including more than 20 mineral elements which are fundamental for a good human health. Among them, we can distinguish macro-elements (P, K, S, Na, Ca, Mg and Cl) and trace elements (I, Mo, Fe, Cu, Zn, Mn, Cr, Se, F, Sn, Si and V). Unfortunately, as previously reported, the evolution of agriculture causes an increase of staple food production and a reduction of fruits, vegetables and legumes which are the main source of micronutrients for humans (Welch and Graham 2004). Fruits and vegetables also have a fundamental role in human health since they comprise important compounds such as vitamins, nutrients and phytochemicals which can decrease the risk of human illnesses.

Information on the main biofortification strategies, with reference to vegetable crops, are provided below.

1.2.2. Strategies of biofortification

Agronomic biofortification

Agronomic biofortification is the simplest methods to biofortify foods. This strategy used fertilizers, sprayed on plant leaves or administered to the soil, which contain the trace element of the biofortification program (Weng et al. 2008). If the

microelements are supplied via the soil, particular attention must be posed to agronomic practices such as tillage and water management which can influence the availability of the nutrients in the soil. For this reason, foliar applications are the better option for agronomic biofortification, and also require less amount of microelement (Prasad et al. 2014). The critical point of this strategy is the definition of the microelement optimal dose. Indeed, these microelements, if supplied in over-dose with toxic effects to the plants. Consequently, many trials must be conducted to establish the best dose for the crop, considering that different varieties might answer differently.

Breeding and biofortification

The conventional breeding biofortification strategy is based on natural variation of crops. For example, it was reported that folate content of vegetables increased two times in new breeding lines (Hanson and Gregory, 2011). There are different criteria which must be respected in breeding programs: (i) the productivity of the crop must be preserved or increased; (ii) the micronutrient concentration must increase in such a way as to provide beneficial effects for human health; (iii) the genetic trait need to be stable between consecutive generations; (iv) the bioavailability of the microelement must be maintained after cooking; (v) the taste of biofortified food has to be accepted by consumers; (vi) the final product of the breeding program must be accepted by farmers. At this regard, it is important to communicate to farmers the importance of growing biofortified crops (Welch and Graham, 2004). The main problem of breeding biofortification is the finding of high natural variability which is less and less present in nature.

Genetic engineering

Biotechnology application in the biofortification field is a powerful tool used worldwide to increase the concentration of vitamin and minerals in crops. Modern genetic engineering techniques allow to integrate the desired trait(s) which is not possible via conventional breeding (Chaudhary et al., 2019; Rana et al. 2019). The main objectives to achieve with this technique are: (i) increase the plant uptake efficiency for minerals, in this case is important to verify if the micronutrient in the

soil is available for plant before absorption, otherwise the enhanced uptake efficiency will be useless; (ii) redistribute the nutrients in the plant especially to the edible portions; (iii) increase the efficiency of the biosynthesis of biochemical compounds; (iv) decrease the concentration of anti-nutritional compounds which interfere with the bioavailability of micronutrients; (v) increase the expression of a gene already enclosed in the plant genome.

1.2.3. Main trace elements used in agronomic biofortification programs

Iodine

Iodine is an essential micronutrient for humans as it is present in the thyroid hormones. The soil contains low quantities of iodine, thus plants have low availability of this trace element. Consequently, the iodine concentration in plants is unsatisfactory for humans in comparison to their nutritional needs (Halka et al. 2019). The deficiency of this element can cause some malfunctions in our organism, and it represents the greatest cause of brain damage in the fetus and newborns, and of delayed psychomotor development in children. In fact, iodine deficiency is the first cause that leads to goiter and cretinism. It is estimated that nearly one billion people suffer from goiter, and that half of the population suffering from this problem is in Asia. About 16.5 million people worldwide suffer from cretinism, and another 49.5 million are likely to suffer from less severe forms, with effects on the brain yet to be quantified.

Diet is the main resource for iodine intake, mainly taken through the consumption of fish. In vegetables, on the other hand, the amount of iodine varies, depending on the soils on which they are grown, but it is often too low compared to human needs. The increase of iodine in the cooking salt is a widely adopted strategy to mitigate the iodine deficiencies in the human diet. Despite this, the consumption of iodized salt is still not widespread in Europe, and even in Italy only a small percentage of the population buys iodized salt. It should be remembered, however, that the intake of salt, even if iodized, must in any case be limited in relation to the risk of cardiovascular diseases.

The increase in the concentration of iodine in vegetables to produce functional foods represents a sector of study of particular interest and with a potential market.

In Italy, recent experiments have developed a procedure that has led to obtaining and trading potatoes enriched in iodine through agronomic biofortification: iodine, brought to potato plants by foliar fertilization, is translocated and effectively accumulated in the tubers in quantities functional to the diet, that is for a minimum content of 15% of the Recommended Daily Allowance on 100 grams of fresh product (Directive 90/496 / EEC of 24/09/90). Interestingly, about 80% of the human's iodine requirement comes from vegetable crops which contain about 99% of bioavailable iodine (Welch and Graham, 2004).

Selenium

Selenium is a trace element which is necessary by human in small quantity but have fundamental functions for human growth and development. Indeed, selenium deficiency can cause many health problems such as cancer and cardiovascular illnesses.

Since 1957, Selenium has been considered an essential element as a component of the enzymes glutathione peroxidase, selenoprotein P, tetraiodothyronine and thyroxine 5 'deiodinase (Turhan et al., 2003). Selenium is a "non-metal" that is present in small quantities in humans. Its presence is important to counteract aging, to maintain the elasticity of the tissues, to eliminate strongly oxidizing substances and to maintain the mobility of spermatozoa.

Selenium is an element that guarantees the normal functioning of an organism (Kieliszek et al., 2013) but, unlike the other elements, it has smaller ranges between food deficiency (< 40 μg day^{-1}) and toxicity (> 400 μg day^{-1}). Gissel-Nielsen (1984) and Marschner (2011) found that the minimum selenium concentration for animals and humans is approximately 50-100 μg Se kg^{-1} DW in forage / food. According to the United States Department of Agriculture (Turhan et al., 2003), the daily human requirement is 50-70 μg. Low dietary selenium intake may be associated with conditions of oxidative stress, hyperthyroidism, immune system weakness, cardiovascular disease, reduced male fertility and increased cancer risk (Rayman et al., 2012; Drutel et al., 2013; Mistry et al., 2012). In contrast, adequate dietary intake has clear health benefits beyond meeting basic nutritional needs. For example, some forms of selenium such as methyl selenocysteine (MSeC) exhibit

anti-carcinogenic activity against different types of cancer (Dennert et al., 2011). Recent studies have demonstrated the role of plants as the main food source of this element; consequently, there has been a growing interest in increasing the Se content in plants used for human nutrition (Chen et al, 2002; Hawkesford and Zhao, 2007; Schiavon et al., 2013). In this regard, the introduction of this element through fertigation is a very effective way to overcome the poor bioavailability.

Although selenium is essential for humans, it is not so important for plant growth and development (Fordyce, 2013). Plant roots can take up selenium as selenate, selenite or organo-selenium compounds, such as selenocysteine (SeCys) and selenomethionine (SeMet), but they cannot absorb the colloidal elemental selenide selenium or the metal (White et al., 2004). Selenium is transported across the plasma membrane of root cells by high affinity sulphate transporters (Terry et al., 2000; White et al., 2016; Broadley et al., 2006; Hawkesford & Zhao, 2007), while selenite is believed to be transported by phosphate transporters (Li et al., 2008). Selenite is rapidly converted into organo-selenium compounds in the root, while selenate is delivered to the xylem and transported to the shoot, where it is assimilated into organo-selenium compounds and redistributed within the plant in a similar way to S (Broadley et al., 2006; Hawkesford & Zhao, 2007; Li et al., 2008).

Selenium concentrations in the shoots tend to increase to the maximum during seedling growth, and then decrease before or after flowering, which is consistent with transcriptional analyses suggesting that Se/S assimilation occurs mainly in the first leaves that a plant produces. Selenium is believed to be redistributed within the plant as selenate and/or organo-selenium compounds via the phloem (White et al., 2016).

Molybdenum

Molybdenum (Mo) is a microelement whose requirement for plants is estimated between 0.5 and 1 mg kg^{-1}. The symptoms that highlight the deficiency of this metal can be difficult to determine since, in some cases, they have strong similarities with the deficiency of N, i.e. they cause yellowing. In other cases, however, a very specific symptomatology may occur, especially in the case of *Brassicaceae*, which

being plants much more subject to this type of deficiency than other families, make the identification of the problem much easier.

Molybdenum turns out to be of considerable importance within the plant since, being moreover made up of four essential enzymes, it is involved in various vital metabolic processes (Mendel and Schwarz, 2002). In plants, Mo is not present in free form, but is complexed into a single molecule called "molybdenum cofactor", which is also identified with the wording Moco, and normally binds to other elements, to form the Mo- enzymes (Hille, 1996). As previously reported, although Mo is a microelement whose requirement is very low, its presence is fundamental for the life of the plant, being the basis of some enzymes involved in various metabolic processes of primary importance, including:

- nitrate reductase: the nitrate reductase enzyme has three functional domains which, allowing the transfer of electrons and therefore the reduction from nitrate to nitrite, represents the first step in organizing mineral nitrogen. One of these three domains is represented by Moco, so, in the absence of Mo and with the reduction of nitrate, nitrogen assimilation would be impossible (Campbell, 1999);
- aldehyde oxidase: it is a cytoplasmic enzyme having FAD, iron and Moco as prosthetic groups (Koshiba et al. 1996). It is involved in the biosynthesis of abscisic acid (Seo et al. 2000) and indole-3-acetic acid (Seo et al. 1998), phytohormones essential for regulating plant growth and development.

Furthermore, the importance of Mo is closely linked to its role in the biosynthetic process of chlorophyll a and b. Therefore, the absence or deficiency of this element interferes with the synthesis of intermediate molecules at the level of the chloroplasts, reducing the amount of chlorophyll inside the leaves. The inefficiency of this process and, consequently, the low level of chlorophyll, weigh on the photosynthetic activity, negatively reflecting on the yield and quality of the product (Yu et al., 2006). Molybdenum deficiency therefore acts on various aspects, such as leaf morphology, fruit quality, plant growth and development, and also interferes with the formation of complete and fertile flowers (Sabatino et al., 2019). The molybdenum cofactor also performs important functions at the level of human health, as it is involved in the activation sites of four molybdenum-enzymes:

sulphite oxidase, aldehyde oxidase, xanthine oxidase and the reducing mitochondrial component amidoxime (mARC, although of this enzyme is still not sufficiently clear how it works). Sulphite oxidase plays an important protective role, as it catalyses the oxidation of sulphites, present in amino acids, into sulphates and thus avoids excessive accumulation with consequent intoxication. Aldehyde and xanthine oxidase, on the other hand, are more involved in catalysing hydroxylation reactions of various molecules toxic to the body, such as aldehydes (Coughlan, 1983). The European Food Safety Authority, which carried out a series of studies in order to identify the dietary reference values (DVR) such as the average requirements, the reference intake for the population and the maximum and minimum tolerable levels assumed, identified for molybdenum an ADI (Acceptable Daily Intake) of 65 μg day^{-1} for adults and pregnant and breastfeeding women, on the other hand, for infants, children and adolescents, an interval between 10 and 65 μg day^{-1} is identified. Mo deficiency leads to the onset of pathologies related to the normal development of the neurological system, as well as a higher incidence of tumors of the esophagus. A medical study conducted by Mudd, Irreverre and Laster (Tan et al., 2005) on the death of a child affected by neurological abnormalities and mental retardation, in fact highlighted deficiencies in the activity of sulphite oxidase in the liver, kidneys and brain, a deficiency normally not present. in healthy individuals. Burrell, Roach and Shadwell (1966) concluded that eating plants grown in some areas could potentially be correlated with the higher incidence of tracheal tumors. In fact, from their study it emerges that the plants in question had significant deficiencies in molybdenum and, in part, also in other elements. In addition, the role played by this deficiency in determining the onset of esophageal cancer in rats was investigated.

The excessive presence of molybdenum, like any other metal, can cause toxic effects, both for plant and animal organisms: the cells, in fact, have a complex regulation system that allows them to accumulate a certain amount of metal without being affected by its toxicity (Luk, Jensen & Culotta, 2003). There is no recommended daily dose. However, safety levels change according to age and are equal to a daily income:

- 0.03-0.06 mg day^{-1} between 0 and 6 months,

- 0.04-0.08 mg day^{-1} between 6 and 12 months,
- 0.05-0.10 mg day^{-1} between 1 and 3 years,
- 0.06-0.15 mg day^{-1} between 4 and 6 years,
- 0.10-0.30 mg day^{-1} between 7 and 10 years
- 0.15-0.50 mg day-1 over 11 years.

1.2.4. Future perspectives

Current researches on biofortification are aiming at increasing vitamin and minerals in horticultural crops, with reference to fruits and vegetables. These efforts will assist developing countries to overcome the hidden hunger problem. To achieve the best possible results in biofortification of vegetables, the collaboration among different specialists (plant physiologist, agronomist, molecular biologist, etc.) is needed. For the agronomic biofortification it is important to establish, via several experiments, dose and method of application. For genetic biofortification, it is important to develop new technology in the field of genetics and genome editing. Nevertheless, for the genetic modified crops (GMO), a regulatory approval from various committee is needed. These new understandings will help biofortification programs to develop and spread all over the world, achieving the aim of reducing hidden hunger in poor and rich countries.

1.3. Herbaceous grafting

1.3.1. Background of the herbaceous grafting development and spread

In the past, the most used and appreciated soil fumigant was the methyl bromide. Its success was due principally to the wide spectrum of activity, the high vapour pressure which facilitate the distribution in the soil, the high cost-efficiency, and a short plant-back period. At first, methyl bromide was used to control soilborne pathogens, but it was found also useful in controlling nematodes and weeds (Wilhelm and Paulus AD, 1980). However, methyl bromide was banned the 1st of January 2008 with the Montreal Protocol that regulate the use of gases with negative effects on atmosphere ozone layer (UNEP, 2006). At that time, Italy was the 1st utilizer of methyl bromide in Europe and the 2nd in the world, consequently, new

chemical and non-chemical strategies were searched such as herbaceous grafting with resistant rootstocks. Although this technique was adopted in fruit trees for many years, the large-scale application on vegetable is quite recent (Sakata et al., 2007). On the other hand, the high requirement of off-season vegetables concomitantly to the intensive cropping systems adoption determined the onset of biotic and abiotic stresses that have a negative effect on plant yield and quality. The vegetable grafting is a reasonable alternative often adopted by farmers in different cultivation conditions. In Mediterranean countries like Greece, Spain, France, and Italy the adoption of this technique is increasing rapidly (Minuto et al., 2007). The use of herbaceous grafting also offers many possibilities since a resistant rootstock confers to the plant tolerance or resistance to abiotic (high temperatures, salinity, heavy metal contamination, etc.) and biotic (e.d. *Verticillium* and *Fusarium*) stresses (Abdelmageed and Gruda, 2009; Estañ et al., 2005; Venema et al., 2008; Rivero et al., 2003a and 2003b; Lee, 1994). Moreover, it was stated that herbaceous grafting is capable of increasing yield and quality. Consequently, it is considered one of the most important tools to ensure plant performance under favourable or unfavourable cultivation conditions.

1.3.2. Biotic stress tolerance

Selected rootstocks have showed tolerance (depending on rootstocks) to soilborne pathogens, such as *Fusarium*, *Verticillium*, *Phytophtora* and nematodes (Ioannou, 2001; Nisini et al., 2002; Morra and Bilotto, 2006). However, the real mechanism of resistance is not clear. The disease tolerance of grafted plants could be simply attributed to the rootstock tolerance to such pathogens. However, it was also reported that compounds associated with pathogen resistance/tolerance move to the scion via the xylem. On the contrary, it is known that susceptible characteristics of the scion are not transferred to the rootstock.

1.3.3. Abiotic stress tolerance

Grafting technique has long been used to confer tolerance/resistance to abiotic stresses. The most common stresses are caused by temperature, salinity, and heavy metals.

Extreme temperatures tolerance is fundamental for fruiting vegetable production. Transplanting in protected cultivation often occurs in the fall-winter period and the harvest usually last until the spring-summer period. Although many greenhouses are heated, there are also farmers who do not have heating systems. These growers face many difficulties to provide optimum temperatures in winter period, especially at the soil level. This situation is stressful for plants, particularly for those with high sensitivity to low temperatures such as watermelon or melon. At this regard, there are several rootstocks which confer tolerance to low temperatures. On the other side, in the summer period, different rootstocks can be used to overcome high temperature stress. Therefore, according to the cultivation period (fall-winter or spring-summer), different rootstocks must be selected (Lee et al., 2010).

Salinity is one of the most relevant stresses that affects vegetables yield and growth in many areas of the world (Colla et al., 2010). It was showed that in comparison to the ungrafted plants, grafted plants cultivated under saline stress often had better yield, higher photosynthesis, higher build-up of abscisic acid and polyamines, greater antioxidant activities of leaves, and lower levels of detrimental ions in shoots such as Na^+ and Cl^- (Santa-Cruz et al., 2002; Colla et al., 2006; Liu et al., 2004; Colla et al., 2010).

Moreover, high concentrations of heavy metal in the soil are considered harmful for plants, humans and for the environment (An et al., 2004; Gratão et al., 2005; Clemens, 2006; Hong-Bo et al., 2010; Raskin et al., 1997). Some of these heavy metals are toxic for the plants even at low concentrations, whereas others can build-up higher quantities in tissues without detrimental effects such as yield reduction (Clemens, 2001; Moustakas et al., 2001; Verkleij et al., 2009). Herbaceous grafting could be useful to reduce the uptake and accumulation of these harmful heavy metals. For instance, Arao et al. (2008), reported that the grafting of eggplant onto *Solanum torvum* reduced the cadmium accumulation in fruits.

1.3.4. Influences on fruit quality and plant yield

It was reported that grafting can affect qualitative and productive traits of vegetables, however, there are contrasting results about the effects (positive or negative) (Proietti et al., 2008). The different results can be attributed to different

environment, agricultural practices, scion/rootstock combination, and harvest date. For instance, grafted watermelon plants showed a higher yield than those ungrafted. Moreover, it was reported that other traits such as fruit shape and colour, skin thickness, and soluble solids content are affected by rootstock. However, in cucumber, the effect of rootstocks on fruit quality traits is not positive as it decreases the sugar content. In general, grafting promotes yield increase in many vegetables. These positive effects are associated with a high plant vigour and diseases tolerance prompted by the rootstock. Yield increases were reported on watermelon, cucumber, melon, pepper, and eggplant (Lee and Oda, 2003).

1.3.5. Grafting technique in vegetable production

Grafting is an agamic propagation technique described as "*the art of joining together two pieces of living plant tissue, so that they develop and grow forming a single plant*". Grafting technique consists of four phases: (i) choice of scion and rootstock species, (ii) execution of the grafting technique, (iii) healing, and (iv) acclimatization of the grafted plants. There are several grafting methods which can vary depending on the kind of crops. The grafting can be accomplished manually (the most spread) or using machines and/or robots.

1.3.6. Grafting methods used for cucurbit species

Tongue approach grafting

After the rootstock fully developed cotyledons and scion emits the first true leaf, a cut with 35°/45° angle is performed at hypocotyl level of the rootstock with a razor blade. Concomitantly, another cut is practiced on the hypocotyl of the scion. At this point, the two cuts are joined together and sealed with aluminium to promote the healing and prevent dehydration. After that, the grafted plantlets are placed in cell trays where they will be watered. The grafted plants will be transferred into a shaded greenhouse at least 1 or 2 days before their transfer into greenhouse growing conditions. The top part of the rootstock is cut after 5 days from grafting, and the bottom part of the scion after a week from the removal of the top part of the rootstock. After all these cuts, 2 days will have to pass for the transplant.

Hole insertion grafting

This technique is often used to graft watermelon plants because the watermelon scion are smaller than rootstock used (squash or bottle gourd). Watermelon seeds are sown a week after the sowing of bottle gourd seeds or 3-4 days after the sowing of squash rootstock seeds. The grafting technique is applied a week after the watermelon sowing. The first phase consists in the remove of the first true leaf and of the growing buds with a sharp probe, then a hole is opened at the top of the rootstock hypocotyl using a needle. The scion is cut with an angle of 35-45° on both side and inserted into the rootstock hole previously made. The cut surfaces are in contact and held together with a clip.

One cotyledon grafting

Rootstock and scion preparation is the same for the hole insertion technique. The grafting is performed when the first true leaf of the rootstock is emitted (about a week after the sowing). One cotyledon and the growing bud are removed from the rootstock with a razor blade operating a cut of 35-45° angle, whereas the scion is cut at the hypocotyl level on a 35-45° angle only on one side.

1.3.7. Grafting methods used for solanaceous species

Tube grafting

This method is the most frequently used for solanaceous plants. This technique is accomplished by carrying out cuts of about 45° on both scion and rootstock, then the two plants were joined by an elastic silicon tube.

Cleft grafting

Originally, cleft grafting was employed in cucurbit species, however, nowadays it is usually used for solanaceous plants. Rootstocks are prepared with a horizontal cut, then a downward direction cut (1-1.5 cm long and ¾ depth of the stem) is performed. The scion is pruned to have 1-3 true leaves and the bottom part is cut to

have a wedge by cut in two opposite sides with an angle of about 45°. After the joint, a clip is applied to foster the union.

1.3.8. Healing phase and acclimatization

After the graft is performed, the seedlings are moved to humidity chamber kept at 30 °C and with a humidity level about 100% to guarantee high uniformity. After the healing phase, grafted plants need an acclimatization that is important to guarantee high survivability. The acclimatization phase was realized inside shaded greenhouses with temperatures of 20-35 °C. Generally, nurseries placed the grafted plants on a greenhouse bench sealed with plastic film to kept high moisture level for 5-7 days. After this period grafted plant are ready to be sell to consumers.

1.4. References

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2.0. Objectives of the research

As already described, the use of biostimulant, biofortification, and herbaceous grafting are agronomic tools capable of increasing yield, quality, and nutraceutical properties of vegetables. Although many studies have been conducted about these subjects, according to the literature, there are few studies on the interaction among these three agronomic practices. For all the above, the aim of the present doctoral thesis was to assess the effects of the mutual application of biostimulant, biofortification, and herbaceous grafting on the yield and quality of some fruiting and leafy vegetables. Therefore, the following studies were performed:

1) Title: rootstock and arbuscular mycorrhiza combinatorial effects on eggplant crop performance and fruit quality under greenhouse conditions. In this research conducted under greenhouse condition, I studied the combined effects of different rootstocks and a microbial biostimulant on eggplant yield and quality.
2) Title: impact of *Ecklonia maxima* seaweed extract and Mo foliar treatments on biofortification, spinach yield, quality and NUE. In this study, the interaction between Mo-biofortification and *E. maxima*-based biostimulant were studied. In particular, I focused my research on spinach yield, quality, and nitrogen use efficiency.
3) Title: protein hydrolysates and Mo-biofortification interactively modulate plant performance and quality of 'canasta' lettuce grown in a protected environment. This research focused on the combined effect of Mo-biofortification and plant protein hydrolysates application on yield and quality of lettuce cultivated in a protected environment.
4) Title: selenium biofortification and grafting modulate plant performance and functional features of cherry tomato grown in a soilless system. This study elucidated the effects of Se-biofortification and grafting on soilless cherry tomato performance and functional features.
5) Title: grafting eggplant onto underutilized solanum species and biostimulatory action of *Azospirillum brasilense* modulate growth, yield,

NUE and nutritional and functional traits. This study focused on the interactive effect of a microbial biostimulant and different eggplant rootstocks on growth, yield, nitrogen use efficiency, nutritional and functional traits of eggplant.

6) Title: agronomic performance and fruit quality in greenhouse grown eggplant are interactively modulated by iodine dosage and grafting. This research evaluated the agronomic performance and the fruit quality of eggplant as influenced by the interaction between I-biofortification and grafting.

All the six researches listed above are already published in peer-reviewed journals. Other studies on the topic were conducted during the three years and, in some cases, published. However, it was preferred to show only the most relevant ones. The experimental part is presented below and each study is composed by six different sections: (i) introduction, (ii) materials and methods, (iii) results, (iv) discussions, (v) conclusions, and (vi) references.

3.0. Experimental part

EXPERIMENT 1

agronomy

MDPI

Article

Rootstock and Arbuscular Mycorrhiza Combinatorial Effects on Eggplant Crop Performance and Fruit Quality under Greenhouse Conditions

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Received: date; Accepted: date; Published: 13 May 2020

Rootstock and arbuscular mycorrhiza combinatorial effects on eggplant crop performance and fruit quality under greenhouse conditions

Introduction

Eggplant (*Solanum melongena* L.) is a major solanaceous vegetable crop cultivated worldwide (Kalloo, 1993). Eggplant originated in the Old World, evolved from *S. insanum*, and it was self-sufficiently domesticated in India and China (Vavilov, 1951; Cericola et al., 2013). With a global production around 49.5 Mt, predominantly in Asia (Toppino et al., 2016), eggplant is a relevant source of minerals and vitamins, and in terms of total nutritional value, it has been compared

to tomato (Kalloo, 1993). *S. macrocarpon* L., also known as African eggplant, and *S. aethiopicum* L., also known as Aethiopicum eggplant, are closely related to *S. melongena* and are mainly distributed in the African continent. In the eggplant cultivation scenario, the Mediterranean Basin represents an important area and Sicily is counted as a secondary eggplant diversification zone (D'Anna e Sabatino, 2013). In Italy, eggplant is grown under either greenhouse or outdoor conditions, and due to the intensive cropping systems commonly used in the vegetable production farms, soilborne diseases and pests widely spread, limiting the yield and growth traits in eggplant (Bletsos et al., 2003). At present, the absence of resistant genotypes, together with the ban on methyl bromide (Maier, 2010), has increased interest in the use of grafted eggplant. There are reports underlining the advantages derived from adopting grafting both in terms of plant biotic/abiotic stress tolerance and yield stability (Daunay, 2008; King et al., 2010; Rouphael et al., 2018a). *Solanum torvum* Sw. is the most used rootstock for eggplant as it allows several soilborne diseases to be overcome, such as *Verticilium dahliae* Klebahn, *Ralstonia solanacearum* (Smith) Yabuuchiet al., *Fusarium oxysporum* (Schlechtend:Fr.) f. sp. *Melongenae* Matuoand Ishigami, and *Meloidogyne* spp. root-knot nematodes (Bletsos et al., 2003; Bogoescu et al., 2014). However, both eggplant's wild and allied relatives and interspecific hybrids are proposed as alternative eggplant rootstocks (Sabatino et al., 2018 and 2019a; Gisbert et al., 2011). The use of tomato rootstock is also suggested to improve yield, fruit visual quality and plant vigour in eggplant (Maršić et al., 2014).

Crop rotations and biological diversity long have been the key factors in successful traditional agricultural production systems. Rotation is essential for minimizing the build-up of pest and soil borne disease problems. However, intensive greenhouse production often precludes vegetable growers from applying rotation. The lack of rotation and tendency towards monocropping in intensive protected vegetable production systems not only increase the prevalence of insects, soil plant pathogens and nematodes but, along with high nutrient levels, can suppress arbuscular mycorrhizal fungi (AMF). AMF are obligate symbionts between plants and fungi belonging to the monophyletic phylum *Glomeromycota* (Schüβler et al., 2001). AMF symbiosis is extremely important for enhancing the assimilation of key

macroelements and microelements (P, Cu and Zn) and improving nutrient uptake and efficiency due to its ability to develop an extended external hypha up to 40–50 times their length (Rouphael et al., 2015). Another prominent and sustainable means of boosting yield is the inoculation of beneficial AMF in specific environments such as soil greenhouses where these fungi are generally suppressed. Moreover, recent findings demonstrated that AM may modulate plant secondary metabolism, enhancing antioxidant activity as well as the accumulation of antioxidant molecules known as phytochemicals (i.e., carotenoids, flavonoids, glucosinolates and phenolic acids) with health promoting properties (Avio et al., 2018). AM fungi have been reported to not only enhance nutritional status and the quality of the produce but also to be able to reduce several forms of plant distress such as thermal stress (Zhu et al., 2011), salinity stress (Cantrell and Linderman, 2001), drought stress (Püschel et al., 2014) and soil heavy metal stress (Ouziad et al., 2005; Shahabivand et al., 2012; Kumar et al., 2015; Rouphael et al., 2015a). Furthermore, there are reports showing an increased resistance to certain diseases in mycorrhized plants (Dugassa et al., 1996).

Although there are several studies on the synergistic effects of grafting and other agronomical or chemical means commonly applied in vegetable crop production (Ioannou, 2001; Katan, 200), few experiments have been conducted on the interactive effects of grafting and AM on plant performance and the nutritive value of vegetables. In particular, Kumar et al. (2015), studying the combined role of grafting and AM on Cd stress tolerance in tomato, observed that vigorous rootstock such as Maxifort successfully alleviate Cd stress symptoms via several physiological and biochemical mechanisms, such as (i) better nutrient absorption and translocation, (ii) higher synthesis of pigments linked to photosynthetic activity and (iii) better capability of producing enzymes (CAT and APX), proline and key metabolites (phytochelatin, fructans and inulins). Furthermore, Oztekin et al. (2013), after studying the effect of grafting and AM on the performance of tomato plants cultivated under different salinity conditions in two growing seasons, conclude that grafting and AM have synergistic effects on tomato plant salinity tolerance. Nevertheless, no specified research has been conducted to study the co-operative effects between using wild and allied eggplant relatives as rootstocks and

AM application in improving eggplant crop performance and nutritive value. On the aforesaid basis, the aims of our investigation were to appraise the concerted action of eggplant wild/allied relatives' rootstocks and AM inoculation on the yield, fruit quality and nitrogen use efficiency of "Birgah" eggplant grown under greenhouse conditions. The outcome of this study should provide useful information on the performance of new eggplant rootstocks and on their response to AMF inoculation.

Materials and Methods

Trial Site, Nursery Production and Growing Settings

The investigation was conducted in 2016 at the experimental farm of the University of Palermo. Seeds of the *Solanum torvum*, *Solanum macrocarpon* and *Solanum paniculatum* rootstocks were sown in 44-cell seedling trays, containing pasteurized peat moss (FAP, Padova, Italia), in a protected environment with a day/night temperature cycle of 25 °C/18 °C. Twenty days after rootstock sowing, seeds of the "Birgah" eggplant cultivar were seeded in 104-cell trays and maintained under the same climatic conditions and with the same planting method as the rootstocks. "Birgah" is an eggplant F_1 hybrid belonging to the violet round shape group, and it is one of the most cultivated eggplants, both in open fields and in protected environments, in Sicily. Seventy-five days after planting, all seedlings attained a hypocotyl diameter of at least 2 mm (the minimum recommended for the tube grafting method). The grafting of "Birgah" scions was performed as reported by Sabatino et al. (2018 and 2019a). Briefly, the rootstock was cut off, 0.5 cm below the cotyledons, at a 45° angle, and a similar cut was performed on the scion. Care was taken to ensure that the cut surfaces fitted perfectly. To complete the grafting procedure, a plastic clip was attached at the grafting point to guarantee that the correct amount of pressure was applied. The grafted plantlets were placed in a greenhouse equipped with a fog system in order to maintain a humidity of 95% and an air temperature of 20 °C for 7 days. After that, the grafted transplants were subjected, for 3 days, to a slow dropping of the humidity, useful for plant acclimatization. Then, they were ready for transplantation. Ungrafted and self-

grafted eggplants were also included. Prior to transplanting, half of the ungrafted, self-grafted and grafted plantlets were treated with 10 g plant^{-1} of micorrhizal inoculum carrying 40 spores g^{-1} of *Rhizophagus irregularis* (formerly *Glomus intraradices*). All plantlets were transplanted on 9 February, 2016 and maintained till the end of May, 2016 in an unheated greenhouse in Typic Rhodoxeralf soil, characterized by the following soil texture: 46.5% sand, 22.3% silt and 31.2% clay. The soil pH was 7.2. Before the experiment, a brassica crop was cultivated. Mulching with a black polyethylene film of 20 μm was installed. Eggplant plantlets were spaced in order to obtain a plant density of 2 plants m^{-2}. Plants were periodically drip irrigated, receiving 250 kg of nitrogen ha^{-1}, 150 kg of phosphorous pentoxide ha^{-1} and 250 kg of potassium oxide ha^{-1}. The cultivation practices reported by Baixauli and Berenjena (2001), suggested for eggplant growth in Mediterranean conditions, were applied regularly subject to crop requirements.

Yield, Nitrogen Use Efficiency, Nutritional Traits and Functional Compounds

Immediately after harvest, the fruits were weighed and separated into marketable and waste production categories. The number of marketable fruits per plant was recorded, and average fruit weight was also calculated. In total, ten harvests were realized, starting on 21 March, 2016.

A digital penetrometer (Trsnc, Forlì, Italy) was used to determine fruit firmness. Fruit firmness was measured based on three replicates of five fruits per scion/rootstock combination. Each fruit was perforated, using a 6 mm diameter stainless steel cylinder probe, on two opposite sides of the fruit equatorial zone. Firmness was expressed in newtons (N).

Soluble solids content (SSC) was recorded based on three replicates of five fruits and was determined using a refractometer (MTD-045nD, Three-In-OneEnterprises Co. Ltd. Taiwan). Prior to the SSC determinations, the fruit juice was adequately clarified.

Three to five commercially mature fruits for each replicate, from the second and third harvests, were used for the analytical determinations. Each sample consisted of the same quantity of apical, equatorial, and distal parts of the fruits. Nutritional and functional determinations were performed on fruits harvested from labelled

flowers at the fruit set stage, and all the fruits were harvested 35 days after labelling (fruit commercial maturity stage).

Fruit dry weight was measured via the dehydration of the sample in a heater at 80 °C until a constant weight was achieved. The fruit dry matter percentage was calculated using the fruit fresh and dry weights. For protein determination, the Kjeldahl method was applied. Specifically, sample was exposed to acid-catalysed mineralization to convert the organic nitrogen into ammoniacal nitrogen. Subsequently, the ammoniacal nitrogen was distilled under alkaline pH. The ammonia produced via the distillation was collected in a boric acid solution and measured through titrimetric dosage. The ammoniacal nitrogen value was multiplied by 6.25. For Ca, Mg, K and Fe determinations, atomic absorption spectroscopy following wet mineralization was adopted as reported by Morand and Gullo (1970). Phosphorus values were assessed using colorimetry as reported by Fogg and Wilkinson (1958).

The nitrogen use efficiency (NUE = yield/N application rate) was calculated and expressed as $t \cdot kg^{-1}$.

The ascorbic acid content was determined as described by Sabatino et al. (2019b) for tomato. Therefore, the determinations were made with a reflectometer (Merck RQflex∗ 10 m) using Reflectoquant Ascorbic Acid Test Strips. Thus, one gram of eggplant fruit juice was mixed with distilled water to a 10 mL total volume. Subsequently, an appropriate test strip was immersed into the prepared sample then inserted into the meter. The results were expressed as mg of ascorbic acid per 100 g of fresh weight (fw).

The anthocyanin extraction procedure applied was that described by Mennella et al. (2012). The determination was conducted on a lyophilized and powdered sample of 200 mg. The flow rate and absorbance units full scale adopted were 0.8 mL min^{-1} and at 0.1, respectively. For RP-HPLC analyses, purified delphinidin-3-rutinoside (D3R, Polyphenols Laboratories AS, Sandnes, Norway) was used as an external standard with a dissimilar retention time (23.9 min) compared to delphinidin-3-(pcoumaroylrutinoside)-5-glucoside (nasunin), which was eluted with a longer retention time (25.8 min for cis-nasunin and 26.1 min for trans-nasunin, respectively). Regarding the nasunin determination, in agreement with Lo Scalzo

et al. (2010), a partially purified standard was used. Total anthocyanins were expressed as mg·100 g^{-1} dry weight (dw), highlighting that the detection limit was 2.00 mg 100 g^{-1} of dw.

For chlorogenic acid determination, the extraction and analysis procedure described by Stommel and Whitaker (2003), with slight variations, was applied. Therefore, a binary mobile phase gradient of methanol in 0.01% aqueous phosphoric acid was provided according to this procedure: 0-15 min, linear increase from 5% to 25% methanol; 15-28 min, linear increase from 25% to 50% methanol; 28-30 min, linear increase from 50% to 100% methanol; 30-32 min, 100% methanol; 32-36 min, linear decrease from 100% to 5% methanol; 36-43 min, 5% methanol. The flow rate was 0.8 mL·min^{-1}. The chlorogenic acid quantification, subsequently conducted by RP-HPLC separation, was based on the absorbance at 325 nm relative to the external standard of chlorogenic acid (Sigma-Aldrich, St.Louis, MO) and expressed as mg·100 g^{-1} of dw.

For glycoalkaloids, the extraction method adopted was that reported by Birner (1969) with some adjustments. Thus, 0.5 g of lyophilized and powdered flesh tissue sample was extracted with 95% ethanol. For glycoalkaloids (expressed as mg·100 g^{-1} dw) analyses, the method described by Kuronen et al. (1999) was applied. Therefore, the analyses were conducted by means of RP-HPLC using purified solasonine and solamargine as the external standards. The detection limit was 0.03 mg 100 g^{-1} of dw.

Root Colonization Assessment

To evaluate mycorrhizal colonization, the method described by Phillips and Haymann (1970) and revised by Torta et al. (2003) was adopted. Briefly, three lateral root samples from mychorrhized plants were collected and marked with acid fuchsin. As reported by Kormanik and McGraw (1991), mycorrhizal colonization (Mycorrhization Index (MI = % of marked tissue, with respect to the hyaline portion, on the unit of the length of the root)) was evaluated on three fragments, attaining the average value.

Experimental Design and Statistical Analysis

The trial was organized in a randomized complete block design with 3 replicates per treatment. Each replicate consisted of 10 plants. Consequently, the total number of plants was 300 (10 treatments × 30 plants per treatment = 300 plants). The data were subjected to GLM (General Linear Model) analysis using the SPSS software package version 20.0. For data expressed in percentages, an arcsin transformation before ANOVA analysis ($Ø = \arcsin(p/100)^{1/2}$) was performed. Tukey's HSD test ($p \leq 0.05$) was used to compare means. Principal component analysis (PCA) was conducted on the whole yield, fruit quality and nitrogen use efficiency data set.

Results

Root Colonization by AM Fungi

AM fungi root colonization was significantly affected by mycorrhizal inoculation (M), whereas the grafting combination (G) did not significantly influence AM root colonization. ANOVA showed no significant interaction (G × M) (Figure 1). Irrespective of the grafting combination, the percentage of root colonization was higher with the inoculated treatment (71.8%) compared to with the non-inoculated one (2.5%) (Figure 1).

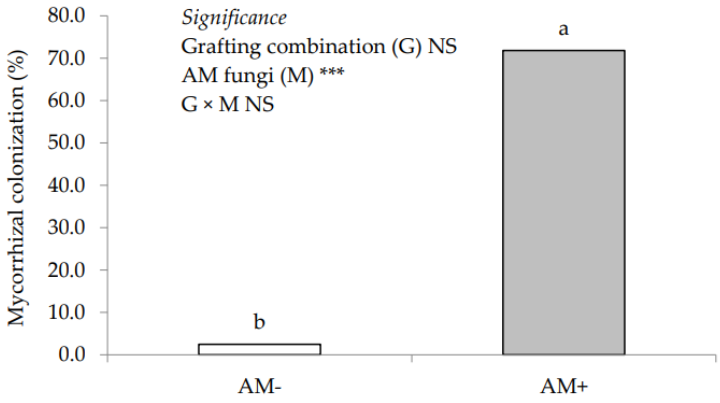

Figure 1. Main effects of arbuscular mycorrhizal inoculation on root colonization. Different letters indicate significant differences according to Tukey HSD test ($p \leq 0.05$). +AM, −AM, mycorrhized and non-mycorrhized eggplants, respectively.

Effect of Grafting Combination and Mycorrhizal Inoculation on Yield, Yield Components and NUE

The marketable yield, number of marketable fruits per plant and percentage of unmarketable production were significantly influenced by the G × M interaction (Table 1).

Table 1. Main effects of the grafting combination and arbuscular mycorrhiza (AM) inoculation on yield and the yield components of greenhouse eggplant.

Treatments	Marketable yield (t ha^{-1})	Fruits number (n. plant^{-1})	Fruit mean weight (g)	Discarded production (%)
Grafting combination (G)				
Birgah ungrafted (B)	36.5 c	6.1 c	505.5 a	6.3 b
Birgah self-grafted (B/B)	37.1 c	6.2 c	501.6 a	6.3 b
Birgah/*S. torvum* (B/T)	48.4 a	8.1 a	500.8 a	5.2 b
Birgah/*S. macrocarpon* (B/M)	40.1 b	6.7 b	500.6 a	10.3 a
Birgah/*S. paniculatum* (B/P)	38.5 bc	6.5 bc	494.7 a	6.3 b
AM fungi (M)				
-AM	37.6 b	6.3 b	499.8 a	9.4 a
+AM	42.6 a	7.1 a	501.4 a	4.4 b
Significance				
G	***	***	NS	***
M	***	***	NS	***
G × M	NS	NS	NS	NS

NS, *** non-significant or significant at $p \leq 0.001$. Data represent mean values of three replicates. Values within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD Test. NS = not significant. +AM, −AM = Mycorrhized and non-mycorrhized eggplants, respectively.

On the other hand, neither the grafting combination nor mycorrhizal treatment had a significant effect on fruit mean weight (avg. 500.6 g fruit^{-1}). When averaged across mycorrhizal treatments, the marketable yield production reached a maximum value with Brigah grafted onto *S. torvum* (B/T), followed by both the B/M and B/P grafting combinations, while the lowest crop productivity was recorded for ungrafted and self-grafted crops (Table 1). Interestingly, the higher marketable fresh yield observed for eggplants grafted onto *S. torvum* and to a lesser extent onto *S. macrocarpon* and *S. paniculatum*, in comparison to both the ungrafted and self-grafted plants, was associated with an increase in the number of eggplant fruits per

plant and not with a change in the mean fruit weight (Table 1). Moreover, the highest discarded production was observed with the B/M grafting combination (Table 1). A significant effect of mycorrhizal treatment on the yield and yield components was also observed (Table 1). Irrespective of grafting combinations, the inoculation of eggplant at the transplantation stage with *R. irregularis* elicited significant increases in the marketable yield and number of fruits per plant of 13.3% and 12.6%, respectively, compared to for the non-inoculated control (Table 1).

Similarly, to the effects on the yield and yield components, the nitrogen use efficiency (NUE) for the B/T grafting combination (0.242 t kg^{-1}) was significantly higher, by 31.2%, than that obtained from the ungrafted and self-grafted eggplant (avg. 0.184 t kg^{-1}; Figure 2). Finally, the inoculation of eggplant with *R. irregularis*, when averaging across grafting combinations, induced a significant increase in the NUE, which was 13.3% higher than for the non-inoculated control treatment (Figure 2).

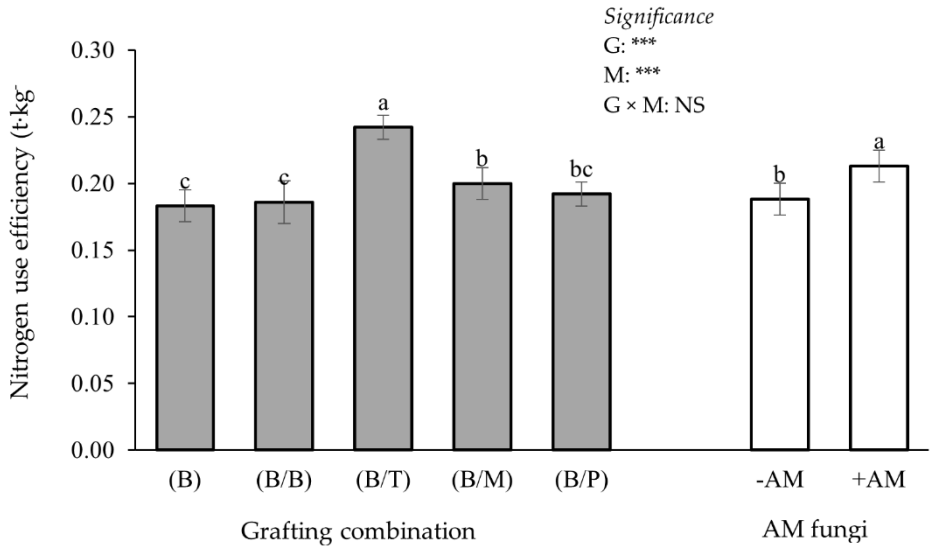

Figure 2. Main effects of grafting combination and AM inoculation on the nitrogen use efficiency (NUE) of greenhouse eggplant. (B): Birgah ungrafted; (B/B): Birgah self-grafted; (B/T): Birgah/*S. torvum*; (B/M): Birgah/*S. macrocarpon*; (B/P): Birgah/*S. paniculatum*.

Effects of Grafting Combination and Mycorrhizal Inoculation on the Mineral Profile and Nutritional and Functional Quality

Neither the grafting combination nor mycorrhizal inoculation had a significant effect on the fruit dry matter content (DM). The soluble solids content (SSC) and K

content of eggplant fruits in the B/T and B/M plots, averaged across mycorrhizal inoculation status, were higher than those for the other grafting combinations (Table 2).

Table 2. Main effects of the grafting combination and AM inoculation on the fruit dry matter (DM) percentage, soluble solids content (SSC) and macromineral content of greenhouse eggplant.

Treatment	Dry matter (%)	SSC (°Brix)	K (mg 100 g^{-1} dw)	Ca (mg 100 g^{-1} dw)	Mg (mg 100 g^{-1} dw)
Grafting combination (G)					
Birgah ungrafted (B)	6.0 a	4.3 b	4.3 b	309 c	18.0 a
Birgah self-grafted (B/B)	5.9 a	4.3 b	4.3 b	312 c	17.0 a
Birgah/*S. torvum* (B/T)	6.0 a	5.1 a	5.1 a	311 c	12.3 c
Birgah/*S. macrocarpon* (B/M)	5.9 a	5.1 a	5.1 a	336 b	16.8 a
Birgah/*S. paniculatum* (B/P)	5.9 a	4.4 b	4.4 b	348 a	14.5 b
AM fungi (M)					
-AM	5.9 a	4.4 b	4.4 b	324 a	16.1 a
+AM	6.0 a	4.9 a	4.9 a	322 a	15.6 a
Significance					
G	NS	***	***	***	***
M	NS	***	***	NS	NS
G × M	NS	NS	NS	NS	NS

NS, *** non-significant or significant at $p \leq 0.001$. Data represent mean values of three replicates. Values within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD Test. NS = not significant. +AM, −AM = mycorrhized and non-mycorrhized eggplants, respectively.

The highest and lowest Ca and Mg fruit contents were recorded in the B/P and B/T plots, respectively. Regardless of the grafting combination, the SSC and K fruit contents in the inoculated plots were 11.4% and 11.4% higher than in the non-inoculated ones, respectively. No significant interaction was observed between the grafting combination and mycorrhizal inoculation in terms of the DM percentage; SSC; and K, Ca, and Mg fruit contents (Table 2).

The grafting combination and mycorrhizal inoculation significantly affected the P and Fe fruit contents, protein content, firmness, ascorbic acid, total anthocyanins, chlorogenic acid and glycoalkaloids (Table 3).

Table 3. Effects of the grafting combination and AM inoculation on the phosphorus (P), iron (Fe), protein, fruit firmness, ascorbic acid, total anthocyanins, chlorogenic acid and glycoalkaloid content of greenhouse eggplant.

Treatment	P (mg 100 g^{-1} dw)	Fe (µg g^{-1})	Proteins (g 100 g^{-1} dw)	Firmness (N)	Ascorbic acid (mg 100 g^{-1} fw)	Total anthocyanins (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Glycoalkaloids (mg 100 g^{-1} dw)
Birgah ungrafted (B) × -AM	418.07 e	21.13 e	10.77 e	43.5 c	6.51 de	7462.0 d	982.37 b	89.38 b
Birgah ungrafted (B) × +AM	431.43 e	22.73 e	12.93 d	53.2 a	7.27 b	7770.4 c	992.6 b	74.16 c
Birgah self-grafted (B/B) × -AM	419.27 e	25.17 d	10.60 e	43.1 c	6.43 e	7428.1 d	982.71 b	89.13 b
Birgah self-grafted (B/B) × +AM	435.53 e	27.40 c	12.70 d	52.2 a	7.19 bc	7772.4 c	984.99 b	73.27 c
Birgah/*S. torvum* (B/T) × -AM	494.90 d	30.17 b	14.03 c	54.7 a	7.27 b	7154.8 e	757.15 e	44.13 d
Birgah/*S. torvum* (B/T) × +AM	525.70 c	30.70 ab	15.23 a	53.8 a	7.63 a	7393.7 de	823.98 d	36.93 e
Birgah/*S. macrocarpon* (B/M) × -AM	383.67 f	31.43 a	9.87 f	46.4 b	6.71 d	7241.6 e	955.27 c	97.2 a
Birgah/*S. macrocarpon* (B/M) × +AM	382.67 f	31.30 a	9.83 f	52.2 a	7.09 c	7222.5 e	948.57 c	90.33 b
Birgah/*S. paniculatum* (B/P) × -AM	553.23 b	29.33 b	13.07 d	41.5 c	6.67 d	9858.4 b	989.16 b	41.94 d
Birgah/*S. paniculatum* (B/P) × +AM	584.30 a	31.33 a	14.90 b	47.3 b	7.19 bc	11163.0 a	1027.86 a	32.43 e
Significance								
G	***	***	***	***	***	***	***	***
M	***	***	***	***	***	***	***	***
G × M	***	***	***	**	***	***	**	***

NS, *** non-significant or significant at $p \leq 0.001$. Data represent mean values of three replicates. Values within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey's HSD Test. NS = not significant. +AM, −AM = mycorrhized and non-mycorrhized eggplants, respectively.

The inoculation treatment elicited a significant increase in the P fruit content in the B/P and B/T plots compared to in the non-inoculated ones. However, this effect was not apparent with the other grafting combinations. A similar positive effect was also observed in eggplant grafted onto *S. paniculatum*, since mycorrhizal inoculation increased Fe concentration in inoculated plants compared to in the non-inoculated ones (Table 3). Moreover, eggplant "Birgah" grafted onto *S. torvum* and inoculated with AM fungi displayed the highest total protein value (Table 3). Except for the B/T grafting combination where no significant changes in fruit firmness were observed, the inoculation of eggplant with AM fungi in all the other grafting combinations (ungrafted, B/B, B/M and B/P) incurred a significant increase in fruit firmness (Table 3). Contrarily, for the latter quality parameters (P, Fe, protein, and firmness), the beneficial effect of AM fungi inoculation on ascorbic acid in eggplant fruits was apparent with all the grafting combinations tested, with the highest values recorded for "Birgah" grafted onto *S. torvum* (Table 3).

The total anthocyanin and chlorogenic acid contents ranged from 7,154 to 11,162 and 757 to 1027 mg 100 g^{-1} dw, respectively. Interestingly, the B/P grafting combination inoculated with AM fungi produced a major amplification of total anthocyanins and chlorogenic acid in comparison to the rest of the treatments (Table 3). Finally, the results indicated that the highest glycoalkaloid content was observed in the non-inoculated B/M grafting combination, whereas the lowest values were recorded in the B/P and B/T combinations inoculated with *R. irregularis* (Table 3).

Principal Component Analysis of All Agronomic and Qualitative Parameters

The principal component analysis (PCA) on the agronomic and qualitative traits of greenhouse eggplant in response to the grafting combination and mycorrhizal inoculation is reported in Figure 3.

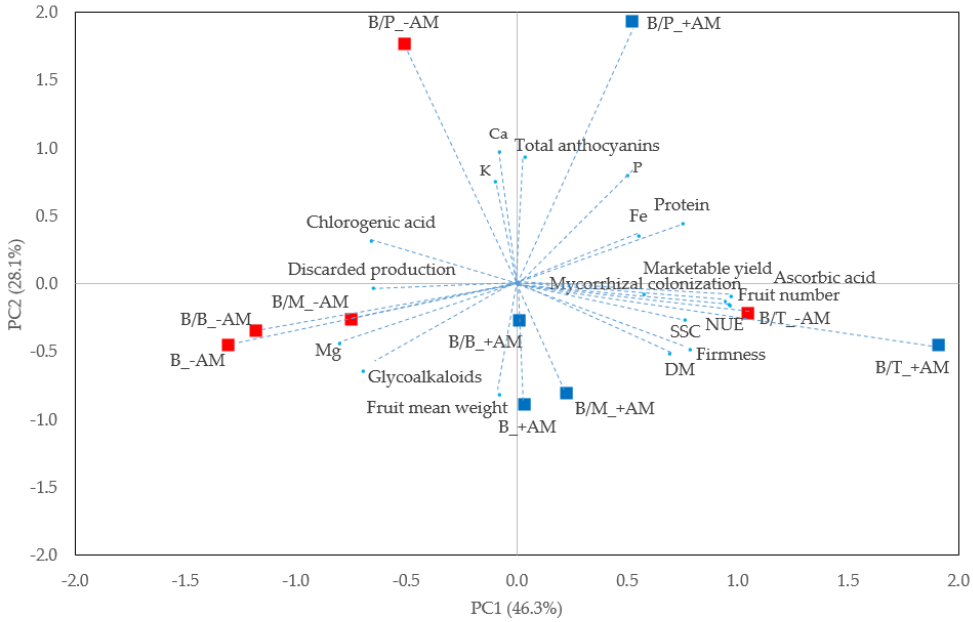

Figure 3. Principal component loading plot and scores of the principal component analysis (PCA) of the yield, yield components, nitrogen use efficiency (NUE), dry matter percentage (DM), protein, firmness, soluble solids content (SSC), Minerals (P, K, Ca, Mg and Fe), bioactive molecules (ascorbic acid, anthocyanins and chlorogenic acid) and glycoalkaloids of eggplant cv. "Birgah" as a function of grafting combination (ungrafted B and self-grafted B/B, B/T, B/M and B/P) and AM inoculation (+AM and –AM).

The first four PCs (i.e., principal components) were related with eigenvalues higher than 1 and explained 94.7% of the total variance (Table 4).

Table 4. Correlation coefficients for each agronomic and qualitative traits, eigenvalues, variance, and cumulative proportions of total variance of the four principal components (PCs).

Variable	PC1	PC2	PC3	PC4
Marketable yield	0.961	-0.157	-0.129	-0.003
Fruit number	0.969	-0.090	-0.156	-0.012
Fruit mean weight	-0.080	-0.813	0.377	-0.041
Discarded production	-0.657	-0.029	-0.727	-0.082
Protein	0.747	0.444	0.360	-0.295
K	-0.097	0.752	-0.394	0.463
P	0.498	0.798	0.193	-0.261
Ca	-0.083	0.972	0.078	0.046
Mg	-0.809	-0.435	0.243	0.290
Fe	0.550	0.359	-0.644	0.345
NUE	0.959	-0.155	-0.130	-0.017
Fruit dry matter	0.691	-0.509	-0.001	-0.001
Firmness	0.780	-0.488	0.088	0.146
SSC	0.757	-0.262	-0.365	0.425
Ascorbic acid	0.940	-0.125	0.210	0.162
Total anthocyanins	0.034	0.936	0.268	0.189
Chlorogenic acid	-0.659	0.319	0.431	0.517
Glycoalkaloids	-0.698	-0.635	-0.155	0.285
Mycorrhizal colonization	0.571	-0.077	0.500	0.636
Eigenvalue	8.793	5.348	2.247	1.601
Variance %	46.281	28.148	11.825	8.427
Cumulative %	46.281	74.429	86.254	94.681

In the current greenhouse experiment, the loading matrix indicates that variation in Ca and K was most closely aligned with the total anthocyanins, whereas the variation in the glycoalkaloids was not correlated to the SSC (Figure 3). The score plot of the PCA superimposed on the agronomic and qualitative parameters demonstrated a strong clustering of the two mycorrhizal treatments along PC1 (with the exception of *S. torvum* × -AM), with +AM plants concentrating marketable yield, NUE, DM, SSC, mineral composition and ascorbic acid (Figure 3). Particularly, the B/T grafting combination inoculated with AM fungi was positioned on the positive side of PC1 in the lower right quadrant of the PCA score plot, and it exhibited the highest crop performance (high yield, number of fruits and NUE) with premium quality due to high concentrations of DM, SSC, and ascorbic acid (Figure 3). Moreover, eggplant grafted onto *S. paniculatum* and inoculated with AM was characterized by higher P, Fe, and total anthocyanin content (Figure 3). Finally, non-inoculated ungrafted and self-grafted plants were positioned in the lower left quadrant, characterized by higher glycoalkaloid content (Figure 3).

Discussions

In the present work, we tested the use of various eggplant grafting combinations and AM fungi, alone or in combination, to boost crop performance and fruit quality in "Birgah" eggplant cultivated in a protected environment. Our results showed that enhancements in terms of yield, yield related traits, NUE, and fruit nutritional and functional quality can be accomplished by harnessing the combined effects of specific grafting combinations and AM fungi. These results are in line with those reported by Sabatino et al. (2018 and 2019a), who - exploring the effects of using eggplant hybrids and allied species as rootstocks on eggplant yield, plant vigor and overall fruit quality - found that *S. paniculatum* and the interspecific hybrid of *S. melongena* × *S. aethiopicum* gr. *Gilo* provided a higher yield performance and a better fruit quality compared to the ungrafted or self-grafted plants. Similarly, our results are in line with those observed by Oztekin et al. (2013), who, studying the influence of AM fungi on salinity tolerance in tomato grafted plants, stated that AM fungi significantly increased the total and marketable yields as well as the average

fruit weight. Several authors (De Pascale et al., 2018; Rouphael and Colla, 2018) report that the phytostimulating effect of the AM fungi could be attributed to numerous mechanisms such as enhancing the uptake and translocation of major and trace elements, inducing a more developed root system, improving the water status and photosynthetic efficiency, bolstering the antioxidative defence system, balancing plant hormones, upregulating nutrient transporter action and promoting the production of enzymes such as phosphatases. Therefore, we might hypothesize that the higher yield and yield related traits of mycorrhized plants could have similar explanations. Our outcomes regarding the NUE agree with those reported by Djidonou et al. (2013), who - investigating the yield, water status and NUE in field-grown, grafted tomato - found that grafting the "Florida 47" tomato onto "Beaufort" or "Multifort" significantly increased N use. Our findings are also consistent with those of Colla et al. (2010), who, studying the influence of selected rootstocks on the plant performance and nitrogen use efficiency of the "Proteo" melon, report that the use of melon grafted onto designated rootstocks would represent a prospective plan for increasing crop productivity and NUE. In the present study, AM fungi increased the NUE of eggplant plants. This accords with the results of Zhu et al. (2015), who - evaluating the effects of AM fungi on growth, nitrogen uptake and NUE in wheat - found that AM symbiosis increased NUE. Our findings are also in line with those presented by Liu et al. (2018), who, studying the influence of AM on NUE in soybean, concluded that AM fungi play an imperative function in increasing NUE. Likewise, Rouphael et al. (2018), in a review, report that plant biostimulants, including microbial plant biostimulants, can enhance NUE. Our study showed that both the *S. torvum* and *S. macrocarpon* rootstocks increased SSC values. This is in congruence with the results of Sabatino et al. (2018), who used the "Birgah" eggplant F1 hybrid as a scion. However, these findings are in contrast to the outcomes reported by Sabatino et al. (2019a), who tested the "Scarlatti" eggplant F_1 hybrid as an eggplant scion. Furthermore, our results for SSC revealed that AM fungi increase the SSC level in eggplant. However, this is in contrast with previous reports (Oztekin et al., 2013) indicating that AM symbiosis does not affect SSC in tomato fruit. Our results on firmness are corroborated by the results of Sabatino et al. (2018), who found that *S. torvum* rootstock increased fruit firmness

in “Birgah” hybrid and by those reported by Miceli et al. (2016), who, assessing the effect of AM fungi and grafting on yield and fruit quality in mini-watermelon, noted that AM symbiosis increases pulp firmness. In our study, at the end of the experiment, the mean mycorrhizal colonization rates were 71.8% and 2.5% in +AM and -AM plants, respectively. There are reports that mycorrhizal hyphae extending into the soil or substrate increase nutrient uptake (Rouphael and Colla, 2018; Colla et al., 2015) thanks to the mycorrhizal hyphae’s capacity to penetrate into very small particles (Al-Karaki, 2006). Our results on fruit mineral composition showed that the B/T, B/M and B/P scion/rootstock combinations improved the K, Ca, and Fe fruit contents. Meanwhile, fruits from the B/M grafts revealed a decrease in terms of P content compared to the fruits from the control plots (ungrafted or self-grafted plants). Moreover, the present study showed that AM fungi treatment increased the K fruit content, without affecting the Ca and Mg fruit content. In addition, our results highlighted that the B/T and B/P grafts benefit significantly from AM fungi in terms of P and Fe fruit concentrations. These outcomes are in accord with those of Kaya et al. (2009) and Oztekin et al. (2013), who supposed that a higher fruit mineral (macro and micro) concentration in grafted and mycorrhized plants could be attributed to a positive impact on water and nutrient uptake. Our trial showed that the *S. torvum* and *S. paniculatum* rootstocks increased fruit protein concentrations. This is in agreement with the results of Sabatino et al. (2018) but in contrast to those of Sabatino et al. (2019a), who found no significant differences in terms of fruit protein content between ungrafted, self-grafted or *S. torvum* grafted plants. These results mark the important role played by the scion in terms of nutritional fruit quality in grafted plants. In addition, our results showed that AM fungi augmented the protein concentration in eggplant fruits. This seems to be in accord with the study of Baum et al. (2015), who asserted that mycorrhizal inoculation induces a higher accumulation of proteins in onion. Contemporary outcomes exposed that AM symbioses are capable to adjust host plant primary and secondary metabolism, encouraging the synthesis of phytochemicals in the root system and shoots of mycorrhized plants (Sbrana et al., 2014). Our data showed that the *S. torvum* rootstock significantly increased ascorbic acid in eggplant fruit, whereas fruits from the B/M and B/P grafts did not show significant differences in

terms of ascorbic acid compared to the fruits from the control plots, demonstrating that the rootstock plays an imperative role in eggplant fruit quality. These results are partially in accord with those reported by Oztekin et al. (2013), who observed that the rootstock factor did not affect vitamin C in tomato fruits, and with those of Miceli et al. (2016), who reported that grafting increases fruit ascorbic acid content in mini-watermelon. Conversely, our results on the positive effect of the AM fungi on fruit ascorbic acid content are fully in accord with those of Oztekin et al. (2013) and Miceli et al. (2016). There is evidence that the increase in secondary metabolites in mycorrhized plants could be correlated to the higher contents of mineral nutrients (Rouphael et al., 2015b). In this regard, overall, our study indicated that AM fungi positively affected total anthocyanins and chlorogenic acid, with the sole exception of with the B/M scion/rootstock combination. Our findings seem to be in line with those reported by Walter et al. (2000), who revealed that some graminaceous crops inoculated with AM fungi produce undergo important biochemical modulation leading to phenolic acid accumulation. Our results are in accord with those of other authors (Copetta et al., 2006; Toussaint et al., 2007; Rasouli-Sadaghiani et al., 2010), who, investigating the influence of AM symbiosis on the production of phytochemicals in *Ocimum basilicum*, found higher concentrations of antioxidants when plants were treated with diverse AM fungi species. Baslam et al. (2011) remarked that mycorrhized lettuce leaves display higher anthocyanin, carotenoid, and phenolic contents than plants from control plots. Castellanos-Morales et al. (2010) found that the strawberry mycorrhized plants produced fruits with a higher content of anthocyanidin cyanidin-3-glucoside. Ceccarelli et al. (2010) state that artichoke, when treated with two AM fungi species, have increased leaf polyphenolic concentrations and antioxidant activity. Our results on glycoalkaloids support the findings reported by Sabatino et al. (2018), who, although not showing a statistically significant effect of the rootstock on glycoalkaloids, highlighted a certain effect of rootstocks on glycoalkaloid content, remarking on the effectiveness of *S. torvum* and *S. paniculatum* in reducing glycoalkaloid concentrations. Furthermore, our results evidenced that +AM treatment had a positive effect on glycoalkaloid reduction which in turn could positively influence human health.

The effectiveness of PCA in interpreting species/cultivar differences across several agronomic and qualitative attributes in response to a wide range of pre-harvest factors such as the genetic material and agricultural practices (i.e., AM inoculation) has been reported previously by several researchers (Colonna et al., 2016; Kyriacou et al., 2015; Carillo et al., 2019). This was also evident in the present study, conducted under protected environment conditions, as the score plot of PCA integrated information on the yield, yield components, NUE, mineral profile and nutritive value of eggplant from five grafting combinations with or without inoculation with AM.

Conclusions

In the present work, eggplant wild/allied relatives' rootstocks and AMF significantly interacted, improving the crop performance and quality traits of "Birgah" eggplant. However, the B/T and B/P grafts combined with AMF stood out as producing the best results in terms of the yield traits, NUE, mineral profile, and nutritional and functional quality. Our findings could be useful information for vegetable crop nurseries interested in introducing new eggplant rootstocks that successfully respond when combined with AMF.

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EXPERIMENT 2

agronomy

MDPI

Article

Protein Hydrolysates and Mo-Biofortification Interactively Modulate Plant Performance and Quality of 'Canasta' Lettuce Grown in a Protected Environment

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Protein hydrolysates and Mo-biofortification interactively modulate plant performance and quality of 'canasta' lettuce grown in a protected environment

Introduction

Nowadays, the agriculture production sector must face both the multiple challenges to serve an increasing global population, and the pressing need to improve food quality and reduce the impact of cultivation on human health and environment (Duhamel and Vandenkoornhu, 2013). In this scenario, the use of plant biostimulants is a technology with innovative application potentiality (Colla et al., 2017). Several authors (Du Jardin, 2015; Yakhin et al., 2017; Rouphael et al., 2017; Sabatino et al., 2019a and 2019b; Sabatino et al., 2020; Rouphael et al., 2020; Consentino et al., 2020; Di Mola et al., 2021) have appraised that plant biostimulants are an eco-friendly means to enhance plant production and quality under favourable or unfavourable growth conditions. In this regard, the protein hydrolysates (PHs), represent a promising group of plant biostimulants (Schaafsma, 2009). Furthermore, PHs, when derived from enzymatic hydrolysis, are suitable plant biostimulants for an organic farm management (Nardi et al., 2016). PHs are either applied by foliar spraying and/or by root fertigation. As reported by

Fernández and Eichert (2009), when furnished via foliar spray, PHs are absorbed by (i) cuticle, (ii) epidermal cells and (iii) stomata, and finally reach the foliar mesophyll. Several studies (Ertani et al., 2009; Matsumiya and Kubo, 2011; Colla et al., 2014; Lucini et al., 2015) have confirmed that PHs elicit crop performances due to hormone-like activities (i.e., gibberellins and auxin). Additionally, PHs enhance minerals absorption and translocation throughout modification of the biomass, density, and lateral branching (Ertani et al., 2009; Halpern et al., 2015; Colla et al., 2015).

Macro- and micro-nutrients are essential to provide a balanced diet and to contribute to human health. Biofortification of edible plants can be achieved via plant breeding improvement (i.e., exploiting the genetic variability of the local populations) or through fertilizer application (Bouis and Welch, 2010; D'Anna and Sabatino, 2013; Azeez et al., 2018). Molybdenum (Mo) is a trace element which is required in minor quantities, less than 100 mg per day (Fraga, 2005; Ierna et al., 2012). As reported by Tsongas et al. (1980) the daily optimal Mo intake ranges from 120 to 240 µg, in relation to age, sex, and income. The importance of the disorder linked with the simple shortage of sulphite oxidase (Bender et al., 2019; Cohen et al., 1971) established the necessity of Mo for standard human health. Furthermore, Johnson et al. (1974) remarked the neurological lesion caused by deficiency of Mo containing enzymes and reinforced the prominence of this metal in human well-being. Moreover, there are reports regarding the fundamental function of Mo in the prevention of esophageal cancer and mammary adenocarcinoma (Gunnison, 1981; Scwarz and Belaidi, 2013). Interestingly, as reported by Schwarz and Belaidi (2013), Mo is the only metal of the 2nd transition row of the periodic table with a biological function for humans. Mo can be found in foods as a trace element in form of soluble molybdates. Legumes, nuts, cereals and cereal derivate are common foods with a high Mo concentration (Pennington and Jones, 1987). Bread and pasta are the main foodstuff suppliers to alimentary Mo intake, followed by dairy products and leafy vegetables (Pennington and Jones, 1987; Rose et al., 2010).

Mo is a crucial constituent of biological structures. However, it can be toxic at concentrations higher than those requested for their biological purposes (Luk et al., 2003). Schwarz et al. (2009) report that Mo is active only when is a portion of an

organic pterin complex named pterin-based molybdenum cofactor (Moco). Currently, four mammalian Mo-enzymes are recognized, all including Moco in their active site. In all of these enzymes, Mo catalyses the transfer of oxygen from or to substrates via water as oxygen donor or acceptor (Scwarz and Belaidi, 2013). Plant benefits from Mo supply are well acknowledged and recognized (Marschner, 2012; Vatansever et al., 2017; Mendel and Shwarz, 1999; Bittner, 2014). Indeed, Mo-enzymes partake in fundamental metabolic processes, comprising the phytohormone biosynthesis, purine metabolism, sulphite detoxification, and nitrate assimilation. Thus, its scarcity or excess obstructs the plant growth and development (Vatansever et al., 2017). Moreover, Mo supply prevents alteration in plant morphology, flower formation reduction and inhibition in plant growth and also improves fruit quality. According to Moncada et al. (2018), Mo supply is effective to increase production and qualitative aspects of lettuce, curly endive, and escarole cultivated in soilless systems. Furthermore, Sabatino et al. (2019c) found that Mo bifortification increases plant performance, yield and quality in different tomato genotypes. Longbottom et al. (2010) also report that Mo foliar application on 'Merlot' grapevines is a promising technique to improve yield in addition to yield-related traits.

Lettuce (*Lactuca sativa* L.) is among the most consumed green leafy vegetable (Li et al., 2010; Luz et al., 2008) although it is frequently underestimated in terms of nutritional and functional value. Lettuce contributes significantly to improve the human diets, being a notable source of minerals, carotenoids, folates, flavonoids, phenolic acids, and vitamins B9, C, and E (Romani et al., 2002; Kim et al., 2016). The species includes numerous horticultural groups displaying a high phenotypical variation. The leaves are used in ready to eat salads and sandwiches, cooked or raw. Lettuce is considered a cool-season crop cultivated on all continents, especially in temperate and subtropical climates (Prohens-Tomàs and Nuez, 2008).

PHs application and Mo biofortification are easy, effective, and concrete approaches to lettuce production. Nevertheless, since plant reaction to PHs and Mo biofortification are influenced by genotype, cultivation conditions and practices, a particular investigation is imperative to appraise methods and doses. Based on the aforesaid evidence, the aim of our study was to assess the interactive influence of

PHs application and Mo supply dose on yield, morphology, as well as the nutritional and functional features of an established lettuce variety in a typical cultivation area.

Materials and Methods

Plant Genetic Material and Experimental Site

The study was performed at the Department of Agricultural, Food and Forestry Sciences of the University of Palermo (SAAF), sited in Palermo (latitude 38°12′ N, longitude 13°36′ E, altitude 65 m). The trial was carried out in a tunnel 30.0 × 5.0 × 2.0 m3, length × width × height, respectively. The tunnel was covered with a transparent polyethylene film and equipped with a drip irrigation system. A data logger placed inside the tunnel collected daily minimum and maximum temperatures (Figure 1).

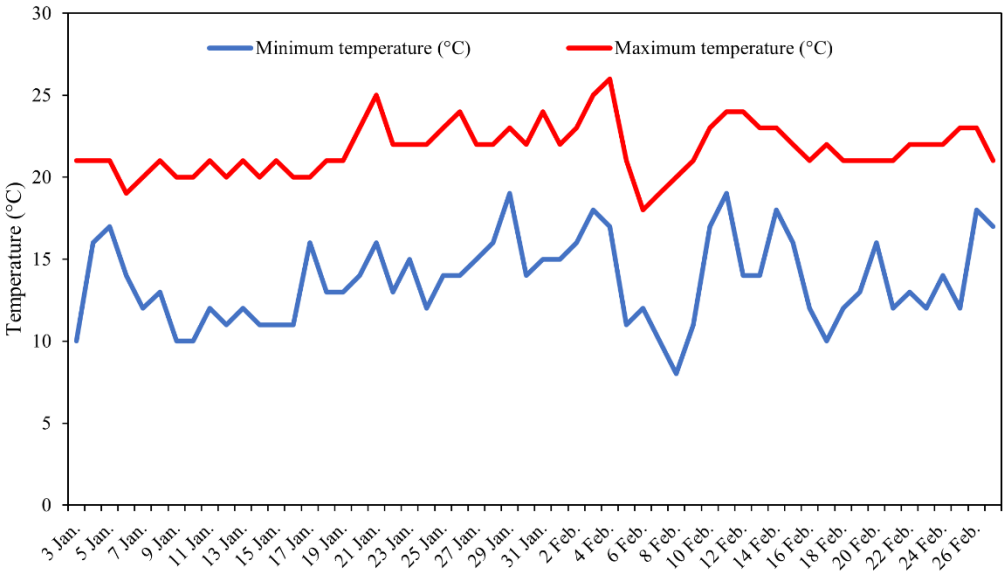

Figure 1. Daily maximum and minimum temperature from 3 January to 26 February at the experimental site.

On 5 January 2020, 360 plug plants of 'Canasta' lettuce (*Lactuca sativa* L.) (Syngenta Seed, Basel, Switzerland), at the stage of four-five true leaves, were transplanted spacing 0.25 m between rows and 0.20 m inter-rows making a plant density of 20 plants m^{-2}. During the entire cultivation period, all lettuce plant needs were assured following all the cultivation recommended practices (Tesi, 2010). The soil was basically sandy clay loam at pH 7.2, composed by 1.6% of total nitrogen

and 3.2% of organic matter. All lettuce plants were harvested 55 days after transplant.

Plant-Derived Protein Hydrolysates Application and Molybdenum Biofortification

The protein hydrolysates (PHs) treatments were performed using TYSON® (Mugavero fertilizers, Palermo, Italy), an organic biostimulant composed by 5% of total nitrogen (4.5% organic nitrogen), 25% of organic carbon and 31% of amino acids, obtained through enzymatic hydrolysis of soy plants at low temperature. PHs treatment was started one week after transplant, and it was accomplished weekly. Two doses of PHs were applied, 0 (control) or 3 mL L^{-1} (standard dose) and distributed via foliar spray. Four different doses of molybdenum (Mo) [0.0 (control), 0.5, 3.0 and 6.0 µmol Mo L^{-1}], in form of sodium molybdate (Na_2MoO_4), were supplied via foliar spray. Mo fertilizations started one week after transplant and were applied every ten days.

Yield, Nutritional and Bioactive Attributes of Lettuce Plants

All the analyses on the agronomic and functional features of the 'Canasta' lettuce plants were carried out on five plant samples, randomly selected from each replicate. Measurements of head fresh weight (HFW), head height (HH), stem diameter (SD), and number of leaves (NL) were taken immediately after plant harvest. To assess soluble solids content (SSC), 120 g of leaf samples were juiced and clarified; the measure of SSC was acquired by a refractometer (MTD-045 nD, Three-In-One Enterprises Co. Ltd. New Taipei, Taiwan). SSC was expressed as °Brix. To determine the head dry matter (HDM) percentage, the samples were placed in a thermo-ventilated oven at 105 °C till reaching steady weight. The HDM values were expressed as percentage. Immediately after plant harvest, colour coordinates were recorded through a colorimeter (Chroma-meter CR-400, Minolta corporation Ltd., Osaka, Japan) on five undamaged leaves arbitrarily designated from each replicate. CIELab colour coordinates a*, b* and L * indicate, respectively, the red-green axis, the yellow-blue axis, and the lightness.

Ascorbic acid concentration was determined in leaf samples implementing a Reflectometer Merck RQflex10 Reflectoquant® (Sigma-Aldrich, Saint Louis, MO,

USA) and Reflectoquant Ascorbic Acid Test Strips (Merck, Darmstadt, Germany). Total phenolic concentration was assessed following the Folin-Ciocalteu method (Meda et al., 2005). Briefly, the leaf sample was mixed with deionized water, sodium carbonate and Folin-Ciocalteau reagent, then the solution was incubated at ambient temperature for 30 min. The 750 nm wavelength was used to measure the mixture's absorbance with the means of a spectrophotometer. After a conversion, total phenolic concentration was expressed as μg g^{-1} fresh weight (fw).

Sugar concentration in lettuce plants was evaluated as reported by Serna et al. (2013). Briefly, three grams of the plant sample were centrifuged at 15,000 × g for 20 min with 10 mL of distilled water. Then, a 10 μL aliquot was analysed by high-performance liquid chromatography (HPLC). Results were shown as a percentage of fresh weight.

Plant Pigments and Mineral Profile

Total chlorophyll and carotenoid concentrations in leaf tissues were evaluated as reported by Costache et al. (2012). Briefly, 1 g of fresh leaf sample was mixed with methanol and homogenized, then the measure was accomplished with a spectrophotometer. Total chlorophyll was expressed as mg 100 g^{-1} fresh weight, whereas carotenoid concentration was shown as μg g^{-1} fresh weight.

Potassium (K), calcium (Ca), and magnesium (Mg) concentrations were determined following the method of Morand and Gullo (1970). The phosphorous (P) concentration in leaf tissues was measured as described by Fogg and Wilkinson (1958). Total nitrogen (N) concentration was assessed following the Kjeldahl method. Molybdenum (Mo) concentration was appraised by inductively coupled plasma-mass spectrometry (ICP-MS) (Plasma Quant MS Elite, Jena, Germany), as detailed by Sabatino et al. (2019c). All the mineral concentrations in leaf tissues were expressed as mg g^{-1} dry weight.

Nitrogen Indices

Nitrogen use efficiency (NUE), nitrogen uptake efficiency (UE) and nitrogen physiological efficiency (PUE) were calculated using the following formulas: NUE

= yield (t)/N application rate (kg); UE = plant N content (kg)/N application (kg); PUE = yield (t)/plant nitrogen content (kg).

Statistics and Experimental Design

A randomized complete block design was adopted for this experiment, with each block replicated three times. Two doses of PHs (0.0 or 3.0 mL L^{-1}) were combined with four doses of Mo (0.0, 0.5, 3.0 or 6.0 µmol Mo L^{-1}) in a two factorial trial displaying eight treatments in total (2 PHs doses × 4 Mo doses). Each treatment included 15 plants, rendering a total of 360 plants. All data were analysed by implementing the SPSS software v.20 (StatSoft, Inc., Chicago, IL, USA) package through a two-way analysis of variance (ANOVA), setting PHs and Mo doses as fix factors. A Tukey honestly significant difference (HSD) test ($p \leq 0.05$) was used to separate the means. Percentage data were converted via arcsin transformation prior ANOVA analysis ($Ø = \arcsin(p/100)^{1/2}$).

To summarize all plant traits, a heat map was also provided through the online program package clustvis (https://biit.cs.ut.ee/clustvis/ (accessed on 15 January 2021).) with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

Results

Crop Yield and Biometric Features

ANOVA for HFW, HH, SD and NL showed no significant interaction between PHs and Mo treatments (Table 1).

Table 1. Effect of PHs treatment and Mo-biofortification on head fresh weight (HFW), head height (HH), stem diameter (SD), and number of leaves (NL) of 'Canasta' lettuce cultivated in a protected environment.

Treatments	HFW (g)	HH (cm)	SD (mm)	NL (no.)
Biostimulant				
Non-treated	500.1 b	23.2 b	27.1 a	26.4 a
PH	630.7 a	26.0 a	26.8 a	26.5 a
Biofortification (µmol Mo L^{-1})				
0.0	572.9 a	24.4 a	29.8 a	25.9 a
0.5	530.1 a	24.8 a	27.8 b	26.7 a
3.0	597.0 a	24.4 a	26.5 c	27.8 a
6.0	561.6 a	24.7 a	23.5 d	25.5 a
Significance				
PH	***	***	NS	NS
Mo	NS	NS	***	NS
PH × Mo	NS	NS	NS	NS

Values in a column followed by different letters are significantly different at $p \leq 0.05$. NS, *** non-significant or significant at 0.001, respectively.

Irrespective of the Mo treatments, PHs significantly increased HFW. Regardless of the PHs application, Mo dosage did not significantly affect HFW of lettuce plants. Data on HH supported the trend described for HFW (Table 1). Disregarding Mo fertilization, PHs application did not significantly influence SD values. Conversely, regardless of the PHs treatment, SD was significantly higher in non-biofortified plants than in plants supplied with Mo (Table 1). ANOVA for NL did not display a significant influence of the main factors and of their interaction (Table 1).

Mineral Profile in Leaf Tissues

ANOVA analysis for P, K, Ca, and Mg did not reveal a significant interaction PHs × Mo (Table 2). PHs application and Mo concentration did not significantly affect P and Ca concentration in plant tissues (Table 2). Irrespective of the PHs treatments, Mo dosage did not influence leaf K concentration. Conversely, regardless of the Mo dosage, plants treated with PHs showed a higher K concentration than non-treated ones (Table 2). Data on Mg concentration sustained the tendency described for K concentration (Table 2).

Table 2. Effect of PH treatment and Mo-biofortification on phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg) concentrations of 'Canasta' lettuce leaves grown in a protected environment.

Treatments	P (mg g^{-1} dw)	K (mg g^{-1} dw)	Ca (mg g^{-1} dw)	Mg (mg g^{-1} dw)
Biostimulant				
Non-treated	4.20 a	32.48 b	1.77 a	10.32 b
PH	4.24 a	40.13 a	1.75 a	11.50 a
Biofortification (µmol Mo L^{-1})				
0	4.23 a	36.09 a	1.72 a	11.12 a
0.5	4.25 a	36.10 a	1.78 a	10.75 a
3	4.20 a	36.87 a	1.76 a	11.26 a
6	4.20 a	36.14 a	1.78 a	10.49 a
Significance				
PH	NS	***	NS	***
Mo	NS	NS	NS	NS
PH × Mo	NS	NS	NS	NS

Values in a column followed by different letters are significantly different at $p \leq 0.05$. NS, *** non-significant or significant at 0.001, respectively.

Plants not treated with PHs and without Mo supply had the highest leaf nitrogen concentration followed by those treated with PHs and without Mo supply which in turn revealed a higher N concentration than those supplied with 0.5 µmol Mo L^{-1} and not treated with PHs. The lowest N concentration was assessed in plants from the combination PHs × 6.0 µmol Mo L^{-1} (Figure 2).

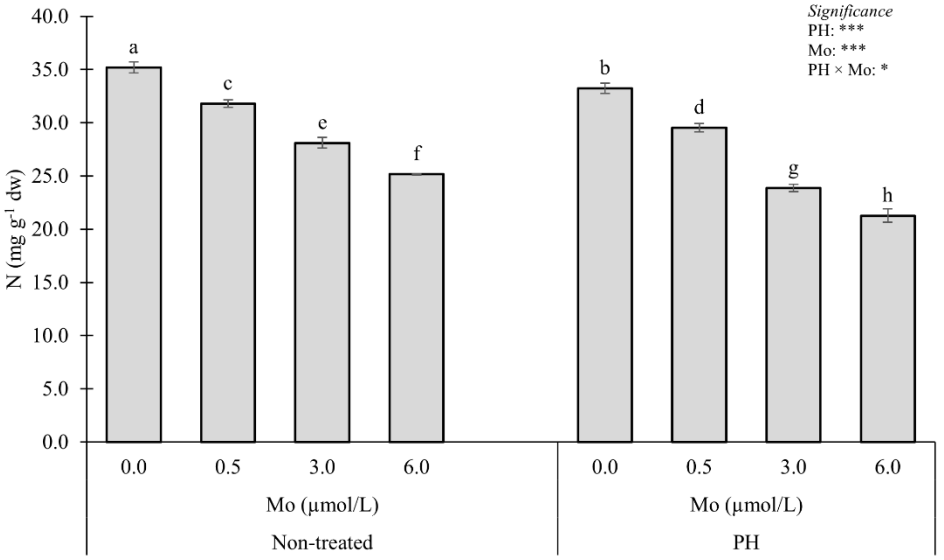

Figure 2. Effects of PH and Mo treatments on nitrogen concentration in 'Canasta' lettuce leaves grown in a protected environment. Values with different letters significantly differ at $p \leq 0.05$. *, *** significant at 0.05 and 0.001, respectively.

Statistical analysis for Mo concentration revealed a significant interaction PHs × Mo (Figure 3); the highest Mo concentration was observed in plants treated with the PHs and 6.0 µmol Mo L^{-1} followed by that recorded in the combination PHs × 3.0 µmol Mo L^{-1}. Meanwhile, the lowest values were recorded in plants not treated with PHs and without Mo supply and in those treated with PHs and without Mo supply (Figure 3).

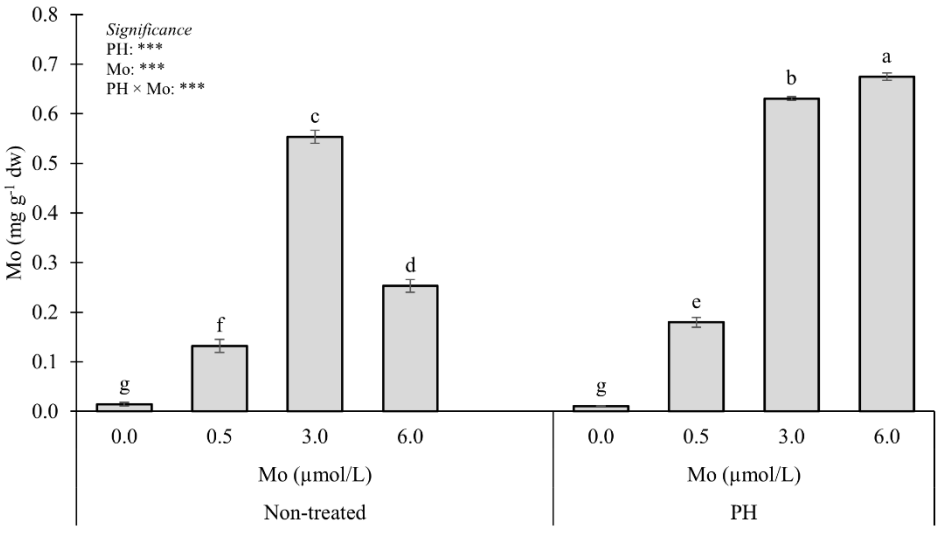

Figure 3. Effects of PH and Mo treatments on molybdenum concentration in 'Canasta' lettuce leaves grown in a protected environment. Values with different letters significantly differ at $p \leq 0.05$. *** significant at 0.001.

Nutritional and Bioactive Compounds

Statistical analysis for ascorbic acid and CIELab parameters displayed no significant interaction PHs × Mo (Table 3).

Table 3. Effect of PH treatment and Mo-biofortification on ascorbic acid and CIELab parameters of 'Canasta' lettuce grown in a protected environment.

Treatments	Ascorbic acid (mg g^{-1} fw)	a*	b*	L*
Biostimulant				
Non-treated	32.80 b	-15.67 a	29.80 a	59.76 a
PH	34.97 a	-18.19 b	28.86 a	57.16 a
Biofortification (µmol Mo L^{-1})				
0	27.62 d	-17.18 a	31.18 a	59.31 a
0.5	32.03 c	-16.84 a	29.79 a	57.83 a
3	36.75 b	-16.46 a	29.08 a	58.47 a
6	39.13 a	-17.29 a	27.34 a	58.22 a
Significance				
PH	***	***	NS	NS
Mo	***	NS	NS	NS
PH × Mo	NS	NS	NS	NS

Values within a column followed by different letters are significantly different at $p \leq 0.05$. NS, *** non-significant or significant at 0.001, respectively.

Disregarding the Mo concentration, PHs treated plants showed a higher ascorbic acid concentration than untreated ones. Irrespective of PHs treatments, our outcomes revealed that augmenting the Mo concentration resulted in a significant increase in ascorbic acid leaf content (Table 3). Results on a* colour parameter showed that, irrespective of the PHs treatment, Mo biofortification did not significantly influence a* coordinate (Table 3). Contrariwise, PHs significantly decreased a* coordinate (Table 3). ANOVA and means separation revealed that both PHs application and Mo concentration did not significantly affect b* and L* CIELab parameters. (Table 3).

ANOVA for HDM, SSC, total sugars and total phenolics showed an interactive effect PHs × Mo (Table 4).

Table 4. Effect of PH treatment and Mo-biofortification on head dry matter (HDM), soluble solid content (SSC), total sugars and total phenolics of 'Canasta' lettuce grown in a protected environment.

Treatments	HDM (%)	SSC (Brix°)	Total sugars (% fw)	Total phenolics (µg g^{-1} fw)
Biostimulant × Biofortification (µmol Mo L^{-1})				
Non-treated × 0.0	14.53 c	2.13 d	1.45 f	37.03 g
Non-treated × 0.5	13.87 d	2.20 d	1.62 e	44.13 f
Non-treated × 3.0	14.53 c	2.30 c	1.92 d	55.07 d
Non-treated × 6.0	15.11 b	2.33 c	2.17 c	75.23 b
PH × 0.0	15.68 a	2.15 d	1.48 f	36.57 g
PH × 0.5	15.11 b	2.40 b	1.92 d	47.07 e
PH × 3.0	14.59 c	2.42 b	2.24 b	59.53 c
PH × 6.0	13.24 e	2.53 a	2.54 a	80.6 a
Significance				
PH	NS	***	***	***
Mo	***	***	***	***
PH × Mo	***	*	***	*

Values within a column followed by different letters are significantly different at $p \leq 0.05$. NS, *, *** non-significant or significant at 0.05 and 0.001, respectively.

Plants of the combination PHs × 0.0 µmol Mo L^{-1} had the highest HDM percentage followed by the plants not treated with PHs and supplied with 6.0 µmol Mo L^{-1}. The lowest HDM value was recorded in plants treated with PHs and supplied with the highest dose of Mo (Table 4). PHs treated plants and subjected to the highest Mo dosage had the highest SSC followed by the plants of the combinations PHs × 0.5 µmol Mo L^{-1} and PHs × 3.0 µmol Mo L^{-1}. In contrast, the lowest SSC values were recorded in plants not treated with PHs and not supplied with Mo and in those treated with PHs and supplied with 0.5 µmol Mo L^{-1} (Table 4). The highest total sugars concentration was exhibited in plants from the combination PHs × 6.0 µmol Mo L^{-1} (Table 4). Meanwhile, the lowest total sugars values were observed in plants not treated with PHs and not supplied with Mo and in those treated with PHs and without Mo supply (Table 4). Plants from the combination PHs × 6.0 revealed the highest total phenolic content, followed by those not treated with PHs and supplied with the highest dosage of Mo. The lowest total phenolic concentration was registered in plants not treated with PHs and not supplied with Mo and in PHs treated plants and without Mo supply (Table 4).

Pigments

ANOVA for carotenoids showed a significative interaction PHs × Mo (Figure 4a); plants from the PHs × 6.0 µmol Mo L^{-1} combination had the highest values, followed by the plants not treated with PHs and supplied 6.0 µmol Mo L^{-1}. However, PHs treated plants and supplied with a dosage of 3.0 µmol Mo L^{-1} did not significantly differ neither from plants belonging to the PHs × 6.0 µmol Mo L^{-1} combination, nor from those not treated with PHs and supplied with 6.0 µmol Mo L^{-1}. The lowest carotenoids concentration was observed in plants not treated with PHs and not supplied with Mo (Figure 4a). Results on leaf total chlorophyll showed no interaction between PHs and Mo treatments (Figure 4b). Irrespective of the Mo treatments, total chlorophyll concentration was higher in plants treated with PHs. Averaged over PHs treatment, total chlorophyll concentration increased with the increase of the Mo concentration (Figure 4b).

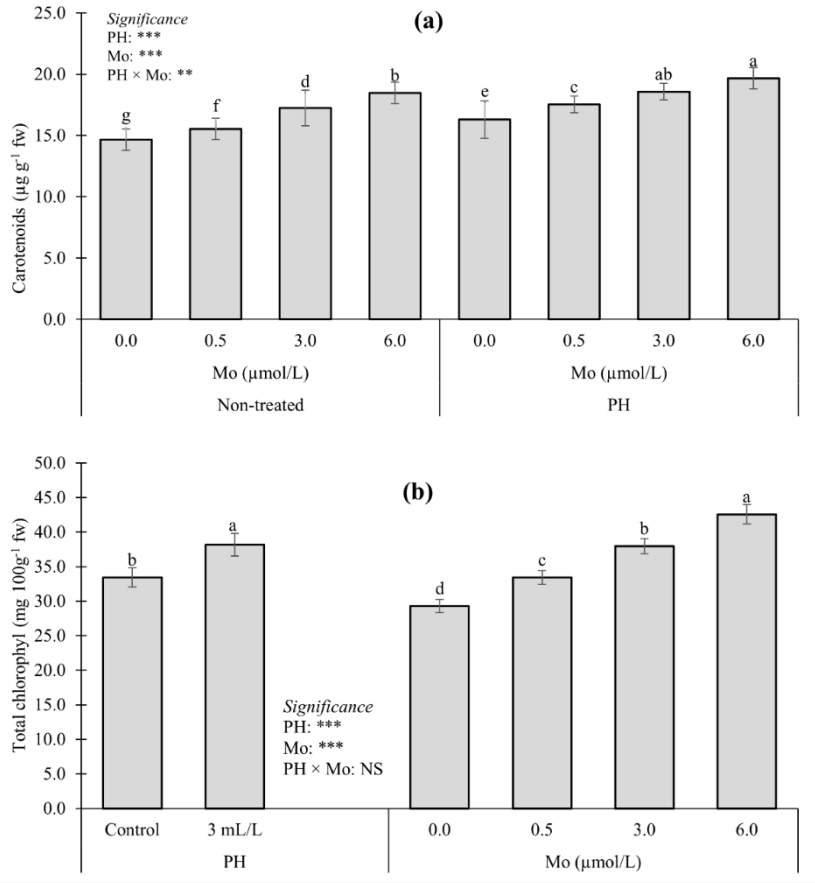

Figure 4. Effects of PH and Mo treatments on carotenoids (a) and total chlorophyll (b) concentration in 'Canasta' lettuce plants. Values with distinct letters significantly differ at $p \leq 0.05$. NS, **,*** non-significant or significant at 0.005 or 0.001, respectively.

Nitrogen Indices

Statistical analysis for NUE did not display a significant interaction PHs × Mo (Figure 5a). Regardless of the Mo treatments, plants supplied with PHs revealed a higher NUE values than non-treated ones. Averaged over PHs, Mo application did not influence the NUE (Figure 5a).

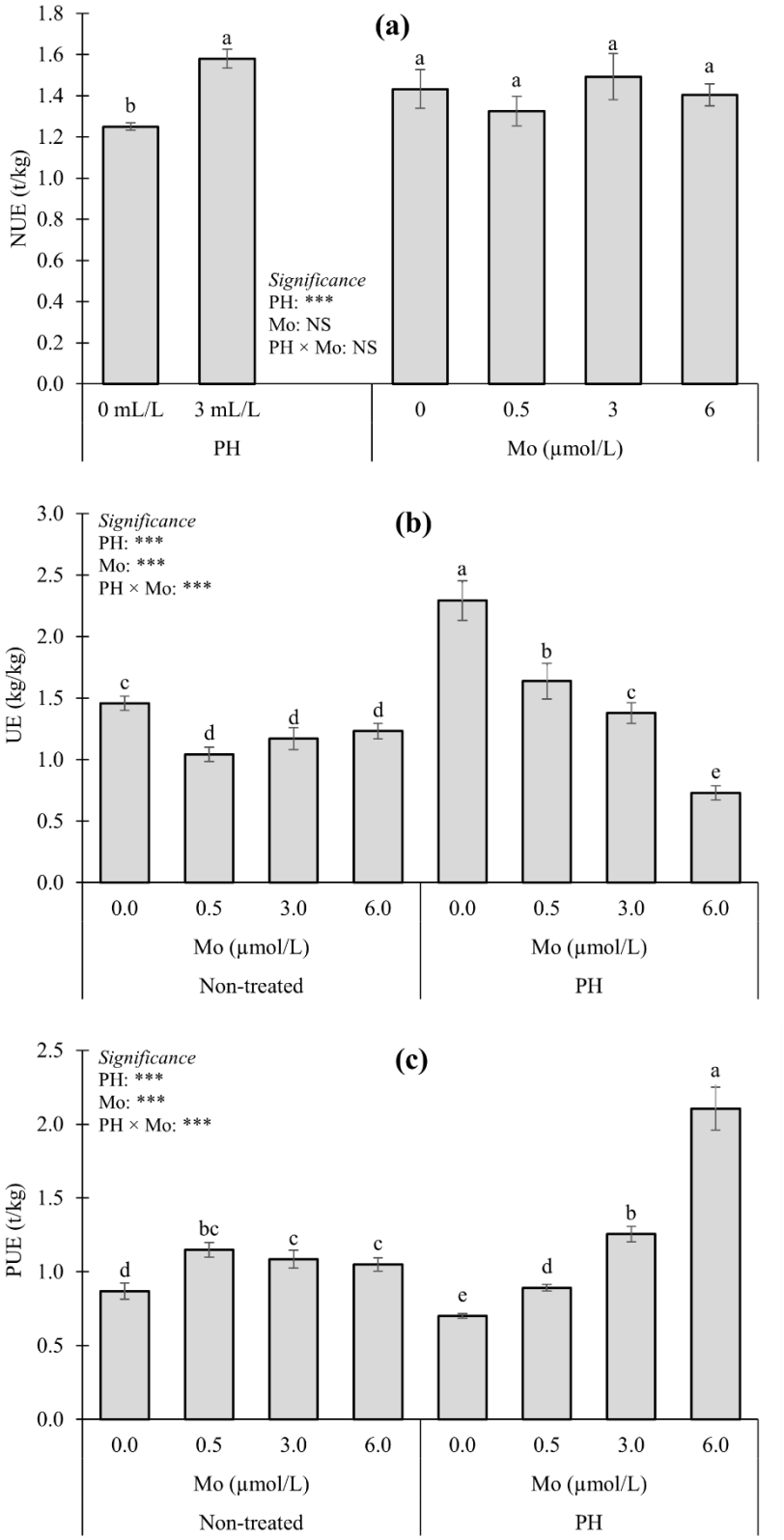

Figure 5. Effects of PH and Mo treatments on nitrogen use efficiency (a), nitrogen utilization efficiency (b) and nitrogen physiological use efficiency (c) of 'Canasta' lettuce plants. Values with distinct letters significantly differ at $p \leq 0.05$. NS, *** non-significant or significant at or 0.001, respectively.

Statistics on UE underlined a significant interaction PHs × Mo (Figure 5b); the highest UE values were obtained in plants treated with PHs and not supplied with Mo, followed by those recorded in plants treated with PHs and supplied with a dosage of 0.5 µmol Mo L^{-1}. Inversely, the combination PHs × 6.0 µmol Mo L^{-1} showed the lowest UE values (Figure 5b). ANOVA for PUE highlighted a significant interaction between PHs and Mo (Figure 5c). The highest PUE values were recorded in the PHs × 6.0 µmol Mo L^{-1} combination, followed by those collected in the PHs × 3.0 µmol Mo L^{-1} combination. However, plants not treated with PHs and supplied with 0.5 µmol Mo L^{-1} did not significantly differ in terms of PUE neither from plants non treated with PHs and supplied with 3.0 or 6.0 µmol Mo L^{-1}, nor from those treated with PHs and supplied with 3.0 µmol Mo L^{-1}. The lowest PUE values were observed in plants treated with PH and not supplied with Mo (Figure 5c).

Heat map analysis of all recorded plant traits

A heat map analysis of all plant traits was carried out to reveal a chromatic assessment of the PHs and Mo treatments on plant traits. The heat map displayed two dendrograms, one situated on the top and called Dendrogram 1, and another placed on the left side, named Dendrogram 2 (Figure 6).

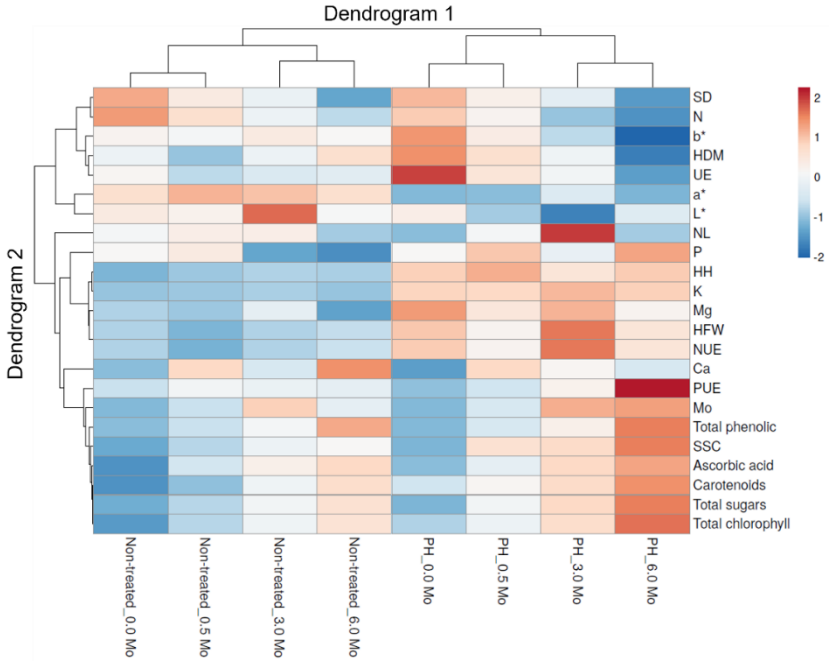

Figure 6. Heat map analysis including all 'Canasta' lettuce plant traits in response of PH and Mo treatments. The heat map figure was created using the https://biit.cs.ut.ee/clustvis/ online program package.

Dendrogram 1 enclosed the combinations of PHs and Mo treatments, whereas Dendrogram 2 showed all investigated traits that affected this distribution. Dendrogram 1 showed two main clusters: on the left, it gathered the PHs untreated × 0.0, 0.5, 3.0, and 6.0 µmol Mo L^{-1} combinations, while on the right side the cluster includes the PHs treated × 0.0, 0.5, 3.0, and 6.0 µmol Mo L^{-1} combinations (Figure 6). In particular, two clusters were recognized on the left side of Dendrogram 1. The first on left side enclosed the PHs untreated × 0.0 µmol Mo L^{-1} and PHs untreated × 0.5 µmol Mo L^{-1} combinations, separated from PHs untreated × 3.0 µmol Mo L^{-1} and PHs untreated × 6.0 µmol Mo L^{-1} combination, which revealed in particularly lower values for SD, N, HDM, UE, NL and P, but higher values for b*, L*, Ca, PUE, Mo, total phenolic, SSC, ascorbic acid, carotenoids, total sugars, and total chlorophyll. The group on the left comprised untreated × 0.0 and non-treated 0.5 combination. Within this cluster, PHs untreated × 0.0 µmol Mo L^{-1} combination is clearly separated by higher SD, N, HDM, UE, Mg, HFW, and NUE and lower a*, P, HH, Ca, PUE, Mo, total phenolic, SSC, ascorbic acid, carotenoids, total sugars, and total chlorophyll. Meanwhile, the grouping on the right contained the PHs untreated × 3.0 µmol Mo L^{-1} and the PHs untreated × 6.0 µmol Mo L^{-1} combinations. Within this cluster, the PHs untreated × 3.0 µmol Mo L^{-1} combination was parted by higher SD, N, b*, a*, L*, NL, P, Mg, and Mo and lower HDM, HFW, NUE, Ca, total phenolic, carotenoids, total sugars, and total chlorophyll. On the right side of the Dendrogram 1 two clusters were identified, the first on the left comprised PHs treated × 0.0 µmol Mo L^{-1} and PHs treated × 0.5 µmol Mo L^{-1} combinations separately from the PHs treated × 3.0 µmol Mo L^{-1} and PHs treated × 6.0 µmol Mo L^{-1} combination, which had, particularly, higher SD, N, b*, HDM, UE and L*, but lower NL, HFW, NUE, PUE, Mo, total phenolic, SSC, ascorbic acid, carotenoids, total sugars, and total chlorophyll. The cluster on the left included the PHs treated × 0.0 µmol Mo L^{-1} combination. In this cluster, the PHs treated × 0.0 µmol Mo L^{-1} combination is noticeably separated by lower NL, P, HH, Ca, PUE, Mo, total phenolic, SSC, ascorbic acid, carotenoids, total sugars, and total chlorophyll, whereas the cluster on the right includes Hs treated × 3.0 µmol Mo L^{-}

^{1} and PHs treated × 6.0 µmol Mo L^{-1} combinations. Inside this cluster, the PHs treated × 3.0 µmol Mo L^{-1} combination is separated by lower L*, P, HH, PUE, total phenolic, SSC, ascorbic acid, carotenoids, total sugars, and total chlorophyll, and higher SD, N, b*, HDM, UE, a*, NL, K, Mg, HFW, NUE, and Ca. The clusters in Dendrogram 2 evidently point out the differential effects of PHs application and Mo supply.

Discussions

In recent years, PHs have acquired importance as plant biostimulants owing to their capacity to enhance the seed germination, yield, and quality of vegetables (Colla et al., 2017). Concomitantly, plant mineral nutrition organization, including the supply of trace elements such as molybdenum, is a key pre-harvest aspect for yield and quality improving many vegetable crops. In the present research, the possible use of PHs and Mo biofortification, alone or combined, to improve yield and quality of lettuce was apprised. The study revealed that PHs significantly increased HFW and HH traits. These results are coherent with those listed by Di Mola et al. (2019a) who, by studying the influence of plant-based biostimulants on agronomical, physiological, and qualitative responses of baby rocket leaves under varied nitrogen levels, found that legume-derived PHs positively affected yield trait. Our outcomes are also in line with those indicated by Consentino et al. (2020) in celery. There are also reports that PHs have a biostimulant role via the variation of plant molecular and physiological mechanisms that elicit growth and plant productivity (Yakhin, 2017; Calvo et al., 2014). PHs effects on plants comprise carbon and nitrogen metabolism stimulation. Furthermore, as reported by a number of authors (Du Jardin, 2015; Nardi et al., 2016; Colla et al., 2015), PHs have a significant role in the regulation of plant nitrogen uptake, via key enzymes included in the nitrogen assimilation mechanism, and in the regulation of the activity of three enzymes involved in the tricarboxylic acid cycle. Moreover, as specified by Colla et al. (2014) and Colla et al. (2015), bioactive peptides included in PHs could also interact with plant hormonal actions. Numerous researches have revealed that PHs can stimulate hormone-like activities (gibberellins and auxin), encouraging plant organ growth, and consequently crop yield (Ertani et al., 2009; Matsumiya and Kubo,

2011; Colla et al., 2014; Lucini et al., 2015). In this study, the improved yield of plants treated with PHs could be also attributed to the certainty that PHs TYSON® contains tryptophan (forerunner of indole-3-acetic acid) which determines shoot and root expansion in plants. Data indicated that although Mo biofortification did not affect HFW, HH, and NL, but it significantly influenced SD. These findings are consistent with those of Moncada et al. (2018) on lettuce and with those of Biacs et al. (1995) and Vieira et al. (2005) on carrot and common bean. Plant N concentration indicated that the PHs application reduced N accumulation in leaf tissue. These findings concur with those reported by Liu and Lee (2012), who stated that amino acids mix treatments significantly reduced nitrate accumulation in several leafy vegetables. Moreover, data presented are in line with that reported by Tsouvaltzis et al. (2014) who found that lettuce nitrate build-up reduces as PHs dose increases. PHs action in limiting the relevant accumulation of nitrogen in plants might be related to the huge regulation attitude of many metabolic pathways involved in the metabolism of nitrogen (Colla et al., 2017). Additionally, other authors (Colla et al., 2015) pointed out that PHs with a high level of free amino acids guide to stout phloem loaded with amino acids which, accordingly, diminish root nitrate absorption and accumulation. Thus, as suggested by Consentino et al. (2020) in celery, the reduction of N concentration in plants treated with PHs could be attributed to the use of their own nitrogen deposits without supplemental nitrogen uptake. Generally, the imperative function of amino acids is the modulation of a number of mechanisms and metabolic pathways related to the nitrogen metabolism in plants (Calvo et al., 2014; Liu and Lee, 2012). The present study revealed that N leaf concentration decreased as Mo dosage increased. These findings are coherent with those obtained by Moncada et al. (2018), who report that a higher Mo dosage is effective in N leaf concentration reduction in lettuce, escarole, and curly endive plants. Our results support those of Zhen et al. (2007) who found that Mo supply reduces N content in lettuce plant tissues. There are reports of a negative relation between Mo dosage and N concentration in spinach and poinsettia plants (Cantliffe et al., 1974; Cox 1992a and 1992 b). As stated by Schwarz (2016), molybdenum cofactors (Moco) contribute to the active site of nitrate reductase which has an imperative function in nitrate absorption and may

enhance the nitrogen use efficiency. PHs did not significantly affect P and Ca leaf concentration. On the contrary, PHs application resulted an effective tool to enhance K and Mg concentration. This is in accordance with the findings of Rouphael et al. (2017), who indicated that plant-derived PHs meaningfully augment K and Mg concentrations in tomato fruits, and it also concurs with the results of Consentino et al. (2020) who found that plant or animal-derived PHs significantly improve K and Mg concentrations in celery, although they do not affect P and Ca concentration. Furthermore, data on mineral profile revealed that Mo-biofortification did not significantly affect P, K, Ca, and Mg concentration in lettuce. PHs application enhanced Mo concentration in plant tissues. This output is in line with other studies (Colla et al., 2017; Ertani et al., 2009; Colla et al., 2015) which report that PHs enhance plant macro- and micronutrient uptake. Additionally, while PHs non-treated plants showed a decrease in Mo concentration when Mo concentration in the nutrient solution was higher than 3.0 µmol Mo L^{-1}, PHs treated plants revealed a positive relation between Mo supply dosage (up to 6.0 µmol Mo L^{-1}) and Mo concentration in leaf tissues. Thus, we may hypothesize that PHs applications improves plant Mo tolerance. PHs significantly increased ascorbic acid concentration. This is in accordance with other studies, which found that foliar application of plant-derived PHs enhances ascorbic acid in tomato and celery (Colla et al., 2017; Rouphael et al., 2017; Consentino et al., 2020). The ascorbic acid concentration could be linked to the improved mineral uptake of plants treated with PHs, which could in turn enhance the synthesis of some amino acids, like tyrosine and phenylalanine (Rouphael et al., 2017). In our study, Mo supply positively affected ascorbic acid content. This is consistent with the report of Moncada et al. (2018) on lettuce, escarole, and curly endive. As described by Valenciano et al. (2011), ascorbic acid concentration can be influenced by Mo supply. In this regard, the intensification of enzymes responsible for ascorbic acid oxidation (phenol oxidase and peroxidase) is associated with Mo plant concentration. Contrariwise, Sabatino et al. (2019c) studying the interactive effect of genotype and Mo supply on tomato yield and fruit quality found a decrease in ascorbic acid concentration with an increase in Mo level. This contrasting result

might be related to the different Mo plant organ distributions among plant species (Brodrick and Giller, 1991).

Colour of vegetables is a fundamental visual quality feature that often influences consumer's choice. Colour parameters revealed that PHs application increased leaf greenish, whereas it did not significantly affect b* and L* colour traits. This is consistent with the findings of Consentino et al. (2020). These results are partially coherent with those of other authors (Caruso et al., 2019; Giordano et al., 2020) who reported that PHs treatment does not significantly influence colour traits in perennial wall rocket. In this study, Mo biofortification did not affect colour traits. Our results partially differ from those of Moncada et al. (2018), who found that Mo-enrichment does not affect L* coordinate, but significantly influences Chroma and Hue angle. HDM indicated that PHs application combined with Mo biofortification (up to 3.0 µmol Mo L^{-1}), enhanced HDM. In contrast, PHs application combined with the highest Mo dosage (6.0 µmol Mo L^{-1}) negatively affected HDM parameter. This is in accordance with the findings of Sabatino et al. (2019c) who revealed that Mo supply decreases fruit water content in tomato. Moreover, the results presented are consistent with those of Moncada et al. (2018), who stated that Mo-biofortification significantly increases head dry matter in lettuce, escarole, and curly endive. Similar results are also reported by Boertje (1969), Valenciano et al. (2011) and Randall et al. (1969) on lettuce, chickpea, and grain, respectively. However, it can be hypothesized that the higher mineral uptake efficiency of PHs treated plants combined with the highest Mo level caused a HDM reduction, which in turn can be considered a plant toxicity signal. SSC was positively influenced by the interactive effect of PHs and Mo and the best results were recorded in plants from the combination PHs × 6.0 µmol Mo L^{-1}. These results are partially in line with those of Moncada et al. (2018) who reported that a Mo supply up to 1.5 µmol Mo L^{-1} positively affects lettuce SSC, whereas a Mo supply at 3.0 µmol Mo L^{-1} reduces SSC content. In the present study, Mo levels up to 3.0 µmol Mo L^{-1} enhanced SSC in PHs non-treated plants, whereas Mo at 6.0 µmol Mo L^{-1} did not determine a further increase. Contrariwise, PHs treated plants showed a positive relation between Mo dosage and SSC values. Thus, it seems that PHs application can boost plant Mo tolerance. PHs and Mo supply significantly interacted

enhancing total sugars. In particular, plants from the PHs × 6.0 µmol Mo L^{-1} combination had the highest sugar content. This is in accordance with the finding of Carillo et al. (2019) who, by investigating the effect of PHs biostimulant and nitrogen fertilization level on morphological and physiological traits of spinach grown in a greenhouse, found that irrespective of the nitrogen fertilization level, PHs significantly increased sucrose concentration in spinach. Furthermore, the study underlined that Mo supply improved total sugars in 'Canasta' lettuce up to the highest dosage (6.0 µmol Mo L^{-1}). Concurrently, the best results were observed in plants treated with PHs and supplied with the highest Mo dosage. This is partially consistent with the reported of Liu et al. (2017) who, studying the effect of Mo-biofortification on nutrition, quality, and volatile compounds of strawberry fruits, found that total sugars increased when Mo supply ranged from 0 to 135 g ha^{-1}, but decreased at the highest dosages. Thus, considering that a dose of 6.0 µmol Mo L^{-1} is not harmful for several plant physiological activities in lettuce and since Xi et al. (2014) found that carbon dioxide supplementation could enhance total sugars concentration with a higher net photosynthesis, we may assume that Mo and carbon dioxide could increase total sugars concentration via the modulation of photosynthesis. In addition, the study demonstrated that Mo-biofortification increased total phenolics in lettuce. However, the highest concentration of total phenolics was recorded in PHs treated plants enriched with 6.0 µmol Mo L^{-1}. There are reports that PHs plant or animal derived, increase total phenolic content in celery (Consentino et al., 2020). Our outcomes also agree with those of Colla et al. (2017) who stated that both amino acids and peptides increase phenolic concentration in banana plants, and they are also coherent with those reported by Sabatino et al. (2019c), who stated that Mo-biofortification increases polyphenolic content in tomato. A similar increase was also reported by Bergmann (1992) and Gupta (1997) in tomato and cauliflower. Thus, it seems that both PHs and Mo supply have a synergistic effect on total phenolics synthesis and accumulation. PHs application and Mo supply improved carotenoids concentration. Similar results were observed in another study on the response of baby rocket to a legume-derived PHs (Di Mola et al., 2019a). We also highlighted that Mo-enrichment improved carotenoid concentration in lettuce. PHs treatment increased total chlorophyll. There are

reports that PHs determine an increase in total chlorophyll in baby rocket and lettuce (Di Mola et al., 2019a and 2019b). This is perhaps due to the high amino acid increase measured in plants subjected to plant-derived PHs, which in turn supports the increase in total chlorophyll concentration and net photosynthesis rate (Luziatelli et al., 2016). Increasing Mo level corresponded a total chlorophyll upsurge. This is in line with the finding of Liu et al. (2017), who report a chlorophyll increase with a Mo increase up to 135 g ha^{-1}. This suggest that, in 'Canasta' lettuce, a Mo dose of 6.0 µmol Mo L^{-1} did not negatively affect the physiological mechanisms involved in chlorophyll synthesis. These results are also coherent with those of Yu et al. (2006) and Zhang et al. (2014) who declared a positive relation between Mo dose and chlorophyll concentration in winter wheat and Chinese cabbage. PHs application positively affected NUE indices. This is in line with the results by Di Mola et al. (2020) who investigated the effect of plant-based biostimulant on NUE indices and crop performance of lamb's lettuce and baby spinach. The current results also underlined that Mo-biofortification enhanced the physiological use efficiency of 'Canasta' lettuce. Based on our acquaintance, this is the first investigation on the influence of Mo biofortification on nitrogen indices in a vegetable crop.

Conclusions

In the current research, the tested PHs significantly improved yield and yield-related features, nutritional and functional traits, as well as nitrogen indices. Simultaneously, Mo biofortification considerably enhanced nutritional and bioactive parameters, like ascorbic acid and SSC. Combining plant-derived PHs with 3.0 or 6.0 µmol Mo L^{-1} meaningfully improved plant nutritional and functional features, such as SSC, total sugars, total phenolics, carotenoids, plant Mo and N concentrations, and nitrogen indices as compared with untreated plants. We may also hypothesize the highest dosage of PHs had a buffer effect on plant Mo toxicity.

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EXPERIMENT 3

plants

MDPI

Article

Impact of *Ecklonia maxima* Seaweed Extract and Mo Foliar Treatments on Biofortification, Spinach Yield, Quality and NUE

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Impact of *Ecklonia maxima* seaweed extract and Mo foliar treatments on biofortification, spinach yield, quality and NUE

Introduction

Currently, modern agriculture must face the double task of nourishing the worldwide population and diminishing the ecological impact of horticultural systems (Colla et al., 2017a; Searchinger et al., 2013). One of the most pioneeristic agronomic practices to meet these challenges is the use of plant biostimulants which can elicit growth and development, productivity, abiotic stress tolerance and quality of plants (Colla and Rouphael, 2015; Du Jardin, 2015). Several authors (Colla et al., 2013; Ertani et al., 2013; Rouphael et al., 2017; Lucini et al., 2018; Rouphael et al., 2020; Sabatino et al., 2020; Consentino et al., 2020; Di Mola et al., 2021) have reported that biostimulants can promote primary and secondary metabolism in vegetables, modulating micro- and macronutrient uptake and assimilation, buildup of phytochemicals and tolerance to abiotic distresses. Among plant biostimulants, seaweed extracts (SEs), especially the brown macro-algae, are often used for their content in signaling molecules such as polysaccharides, betaines, macro- and micronutrients and phytohormones which enhance plant performance (Khan et al.,

2009; Craigie, 2011). The upsurge of crop yields prompted by SE application under optimal or unfavourable cultivation conditions has been linked with a number of physiological and biochemical mechanisms, including the elicitation of enzymes included in carbon and nitrogen metabolic pathways, the Krebs cycle and glycolysis, the stimulation of phytohormones and the boost of mineral uptake and accumulation of treated plants via root morphology alterations (Colla et al., 2015 and 2017b; Battacharyya et al., 2015). Along with the persistent concern of maximizing the yield of horticultural crops, there is an urgent request for vegetables of high quality. This is motivated by the increasing attention of consumers to vegetables containing high amounts of nutritional and biofunctional compounds. Furthermore, the enrichment of vegetables with micronutrients (agronomic biofortification) is an essential tool to overcome mineral malnourishment in humans (Sabatino et al., 2019a, 2021a and 2021b). Molybdenum (Mo) is a valuable and indispensable trace element to avoid disorders related to the simple deficiency of sulphite oxidase (Mudd et al., 1967; Cohen et al., 1971). Tsongas et al. (1980) communicated an optimal Mo consumption of 120–240 μg per day, dependent on sex, age, and income. Generally, Mo can be detected in foods such as legumes, nuts, cereals, and cereal derivatives, in form of soluble molybdates (Pennington et al., 1987). Furthermore, as specified by Pennington et al. (1987) and Rose et al. (2010), bread and pasta are the principal food providers of dietary Mo ingestion, followed by vegetables. The benefits of Mo for higher plants are well-known and documented (Arnon and Stout, 1939; Mulder, 1954; Mendel and Schwarz, 1999; Zimmer and Mendel, 1999). Plants use Mo in specific enzymes (Schwarz, 2016) which conduct redox reactions, specifically, in processes comprising nitrogen metabolism (Kaiser et al., 2005). Mo biofortification promotes plant performance in fruiting and green leafy vegetables (Moncada et al., 2018; Sabatino et al., 2019b). Moreover, Mo foliar supply in grapevines has proven itself a promising practice to enhance yields (Longbottom, 2010). Among green leafy vegetables, spinach (*Spinacia oleracea* L.) is a main crop largely cultivated in the Mediterranean area, both in open-field and protected environments. Its leaves are usually consumed either fresh or after storage using specific preservation techniques. Spinach is one of the less efficient green leafy vegetables in terms of nitrogen uptake and

utilization (Biemond et al., 1996), and concomitantly, it requires huge nitrogen supplies to develop and acquire a dark green foliage (Smolders et al., 1993). This leads spinach to build up large quantities of nitrate in its edible plant part (Biemond et al., 1996). Considering that: (i) SE application and Mo supply are both simple, effective and practical methods to improve spinach production; (ii) both SE application and Mo biofortification may improve nitrogen use efficiency in green leafy vegetables like spinach (Kaiser et al., 2005; Rouphael et al., 2018); (iii) Mo-biofortification enhances the functional aspect of vegetables (Moncada et al., 2018; Sabatino et al., 2019b); (iv) SEs increase plant performance and mineral uptake (Khan et al., 2009; Craigie, 2011); (v) SEs may improve mineral stress tolerance in plants (Colla and Rouphael, 2015; Du Jardin, 2015), specific investigations are crucial to appraise the combined effect of SE application and Mo-biofortification on spinach plant responses (direct and/or indirect). To the best of our knowledge, no research has been conducted on the combined influence of SE application and Mo biofortification on vegetables. Additionally, the possible influences of SE on productivity and nutritive quality of vegetable crops, including spinach, were mostly studied in soilless systems. Thus, the aim of the current study was to appraise the impact of SE and Mo biofortification foliar treatments on yield and yield-related parameters, minerals, nutritional and functional traits, as well as NUE indices of spinach plants grown in a protected environment.

Materials and Methods

Plant Genetic Resource and Research Location

The research was conducted in Palermo (Italy), at an experimental field of the Department of Agricultural, Food and Forestry Sciences of the University of Palermo (latitude 38.12° N, longitude 13.36° E, altitude 65 m). The study was accomplished in a tunnel covered with a transparent polyethylene film (0.05 mm in thickness) and provided with a micro-flow irrigation system with dripping wings placed on the ground. Daily maximum and minimum temperatures during the growing cycle were monitored and collected via a data logger located inside the tunnel, 1.5 m from the ground (Figure 1).

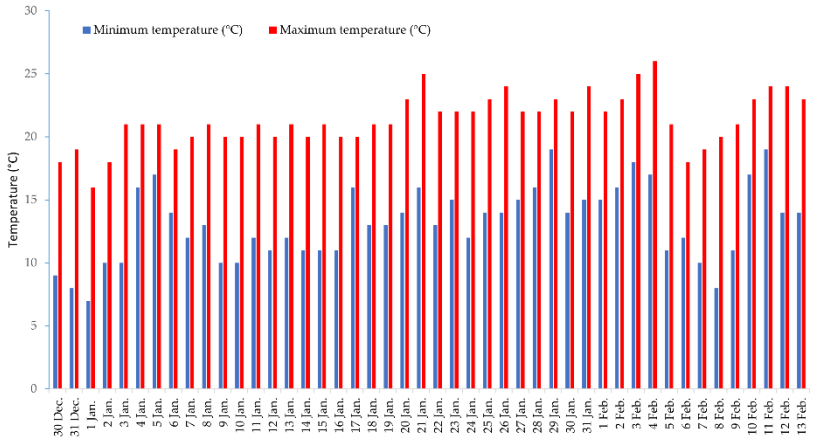

Figure 1. Maximum and minimum temperatures registered daily from 30 December to 13 February at the experimental station (latitude 38.12° N, longitude 13.36° E, altitude 65 m).

On 30 December 2020, plug plants of spinach (*Spinacia oleracea* L.) Spargo F1 (Fratelli Ingegnoli, Milan, Italy), were transplanted at the 4–5 true leaves stage with a plant density of 25 plants per m^{-2} (20 cm between and inter rows). Through the whole cultivation cycle, spinach plant demands were ensured following all conventional cultivation practices (Tesi, 2010). All plants were harvested 45 days after transplant.

Experimental Set-up and Design

The seaweed treatment was accomplished using a liquid extract of Ecklonia maxima (Kelpstar®, Mugavero fertilizers, Palermo, Italy) produced through a cold micronization process, which does not use heat or chemicals. It contains organic nitrogen (1%), organic carbon (10%), phytohormones; mainly auxin (11 mg L^{-1}) and cytokinin (0.03 mg L^{-1}) and organic substances with nominal molecular weights <50 kDa (30%). The pH of Kelpstar® is 6.0. Foliar spray SE treatments started seven days after transplant and were distributed weekly. Mo was distributed via foliar spray in form of sodium molybdate (Na_2MoO_4). Mo treatments started ten days after transplant and were accomplished every ten days. For every foliar spray application, the volume used was 1.0 L m^{-2}. Two doses of SE [0 (control treatment) or 3 mL L^{-1} (recommended dosage)] were combined with five molybdenum (Mo) doses [0.0 (control), 0.5, 2.0, 4.0 or 8.0 µmol L^{-1}]. The treatments were replicated 3

times (15 plants each one) and organized in a randomized complete block design, rendering 30 experimental units (2 SE × 5 Mo × 3 replicates).

Production, Nutritional, Nutraceutical Implications and Carotenoid Concentration of Spinach Plants

Five samples, randomly selected from each replicate, were used for carrying out all agronomic and qualitative traits of Spargo F_1 spinach. Immediately after harvesting, all plants were washed with deionized water to remove residual components of the treatments. Values of head fresh weight (head FW), head height (head H), stem diameter (SD) and number of leaves (N. leaves) are considered the main yield and yield-related traits in spinach, thus, they were collected instantaneously after the harvest. Since spinach is a green leafy vegetable, we did not consider it relevant to collect data on days to flowering. Our study focused on the assessment of the most critical nutritional and functional features of spinach. To appraise soluble solid content (SSC), 100 g of leaf sample was squeezed and filtered, then the measurement was carried out via a digital refractometer (MTD-045 nD, Three-In-One Enterprises Co. Ltd. New Taipei, Taiwan). The SSC value is reported as Brix°. After plant harvest, CIELab color parameters (a*, b* and L*) were measured via a colorimeter (Chroma-meter CR-400, Minolta corporation Ltd., Osaka, Japan) on five intact leaves casually chosen from each replicate. To evaluate percentage of dry matter (percentage DM), leaf samples were dried in an oven at 105 °C up to constant mass. The concentration of ascorbic acid in leaves was revealed using a reflectometer (Merck RQflex10 Reflectoquant®, Sigma-Aldrich Saint Louis, MO, USA) and reflectoquant ascorbic acid test strips (Merck, Darmstadt, Germany). Ascorbic acid value is expressed as mg 100 g^{-1} fresh weight. Polyphenol concentration was assessed by the Folin-Ciocalteau method (Meda et al., 2005). In brief, leaf tissue samples were mixed with Folin-Ciocalteau reagent, distilled water and sodium carbonate, then the mixture was kept in room temperature for 30 min. After that, the absorbance of the solution was evaluated at 750 nm via a spectrophotometer. The polyphenol concentration is expressed as gallic acid equivalents (GAE) 100 g^{-1} dry weight. Plant carotenoid concentration was assessed following the method reported by Costache et al. (2012). Briefly, a sample of 1 g

was mixed with methanol, then the measurement was conducted via a spectrophotometer. The carotenoid concentration value is expressed as μg g^{-1} dry weight.

Mineral Profile

For calcium (Ca), magnesium (Mg) and potassium (K) concentrations, the method described by Morand and Gullo (1970) was applied. Phosphorous (P) concentration was determined using the Fogg and Wilkinson (1958) method. Nitrogen (N) concentration in leaf tissues was measured following the Kjeldahl method. Molybdenum (Mo) concentration was evaluated as reported by Sabatino et al. (2019b) via inductively coupled plasma-mass spectrometry (ICP-MS) (Plasma Quant MS Elite, Jena, Germany). The concentrations of N, P, K, Ca and Mg are expressed as g kg^{-1} dry weight, while the Mo concentration is shown as mg kg^{-1} dry weight.

Calculation of Nitrogen Indices

Nitrogen use efficiency (NUE), nitrogen uptake efficiency (NUpE) and nitrogen physiological use efficiency (NiPUE) were determined as follows: NUE = yield (t)/N application rate (kg); NUpE = plant N content (kg)/N application (kg); NiPUE = yield (t)/plant nitrogen content (kg).

Statistics and Heat Map

The SPSS software v.20 (StatSoft, Inc., Chicago, USA) package was used to analyze all datasets through a two-way Analysis Of Variance (ANOVA). Tukey Honestly Significant Difference (HSD) test ($p \leq 0.05$) was adopted for multiple comparisons of means. Data expressed as percentages were converted via arcsine transformation prior to ANOVA analysis ($Ø = \text{arcsine}(p/100)^{1/2}$). A heat map revealing the agronomical, qualitative and NUE responses of spinach to SE applications and Mo supply dosages was also created.

Results

The biostimulant action of brown seaweed extract from *Ecklonia maxima* can vary depending on multiple interacting parameters such as genus and species, growth conditions (greenhouse versus open field) and foliar feedings of micronutrients such as Mo. Taking this into account, the overall objective of the current work was a composite examination of yield, quality attributes and NUE in greenhouse spinach through a factorial analysis of the relative effects of seaweed-based biostimulant use and Mo biofortification.

Production and Biometric Features of Spinach Plants

The combined effect of *Ecklonia maxima* SE and Mo doses on head fresh weight (FW), head height (H) and stem diameter (SD) are shown in Table 1. Statistical analysis for head FW, head H and SD displayed no significant interaction between SE and Mo treatments (Table 1).

Table 1. Impact of SE application and Mo-biofortification on head fresh weight (head FW), head height (head H) and stem diameter (SD) of spinach plants cultivated in a protected environment.

Treatments	Head FW (g)	Head H (cm)	SD (mm)
SE (ml L^{-1})			
0.0	39.7 b	15.6 a	9.8 a
3.0	45.4 a	15.5 a	8.2 b
Mo (µmol L^{-1})			
0.0	32.1 c	13.3 c	8.0 c
0.5	35.1 bc	14.4 bc	8.4 bc
2.0	42.3 b	16.7 ab	9.2 ab
4.0	49.7 a	17.4 a	10.0 a
8.0	53.5 a	16.1 ab	9.6 a
Significance			
SE	***	NS	***
Mo	***	***	***
SE × Mo	NS	NS	NS

Values in a column with different letters significantly differ at $p \leq 0.05$. NS, *** not significant or significant at 0.001, respectively.

When averaged over Mo-biofortification, SE significantly augmented head FW. Not considering the SE application, the highest head FW values were observed in

plants supplied with 4 or 8 µmol Mo L^{-1}, while the lowest values were documented in control plants (Table 1). Irrespective of Mo treatments, SE did not significantly affect head H. Conversely, plants treated with 2, 4 or 8 µmol Mo L^{-1} exhibited the highest head H values, whereas control plants and plants supplied with 0.5 µmol Mo L^{-1} had the lowest (Table 1). Regardless of the Mo supply, SE application significantly decreased SD (Table 1). Specifically, plants supplied with a dosage of 2, 4 or 8 µmol Mo L^{-1} had the highest SD values. Control plants and plants treated with 0.5 µmol Mo L^{-1} had the lowest SD (Table 1).

The combined effects of *Ecklonia maxima* SE and Mo dose on number of leaves (N. leaves) are presented in Figure 2. ANOVA for N. leaves showed a significant interaction between SE × Mo; plants treated with SE and supplied with 2, 4 or 8 µmol Mo L^{-1} had the highest leaf number, followed by those not treated with SE but fed with 2 µmol Mo L^{-1}. The lowest leaf number was recorded in plants not treated with SE and biofortified with 0.5 µmol Mo L^{-1}.

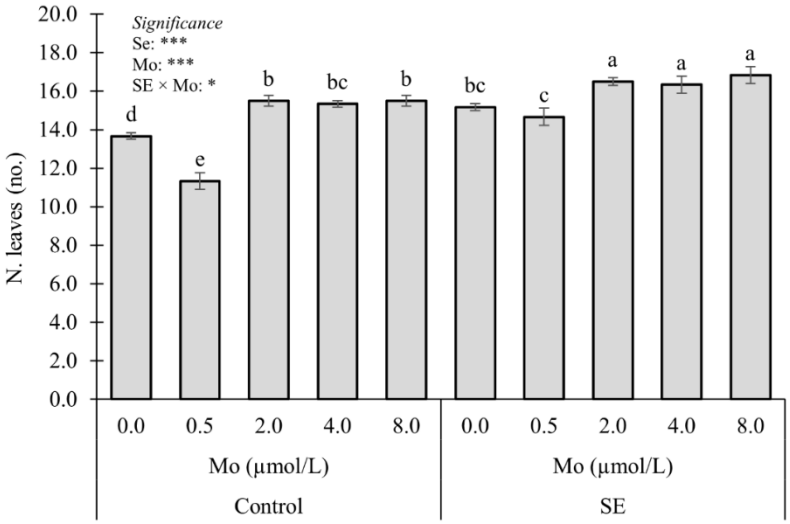

Figure 2. Impact of SE application and Mo-biofortification on number of leaves (N. leaves) of spinach plants cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. *, *** significant at 0.05 or 0.001, respectively. Bars indicate mean ± standard error of 3 replicates of 5 samples.

2.2. Nutritional and Nutraceutical Parameters and Carotenoid Concentration

The effect of *Ecklonia maxima* SE and Mo dose on head dry matter (DM) is presented in Figure 3. Statistical analysis for head DM indicated a significant interaction between SE application and Mo-biofortification.

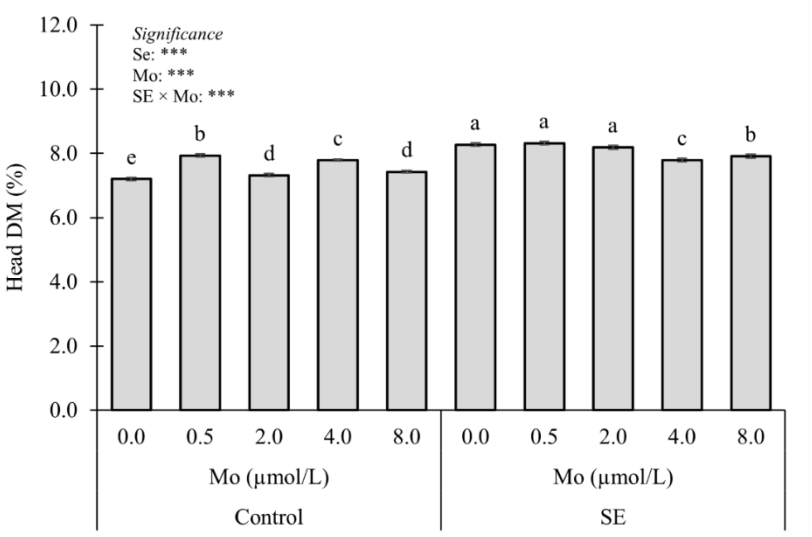

Figure 3. Impact of SE application and Mo-biofortification on head dry matter (head DM) of spinach plants cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. *** significant at 0.001. Bars indicate mean ± standard error of 3 replicates of 5 samples.

Plants treated with SE and supplied with Mo at 0, 0.5 or 2 µmol L^{-1} had the highest head DM, followed by those untreated with SE and supplied with 0.5 µmol Mo L^{-1} and by those treated with SE with the highest Mo dosage (Figure 3). Untreated plants exhibited the lowest head DM (0 mL SE L^{-1} × 0 Mo µmol L^{-1}). The current study also investigated the impact of *Ecklonia maxima* SE and Mo treatments on colour parameters, soluble solids content (SSC), ascorbic acid, polyphenols and carotenoids. Statistics on these parameters indicated no significant interaction between SE × Mo.

Treatments had no significant effect on either CIELab parameters or on SSC (Table 2).

Table 2. Effect of SE application and Mo-biofortification on CIELab parameters (a*, b* and L*), soluble solid content (SSC), ascorbic acid, polyphenols and carotenoids of spinach plants cultivated in a protected environment.

Treatments	a*	b*	L*	SSC (Brix°)	Ascorbic acid (mg 100 g^{-1} fw)	Polyphenols (GAE 100g^{-1} dw)	Carotenoids (µg g^{-1} dw)
SE (ml L^{-1})							
0.0	-15.73 a	16.79 a	34.3 a	3.0 a	87.9 b	29.27 b	5.48 a
3.0	-17.22 a	17.11 a	36.3 a	3.0 a	137.4 a	31.63 a	5.47 a
Mo (µmol L^{-1})							
0.0	-14.92 a	16.72 a	34.9 a	3.1 a	97.1 e	25.98 e	5.19 e
0.5	-16.87 a	17.38 a	35.5 a	3.0 a	107.9 d	28.62 d	5.36 d
2.0	-16.27 a	16.91 a	34.6 a	3.0 a	113.0 c	30.05 c	5.46 c
4.0	-16.06 a	16.67 a	35.6 a	3.0 a	119.9 b	32.31 b	5.58 b
8.0	-16.25 a	17.06 a	36 a	3.0 a	125.2 a	35.28 a	5.78 a
Significance							
SE	NS	NS	NS	NS	***	***	NS
Mo	NS	NS	NS	NS	***	***	***
SE × Mo	NS	NS	NS	NS	NS	NS	NS

Values in a column with diverse letters significantly differ at $p \leq 0.05$. NS, *** not significant or significant at 0.001, respectively.
Each value is the mean of 3 replicates of 5 samples each.

Regardless of Mo supply, SE significantly increased ascorbic acid concentration (Table 2). Irrespective of SE application, ANOVA analysis found a positive relation between Mo dosage and ascorbic acid concentration, up to 8 μmol Mo L^{-1} (Table 2). Data on polyphenols supported the trend recognized for ascorbic acid (Table 2). Irrespective of the Mo supply, SE application did not influence carotenoid concentration in leaf tissues. Conversely, ignoring the SE treatments, data on carotenoids sustained the trend previously reported for ascorbic acid and polyphenols (Table 2).

Mineral Concentrations in Leaf Tissues

The analysis of mineral concentrations in leaf tissues was performed to evaluate the influence of *Ecklonia maxima* SE and Mo doses on important nutritional values (Table 3). Statistical analysis for N, P, K, Ca, and Mg concentrations did not indicate a significant interaction between SE × Mo.

Table 3. Impact of SE application and Mo-biofortification on mineral profile (N, P, K, Ca and Mg) of spinach plants cultivated in a protected environment.

Treatments	N (g kg^{-1} dw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)
SE (ml L^{-1})					
0.0	5.35 b	3.61 b	70.38 b	12.79 a	7.67 b
3.0	5.50 a	3.70 a	82.51 a	12.78 a	8.66 a
Mo (μmol L^{-1})					
0.0	5.78 a	3.67 a	76.62 a	12.67 a	8.13 a
0.5	5.54 b	3.71 a	77.25 a	12.95 a	8.21 a
2.0	5.40 c	3.69 a	75.83 a	12.68 a	8.25 a
4.0	5.27 d	3.61 a	77.05 a	12.68 a	8.14 a
8.0	5.14 e	3.58 a	75.48 a	12.97 a	8.20 a
Significance					
SE	***	*	*	NS	***
Mo	***	NS	NS	NS	NS
SE × Mo	NS	NS	NS	NS	NS

Values in a column with diverse letters significantly differ at $p \leq 0.05$. NS, *, *** not significant, significant at 0.05 or 0.001, respectively. Each value is the mean of 3 replicates of 5 samples each.

Mo leaf enrichment was one of the main purposes of the study. Figure 3 shows that irrespective of the Mo-biofortification, SE treatments significantly augmented N leaf concentration. Control plants had the highest N content followed by plants supplied with 0.5 μmol Mo L^{-1}, which in turn had a higher N concentration than those biofortified with Mo at 2 μmol L^{-1}. The lowest N leaf concentration was observed in plants treated with the highest Mo dosage (Table 3). Not considering the Mo treatments, SE application significantly increased P concentration. Regardless of SE treatments, Mo-biofortification did not significantly affect P concentration. Results on K and Mg concentrations support the tendency described for P concentration (Table 3). SE application and Mo-biofortification did not affect Ca concentration in leaf tissues (Table 3). ANOVA and mean separation for leaf Mo concentration displayed a significant interaction SE × Mo (Figure 4). Plants treated with SE and supplied with the highest dosage of Mo had the highest Mo concentration, followed by those non-treated with SE and biofortified with 8 μmol Mo L^{-1}, which in turn displayed a higher value than those treated with SE and supplied with 4 μmol Mo L^{-1}. The lowest Mo concentration was detected in Mo-untreated plants (Figure 4).

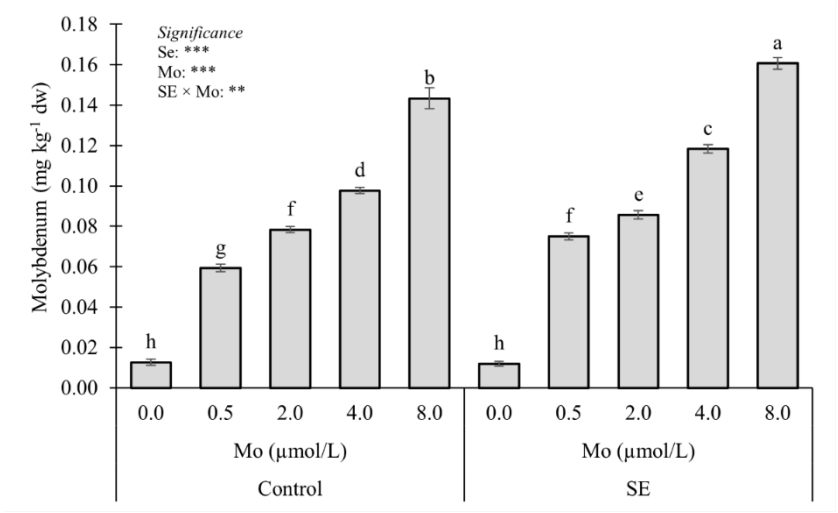

Figure 4. Impact of SE application and Mo-biofortification on molybdenum concentration in leaves of spinach plants cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. **, *** significant at 0.005 or 0.001, respectively. Bars indicate mean ± standard error of 3 replicates of 5 samples.

Nitrogen Indices

Since spinach is one of the less efficient green leafy vegetables in terms of nitrogen uptake and utilization, NUE indices represented in this experiment are valid indicators to evaluate the impact of *Ecklonia maxima* SE and Mo doses. ANOVA for NUE, NUpE and NiPUE did not indicate a significant interaction between SE application and Mo doses (Table 4).

Table 4. Impact of SE application and Mo-biofortification on nitrogen use efficiency (NUE), nitrogen uptake efficiency (NUpE) and nitrogen physiological use efficiency (NiPUE) of spinach plants cultivated in a protected environment.

Treatments	NUE (t kg^{-1})	UE (kg kg^{-1})	PUE (t kg^{-1})
SE (ml L^{-1})			
0.0	0.124 b	0.050 b	0.187 a
3.0	0.142 a	0.063 a	0.182 b
Mo (µmol L^{-1})			
0.0	0.100 c	0.045 c	0.173 e
0.5	0.109 c	0.049 bc	0.180 d
2.0	0.132 b	0.056 ab	0.184 c
4.0	0.156 a	0.064 a	0.189 b
8.0	0.167 a	0.066 a	0.194 a
Significance			
SE	***	***	***
Mo	***	***	***
SE × Mo	NS	NS	NS

Values in a column with diverse letters significantly differ at $p \leq 0.05$. NS, *** not significant or significant at 0.001, respectively. Each value is the mean of 3 replicates of 5 samples each.

Irrespective of Mo-biofortification, SE application significantly increased NUE. Regardless of SE application, plants biofortified with Mo at 4 or 8 µmol L^{-1} had the highest NUE indices, while control plants and plants treated with 0.5 µmol Mo L^{-1} had the lowest. (Table 4). Regardless of Mo-biofortification, NUpE index was significantly increased by SE application. Regardless of SE treatment, plants supplied with 2, 4 or 8 µmol Mo L^{-1} had the highest NUpE index, whereas control plants the lowest (Table 4). Not considering Mo-biofortification, SE application significantly decreased the NiPUE index. Notwithstanding SE application, plants biofortified with 8 µmol Mo L^{-1} had the highest NiPUE index and control plants had the lowest (Table 4).

Heat Map Analysis of all Spinach Plant Features

A grouped data heat map analysis of all agronomic, nutritional, functional and physiological plant traits was carried out to expose a chromatic evaluation of the SE application and Mo-biofortification on spinach plants. In Figure 5, the heat map analysis exposes a couple of dendrograms, one placed on the top and named Dendrogram 1, and the other sited on the left, named Dendrogram 2.

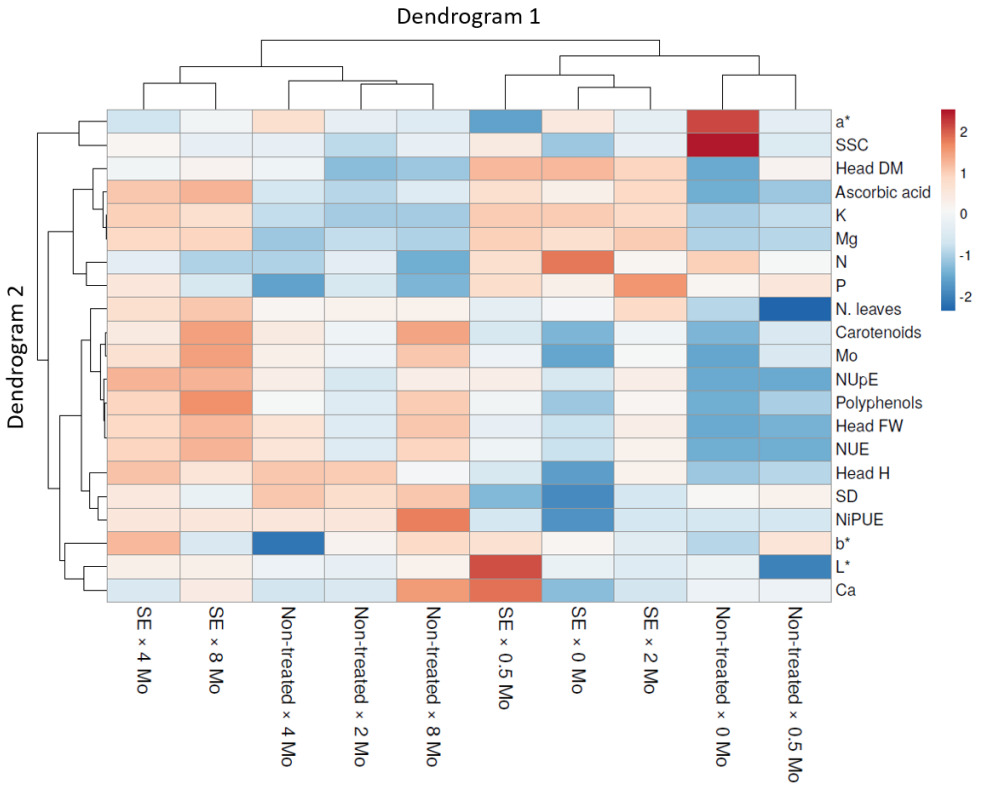

Figure 5. Heat map analysis comprising all spinach plant traits in response to SE application and Mo-biofortification. The heat map picture was made via the https://biit.cs.ut.ee/clustvis/ (accessed on 27 April 2021) online program package.

Dendrogram 1 comprises the combinations of SE and Mo treatments, while Dendrogram 2 exhibits all studied features which modified the distribution. Dendrogram 1 displays two principal groups, the one on the left collected the non-treated × 2, 4 and 8 µmol Mo L^{-1} and SE × 4 and 8 µmol Mo L^{-1} combinations. The other on the right side of the cluster includes the non-treated × 0 and 0.5 µmol Mo L^{-1} and SE × 0, 0.5 and 2 µmol Mo L^{-1} combinations (Figure 5). Specifically, two

groups were identified on the left side of Dendrogram 1. The first on the left comprises the SE × 4 μmol Mo L^{-1} and SE × 8 μmol Mo L^{-1} combinations, parted from non-treated × 2, 4 and 8 μmol Mo L^{-1} combinations. These exhibited particularly low values for SSC, head DM, ascorbic acid, K, Mg, N, P, N. leaves, carotenoids, Mo, NUpE, polyphenols, head FW, NUE, b*, L* and Ca. The group on the left encloses SE × 4 Mo and SE × 8 Mo combinations. Inside this group, the SE × 4 μmol Mo L^{-1} combination is evidently separated by higher SSC, K, N, P, head H, SD and b*, whereas the clustering on the right side comprises the non-treated × 2, 4 and 8 μmol Mo L^{-1} combinations. Within this group, the non-treated × 4 μmol Mo L^{-1} combination was parted by higher a*, head DM, K, NUpE and head H and lower Mg, P, N. leaves, polyphenols, NiPUE, b* and Ca. The right side of this cluster encloses non-treated × 2 and 8 μmol Mo L^{-1} combinations. The non-treated × 2 μmol Mo L^{-1} combination was separated by lower SSC, head DM, ascorbic acids, carotenoids, Mo, NUpE, polyphenols, head FW, NUE, SD, NiPUE, b*, L* and Ca (Figure 5). Looking to the right side of the Dendrogram 1, two clusters were identified. The first cluster on the left encloses SE × 0, 0.5 and 2 μmol Mo L^{-1} combinations separately from the non-treated × 0 and 0.5 μmol Mo L^{-1} combinations, which had higher head DM, ascorbic acid, K, Mg, N, P, N. leaves, carotenoids, Mo, NUpE, polyphenols, head FW, NUE, b*, L* and Ca, but lower a*, SSC, head H, SD and NiPUE. The cluster on the left includes the SE × 0.5 μmol Mo L^{-1} combination; in this group the SE × 0.5 μmol Mo L^{-1} combination is markedly parted by higher SSC, head DM, ascorbic acid, K, Mg, carotenoids, Mo, NUpE, polyphenols, b*, L* and Ca. The right side of this cluster comprises SE × 0 and 2 μmol Mo L^{-1} combinations; the SE × 0 μmol Mo L^{-1} combination was separated by lower SSC, ascorbic acid, Mg, P, N. leaves, carotenoids, Mo, NUpE, polyphenols, head FW, NUE, head H, SD, NiPUE and Ca (Figure 5). The group on the right side encloses the non-treated × 0 and 0.5 μmol Mo L^{-1} combination. Within this cluster, the non-treated × 0 μmol Mo L^{-1} combination is divided by lower head DM, ascorbic acid, P, carotenoids, Mo, polyphenols, head H, SD and b*, and higher a*, SSC, N, N. leaves and L*. Intriguingly, the groups in Dendrogram 2 manifestly highlight the diverse influence of SE application and Mo-biofortification (Figure 5).

Discussions

Nowadays, SEs have a reputation as plant biostimulants due to their ability to improve performance, mineral uptake, and mineral stress tolerance in vegetable crops (Colla and Rouphael, 2015; du Jardin, 2015; Khan et al., 2009; Battacharyya et al., 2015). Concurrently, plant fertilization management, such as counting trace elements like molybdenum, is an essential cultivation step to the enhance functional aspect (direct advantage) as well as yield and quality (indirect advantages) (Moncada et al., 2018; Sabatino et al., 2019b). Furthermore, as reported by several authors (Kaiser t al., 2005; Rouphael et al., 2018a), both SE application and Mo-biofortification may improve nitrogen use efficiency in green leafy vegetables, such as spinach. Thus, in the present study the impact of *Ecklonia maxima* SE and Mo-biofortification foliar treatments, alone or combined, on yield and yield-related parameters, minerals, nutritional and functional traits, as well as NUE indices of spinach was evaluated. Our results on yield showed that SE enhanced plant productivity. These findings are supported by Rouphael et al. (2018a) who, by studying the impact of plant and seaweed extracts on spinach cultivated in a protected environment, found that *E. maxima* SE significantly improved yield by 53%. Analogous results were also observed by Di Mola et al. (2019), who investigated baby lettuce grown under diverse nitrogen regimes. The higher yield could be related to the SE polysaccharide content. Indeed, sugars are recognized to increase plant productivity by eliciting endogenous hormone homeostasis (Rolland et al., 2002). We found that the highest Mo supply improved yield and yield-related traits. This is in accordance with the reports of Moncada et al. (2018), Biacs et al. (1995) and Vieira et al. (2005) on leafy vegetables (lettuce, escarole and curly endive, respectively), carrot and common bean. Our study revealed that SE enhanced head DM percentage. These findings, although in contrast with those of Colla et al. (2017a) on tomato, are in harmony with those indicated by Rouphael et al. (2018a) on spinach. These dissimilar results could be related to the different plant organs (leaves vs fruits) and species tested. Our results are in accordance with those reported by Boertje (1969), who found that Mo-shortage reduces dry matter in lettuce. Plants exposed to SE application had a head DM percentage which did not differ from that of plants treated with 0, 0.5 or 2 μmol Mo L^{-1}. Thus, it seems

that in our study the SE biostimulant effect was much higher than that of Mo-biofortification and, consequently, SE application produced a buffer effect towards Mo supply. Furthermore, our study underlined that SE-treated plants supplied with the highest Mo dosage (8 μmol L^{-1}) displayed a higher head DM percentage than SE-untreated plants. Consequently, we may speculate that SE application enhanced spinach Mo tolerance. Leaf colour parameters were not influenced by SE application. Our results are sustained by those of Rouphael et al. (2018a) who found no significant effect of *E. maxima* SE on CIELab parameters in spinach leaves. However, our findings are partially consistent with those reported on baby lettuce plants by Di Mola et al. (2019) who found that L* and b* coordinates increased with SE application. Thus, we may hypothesize that the effect of SE treatment on leaf colour is a species-related feature. Our results also revealed that Mo biofortification did not significantly influence a*, b* and L* colour coordinates. These results diverge with those by Moncada et al. (2018) on lettuce, escarole, and curly endive. Both SE application and Mo-biofortification did not affect SSC content. These results support those of Colla et al. (2017a), who detected no significant effect of SE application on total soluble solids content in tomato but differ with those of Moncada et al. (2018), who found that Mo supply increased total soluble solids content. These dissimilarities could be related to the different effects of Mo availability on the metabolism of diverse species. This hypothesis is supported by Kaiser et al. (2005) who specified that, since Mo is an element included in several enzymatic mechanisms, it can be difficult to identify a definite plant reaction to its deficiency. Our results revealed that SE application improved ascorbic acid and polyphenol content in spinach leaves. These outcomes support the findings of Rouphael et al. (2018a) on spinach and those of Abbas et al. (2020) on onion. The elicitation of the secondary metabolism, resulting in an augment of bioactive molecules (i.e., total phenols and ascorbic acid), could be related to the key enzyme activity (chalcone isomerase) in phytochemical homeostasis (Ertani et al., 2014; Rouphael et al., 2018b). Furthermore, the secondary metabolism stimulation could be linked to the modification of the plant nutritional status (indirect effect) (Rouphael et al., 2017). Likewise, as reported by Fan et al. (2011), SE of *A. nodosum* application improved flavonoid concentration in spinach treated

plants compared to the control. Our results also displayed that ascorbic acid and polyphenol concentrations increased as Mo dosage increased. Similar findings are reported on lettuce, cauliflower, tomato and potatoes (Sabatino et al., 2019b; Boertje, 1969; Agarwala and Hewitt, 1954; Munshi and Mondy, 1988). Ascorbic acid has a significant role in chloroplast protection. According to Valenciano et al. (2011), the reduction in ascorbic acid concentration in Mo-deficient plants might be connected to the chloroplast inefficiency that occurs in plants with Mo deficiency. Our results displayed that SE application did not significantly affect carotenoid concentration. This is in contrast with the outcome of Di Mola et al. (2019), who reported a beneficial effect of the SE-based biostimulant on baby lettuce. However, Carillo et al. (2019), by studying the influence of protein hydrolysate (PH) and different nitrogen levels in spinach, found that carotenoid content was negatively affected by PH application. Thus, we may assume that plant- or seaweed-based biostimulants could affect carotenoid content differently based on genotype. Furthermore, our study highlighted that a higher Mo concentration in the nutrient solution increased carotenoid concentration. Since Mo supply improves plant nitrogen metabolism (Marschner, 2012), and considering that nitrogen fertilization enhances carotenoid concentration in plants (Di Mola et al., 2019; Chenard et al., 2005), we hypothesized that in our study Mo supply indirectly increases carotenoid concentration. Regarding the mineral profile of spinach, we found that SE application enhanced N, P, K and Mg concentration in leaves, whereas it did not affect Ca concentration. Our outcomes are partially consistent with those by Rouphael et al. (2018a), who found that SE application causes an increase in protein, K and Mg, without affecting Ca and P concentrations. Our outcomes were also in line with those of Di Mola et al. (2019), who reported that, notwithstanding the nitrogen rates, SE improved nitrate concentration in baby lettuce leaves. These results could be attributed to the fact that SE changes the root architecture, resulting in improved plant nutrient uptake (Battacharyya et al., 2015). Furthermore, as reported by Luthje and Bottger (1995), SE contains a component called kahydrin, a derivative of vitamin K1, which alters the plasma membrane proton pumps and stimulates the secretion of H^+ ions into the apoplast, leading to rhizosphere acidification. This condition modifies the soil redox state and metal ion

solubility, increasing their availability to the plant (Krouk et al., 2010; Castaings et al., 2011). Our findings reveal that Mo supply did not significantly influence P, K, Ca and Mg concentrations in spinach plants, but significantly decreased N concentration in leaf tissues. Our outcomes are consistent with those of Moncada et al. (2018), who found a reduced nitrate content in Mo-biofortified green leafy vegetables. Our findings are also corroborated by those of Zhen et al. (2007), Cantliffe et al. (1974) and Cox (1992). Moreover, molybdenum cofactors (Moco) partake in the active site of nitrate reductase, which modulates nitrate uptake and may improve nitrogen use efficiency (Schwarz, 2016). A significant influence of the interaction SE application × Mo supply on leaves Mo concentration was found in this study. In particular, plants supplied with the highest Mo dosage and treated with SE provided the highest Mo concentration in leaves. Conversely, control plants (0 μmol Mo L^{-1}) treated or not with SE showed the lowest Mo content. Our findings are corroborated by those of Colla et al. (2017a), who stated that plant-based biostimulants improve plant macro- and micronutrient absorption and accumulation. Our results are also in line with those of Moncada et al. (2018), who found a positive correlation between Mo concentration in the nutrient solution and Mo leaves tissue content. However, in our study when plants were not biofortified with Mo, SE application did not enhance Mo concentration. Thus, we assume that to optimize Mo content in spinach plants, the combined use of Mo-biofortification and SE application is crucial. Our results indicate that both SE application and Mo supply enhanced nitrogen use efficiency indices. These outcomes could be linked to the fact that SE modifies the roots architecture, enhancing the efficiency of plant mineral absorption (Battacharyya et al., 2015). Furthermore, it is known that Mo supply, via the Moco participation in nitrate reductase, modulates nitrogen uptake (Valenciano et al., 2011) and consequently improves nitrogen use efficiency.

Conclusions

The continuous need to maximize yield and functional action of green leafy vegetables imposes a challenge for growers, extension specialists and researchers in their search for eco-friendly tools to combine high production with premium quality. In the present study, *E. maxima* SE significantly enhanced yield parameters,

mineral profile, nutritional and functional features, and NUE indices. Concurrently, Mo supply substantially increased productivity, NUE indices, nutritional and bioactive features such as ascorbic acid, polyphenols, Mo leaf concentration and carotenoids, while it decreased N content in leaf tissues. Despite the remarkable current literature on the use of SE biostimulants alone as well as that of biofortification with trace elements, information regarding the combined effect of both agronomic practices is limited. In view of the above considerations, our results showed that combining SE with 2 μmol Mo L^{-1} notably ameliorated leaf number and head DM, whereas, combining SE with 8 μmol Mo L^{-1} significantly enhanced leaf Mo concentration. Overall, our novel outcomes recommend that a mutual application of SE and Mo supply at 4 or 8 μmol L^{-1} may efficiently increase crop performance and the nutritional and functional quality of spinach.

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EXPERIMENT 4

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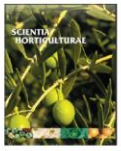

Selenium biofortification and grafting modulate plant performance and functional features of cherry tomato grown in a soilless system

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Selenium biofortification and grafting modulate plant performance and functional features of cherry tomato grown in a soilless system

Introduction

Solanum lycopersicum L. is the world preeminent vegetable crop accounting for a production of around 180 million tonnes and a cultivation surface of over 4.7 million hectares in 2018 (FAO STAT, 2018; Davino et al., 2017). In Europe, Spain and Italy are the primary tomato producers with a production of above 5 million tonnes (FAO STAT, 2018). Tomato is highly valued for its health promoting compounds (nutritional and functional), low content in fat and calories, richness in vitamins E and C, folic acid, potassium and for its exemption from cholesterol (Kim et al., 2011). Therefore, it is considered an imperative component of the human diet. Likewise, tomato is an outstanding font of functional compounds especially lycopene, which concurs in preventing some types of cancer and heart failure (Zhang et al., 2009; Silaste et al., 2007). Vegetable grafting is considered a suitable approach to enhance fruit quality either under favourable or stressful cultivation conditions (biotic or abiotic) (Sabatino et al., 2019a; Singh et al., 2020). Consequently, the use of grafted plants has become a pervasive agricultural practice in several areas of the world (Turhan et al., 2011). In the Mediterranean region, grafted plants are largely adopted in greenhouse tomato cultivation, as there is

evidence that their vigorous root system increases the uptake of minerals and water compared with that of ungrafted plants (Lee and Oda, 2003). There are reports that the intrinsic and extrinsic fruit quality properties fruiting vegetables may be variously affected by the grafting genotypes (both scion and rootstock), the respective rootstock-scion combinations and their compatibility, as well as the growth conditions of the cultivated plants (Lee and Oda, 2003; Kyriacou et al., 2017). It is also known that yield and fruit quality traits of vegetables may be improved through biofortification i.e the enrichment of the nutrient solution with essential micronutrients. In this regard, there are findings that selenium (Se) supply has a double benefit: i) producing Se-enriched food and ii) eliciting the production of secondary metabolites in fruiting vegetables (Schiavon et al., 2013; D'Amato et al., 2020). Selenium is an indispensable micronutrient for humans, and an adequate Se intake, ranging from 40 μg to 400 μg day^{-1}, is beneficial to maintain human health (Gupta and Gupta, 2017). Based on the U.S. Department of Agriculture (2003) data, the daily human Se need is 50–70 μg. Reduced Se dietary intake can be correlated with health dysfunctions such as cancer, keshan disease, diabetes, viral infections, hyperthyroidsim and heart diseases (Finley, 2005; Malagoli et al., 2015). The importance of Se has been stressed by Dennert et al. (2011) who report that some organic forms of Se such as methyl-selenocysteine (MSeC) present anti-carcinogenic action against diverse forms of cancer. This report also elucidated the role of vegetables as the major dietary source of this element. Chun et al. (2010) indicate that US and Canadian population do not suffer from Se shortage, differently from New Zealand, China and part of Europe and Russia whose inhabitants display an unsatisfactory intake of this micronutrient. This shortage is due, according to Kipp et al. (2015), to low levels of Se in the soil and, consequently, in the food. Mineral malnutrition can be addressed by appropriate dietary variation, mineral supply, food fortification and also by enhancing the mineral profile in edible species/crops (White and Broadley, 2009; Sabatino et al., 2019b). With regard to this aspect there are recent studies aimed at Se content in fruiting vegetables (Sabatino et al., 2019a; Hern´andez-Hern´andez et al., 2019; Turakainen et al., 2004). In a soilless cultivation system, fertigation, rather than foliar spray, is the more effective Se supply method (Sabatino et al., 2019a;

Puccinelli et al., 2017). By adopting this approach in closed soilless systems, Se loss in the environment is minimalized and Se uptake is aggravated by the lack of the soil buffer effect. In addition, continuous root exposure to water/nutrient solution overcomes reduced Se supply, simplifies the standardization of food feature, and assures the safe consumption of vegetables (Puccinelli et al., 2017). However, since root absorption is affected by the type of plant (grafted or self-rooted) and by the growing conditions, specific studies are indispensable to evaluate the effects of grafting combined with Se doses. To date, the literature lacks scientific evidence both on the interaction between grafting and Se fertilization dosage in cherry tomato, and on related outcomes concerning crop performance and qualitative features. Taking the aforementioned into consideration, a greenhouse experiment was carried out to evaluate the effect of Se supply at four diverse concentrations on plant vigour, yield traits, functional properties, mineral composition and nitrogen use efficiency (NUE) of grafted or ungrafted cherry tomato plants grown in a soilless culture.

Materials and methods

Experimental site, plant material and cultivation conditions

The trial was carried out at Ragusa (longitude 14°43′ E, latitude 36°55') in south-east Sicily (Italy). A multispan greenhouse (27 m × 50 m), was adopted for the experiment. Polyvinyl chloride was installed as greenhouse covering. The greenhouse was equipped with an automated climate control system. On 15 February 2018 plug plants of 'TyTy' F_1 cherry tomato (Syngenta, Switzerland), grafted or not onto 'Interpro' F_1 (*S. lycopersicum* × *S. habrochaites*) (Vilmorin, Italy, Bologna), were transplanted in 33 l perlite grow-bags. Each grow bag supported four cherry tomato plantlets. Grow bag were spaced 1.20 m between rows, obtaining 3.3 plants m^{-2}. Single branches cherry tomato plants were sustained by trellising supporting twine. Plants were exposed to four levels of Se in the nutrient solution (0.0, 1.0, 2.0 or 4.0 μmol·L^{-1}) administrated via fertigation, throughout the trial. The diverse Se concentrations were accomplished by adding appropriate quantities of selenium dioxide (SeO_2) to the nutrient solution (NS). The

basic NS adopted for all treatments was the one reported by Sonneveld and Voogt (2009) expressed in mM for macronutrients and μM for micronutrients: 1.2 ammonium, 9.5 potassium, 5.4 calcium, 2.4 magnesium, 16.0 nitrate, 4.4 sulphur, 1.5 phosphorus, 15.0 iron, 10.0 manganese, 5.0 zinc, 30 boron, and 0.75 copper. The water used for irrigation contained 9.0 mM of Cl and 9.5 mM of Na. The NS electrical conductivity was 3.6 mS cm^{-1} and the pH 5.6. Once a day, the NS pH was checked and corrected to pH 5.6–5.7 by adding HNO_3. Plants were fertigated by a comprehensive NS given daily through a drip irrigation system (emitters having a flow rate of 2.0 L h^{-1}). The volume of irrigation water was provided, as reported by Gül and Sevgican (1992), according to the solar radiation of the preceding day. Plants were fertigated adopting a leaching fraction of 30 %, where the drainage was not reutilised (open cycle management). The trial ended on 15 June 2018. The greenhouse microclimate settings were monitored and adjusted through a computer controller. Air temperature was fixed at 24 ± 1 and 16 ± 1 °C (day/night) and relative humidity was maintained at 60-70 %. The cumulated greenhouse global radiation measured at the plant canopy level was 1825.1 MJ m^{-2}.

Plant growth, yields, shoot nitrogen uptake and nitrogen use efficiency

Cherry tomato plant vigour was evaluated on all plants through the measurement of plant height at 50 days after the transplant (DAT). The first truss emission (emission of the first inflorescence) was recorded on all plants and expressed as DAT. Shoot dry weight was assessed at the beginning of the fruits production phase in fifteen samples (five for each replication) dehydrated at 100 °C until reaching a constant weight. All fruits were harvested after 35 days from truss emission (fruit commercial maturity stage). Immediately after harvesting (four harvests in total), fruits were weighed. Total and marketable yields (t ha^{-1}), number of fruits (no. $plant^{-1}$) and average weight of fruit (g) were measured. Nitrogen efficiency indices were calculated after harvest. The nitrogen use efficiency was calculated by dividing yield (t) by N application rate (kg) and was expressed as t kg^{-1}. The N uptake efficiency was calculated as the ratio between N content of the plant (kg) and N administration rate (kg) and expressed as kg kg^{-1}.

Nutritional properties and mineral composition

The fruits used for nutritional assessments were harvested from previously labelled flowers (at fruit set phase) and at 35 days from labelling, corresponding to full red coloured fruits within the infructescence (maturity stage). Fifteen samples (randomly selected) for each measurement were used (five for each replication). Water content of the fruits was calculated from the difference in the pre- and post-drying weight, dried at 105 °C. In order to determine the soluble solid content (SSC) a digital refractometer was used (MTD-045nD, Three-In-One Enterprises Co. Ltd. Taiwan) and the results were expressed as °Brix. Fruit firmness was measured with a digital penetrometer (Trsnc, Forlì, Italy), puncturing each fruit at the equatorial zone with a 6 mm diameter stainless steel cylinder probe. Firmness was expressed as newton (N). For fruit redness (a*) determination, a tristimulus Minolta Chroma meter (CR-400 model, Minolta Corporation, Ltd., Osaka, Japan) was used. Fruit nitrogen concentration was assessed by the Kjeldahl method. Fruit phosphorus content (P) was evaluated via colorimetric determination using the procedure reported by Fogg and Wilkinson (1958). As reported by Morand and Gullo (1970), a wet mineralization followed by atomic absorption spectroscopy (SavantAA, 200 ERRECI, Milan, Italy) was used to determine concentrations of magnesium (Mg), calcium (Ca) and potassium (K). Total S concentration was also measured by atomic absorption spectrophotometer. Se concentration in shoot, root as well as fruit occurred in an analytical microwave oven, via a digestion of 25 mg of dried tissue by adding 2.5 ml of HNO_3 and 1 ml of H_2O_2. According to Pedrero et al. (2006), 25 ml of deionized water was added to the resultant solution. An ICP-MS (Plasma Quant MS Elite, Jena, Germany) was used to assess Se concentration that was expressed as mg kg^{-1} of dry weight (dw). Hazard quotient (HQgv) of the vegetables was calculated based on the Protocol (2011) of the United States Environmental Protection Agency, implementing the following formula: HQgv = ADD/RfD ADD: the average daily dose of Se, expressed as μg Se day^{-1}; RfD: the recommended dietary acceptable upper intake Se level, expressed as μg Se day^{-1} set at 400 μg day^{-1}, as reported by Johnson et al. (2003), attributing to the risk for human health of a

adult, weighing 70 kg as a result of intaking Se, when a 150 g portion of fresh cherry tomato is consumed.

Bioactive compounds

Sampling for fruit functional properties determination was done of five fruits for each replication. Fruit ascorbic acid concentration was determined with a reflectometer RQflex 10 Reflectoquant through Ascorbic Acid Test Strips (Merck, Darmstadt, Germany). Ascorbic acid values are presented as mg of ascorbic acid 100 g^{-1} of dw. For determination of fruit polyphenol concentration, 50 ml of methanol were used to extract 2 g of fruit sample at 60 °C by stirring for 60 min. The mix was clarified and placed in darkness until analysis. The Folin-Ciocalteu method, described by Slinkard and Singleton (1997) was used with minor adjustments to determine polyphenol content. Absorption was measured at 765 nm via a spectrophotometer. For calibration, a gallic acid standard was used. Consequently, the outcomes were expressed as gallic acid equivalent (mg·100 g^{-1} dw basis). For lycopene concentration, the method detailed by Sadler et al. (1990) was used. The absorbance was measured at 472 nm. Lycopene concentration values were expressed as mg·100 g^{-1} of dw. Fruit carotenoid concentration was assessed according to Tonucci et al. (1995) with minor changes reported by Leonardi et al. (2000). Flow rate of 0.8 ml min ± 1 was adopted for HPLC separation. Carotenoid quantification was determined via standard curves based on HPLC purified lycopene or on commercial β-carotene (Fluka). Extinction coefficient for the estimated recovery was adopted for calculation of the standards concentrations. For glycoalkaloid content, the extraction method described by Friedman and Levin (1998) was followed. Thus, 1 g of lyophilized tomato sample was immersed for 2 h in 20 ml of 1% acetic acid. The purification of the extract was carried out by a Sep-Pakcolumn, as recommended by Friedman and Levin (1992). HPLC analysis with UV detection (200 nm) was accomplished.

Statistical analysis and design

The four Se doses (0.0, 1.0, 2.0 or 4.0 μmol Se L^{-1}) were combined with grafting (ungrafting *vs* grafting) in a two-factor split-plot randomized block design with Se treatments as plots and grafting or ungrafting as sub-plots. Three replicates (eight plants each) were adopted for each treatment, accounting for a total of 192 cherry tomato plants. The effect of the diverse treatments was assessed by the Analysis Of Variance (ANOVA), while Tukey HSD ($p \leq 0.05$) was used for the mean separation. Principal component analysis (PCA) was made on the entire data set. The ANOVA and PCA analyses were calculated using SPSS software package.

Results

Implications of selenium application for growth, yield responses and N efficiency parameters of grafted and ungrafted tomato

The marketable yield and fruit mean mass of tomato were influenced by Se concentration and grafting combination (G) with no Se × G interaction, whereas plant height at 50 DAT, fruit truss emission, shoot dry biomass, total yield, number of fruits, N-use efficiency as well as N-uptake efficiency showed a significant Se × G interaction (data not shown). Our data indicate that the addition of Se at 1 and 2 μM increased plant height in both ungrafted and grafted tomato compared to the control, while an opposite effect was observed in both grafting combinations at 4 μM Se treatment with a reduction in ungrafted and grafted tomato, respectively compared to both the1 and 2 μM concentration treatments (Figure 1).

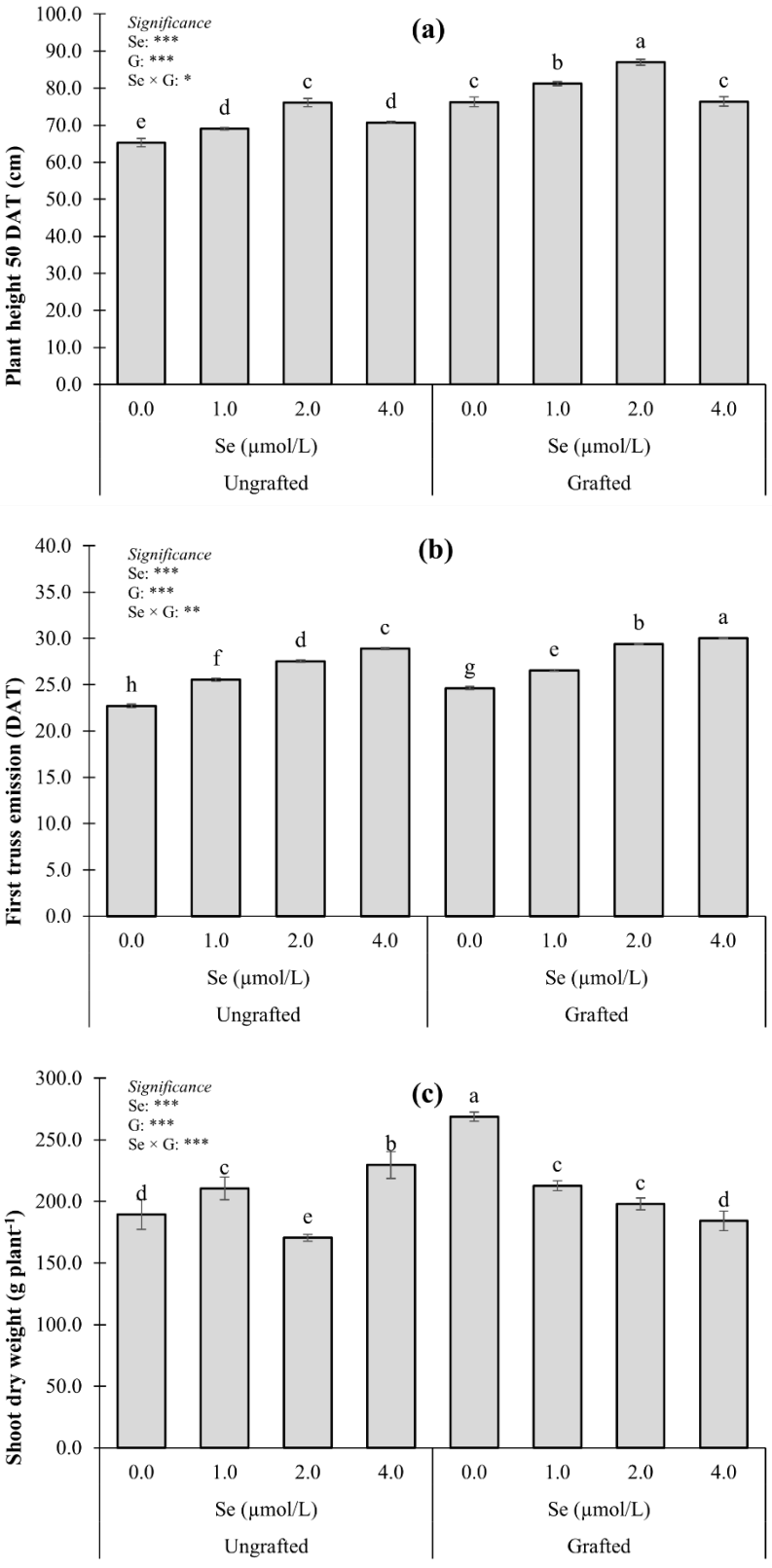

Figure 1. Plant height at 50 DAT (a), first truss emission (b) and shoot dry weight (c) (at the beginning of the fruits production phase) as influenced by combining Se enrichment and grafting

in cherry tomato. Values with different letters are significantly different at $p \leq 0.05$. Each value is the mean of three replicates of 8 plants each.

Moreover, increasing Se concentration in the NS from 0 to 4 μM Se provoked a significant and linear increase in fruit truss emission in both grafted and ungrafted tomato, with the highest values registered in grafted tomato at 4 μM Se (Figure 1). The shoot dry biomass of ungrafted and grafted tomato plants behaved differently to increasing Se concentration in the NS. With the exception of the 2 μM Se, increasing Se concentration from 0 to 4 μM increased the biomass production in ungrafted plants, while the opposite trend was recorded in grafted plants (Figure 1). Se concentrations up to 2 μM improved total yield in ungrafted tomato plants, but the addition of 4 μM Se in the NS reduced total yield by 15.0 % compared to 2 μM Se. In contrast, a beneficial outcome was noted for grafted tomato plants with a significant and linear increase in total yield in response to Se addition in the NS (Figure 2).

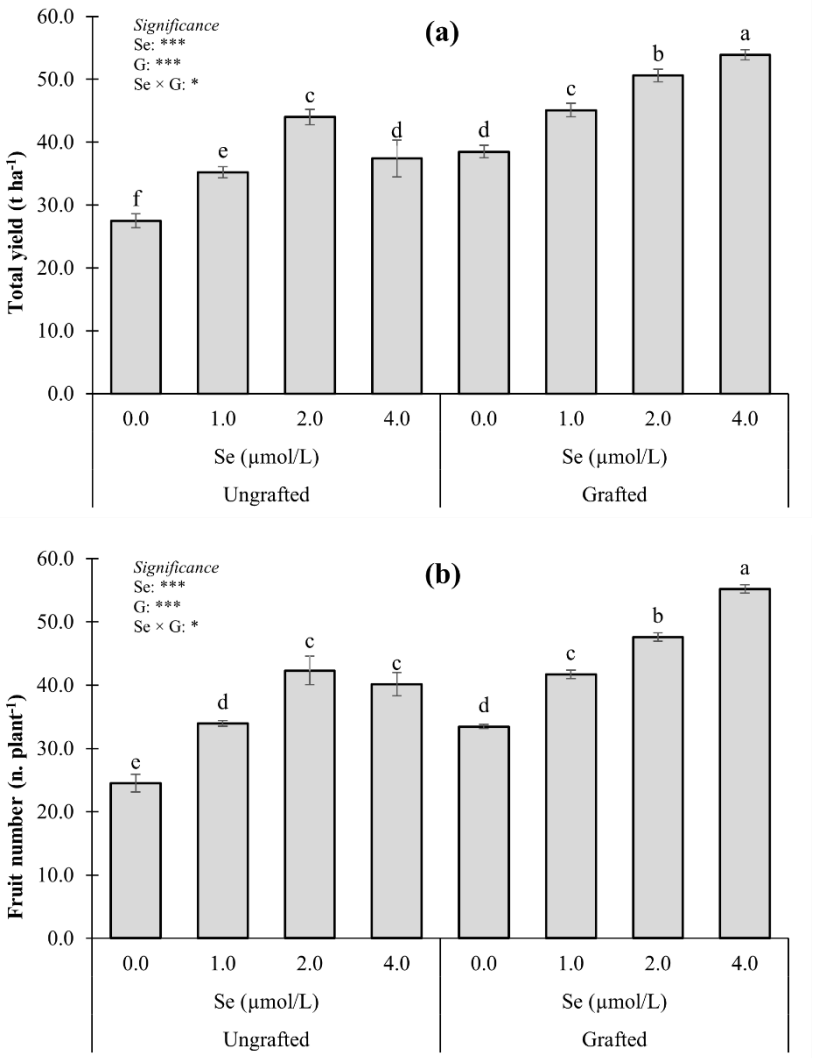

Figure 2. Total yield (a) and fruit number (b) as influenced by combining Se enrichment and grafting in cherry tomato. Values with different letters are significantly different at $p \leq 0.05$. Each value is the mean of three replicates of 8 plants each.

As for total yield, the number of marketable numbers of fruits in both grafting combinations treated with Se were significantly greater than that for untreated plants. Grafted tomatoes treated with 4 µM Se gave the highest value (Figure 2). Marketable yield in the Se-treated plants exposed to 2 and 4 µM Se concentrations was higher by 39.3 % and 37.7 %, respectively compared to untreated control (Figure 3), while fruit mean mass showed an opposite trend. The lowest values were registered at 4 µM Se (Figure 3). Averaged over the Se concentrations, marketable yield and average fruit weight were increased significantly by 31.2 % and 4.5 % in grafted compared to ungrafted plants (Figure 3).

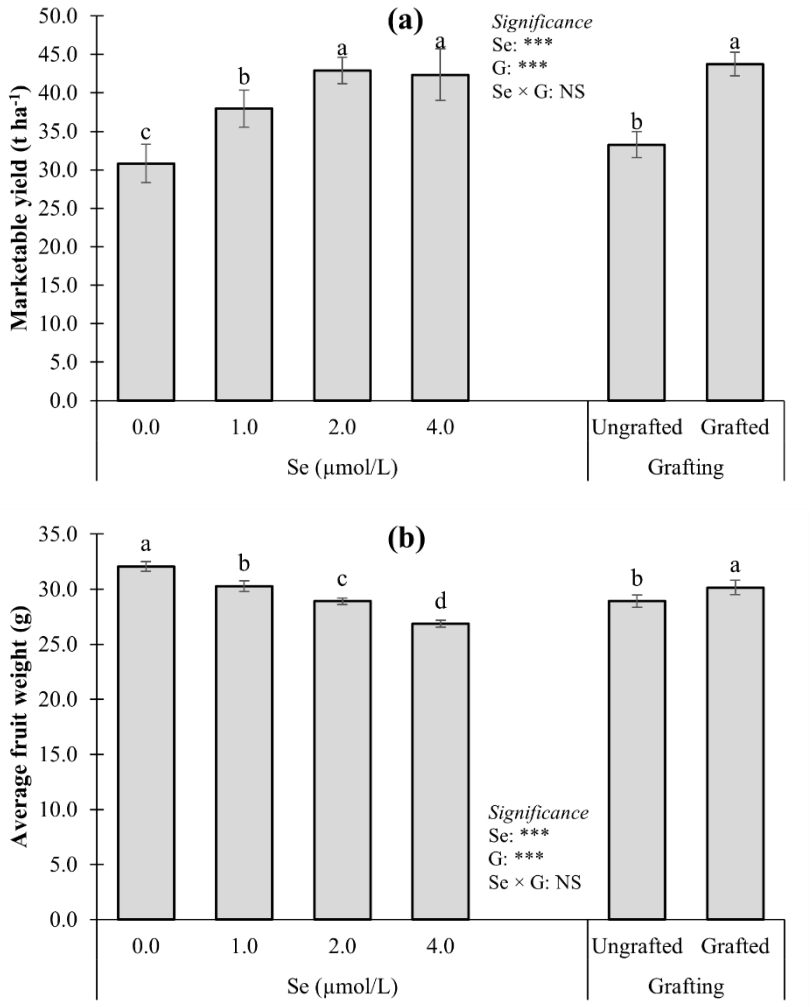

Figure 3. Marketable yield (a) and average fruit weight (b) as influenced by combining Se enrichment and grafting in cherry tomato. Values with different letters are significantly different at $p \leq 0.05$. Each value is the mean of three replicates of 8 plants each.

The higher marketable production noted in plants treated with 2 or 4 µM was due to an increase in the fruit number, while the higher crop productivity of grafted tomato compared to ungrafted ones was attributed to an increase in both fruit mean mass and fruit number. Finally, there was a significant increase of N-use efficiency and N-uptake efficiency under Se biofortification in both ungrafted (expect at 4 µM for N-use efficiency) and grafted plants (Figure 4).

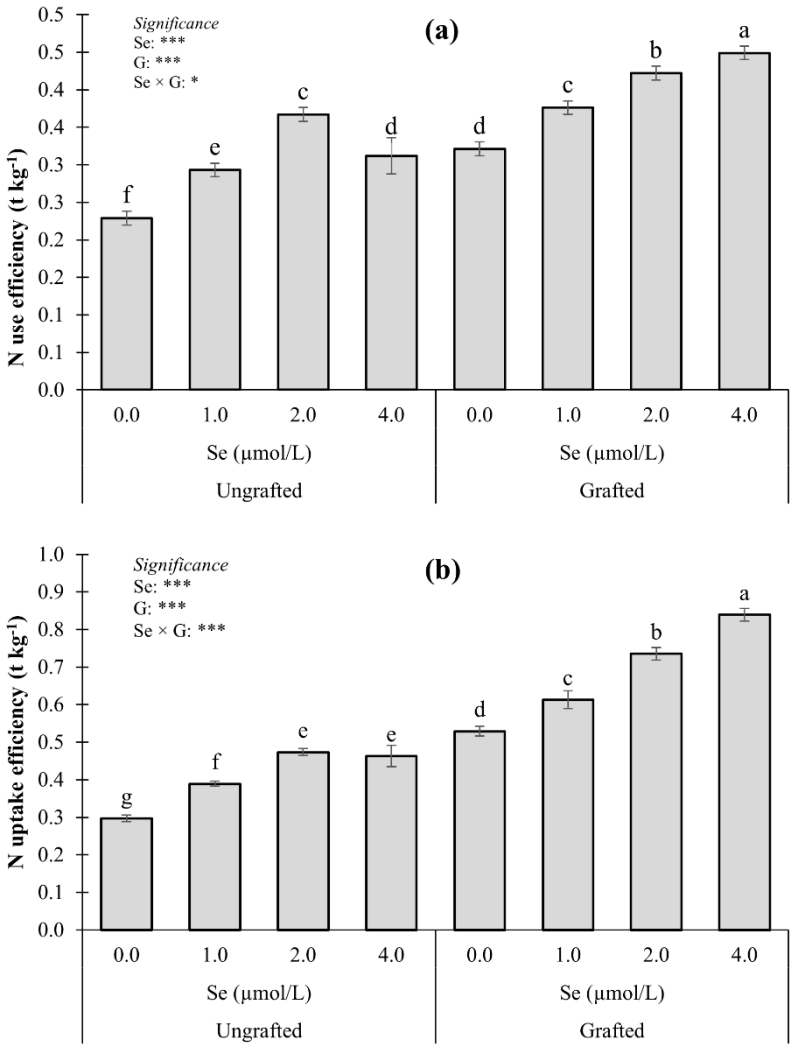

Figure 4. N use efficiency (a) and N uptake efficiency (b) as influenced by combining Se enrichment and grafting in cherry tomato. Values with different letters are significantly different at $p \leq 0.05$. Each value is the mean of three replicates of 8 plants each.

Implications of Se concentration on fruit mineral composition Se biofortification and consumer safety of grafted and ungrafted tomato

With regard to the mineral composition, potassium was the most present macronutrient in tomato fruit (average 40.4 g kg^{-1} dw), followed by nitrogen (average 17.8 g kg^{-1} dw), phosphorus (average 5.7 g kg^{-1} dw), magnesium (average 2.5 g kg^{-1} dw), calcium (average 2.0 g kg^{-1} dw), and sulphur (average 1.8 g kg^{-1} dw) (Table 1).

Table 1. N, P, K, Ca. Mg and S as influenced by combining Se enrichment and grafting.

Treatments	N (g·kg^{-1} dw)	P (g·kg^{-1} dw)	K (g·kg^{-1} dw)	Ca (g·kg^{-1} dw)	Mg (g·kg^{-1} dw)	S (g·kg^{-1} dw)
Se concentration (µmol·L^{-1})						
0.0	18.0 a	5.6 a	39.1 c	1.9 a	2.4 a	1.2 d
1.0	17.8 a	5.7 a	40.3 b	2.0 a	2.5 a	1.6 c
2.0	17.8 a	5.7 a	41.1 a	1.9 a	2.4 a	2.0 b
4.0	18.0 a	5.7 a	41.0 ab	1.9 a	2.4 a	2.6 a
Grafting						
Grafted	19.1 a	6.4 a	42.2 a	2.2 a	2.5 a	2.1 a
Ungrafted	16.5 b	5.0 b	38.6 b	1.7 b	2.4 b	1.6 b
Significance						
Se concentration (Se)	NS	NS	***	NS	NS	***
Graft (G)	***	***	***	***	*	***
Se × G	NS	NS	***	NS	NS	NS

Data within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey HSD Test. The significance is designated by asterisks as follows: *, statistically significant differences at *p*-value below 0.05; ***, statistically significant differences at p-value below 0.001; NS = not significant.

The fruit mineral profile was chiefly affected by grafting and to a lesser extent by the Se concentration. When averaged over grafting combination, sulphur and potassium concentration in fruit tissue increased significantly and linearly with Se concentration ranging from 1.2 to 2.6 g kg^{-1} dw and from 39.1 to 41.1 g kg^{-1} dw, respectively (Table 1). Moreover, grafting of tomato plants, averaged over Se levels, increased the N, P, K, Ca, Mg and S concentrations in fruit tissue by 15.7 %, 22.0 %, 9.3 %, 29.4 %, 5.0 % and 31.2 %, respectively compared to ungrafted plants (Table 1). ANOVA for K concentration showed a significant interaction Se concentration × grafting (Table 1); fruits from grafted plants enriched with 4.0 µmol Se L^{-1} showed the highest values followed by those from grafted cherry tomato plants fed with 2.0 µmol Se L^{-1}, which in turn displayed higher values than cherry tomato fruits from grafted plants supplied with 1.0 µmol Se L^{-1} (Figure 5).

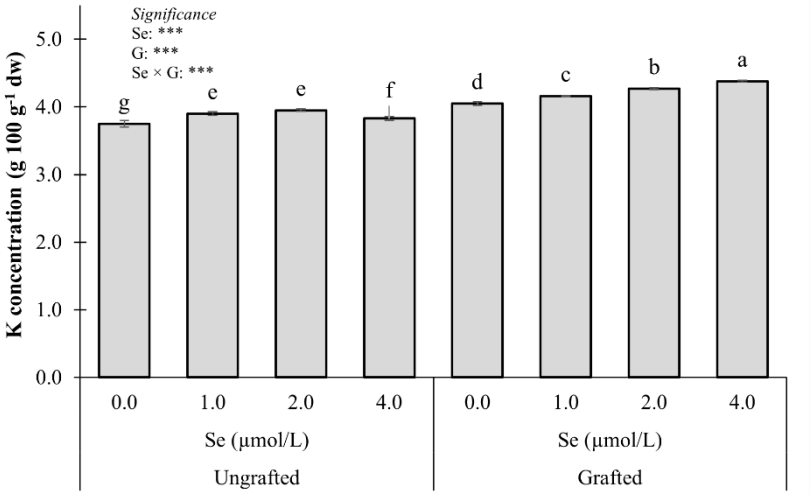

Figure 5. Potassium (K) concentration as influenced by combining Se enrichment and grafting in cherry tomato. Values with different letters are significantly different at $p \leq 0.05$. Each value is the mean of three replicates of 5 fruits each.

The lowest values in terms of fruit K concentration were recorded in fruits harvested from control plants (0.0 µmol Se L^{-1} × ungrafted) and ungrafted plants treated with 4.0 µmol Se L^{-1}. For Se distribution in plant tissues, Se accumulated mainly in root tissue and to a lesser extent in by shoot tissue. The lowest Se concentration was observed in tomato fruit (Table 2).

Table 2. Shoot, root and fruit Se concentration and HQgv as influenced by combining Se enrichment and grafting.

Treatments	Shoot Se concentration (mg kg^{-1} dw)	Root Se concentration (mg kg^{-1} dw)	Fruit Se concentration (mg kg^{-1} dw)	HQ_{gv} with 150 g fw of cherry tomato
Ungrafted × 0.0 µmol Se L^{-1}	0.12 g	0.17 g	0.09 g	0.002 g
Ungrafted × 1.0 µmol Se L^{-1}	3.89 f	4.16 f	1.77 f	0.045 f
Ungrafted × 2.0 µmol Se L^{-1}	8.23 d	10.29 d	3.84 d	0.104 d
Ungrafted × 4.0 µmol Se L^{-1}	14.07 b	28.44 b	8.91 b	0.255 b
Grafted × 0.0 µmol Se L^{-1}	0.20 g	0.21 g	0.08 g	0.002 g
Grafted × 1.0 µmol Se L^{-1}	4.78 e	6.4 e	2.32 e	0.067 e
Grafted × 2.0 µmol Se L^{-1}	11.36 c	14.55 c	5.16 c	0.168 c
Grafted × 4.0 µmol Se L^{-1}	16.03 a	32.9 a	9.88 a	0.353 a
Significance				
Se concentration (Se)	***	***	***	***
Grafting (G)	***	***	***	***
Se × G	***	***	**	***

Data within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey HSD Test. The significance is designated by asterisks as follows: **, statistically significant differences at p-value below 0.01; ***, statistically significant differences at p-value below 0.001; NS = not significant.

Comparing the two grafting combinations, grafted plants accumulated on average 23.1 %, 25.5 % and 19.4 % more Se in shoot, root and fruits, respectively, compared to the ungrafted ones (Table 2). Selenium shoot, root, and fruit concentration was significantly influenced by grafting, Se treatments and their interaction. Moreover, in both grafted and ungrafted plants Se concentration peaked at the highest dose (4 μM). Furthermore, Se concentration in the fruits gradually increased with the supplied amount of Se in the two grafting combinations: in ungrafted tomato 0.09, 1.77, 3.88 and 8.91 mg kg^{-1} dw at 0, 1, 2 and 4 μM doses, respectively; whereas in grafted plants 0.08, 2.32, 5.16 and 9.88 mg kg^{-1} dw, at 0, 1, 2 and 4 μM doses, respectively (Table 2). Finally, HQgv increased with the application of Se rate in the NS, ranging overall from 0.002 to 0.225 and from 0.002 to 0.353 in ungrafted and grafted plants, respectively (Table 2). Consequently, the 150 g fraction of biofortified cherry tomato can be regarded safe because the HQgv in the six biofortification treatments were less than one (Table 2).

Implications of selenium for nutritional and functional quality of grafted and ungrafted tomato

Neither grafting combination nor Se treatment had significant effect on titratable acidity in fruit tissue (average 0.57 %). Fruit water content, firmness, redness (a*), soluble solids content (SSC), total polyphenols, carotenoids and glycoalkaloids were significantly influenced by grafting combination, Se treatment and their interaction, while total ascorbic acid was affected only by Se concentration. Lycopene was significantly influenced by both tested factors with no G × Se interaction (Table 3).

Table 3. Fruit water content, firmness, a*, SSC, Polyphenol content, total carotenoids, glycoalkaloids, ascorbic acids, and lycopene as influenced by combining Se enrichment and grafting.

Treatments	Fruit water content (%)	Firmness (N)	a*	SSC (Brix°)	Polyphenol content (mg gallic acid eq. 100 g^{-1} dw)	Total carotenoids (mg 100g^{-1} dw)	Glycoalkaloids (mg kg^{-1} dw)	Ascorbic acid (mg100g^{-1} dw)	Lycopene (mg 100g^{-1} dw)
Ungrafted × 0.0 µmol Se L^{-1}	94.2 a	16.3 g	13.4 d	8.4 e	17.00 e	171.90 c	174.40 b	1571.10 a	859.70 a
Ungrafted × 1.0 µmol Se L^{-1}	93.7 b	18.3 e	13.5 c	8.7 d	17.40 d	180.47 b	122.67 e	1728.30 a	899.33 a
Ungrafted × 2.0 µmol Se L^{-1}	93.3 d	20.9 c	13.7 b	9.1 c	18.37 b	188.63 a	117.20 f	1788.37 a	930.77 a
Ungrafted × 4.0 µmol Se L^{-1}	92.9 e	22.0 b	14.1 a	9.4 b	18.90 a	188.23 a	110.83 g	1805.20 a	957.80 a
Grafted × 0.0 µmol Se L^{-1}	93.5 c	16.9 f	12.6 e	8.4 e	16.57 f	131.83 f	194.70 a	1558.47 a	810.27 a
Grafted × 1.0 µmol Se L^{-1}	92.8 e	20.2 d	13.7 b	9.2 c	17.10 e	142.97 e	157.27 c	1723.47 a	862.47 a
Grafted × 2.0 µmol Se L^{-1}	92.0 f	22.2 b	13.7 b	9.5 ab	17.47 d	157.83 d	134.33 d	1781.23 a	892.73 a
Grafted × 4.0 µmol Se L^{-1}	91.3 g	22.7 a	14.1 a	9.7 a	18.13 c	172.53 c	118.10 f	1807.70 a	913.37 a
Significance									
Se concentration (Se)	***	***	***	***	***	***	***	***	***
Grafting (G)	***	***	**	***	***	***	***	NS	***
Se × G	***	***	**	*	*	***	***	NS	NS

Data within a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Tukey HSD Test. The significance is designated by asterisks as follows: *, statistically significant differences at *p*-value below 0.05; **, statistically significant differences at *p*-value below 0.01; ***, statistically significant differences at *p*-value below 0.001; NS = not significant.

Increasing Se concentration to 4 μM significantly increase fruit firmness, colour redness and SSC, with more beneficial effect recorded on grafted tomato plants, whereas fruit water content significantly decreased with the application of Se in the NS (Table 3). Moreover, increasing the Se concentration in the NS from 0 to 4 μM resulted in a significant accumulation of bioactive compounds such as total polyphenols and carotenoids, with the highest values recorded in ungrafted plants treated with the highest Se dose (4 μM for polyphenols; 2 and 4 μM for total carotenoids). In contrast to the bioactive compounds pattern, the glycoalkaloids decreased significantly in response to Se treatments from 1 to 4 μM compared to the untreated control.

Principal component analysis-PCA

The PCA of all agronomical, nutritional and functional quality parameters of grafted and ungrafted plants treated with four increasing concentrations of Se in the NS, featured that the first three principal components (PCs) explained 95.7 % of the total variance, with PC1, PC2 and PC3 resulting a percentage of 59.8 %, 31.5 % and 4.3 % respectively (Table 4).

Table 4. Eigenvalues, proportion of total variability and correlation between the 28 variables and the first four principal components (PCs).

Variable	PC1	PC2	PC3
Total yield	0.878	0.409	-0.198
Marketable yield	0.869	0.447	-0.15
Fruit number	0.969	0.187	-0.085
Average fruit weight	-0.751	0.651	-0.087
Plant heigth 50 DAT	0.56	0.656	-0.284
First truss emission	0.98	-0.056	0.038
Fruit water content	-0.946	-0.281	-0.059
Shoot dry weight	-0.349	0.399	0.719
Shoot Se concentration	0.95	-0.263	0.141
Root Se concentration	0.883	-0.284	0.343
Fruit Se concentration	0.904	-0.301	0.289
HQgv	0.918	-0.208	0.274
SSC	0.978	-0.039	0.006
Firmness	0.972	-0.124	-0.051
a*	0.768	-0.584	-0.029
N	0.376	0.909	0.169
P	0.451	0.883	0.043
K	0.708	0.669	-0.115
Ca	0.4	0.912	0.055
Mg	0.256	0.893	-0.019
S	0.979	0.003	0.197
Ascorbic acid	0.874	-0.36	-0.186
Polyphenol content	0.647	-0.718	0.1
Lycopene	0.615	-0.773	-0.054
Total carotenoids	0.236	-0.948	-0.155
Glycoalkaloids	-0.704	0.635	0.167
N use efficiency	0.878	0.409	-0.198
N uptake efficiency	0.832	0.539	0.006
Eigenvalue	16.759	8.826	1.204
Variance %	59.853	31.522	4.299
Cumulative %	59.853	91.374	95.676

Se application and grafting contributed to the separation of the first two PC, disclosing common trait variations among the two tested factors. The PCA scores showed in Fig. 6 present an insight on cherry tomato performance and nutritive value in response to the eight Se-grafting treatments. The first two PCs score plots separated grafting and Se application treatments into four groups (Figure 6).

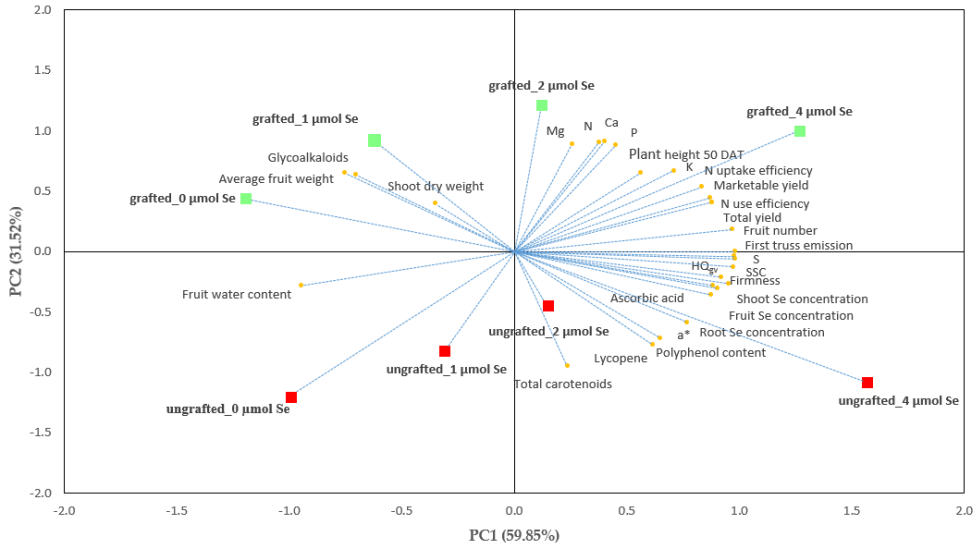

Figure 6. Scores and loading plots of PCA of cherry tomato yield and yield related traits (total and marketable yield, fruit number and average fruit weight), NUE indices (nitrogen use efficiency and nitrogen uptake efficiency), plant height 50 DAT, first truss emission, shoot dry weight, plant Se concentration (shoot, root and fruit Se concentration and HQgv), fruit water content, SSC, firmness, fruit redness (a*), minerals (N, P, K, Ca, Mg and S), functional compounds (ascorbic acid, polyphenol content, lycopene and total carotenoids) and glycoalkaloids as influenced by combining Se enrichment (0.0, 1.0, 2.0 or 4.0 µmol·L^{-1}) and grafting (ungrafted or grafted).

The upper right quadrant/group of the positive side of PC1 contains the grafted tomato with 2 and especially 4 µM Se that delivered cherry tomato fruit of high mineral composition, N efficiency indices as well as highest crop performance (Figure 6). The ungrafted plants fertigated with 2 and especially 4 µM Se were positioned on the lower right quadrant of the positive side of PC1 constituting fresh tomato with higher polyphenols and total carotenoids concentrations. Finally, the lower and higher left quadrants outlined the untreated control and 1 µM Se treatments which were characterized by the lowest quality attributes but higher glycoalkaoids concentration (Figure 6).

Discussions

The correlation between foodstuff consumption and human health is a contemporary research subject as considerable findings emphasize that food features can influence human biological mechanisms. Hence, vegetables improved in nutritional and/or nutraceutical properties, called ‘functional foods’, are of particular interest in prevention of human dysfunctions (Di Gioia et al., 2017). Nowadays, the production of trace elements enriched vegetables represents a way

to enhance intrinsic quality of fruiting and green leafy vegetables (Sabatino et al., 2019a; Pannico et al., 2020; Sabatino et al., 2019b). Among trace elements, Se is one of the imperative for the prevention of several human disorders such as cardiovascular illnesses, hypothyroidism, weakened immune system, male fertility and cancer (Hatfield et al., 2014; Malagoli et al., 2015). Along with the production of trace elements enriched vegetables, grafting is considered a practice for safeguarding or improving production constancy and fruit quality under favourable or unfavourable cultivation conditions (Sabatino et al., 2019a; Rouphael et al., 2018a). Our findings revealed that a moderate Se-enrichment (2.0 μmol·L^{-1}) supplied via nutrient solution enhance plant vigour traits such as plant height at 50 DAT. These results are in line with those reported by several authors (Marschner, 2012; Schiavon et al., 2013) who found an increased plant growth and development when both Se hyper-accumulator and non-hyper-accumulator species were cultivated under low doses of Se. As reported by Hartikainen (2005), these effects appear to be connected with improved activity of glutathione peroxidase and a reduced lipid peroxidation. Moreover, Marschner (2012) report that Se-enriched plants have a higher total respiratory activity in the leaves, resulting in an enhanced plant growth. Additionally, in the present study, grafting positively affected plant height at 50 DAT; the best result achieved with the grafting × 2.0 μmol Se L^{-1} combination. Our observations are partially in line with those of Mauro et al. (2020), who observed an increased plant vigour in tomato grafted onto Arnold F_1, Beaufort F_1, Maxifort F_1 or Top Pittam F_1 rootstocks. Moreover, our results agree with those of other authors (Kyriacou et al., 2017; Rouphael et al., 2018a), who found that grafting enhances plant vigour traits in vegetable crops. In our study both Se biofortification and grafting delayed flower emission. This may be related to the greater vigour of the grafted biofortified plants which therefore manifested a longer vegetative phase compared to the control plants. Overall, shoot dry weight in ungrafted plants showed a positive relation with Se-dosage. This finding is partially in line with that of Castillo-Godina et al. (2016), who by assessing the influence of Se on tomato plants, found that increasing Se concentration results in increased leaf dry matter. In our study, Se-biofortified grafted plants grew faster than ungrafted one. Pannico et al. (2020) found that different microgreen genotypes behave

differently in relation to Se-dosage in terms of plant dry matter. Similar results were obtained in this study. Here, Se positively affected yield traits of ungrafted plants up to a concentration of 2.0 μmol L^{-1}, whereas increasing its concentration to 4.0 μmol L^{-1} caused a substantial yield reduction. Conversely, grafted plants showed a positive relation between Se concentration (from 0.0 to 4.0 μmol Se L^{-1}) and yield traits with the exception of average fruit weight. It is known that Se is assimilated in plants via the S-assimilation pathway (Marschner, 2012). Since, selenocysteine and selenomethionine are integrated into proteins in non-accumulator plants, the non-specific replacement of cysteine and methionine, respectively, causes toxicity to plants because the proteins become non-functioning or are less functional as enzymes than the corresponding S-containing proteins. Thus, based on our findings, seems that ungrafted tomato plants are less tolerant to high dose of Se than grafted ones. Our results are in accordance with those of Nancy and Arulselvi (2014) studying Se-enrichment on the biochemical activities of tomato, found a yield increase with increasing concentrations of selenium in the soil, seed, or foliar application. The results of this investigation are also in line with previous studies (Germ et al., 2005) investigating the interactive effects of UV-B exclusion and Se application on the efficiency of PSII and production performance in zucchini squash. Other authors report that Se application improves the yield of lettuce (Xue et al., 2001), curly endive (Sabatino et al., 2019a), soybean (Djanaguiraman et al., 2004), rice (Wang et al., 2013), coriander and tatsoi (Pannico et al., 2020). However, Becvort-Azcurra et al. (2012), who examined the effect of Se application (2.5 and 5 mg L^{-1} of Se) on growth, production and antioxidants in tomato fruits, found no significant differences in terms of fruit production. These contrasting results could be attributed to tomato genotype. In our experiment, grafting was proven to be a valuable technique to improve tomato yield. Similar cases have been reported previously (Kyriacou et al., 2017) where grafted tomato plants were more productive than self-rooted plants. We should point out also that according to previous studies fruit yield in grafted tomato plants is rigorously correlated to the scion-rootstock combination (Riga, 2015; Schwarz et al., 2013). Se-biofortification up to 2.0 μmol L^{-1} enhanced NUE values in cherry tomato plants. This is in line with the findings of Ríos et al. (2010), who found that lettuce NUE indices are

positively affected by a higher Se rate when Se is applied in form of selenate. Likewise, our outcomes are in accordance with those of Sung and Chen (2018), who testing in pak-choi the response of N metabolism under different Se (sodium selenite) concentrations, report that Se supply augments the enzymatic actions (glutamine synthetase and glutamate synthase) in lettuce plants, and thus enhancing NUE and reducing NO_3^- concentration. Colla et al. (2010) found that grafting improves melon NUE indices. Similar results were observed in this study. Moreover, our findings are in line with those of Djidonou et al. (2013), who by studying yield, water, and NUE in field-grown, tomatoes, found that grafting onto interspecific tomato hybrid rootstocks (Beaufort and Multifort) significantly enhance NUE. Minerals are imperative for human fitness as constituents of our bone structure while also crucial for numerous body mechanisms, functioning as co-factors for some enzymes (Gupta and Gupta, 2014). Our data showed that grafting increased nitrogen, phosphorus, potassium, calcium, magnesium and sulphur fruit concentration. This is in line with previous studies by Lee and Oda (2003) but, when nitrogen fruit concentration values were averaged over Se concentration, no statistically significant differences were found. These findings are in accord with those of Castillo-Castillo-Godina et al. (2016), but in contrast with those reported by Sabatino et al. (2019a) who, by studying the effect of Se biofortification and administration type on yield and functional properties of curly endive grown in a floating raft system, found a positive relation between Se supply and plant nitrogen concentration. Our findings are also in opposition to those of Pannico et al. (2020), studying nitrate concentration in relation to Se-supply in coriander, green basil, purple basil and tatsoi, observed that a higher Se dosage determines a reduction in nitrate concentration. In relation to the interactive mechanisms between Se accumulation and other metabolic pathways, Schiavon et al. (2017) clarify that selenium modify molybdenum (Mo) uptake, an important cofactor of nitrate reductase, thus causing an effect on the assimilation of N into proteins and amino acids. Thus, we might hypothesize that the different response of tomato compared to green leafy vegetables or microgreens is probably due to the different plant organs nitrogen accumulation capacity. Regardless of the grafting, Se concentration did not significantly affect P fruit concentration. These results are

in agreement with those of Castillo-Godina et al. (2016), who stated that Se concentration does not interfere with the absorption of P. Regardless of grafting, higher Se concentrations positively affected K fruit concentration. Our results are consistent with those of Pannico et al. (2020) who found that Se-enrichment in microgreens determines an increase in K tissue concentration. Conversely, other authors found that Se biofortification does not affect K level in plant tissue (Sabatino et al., 2019a; Castillo-Godina et al., 2016). Furthermore, our study showed that fruit Ca concentration was not influenced by Se-enrichment. These results agree with those of Schiavon et al. (2013) who, assessing the influences of Se-biofortification on antioxidant constituents and phytochemicals of tomato, established that increasing dosage of Se does not influence Ca concentration in plant tissue. Moreover, our results are similar with those of Castillo-Godina et al. (2016) and Smolen et al. (2014) who, investigating the effect of biofortification with selenium and iodine in lettuce grown in nutrient film technique, found a decrease in the levels of Ca in plant tissue. Conversely, Pannico et al. (2020) mentioned an augment in leaf Ca concentration as Se concentration increases in the NS. According to these contrasting results, we may speculate that Ca concentration of cherry tomato is strictly related to several factors such as cultivation conditions growth environment and mostly genotype. Se doses did not influence Mg fruit concentration. This is in contrast to the finding of Islam and Ho-Min (2018) who, by investigating iron (Fe), iodine (I) and Se effects on cherry tomato shelf-life and functional quality, found that Se-enrichment determines an increase in terms of Mg fruit concentration. Additionally, studying on coriander, green basil and purple basil, Pannico et al. (2020), found an increase in Mg concentration due to Se-enrichment. However, these authors observed an opposite trend in tatsoi. Thus, it seems that the response in Mg concentration in relation to Se-biofortification is a genotype-related response. Se can be assimilated via the sulphur (S) assimilation pathway into Se-amino acids (Schiavon et al., 2013). In our study Se concentration positively affected S fruit concentration. This is consistent with the results of others (Ríos et al., 2008; Hawrylak-Nowak, 2013) who reported that increasing Se concentration in the nutrient solution, greatly enhances S shoot concentration in *Stanleya pinnata*, lettuce, *Arabidopsis thaliana* and some Brassica species. Similar

results were reported by Boldrin et al. (2013) who by evaluating the outcome of Se rice biofortification via foliar spray and/or via fertigation, reported that high Se dosage determines an increase of S tissue concentration. The positive relation between Se and S was, also, demonstrated by White et al. (2004) in *Arabidopsis thaliana*. The outcomes of the present work pointed out that Se supply stimulate sulphate accumulation in the fruits, probably by avoiding a decrease in the abundance and/or activity of sulphate transporters by sulphate and its metabolites. In our experiment shoot, root and fruit Se concentrations increased due to Se-biofortification. Our results are in line with those of Schiavon et al. (2013), who reported that the concentration of Se in plant tissues was more noticeable when plants were fed with high rates of Se (25 and 50 μM). Our findings are also in agreement with Castillo-Godina et al. (2016) who stated that Se accumulation in leaves, roots or fruits is directly proportion to its availability. Additionally, several authors (Sabatino et al., 2019a; Ferrarese et al., 2012; ´Avila et al., 2014) found an enhancement of Se plant tissue concentration when plants are exposed to Se-biofortification. Of note grafted plants performed better than ungrafted ones in terms of Se concentration in plant tissues. According to Lee and Oda (2003), these results should be related to the greater mineral accumulation capacity of the grafted plants compared to the ungrafted ones. To evaluate the dangers to human health, a calculation of HQgv was performed according to the protocol (2003) of USEPA. Our data showed an HQgv increase correlated with Se fertigation and grafting, ranging from 0.002 to 0.353. Consequently, the 150 g daily portion of Se-enriched cherry tomato can be considered safe, since the HQgv values were always less than one. Water fruit content was affected by Se-dose and grafting; a decrease of fruit water content was evident with an increase of Se dose and grafting. In a similar manner, Pannico et al. (2020) found that dry matter percentage in green basil, increases with Se enrichment. However, Businelli et al. (2015), investigating the effects of Se-biofortification on cucumber, lettuce and tomato, found an increasing trend of fruit water content with an increase of Se-doses. Moreover, we found that fruit firmness was increased by Se-enrichment, in line with Islam and Ho-Min (2018), who report an increase in fruit firmness in cherry tomato harvested from Se-biofortified plots. Our findings are also similar to those of Castillo-Godina et al.

(2016) who reported an increase of fruit firmness, ranging from 2.9 to 4.5 kg cm^{-2}, when Se doses increased from 0 to 5 mg L^{-1}. These results may be related to the higher total respiratory activity of Se-enriched plants (Marschner, 2012), resulting in a lower fruit water consent and, consequently, higher fruit firmness. Although Khah et al. (2006) and Riga (2015) did not observe changes in terms of fruit firmness between fruits from ungrafted and self-grafted tomato, our study demonstrated that fruit firmness increased by grafting alone or by combining grafting and Se-biofortification. In our study both Se concentration and grafting affected a* colour coordinate. The highest redness values were recorded in fruits from biofortified plants (14.1 for both ungrafted and grafted plants), a result most probably correlated to the higher fruit lycopene concentration in plants enriched with higher Se doses. Moreover, increasing Se dosage in the NS increased fruit SSC; these outcomes are consisted with those of Castillo-Godina et al. (2016) and Sabatino et al. (2019a). Some authors (Riga, 2015; Kumar et al., 2015) report that in numerous grafting combinations, rootstocks decreased SSC and sugar concentration in the fruits, but in our study the highest SSC values were registered in fruits from grafted plants. However, Kyriacou et al. (2017) stated that the decline triggered by grafting is insignificant compared to the potential increase acquired by using a designated scion. Polyphenols such as caffeic and chlorogenic acids underpin anti-cancer properties of tomato, which have been demonstrated using cellular models (Weng and Yen, 2012). In our study polyphenols fruit concentration increased progressively with increasing Se concentration in the NS. This is presumably due to the fact that Se can modify the uptake of Mo, and consequently can exert an influence on the assimilation of N into phenolics. Our results agree with those of Schiavon et al. (2013) who report that Se influences the amount of phenolic acids in tomato peels, particularly cinnamic acid derivatives. Our results are also confirmed by those of Sabatino et al. (2019a), who found that polyphenols synthesis is evidently encouraged at 4 μmol Se L^{-1} dose through fertigation or at 8 μmol Se L^{-1} dose via foliar spray, suggesting a promising valuable influence of Se in curly endive plants. Furthermore, our data revealed that fruits from grafted plants accumulated less polyphenols than fruits from ungrafted plants, which aligns with the outcomes of Vinkovic-Vrcek et al. (2011) who stated that grafting significantly

decreases the concentration of total phenolics in tomato. However, Riga et al. (2016) comparing nine different rootstocks, stated that the effect on flavonoids content evidently depends on the rootstock selection when the identical scion cultivar is adopted. Thus, the rootstock influence on flavonoids concentration remains unclear. However, since stress conditions stimulate phenolics accumulation (Moglia et al., 2008), we hypothesize that, because of Se-enrichment, grafted plants were less stressed than grafted ones due to the rootstock effect. Zu et al. (2014) proposed that a higher ingestion of lycopene was inversely linked with prostate cancer. Our study showed that Se-biofortification increased the total carotenoid concentration in cherry tomato fruit. This result is sustained by those of Schiavon et al. (2017), who reported that Se stimulates the plant cell antioxidant capacity through the amelioration of the antioxidant enzymes activity and the synthesis of some compounds, including carotenoids. Kyriacou et al. (2017) report that carotenoid concentration in tomato fruit, mostly lycopene and β-carotene, is affected by grafting. Indeed, these parameters are subject to the influence of rootstock-scion interactions. In line with the aforesaid study, our data showed that grafting negatively affected total carotenoids. These results are corroborated by other authors (Brajovi´c et al., 2012; Nicoletto et al., 2013), who declared that tomato lycopene concentration tends to decrease with grafting. Contrariwise, Miskovic et al. (2016) stated that when 'Madonna' eggplant rootstock was used, tomato lycopene concentration increases. Thus, this contrasting result can be related to the different response of the rootstocks used. Our study demonstrate that higher Se dose reduces the glycoalkaloids accumulation in the fruit, resulting, as reported by Krits et al. (2007), in a positive effect on human health. However, Interpro F_1 rootstock increased fruit glycoalkaloids concentration and consequently this is in line with the finding of other authors (Sabatino et al., 2019) assuming a scion-rootstock relation. Additionally, tomato fruit contains noteworthy quantities of ascorbic acid. In our work, ascorbic acid was not affected by Se concentration. There was, however, a positive grafting effect, which is in accordance with those of Schwarz et al. (2013) on grafted tomato.

Conclusions

In this study, Se-enrichment at 2.0 or 4.0 μmol L^{-1} and grafting effectively enhance plant performance, mineral accumulation, including Se and SSC fruit content, as well as some nutraceutical traits such as polyphenol content, total carotenoids, ascorbic acid and lycopene concentration. Our results, also, revealed that fruit derived from Se-biofortification is not harmful for human consumption up to 4 μmol L^{-1}. Overall, we can conclude that the combination of Se biofortification and grafting of cherry tomato grown in a soilless system are attractive and convenient tools for improving Se dose in human diet.

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EXPERIMENT 5

Scientia Horticulturae 295 (2022) 110891

Contents lists available at ScienceDirect

Scientia Horticulturae

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Agronomic performance and fruit quality in greenhouse grown eggplant are interactively modulated by iodine dosage and grafting

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Agronomic performance, functional quality, mineral profile, and browning potential in greenhouse grown eggplant are interactively modulated by iodine dosage and grafting

Introduction

Eggplant is among the highly appreciated fruiting vegetables grown all over the world (Faostat, 2019). Cultivated for centuries in Asia, Africa, and Europe it is now considered a global relevant species (Sabatino et al., 2018). Italy is among the top European producers of eggplant, especially in the southern areas of the country (FAOSTAT, 2019) where the cultivation is generally grown in open field, amid the spring-summer season or under protected environment, amid the fall-winter season for an early crop production (Tesi, 2010). Although eggplant is a fruiting vegetable native to the Old World, the Mediterranean Basin is recognized as a secondary centre of diversification, whereas Sicily is considered as one of the most important world producers (D'Anna et al., 2013; Sabatino et al., 2019). Nevertheless, the incessant cropping cultivation schemes - conventionally adopted in the protected environment - have caused a build-up of agronomic negative factors, which, partially or totally, may compromise production and fruit quality (Bletsos et al., 2003; Sabatino et al., 2019).

Herbaceous grafting is a proper propagation technique to enhance plant performance in fruiting vegetables under both, optimal or not-optimal growing conditions (Singh et al., 2020; Sabatino et al., 2020). Thus, the usage of grafted plants has turned into a worldwide horticultural practice (Turhan et al., 2011). Concomitantly, there is an increasing interest for vegetables enriched in nutritional and/or bioactive compounds for their role in the avoidance of human disorders, like mineral malnourishment. In this respect, several authors (Blasco et al., 2008; La Bella et al., 2021; Sabatino et al., 2021) suggest biofortification as a promising, eco-friendly and sustainable tool able to enhance micro- and trace element concentrations in leafy and fruiting vegetables. In addition, Sabatino et al. (2021) affirmed that grafting might interrelate with trace elements, such as selenium (Se), to enhance yield and quality of cherry tomato fruits.

Iodine (I) is a microelement fundamental for the normal functions of thyroid hormones in humans (Grasberger and Refetoff, 2011; Zimmermann, 2009) and its shortage is one the most widespread mineral deficiencies, causing important human disorders like cretinism, goiter, reproductive failure, and diverse types of brain injury (Andersson et al., 2005; Delange, 2000; Dillon and Milliez, 2000). As suggested by the European Food Safety Authority (EFTSA NDA Panel, 2014), the recommended daily ingestion of I is 90-120, 150 and 290 μg for kids, adult men or women, and for prenatal or lactating women, respectively. Most of the food, except the sea-derived ones, has a low I concentration (30-100 μg kg^{-1} of fresh weight) which is unsatisfactory to furnish the recommended daily allowance dose (Zimmerman, 2017). In the past century, iodized salt ingestion was considered the main strategy to overcome I deficiency in human diet. However, as reported by Mottiar and Altosaar (2011) and Aburto et al. (2014), non-organic I is volatile and its loss is frequent throughout transport, storage and cook. In addition, Kiferle et al. (2013) indicated that relevant quantity of iodized salt is not suggested for people suffering from hypertension and cardiovascular illnesses. Thus, the daily recommended I ingestion is - generally - not assumed. The World Health Organization (WHO, 2007) has endorsed the ingestion of I via seafood and biofortified food like leafy or fruiting vegetables, since the I contained inside is easily integrated by humans (Weng et al., 2008; Gonzali et al., 2017). Although I is

indispensable for humans, it is considered only beneficial for plants (Puccinelli et al., 2021) which assimilate it by root, stem, or leaves (Tschiersch et al., 2009). Nowadays, it is well recognized that plant answer to I biofortification is linked to numerous aspects like chemical form, nutrient solution concentration, and cultivation practice (Duborská et al., 2018; Medrano-Macías et al., 2016). Nonetheless, to the author's knowledge, the scientific literature requires specific studies concerning the effect of biofortification and grafting on eggplant. Taking all the above into account, an experiment was designed to estimate the interactive impact of I dosage and grafting on growth, yield, nutritional properties, mineral content, and pulp browning of 'Birga F_1' eggplant cultivated in a protected environment.

Materials and methods

Experimental location, plant material and grafting

The experiment was set up in Marsala (longitude 12°26' E, latitude 37°47' N, altitude 37 m), at the Department of Agricultural, Food, and Forestry Sciences of the University of Palermo. A hybrid scion of eggplant (*Solanum melongena*) 'Birgah F_1' was grafted onto *Solanum torvum* rootstock using the tube grafting technique. On 21 February 2020, after the healing phase, plants were transplanted in a climate-controlled greenhouse covered with polyvinyl chloride. Plantation density was about 2 plants m^{-2} (1 m × 0.5 m). The soil was composed by sand (<80%) at 8.5 pH and limestone (8.8%). Exchangeable K_2O, phosphorous, total nitrogen and organic matter were as follows: 65 mg kg^{-1}, 70 mg kg^{-1}, 2% and 10 t ha^{-1}, respectively. Above the drip irrigation system, a black PE mulching film (30 µm) was placed. During the whole study, the plants were grown based on the recommended practices for eggplant cultivation (Tesi, 2010).

Air temperature and the relative humidity level inside the greenhouse have been set to 18/26 ± 1°C throughout the night/day and 60-70%, respectively, during the whole cultivation period.

Biofortification treatments

The iodine (I) biofortification started ten days after transplant (DAT) and was distributed fortnightly. All plants were subjected to four different doses of I (0, 100, 300 or 600 mg L^{-1}). Iodine was provided via foliar spray to the plants in the form of potassium iodate (KIO_3). For each I level, 1.0 L m^{-2} of solution was applied. Not-biofortified plants (0 mg I L^{-1}) received only water applied via foliar spray (control plants).

Plant growth and yield

Eggplant growth and yield features were recorded on all plants. Plant height 40 DAT (cm), collar diameter 40 DAT (mm) and leaf number 40 DAT ($plant^{-1}$) were recorded. Fruits at commercial maturity stage were harvested (six harvest in total) according to fruit dimension, brightness and colour, as mentioned by Sabatino et al. (2018). Immediately after harvest, the fruits were grouped in marketable and unmarketable production. Thus, total, and marketable yield as well as number of marketable fruits were recorded and expressed as kg $plant^{-1}$, kg $plant^{-1}$ and n. fruits $plant^{-1}$, respectively. Mean mass of fruit (g) was also calculated.

Proximal composition, mineral profile, and functional compounds

All nutritional and nutraceutical analyses were carried out on fruits belonging to the 2^{nd}-4^{th} harvest. Fruit firmness was measured on five marketable fruits, randomly selected from each replicate, using a penetrometer (Trsnc, Italy), which was applied at the centre of the fruit at two opposite points. The firmness value was expressed as Newton.

For soluble solids content (SSC) estimation, fruit pulp samples (100 g) were squeezed and filtered, then the SSC value was appraised with a refractometer (MTD-045 nD, Three-In-One Enterprises Co. Ltd. Taiwan) and expressed as °Brix. Titratable acidity (TA) was measured as described by Han et al. (2008) and was expressed as percentage.

pH values were assessed via a pH-meter (Mettler Toledo, FiveGo F2, Milan, Italy). Fruit dry matter was obtained by dehydrating 200 g of sample at 105 °C until a steady mass was reached. Fruit dry matter content was expressed as percentage.

Fruit nitrogen concentration (N) was estimated following the Kjeldahl procedure and was expressed as protein content (g 100 g^{-1} DW). Phosphorous (P) was measured following the protocol of Fog and Wilkinson (1958). Potassium (K), magnesium (Mg), Calcium (Ca), zinc (Zn), copper (Cu), and iron (Fe) concentrations were obtained utilizing atomic absorption spectroscopy (SavantAA, 200 ERRECI, Milan, Italy) as delineated by Sabatino et al. (2021a) and Subramanian et al. (2012). The macro- and microelement concentrations were expressed as mg 100 g^{-1} DW, and µg g^{-1} DW, respectively.

For the evaluation of iodine (I) fruit concentration, an extraction of the samples was conducted using tetramethylammonium hydroxide, then an examination through inductively coupled plasma mass spectrometry was performed (ICP-MS). Iodine concentration in fruit was expressed as mg kg-1 DW.

Ascorbic acid concentration was determined with a reflectometer (Merck RQflex® 10) using Test Strips for ascorbic acid. Data on ascorbic acid concentration were presented as mg 100 g^{-1} FW.

The determination of total anthocyanins concentration in fruit was performed as stated by Mennella et al. (2012). Briefly, 0.2 g of lyophilized peel was mixed with 10 mL of methanol, while the analysis was realized via an HPLC system. The results of these analyses were showed as mg 100 g^{-1} DW.

To measure the chlorogenic acid concentration, the procedure reported by Stommel and Whitaker (2003) was applied and the data were presented as mg·100 g^{-1} of dw.

Glycoalkaloids concentration in fruits was measured as explained by Sabatino et al. (2018). Briefly, glycoalkaloids extraction was accomplished on 0.5 g of lyophilized fruit sample using ethanol, whereas the analyses were carried out by high-performance liquid chromatography (HPLC) (Agilent, 6546 Quadrupole Time of Flight LC/MS, Santa Clara, US). Data on glycoalkaloids concentration were presented as mg 100 g^{-1} DW.

Pulp browning potential

For the determination of the pulp browning, the colorimeter parameter L* (lightness) was measured via a digital colorimeter (Minolta Chroma meter CR-400). Eggplant fruits were cut in the equatorial region and the L* value was immediately

measured (L_0), then the measurement was repeated 30 minutes after cutting (L_{30}). The potential of oxidation was calculated using the equation $\Delta L_{30} = (L_{30} - L_0)$ (Larrigaudiere et al..1998; Concellòn et al., 2007).

Experimental design and statistical analyses

The 12 treatments [iodine dosage (0, 100, 300 or 600 mg L^{-1}) × type of plant (grafted, self-grafted or ungrafted)] were arranged in a completely randomized block design while each block was repeated 3 times. For each replicate, 10 plants were disposed, obtaining a total of 360 plants. A two-way Analysis Of Variance (ANOVA) (iodine dosage × type of plant) was used to analyse the recorded data. Mean separation was performed via Tuckey HSD test at $p \leq 0.05$. Data expressed as percentage were subjected to arcsin transformation [$Ø = \arcsin (p/100)^{1/2}$] prior ANOVA analysis. Principal Component Analysis (PCA) was performed on all experimental data. All statistical evaluations were performed utilizing the SPSS v.20 software (StatSoft, Inc., Chicago, USA).

Results

Plant growth and yield

ANOVA analysis for plant height 40 DAT, root collar diameter 40 DAT and number of leaves 40 DAT showed a significant interaction T × I (Figure 1). Grafted plants exposed to 0, 100 and 300 mg I L^{-1} revealed the highest plant height 40 DAT (Figure 1a) while the reverse was the case for self-grafted and ungrafted plants receiving an I concentation of 600 mg I L^{-1}. Data collected on root collar diameter 40 DAT and number of leaves 40 DAT supported the trend established for plant height 40 DAT (Figure 1b and c).

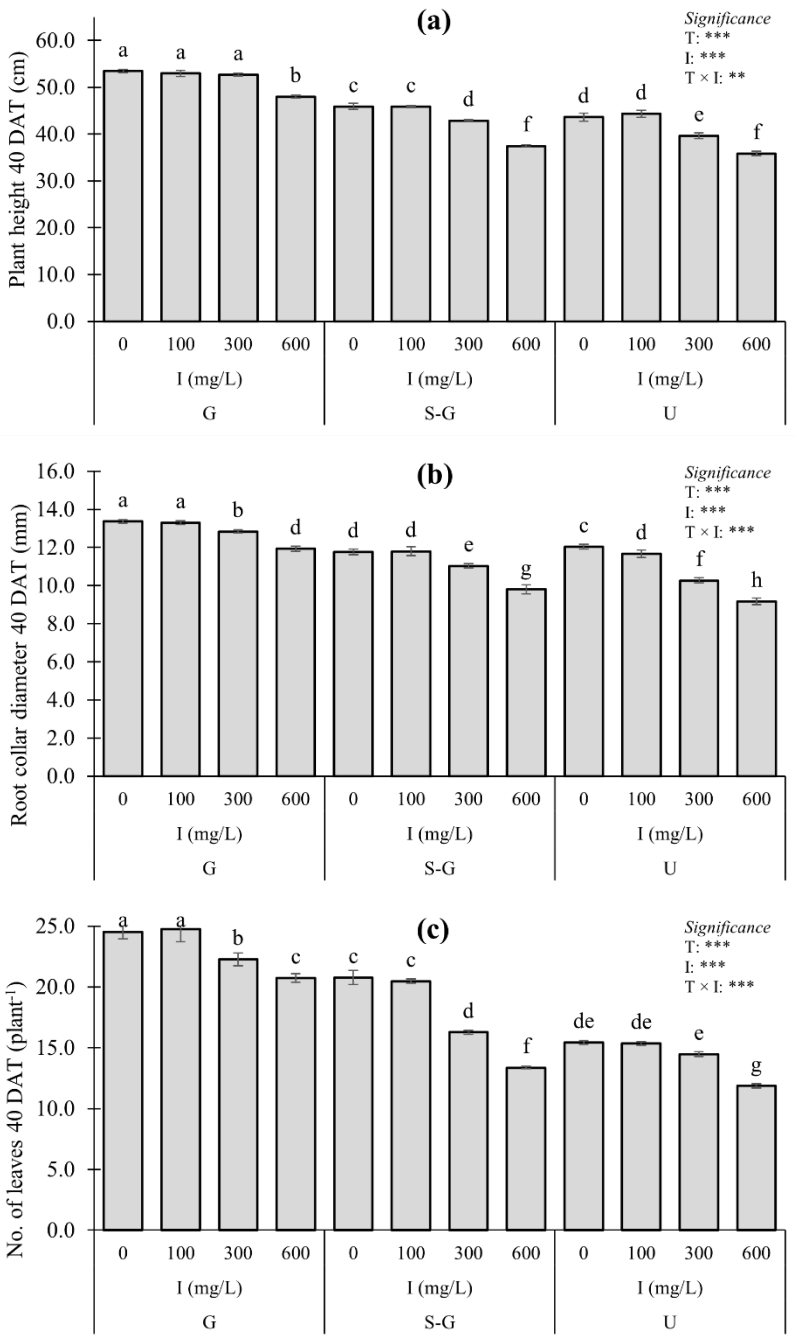


Figure 1. Impact of grafting and I-biofortification on plant height 40 DAT (a), root collar diameter 40 DAT (b) and number of leaves 40 DAT (c) of eggplant cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. **, *** significant at 0.01 and 0.001, respectively. Bars represent the standard error.

No significant effect of the interaction T × I for yield components was found (Table 1).

Table 1. Impact of grafting and I-biofortification on total yield, marketable yield, number of marketable fruits and mean mass of marketable fruits of eggplant cultivated in a protected environment.

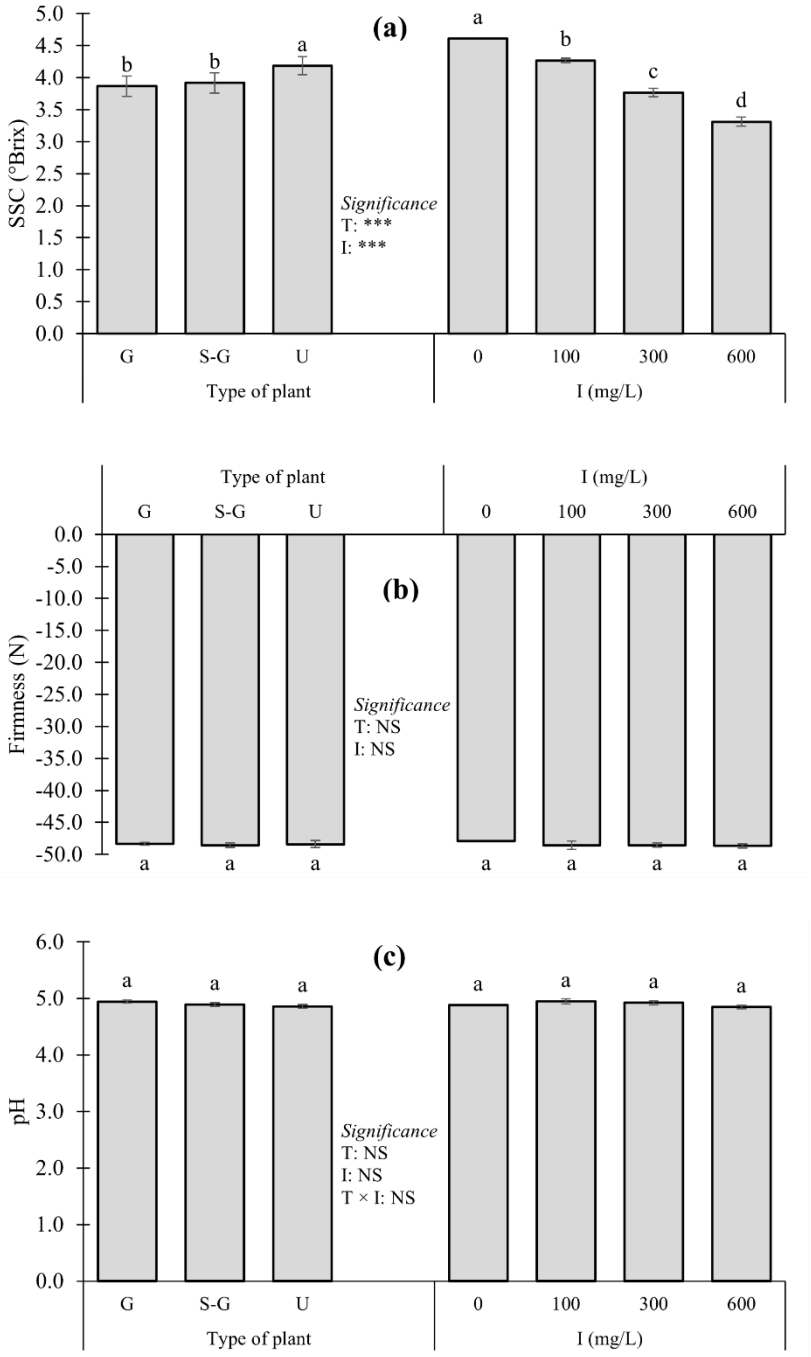
Treatments	Total yield (kg plant $^{-1}$)	Marketable yield (kg plant^{-1})	Number of marketable fruits (no. plant^{-1})	Mean mass of marketable fruits (g)
Type of plant (T)				
Grafted	4.5 a	3.8 a	9.2 a	417.2 a
Self-grafted	4.2 a	3.6 a	8.7 a	420.7 a
Ungrafted	2.7 b	2.5 b	5.7 b	447.0 a
I-dosage (mg I L^{-1})				
0	3.8 ab	3.2 ab	8.0 a	395.6 b
100	4.5 a	4.1 a	9.3 a	446.3 ab
300	3.9 ab	3.5 a	8.6 a	404.6 b
600	2.9 b	2.5 b	5.5 b	466.6 a
Significance				
T	***	***	***	NS
I	**	***	***	*
G × B	NS	NS	NS	NS

Values in the same column with different letters are significantly dissimilar at $p \leq 0.05$. NS, *, **, *** non-significant or significant at 0.05, 0.01 and 0.001, respectively.

Regardless of the I-dosage, grafted and self-grafted plants showed higher yield components (except for mean mass of marketable fruits) than ungrafted plants. Irrespective of the type of plant, plants supplied with 100 mg I L^{-1} presented the highest total yield, while the lowest total yield was detected in plants biofortified with I at 600 mg L^{-1}. Total yield in plant supplied with 0 and 300 mg I L^{-1} did not significantly diverge neither from plants supplied with 100 mg I L^{-1} nor from those supplied with 600 mg I L^{-1}. Without regard of the type of plant, plants sprayed with 100 or 300 mg I L^{-1} had the highest marketable yield, whereas plants supplied with 600 mg I L^{-1} had the lowest one. Regardless of the type of plant, plants fertigated with a I-dosage of 0, 100 and 300 mg I L^{-1} revealed the highest number of marketable fruits compared to those biofortified with 600 mg I L^{-1}. Regardless of the I-dosage, plant type did not significantly affect mean mass of marketable fruits. Irrespective of the plant type, plants sprayed with 600 mg I L^{-1} had the highest values. The lowest marketable fruits mean mass was detected in plants exposed to 0 and 300 mg I L^{-1}.

Nutritional parameters and functional features

ANOVA analysis for SSC, firmness, pH and TA did not display a significant interaction T × I (Figure 2).



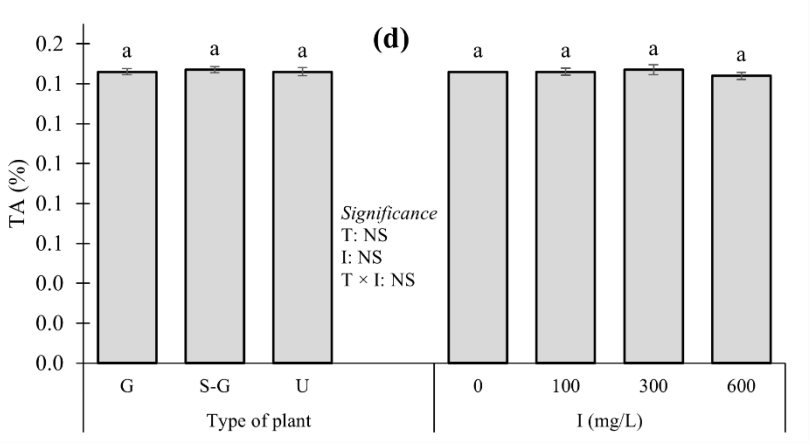


Figure 2. Impact of grafting and I-biofortification on soluble solid content (a), Firmness (b), pH (c) and titratable acidity (d) of eggplant cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. NS, *** non-significant and significant at 0.001, respectively. Bars represent standard error.

Independently of I-dosage, fruits from ungrafted plants exhibited the highest SSC. Regardless of the type of plants, increasing I concentration in the nutrient solution from 0 to 600 mg L^{-1} induced a significant and linear decrease in SSC (Fig. 2a). The treatments tested had no significant effects on firmness, pH and TA (Fig. 2b, c and d). Fruit dry matter, ascorbic acid, total anthocyanins, chlorogenic acid and glycoalkaloids, were significantly affected by the interaction grafting × I-dosage (Table 2).

Table 2. Impact of grafting and I-biofortification on fruit dry matter, ascorbic acid, total anthocyanins, chlorogenic acid and glycoalkaloids of eggplant cultivated in a protected environment.

Treatments	Fruit dry matter (%)	Ascorbic acid (mg 100 g^{-1} FW)	Total anthocyanins (mg 100 g^{-1} DW)	Chlorogenic acid (mg 100 g^{-1} DW)	Glycoalkaloids (mg 100 g^{-1} DW)
Type of plant × I-dosage (mg I L^{-1})					
Grafted × 0	5.5 ab	7.2 d	7128.2 f	764.9 h	44.4 f
Grafted × 100	5.0 bc	7.3 c	7204.6 e	774.7 gh	46.6 f
Grafted × 300	6.3 a	7.5 b	7342.9 e	789.5 g	70.4 e
Grafted × 600	5.8 ab	7.8 a	7503.7 d	816.5 f	81 d
Self-grafted × 0	4.2 c	6.5 h	7491.3 d	936.7 e	85 d
Self-grafted × 100	5.3 b	6.8 g	7639.9 c	955.1 d	93.9 c
Self-grafted × 300	4.6 bc	6.9 f	7864.9 b	970.6 c	104 b
Self-grafted × 600	4.4 c	7.1 e	8132.9 a	1020.2 b	130.6 a
Ungrafted × 0	4.3 c	6.5 h	7496.7 d	939.1 de	85.3 d
Ungrafted × 100	5.8 ab	6.8 f	7655.1 c	952.6 d	95.2 c
Ungrafted × 300	3.9 cd	6.9 f	7894.6 b	970.6 c	104.6 b
Ungrafted × 600	3.3 d	7.1 d	8176.9 a	1040.6 a	133.1 a
Significance					
T	***	***	***	***	***
I	*	***	***	***	***
G × B	**	*	***	*	***

Values in the same column and with different letters are significantly dissimilar at $p \leq 0.05$. *, **, *** significant at 0.05, 0.01 and 0.001, respectively.

The combination grafting × 300 mg I L^{-1} gave the highest fruit dry matter, whereas the combination grafting × 600 mg I L^{-1} gave the lowest. Nevertheless, fruit dry matter recorded in grafted plants supplied with 0 or 600 mg I L^{-1} and in ungrafted plants treated with 100 mg I L^{-1} did not diverge from that found in the combination grafting × 300 mg I L^{-1} (Table 2). Fruits from the combination grafting × 600 mg I L^{-1} had the highest ascorbic acid concentration, followed by those from grafted plants supplied with 600 mg I L^{-1}, which in turn revealed a higher concentration than fruits from grafted plants fertigated 100 mg I L^{-1}. The lowest ascorbic acid concentration was found in fruits from self-grafted or ungrafted plants supplied with 0 mg I L^{-1} (Table 2). Fruits from self-grafted or ungrafted plants exposed to the

highest I-dosage manifested the highest total anthocyanins content, followed by those from self-grafted or ungrafted plants supplied with 300 mg I L^{-1}. The combination grafting × 0 mg I L^{-1} had the lowest total anthocyanins concentration (Table 2). Fruit belonging to ungrafted plants supplied with 600 mg I L^{-1} had the highest chlorogenic acid concentration, followed by those from self-grafted plants enriched with 600 mg I L^{-1}. Whereas fruits from non-biofortified grafted plants revealed the lowest chlorogenic acid concentration (Table 2). Fruits from self-grafted and ungrafted eggplants exposed at 600 mg L^{-1} had the highest glycoalkaloids concentration, followed by those from self-grafted and ungrafted plants supplied with 300 mg I L^{-1}. Fruits from grafted plants exposed to I at 0 or 100 mL^{-1} had the lowest glycoalkaloids concentration (Table 2).

Mineral profile and iodine concentration

Proteins, phosphorous, potassium, calcium, magnesium, iron, and copper concentrations were not significantly affected by the interaction T × I (Table 3).

Table 3. Impact of grafting and I-biofortification on proteins, phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu) and zinc (Zn) concentrations of eggplant cultivated in a protected environment.

Treatments	Proteins (g 100 g^{-1} DW)	P (mg 100 g^{-1} DW)	K (mg 100 g^{-1} DW)	Ca (mg 100 g^{-1} DW)	Mg (mg 100 g^{-1} DW)	Fe (µg g^{-1} DW)	Cu (µg g^{-1} DW)	Zn (µg g^{-1} DW)
Type of plant (T)								
Grafted	14.5 a	506.2 a	355.8 a	102.9 a	15.5 b	30.4 a	3.3 a	9.9 a
Self-grafted	11.6 b	495.5 a	339.4 b	102.9 a	17.4 a	25.4 b	2.4 b	9.7 b
Ungrafted	11.8 b	458.6 a	331.8 c	103.0 a	17.5 a	25.3 b	2.3 b	9.7 b
I-dosage (mg I L^{-1})								
0	12.7 a	494.9 a	341.3 a	103.5 a	16.8 a	27.0 a	2.7 a	8.7 d
100	12.6 a	494.2 a	342.8 a	103.1 b	16.8 a	26.9 a	2.7 a	9.0 c
300	12.5 a	462.5 a	341.9 a	102.8 c	16.8 a	27.1 a	2.7 a	10.3 b
600	12.6 a	495.5 a	343.4 a	102.5 d	16.8 a	26.9 a	2.7 a	11.0 a
Significance								
T	***	NS	***	NS	***	***	***	***
I	NS	NS	NS	***	NS	NS	NS	***
G × B	NS	NS	NS	NS	NS	NS	NS	*

Values in the same column and with different letters are significantly dissimilar at $p \leq 0.05$. NS, *, *** non-significant or significant at 0.05 and 0.001, respectively.

Independently of the I-dosage, fruits from grafted plants revealed a higher proteins concentration than those from self-grafted or ungrafted plants. Conversely, I-dosage did not have a significant influence on fruit proteins concentration (Table 3). Both main factors tested had no significant effects on P. Without regard of the I-dosage, fruits from grafted plants revealed the highest K concentration, while those from ungrafted plants revealed the lowest K concentration. I-treatment had no significant effect on K fruit concentration (Table 3). Regardless of the I-dosage, plant type did not significantly affect Ca concentration. Contrariwise, I-dosage significantly affected Ca concentration which was highest in fruits from untreated plants (0 mg I L^{-1}) and lowest in fruits from plants sprayed with 600 mg I L^{-1} (Table 3). Plant type significantly affected Mg concentration; the highest value was recorded in fruits from self-grafted and ungrafted plants and the lowest in fruits from grafted plants. Regardless of the type of plants, ANOVA analysis did not underline a significant effect of I-dosage on Mg concentration. Data recorded on Fe and Cu concentrations sustained the tendency recognized for Mg concentration. Zn concentration was significantly affected by the interaction T × I (Table 3). The highest Zn concentration was observed in fruits from the grafting × 600 mg I L^{-1} combination, followed by that from self-grafted and ungrafted plants treated with identical dosage (Table 4).

Table 4. Impact of grafting and I-biofortification on zinc (Zn) concentrations of eggplant cultivated in a protected environment.

Treatments	Zn (µg g^{-1} DW)
Type of plant × I-dosage (mg I L^{-1})	
Grafted × 0	8.9 d
Grafted × 100	9.1 d
Grafted × 300	10.4 c
Grafted × 600	11.3 a
Self-grafted × 0	8.6 e
Self-grafted × 100	9.2 d
Self-grafted × 300	10.3 c
Self-grafted × 600	10.9 b
Ungrafted × 0	8.7 e
Ungrafted × 100	9.0 d
Ungrafted × 300	10.3 c
Ungrafted × 600	10.9 b
Significance	
T	***
I	***
G × B	*

Values in the same column and with different letters are significantly dissimilar at $p \leq 0.05$. *, *** significant at 0.05 and 0.001, respectively.

The lowest Zn concentration was depicted in fruits belonging to self-grafted and ungrafted plants exposed to 0 mg I L^{-1}. ANOVA analysis for I fruit concentration indicated a significant interaction between plant type and I-dosage (Figure 3).

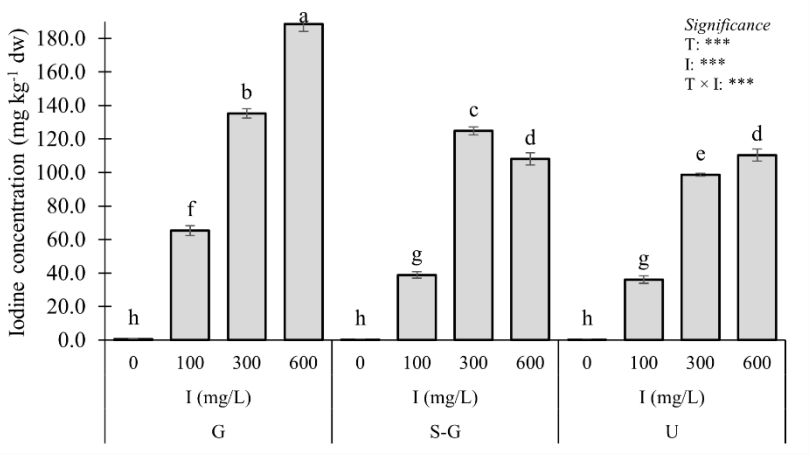


Figure 3. Impact of grafting and I-biofortification on iodine fruits concentration of eggplant cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. *** significant at 0.001. Bars represent the standard error.

The highest I concentration was observed in fruits from the grafting × 600 mg I L^{-1} combination, followed by that from grafted plants supplied with 300 mg I L^{-1} that in turn manifested a higher I concentration than that from self-grafted plants exposed to 300 mg I L^{-1}. Fruits from non-enriched plants (grafted, self-grafted, and ungrafted) exhibited the lowest I concentration (Figure 3).

Pulp browning

ANOVA analysis for L_0 did not present a significant interaction T × I (Fig. 4a).

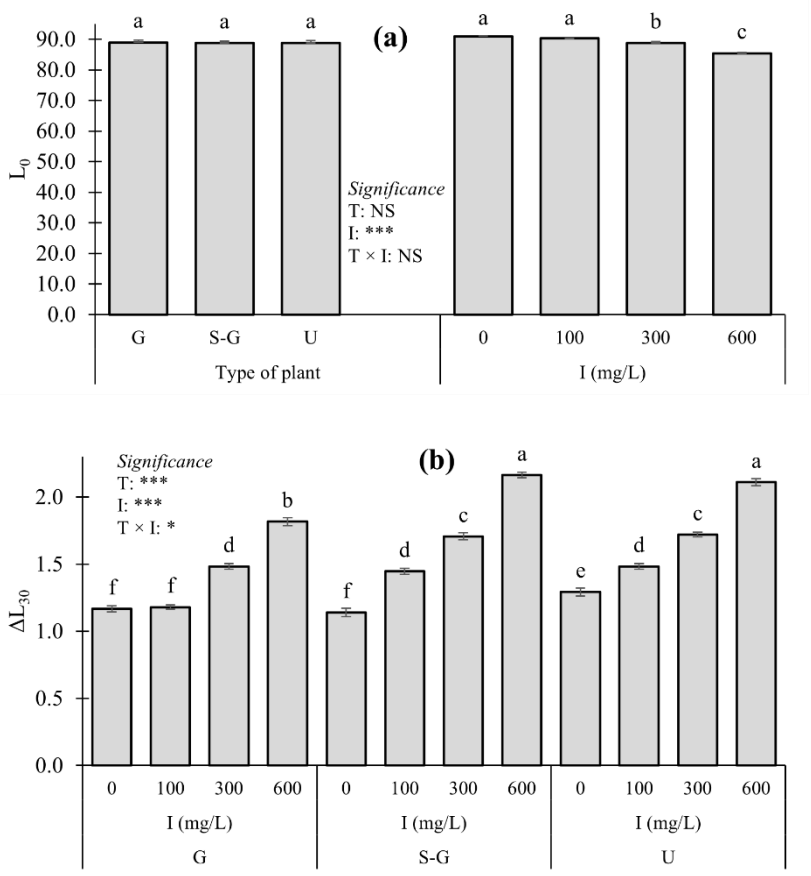


Figure 4. Impact of grafting and I-biofortification on L_0 (a) and ΔL_{30} (b) of eggplant cultivated in a protected environment. Values with diverse letters significantly differ at $p \leq 0.05$. NS, *, *** non-significant or significant at 0.05 and 0.001, respectively. Bars represent the standard error.

Regardless of the I-dosage, the plant type did not significantly influence L_0. Conversely, I-dosage significantly affected L_0 parameter which was highest in fruits from plants supplied with 0 or 100 mg I L^{-1} and lowest in fruits from plants treated with the highest I-dosage (Figure 4a). Statistical analysis for ΔL_{30} underlined a significant interaction between plant type and I-dosage (Figure 4b); the highest value was registered in fruits from self-grafted and ungrafted plants supplied with 600 mg I L^{-1}, followed by those from grafted plants exposed to the highest I-dosage. The lowest ΔL_{30} values were found in the combinations grafting × 0 mg L^{-1}, grafting × 100 mg L^{-1} and self-grafting × 0 mg L^{-1} (Figure 4b).

Discussions

In the vegetable production sector, a nutrient solution accurate management is the main task to accomplish a satisfactory yield and product quality (Islam et al., 2018). Simultaneously, the relationship between human health and vegetables consumption is a contemporary investigation topic as significant outcomes stress that intrinsic and extrinsic vegetable properties can affect human biological mechanisms. Thus, leafy and fruiting vegetables upgraded in nutritional and/or functional traits have a primary importance in human dysfunctions prevention (Di Gioia et al., 2019). In this scenario, biofortified vegetables production can be a feasible approach to improve yield, nutritional and functional features of vegetables (Sabatino et al., 2019a; Sabatino et al., 2019b; La Bella et al., 2021; Sabatino et al., 2021; Sabatino et al., 2021a; Sabatino et al., 2021b). Iodine is an indispensable microelement for human and the assimilation is mainly related to seafood and biofortified food consumption, for example vegetables (WHO, 2007). Definitely, there are a number of researches evidencing that I deficiency causes various dysfunctions in humans, such as insufficient thyroid hormone production (Andersson et al., 2005; Delange, 2000; Dillon and Milliez, 2000). In addition to vegetables biofortification, grafting is a valid toolbox for securing and enhancing yield, nutritional and bioactive compounds under favorable or stressful growth conditions (Sabatino et al., 2018; Sabatino et al., 2019; Rouphael et al., 2018). Our outcomes showed that a medium I supply (100 mg L^{-1}) did not significantly affect plant vigor-related traits, like plant height 40 DAT, root collar diameter 40 DAT and number of leaves 40 DAT. A similar response was found in carrot by Signore et al. (2018). On the other hand, there are evidences that I supply encourages plant growth response (Borst Pauwels, 1961; Zhu et al., 2003; Li et al., 2017). Our data, also, underlined that I-dosage at 600 mg L^{-1} negatively affected plant vigour traits in ungrafted, self-grafted and grafted plants. These findings agree with those of Sabatino et al. (2021b), who reported that I-enrichment result in a significant decrease in plant vigour-related traits in curly endive. Our data are also reinforced by those of Lawson et al. (2015) who, studying the effect of soil *vs* foliar I-biofortification, found a decrease in butterhead lettuce head size of biofortified plants. In the present study, grafting positively affected plant height, root collar

diameter and number of leaves 40 DAT; the highest response was accomplished when grafted plants were supplied with 0 and 100 mg I L^{-1}. Our findings are in agreement with those of Sabatino et al. (2013), Sabatino et al. (2018) and Sabatino et al. (2019), who evidenced an increased plant vigour in eggplant grafted onto *S. torvum*. Our study is also in agreement with those of Mauro et al. (2020) who, by assessing the impact of grafting and long-term oxygen root zone deprivation on physiological parameters of tomato plants, found that Top Pittam F_1, Maxifort F_1, Beaufort F_1 or Arnold F_1 rootstocks increase plant vigour in tomato plants. In addition, grafting improved plant vigour in various fruiting vegetables (Kyriacou et al., 2017). Thus, according to these considerations our experience suggests that the lowest I concentration tested (100 mg L^{-1}) is within the threshold value of eggplant I-tolerance. Furthermore, it seems that grafting can mitigate the I toxic effect in eggplant. In the present study, a moderate I dosage (100 or 300 mg L^{-1}) enhanced yield and yield-related traits, such as marketable yield and number of marketable fruits. These results tie well with previous studies (Lawson et al., 2015) wherein lettuce and radish plants biofortified with I displayed a significant variation in terms of yield compared with the control plants. However, this research is partially in line with that of Sabatino et al. (2021b), who noted that boosting I dosage in the nutrient solution resulted in a significant reduction of curly endive yield. Thus, it seems that I effect and tolerance is a species related trait. Moreover, since I is mainly stored in the chloroplasts (Weng et al., 2008a) and considering that plant vigour traits were reduced at the highest I doses, we may hypothesize that the yield reduction recorded in plants treated with 600 mg I L^{-1} could be related to a photosynthesis negative effect. Our outcomes displayed that grafting significantly improved total and marketable yield and marketable fruits number. These outcomes are endorsed by those of Sabatino et al. (2018), who investigating the use of hybrid and allied species as potential rootstocks for eggplant, found that grafted plants produced higher marketable yield than ungrafted ones, and that the yield increase was related to a higher number of marketable fruits. Similar results are reported by Sabatino et al. (2019), Sabatino et al. (2016) and Gisbert et al. (2011) on eggplant. The higher grafted plants yield was correlated to an increase in mineral and water uptake (Lee, 1994; Colla et al., 2006). In our study SSC was inversely related to I dosage. This

result agrees with that of Sabatino et al. (2021b) on curly endive plants. Our observations are, also, in accordance with those of Budke et al. (2020) and Li et al. (2017), who noticed that I supply decreases SSC in strawberry. In the present study, grafting decreased SSC in the fruits. Although this is in line with previous studies (Riga et al., 2015; Kumar et al., 2015), it was also proved that the reduction provoked by grafting is minimal in comparison to the potential increase attained by using a chosen scion (Kyriacou et al., 2017). Fruit firmness, juice pH and TA were not affected neither by I-dosage nor by grafting. This is in accordance with the findings of Budke et al. (2020) and Sabatino et al. (2019). I-dosage and grafting interacted significantly on fruit dry matter; the best results were detected in fruits harvested from grafted plants, highlighting that in self-grafted and grafted plants, the highest I dosage did not significantly affect dry matter percentage when compared with the control; whereas, in ungrafted plants, fruit dry matter decreased as I concentration increased. These outcomes are coherent with those stated by Incrocci et al. (2019), who found a reduction of dry matter when I concentration in the nutrient solution was higher than 50 μM. Our outputs disagree with those of Sabatino et al. (2018) and Sabatino et al., (2019) who assessed that the scion-rootstock combinations tested had no effect on fruit dry matter percentage. This discrepancy could be related to the type of cultivation (open field *vs* controlled environment). Our results on ascorbic acid revealed that I biofortification and grafting increased ascorbic acid concentration. Overall, these results are in accordance with those of Blasco et al. (2010) in lettuce. Sabatino et al (2021b) also found that increasing I concentration in the nutrient solution from 0 to 600 mg L^{-1} trigger a significant and linear increase in ascorbic acid concentration in curly endive. Similarly, Sabatino et al. (2020), assessing the effect of arbuscular mycorrhiza fungi (AMF) and grafting on eggplant yield and quality of the fruits, observed that grafting onto *S. torvum* rootstock promoted fruit ascorbic acid concentration. Nevertheless, Oztekin et al. (2013) stated that grafting does not significantly affect vitamin C in tomato fruits. These contrasting results could be attributed to the different rootstock used. Accordingly, tomato grafted onto 'Maxifort', 'Beaufort' or 'King Kong,' rootstocks had a higher ascorbic acid concentration than tomato self-grafted or grafted onto 'Brigeor' and plant tissues is

positively related to ascorbic acid concentration (Blasco et al., 2008) and considering that grafting enhances I accumulation in eggplant fruits, we may assume that I-biofortification at the highest dosage combined with grafting elicit ascorbic acid biosynthesis. Anthocyanins are the most important compounds within flavonoids group, and they affect eggplant peel colour (Sadilova et al., 2006). In our study, anthocyanins and chlorogenic acid concentration showed a significant increase as I-dosage increased. This is probably due to the fact that phenols and anthocyanins are secondary metabolites included in plant defence system against various biotic and/or abiotic distress, comprising mineral toxicity (Pardossi et al., 2015; Medrano-Macías et al., 2016). Concomitantly, our findings also revealed that grafting significantly decreased total anthocyanins concentration. Since anthocyanins biosynthesis and accumulation is strongly light-exposure-dependent (Manach et al., 2004; Awad et al., 2001) and considering that grafting enhances plant vigour traits, we can suppose that this result was an outcome of more shaded fruits. Fruits from grafted plants had a lower chlorogenic acid concentration than those from ungrafted plants. Considering that: i) I-enrichment is a distress for higher plants, since iodide can be oxidized to elemental I and, thus, can permanently injure the root cell membranes and oxidize chlorophylls and carotenoids, causing chlorosis of leaf and reduced CO2 assimilation (Blasco et al., 2010; Lawson et al., 2015; Duborsk´a et al., 2018); ii) plant distress elicit phenolic biosynthesis (Moglia et al., 2008; Marˇsiˇc et al., 2014), we may hypothesized that the lower chlorogenic acid concentration observed in fruits harvested from grafted plants can be related to the abiotic tolerance capacity of grafted plants. Our results showed that I-biofortification enhanced glycoalkaloids concentration in eggplant fruits. There are no reports on the impact of the I-enrichment on glycoalkaloids biosynthesis, thus, no comparisons can be presented. However, the results are in line with those by Papathanasiou et al. (1999), who investigating the impact of environmental distress on glycoalkaloids accumulation in potato, found that unfavorable cultivation condition and practices might increase glycoalkaloids concentration in potato tubers. Furthermore, it is relevant that grafted plants biofortified with a dosage of 100 mg I L^{-1} did not show significant differences compared with non-biofortified plants in terms of glycoalkaloids concentration. Thus, we might suppose that

grafted plants are less stressed than ungrafted ones when subjected to I-biofortification. Our results on the effect of grafting on glycoalkaloids concentration are fully in agreement with those described by Sabatino et al. (2019). As stated by Gupta and Gupta (2014), quantitative aspect of the mineral profile in vegetables is fundamental for human health since minerals are bone structure constituents while, as co-factors for some enzymes, play an imperative role for several physiological mechanisms. Regardless of the I-dosage, our data on mineral profile totally concurred with those described by Sabatino et al. (2018) who found that grafting enhances proteins, K, Fe and Cu, decreases Mg, and does not significantly affect P and Ca concentrations. In the current paper, we showed that grafting improved Zn concentration in eggplant fruits. I-dosage did not significantly influence proteins, P, K, Mg, Fe and Cu. Our outcomes also showed a linear decrease of Ca concentration as I-dosage increased and a positive relation between Zn concentration and I-dosage. Our findings on proteins, P, K, Mg concur with those of Sabatino et al. (2021b) on curly endive. Our outcomes on Cu and Zn are in accordance with those by Incrocci et al. (2019) who observed that I supply does not significantly affect Cu concentration in sweet basil and, simultaneously, enhances Zn concentration. Therefore, we might presume that the diverse response of eggplant compared to other green leafy vegetables is apparently a result of the dissimilar capacity of plant organs mineral accumulation. The results obtained by I-biofortification on mineral profile are supported by Kato et al. (2013), who found that I supply may elicit reductase activity in the root which can influence the mineral uptake and accumulation. As documented by Tschiersch et al. (2009), higher plants can intake I by different plant organs like roots or stem and leaves. Plant I intake occurs via ionic channels and chloride transporters (White and Broadley, 2009). Furthermore, Lawson et al. (2015) found that I-enrichment by foliar spray treatments is more functional than soil implementation to enhance I presence in the tissues of plants. However, there are reports that fruits are poor in iodine (Budke et al., 2020) and, as supposed by Herrett et al. (1962), this is probably related to I low mobility in plants. Accordingly, Li et al. (2017) found that iodine concentration in strawberry fruits from plants cultivated in a hydroponic system was about 7-9 times lower than in leaves when I was added in the nutrient solution. Similarly, a low

translocation of iodine from roots to fruits and seeds was remarked (Cakmak et al., 2017). Noticeable, Hocking et al. (2016) documented that the I transport from roots to fruits is - probably - essentially limited to the xylem sap influx happening in the early stage of fruit development, as described for other phloem-immobile elements like Ca. In our trial, I concentration increased due to I-biofortification. Our results also highlighted that self-grafted plants treated with 300 mg I L^{-1} had the highest iodine concentration, whereas, grafted plants had the highest I concentration in fruits biofortified with the highest dose. Thus, taking into account the aforesaid considerations, we might suppose that *S. torvum* rootstock improved I uptake and accumulation in eggplant fruits, enhancing eggplant I toxicity tolerance. Regarding the antioxidant activity, eggplants are among the top ten vegetables. Indeed, as reported by several authors (Mishra et al., 2013; Gisbert et al., 2011), a high browning potential could be estimated. Mishra et al. (2013) documented that browning in eggplant pulp acts on cutting, when the enzyme polyphenol oxidase (PPO) is released when cellular structures are disrupted. This enzyme oxidizes phenolics and, with oxygen presence, *o-quinones* are polymerized, producing pigments of brown colour. In line with other studies highlighting the implication of diverse factors on browning pulp (Prohens et al., 2007; Mishra et al., 2013), the browning potential in our trial showed a significant effect of the I-biofortification and grafting interaction. In fact, browning potential was increased by I-dosage and decreased by *S. torvum* rootstock. Thus, since i) IO_3^- has a toxic effect in higher plants, ii) information on phytotoxic effect are sparse, iii) grafting onto *S. torvum* mitigate the I-toxicity and iv) propensity to browning of eggplant pulp is ordinarily considered an important quality index for this produce, we may hypothesize that grafting plants onto *S. torvum* rootstock, is more suitable for I-biofortified eggplant production.

Conclusions

In this study grafting combined with I-biofortification significantly affected plant vigour traits, yield, and quality in eggplant. The damaging effects of high I dosages in the nutrient solution on 'Birgah F_1' eggplant were related to a large iodine

accumulation in leaf tissues and a marked reduction of plant vigour features. The better tolerance to I-toxicity found on grafted plants was linked to their capacity to stand higher concentrations of I, rather than to an exclusion mechanism. High concentration of chlorogenic acid could play an imperative role in I-tolerance of 'Birgah F_1' eggplant. Overall, our findings suggested that combining grafting onto *S. torvum* and I dosages of 300 or 600 mg L^{-1} may effectively increase plant growth, yield, nutritional and functional quality of eggplant.

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EXPERIMENT 6

horticulturae

MDPI

Article

Grafting Eggplant Onto Underutilized *Solanum* Species and Biostimulatory Action of *Azospirillum brasilense* Modulate Growth, Yield, NUE and Nutritional and Functional Traits

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Grafting eggplant onto underutilized solanum species and biostimulatory action of *Azospirillum brasilense* modulate growth, yield, nue and nutritional and functional traits

Introduction

Three eggplants species attracted human attention and were domesticated (Toppino et al., 2021). Among them, the most significant is the common eggplant (*Solanum melongena*), whose first cultivation started in Southeast Asia (Meyer et al., 2012), whereas *S. aethiopicum* (scarlet eggplant) and *S. macrocarpon* (gboma eggplant) are the other species, which were domesticated in Africa (Lester and Daunay, 2003). Scarlet and gboma eggplants are less notorious than common eggplant and, as reported by Schippers (2000), are frequently grown in sub-Saharan Africa and sporadically in other world locations, such as Southeast Asia. Noteworthy, Sunseri et al. (2010) reported that scarlet eggplant is also cultivated in the Caribbean, Brazil and in a few areas of Southern Italy. Taher et al. (2017) declared that, in line with the AVGRIS and Genesys databases, there are 6632 accessions of cultivated eggplants preserved in gene banks (5665 S. melongena, 798 *S. aethiopicum* and 169 *S. macrocarpon*). As concerning wild eggplant relatives, among the 1304 accessions deposited in germplasm banks, *S. incanum* is the most representative species (167 accessions), followed by *S. torvum* (132 accessions). Within the Mediterranean Basin, Sicily represents one of the most important centres of out-of-season cultivation, and it is considered an important area of eggplant ecotypes

conservation (D'Anna and Sabatino, 2013). Currently, due to the absence of resilient genotypes to biotic and/or abiotic distress, grafting is recognised as a valid toolbox for ensuring production and fruit quality under favourable or unfavourable grown conditions (Rouphael et al., 2018; Sabatino et al., 2013, 2016 and 2021a; Mauro et al., 2020; Consenino et al., 2022a). Currently, the vegetable plug plant sector is highly specialised. However, the higher labour and time required for propagation determined an increase in the grafted vegetable plug plant cost compared with the not grafted ones. Although grafted plantlets have a higher initial cost, they permit an increased yield and reduced cost of the control measures for soilborne diseases and pests. *Solanum torvum* is the most widespread rootstock for *S. melongena*, as it permits to overcome different soilborne diseases (Bletsos et al., 2003; Bogoescu et al., 2014). Nevertheless, other authors (Sabatino et al., 2018 and 2019; Murata et al., 2022) have proposed wild eggplant relatives or interspecific hybrids as potential rootstocks for eggplant. In agreement with the European Green Deal strategies, biostimulants, comprising nonmicrobial and microbial ones, are considered sustainable means to support the yield and quality of vegetables (Consentino et al., 2020, 2021 and 2022b; Di Mola et al., 2021; Sabatino et al., 2021b and 2022; La Bella et al., 2021; Giordano et al., 2022). Among the microbial biostimulants, plant growth-promoting bacteria (PGPB) contain a cluster of microorganisms with an aptitude for colonising the root apparatus, the rhizosphere and the internal part of the plant tissues (Davison, 1988; Kloepper et al., 1989). As reported by Cassan et al. (2008), PGPB can provoke plant growth by inducing a number of mechanisms, such as nitrogen fixation and nitrate reductase activity. Furthermore, significant actions on hormones biosynthesis, the solubilisation of phosphate and organic plant defence were reported (Tien et al., 1979; Bottini et al., 1989; Strzelczyk et al., 1994; Rodriguez et al., 2004; Correa et al., 2008). Currently, an eclectic collection of PGPB was documented, including the *Azospirillum* genus (Weller and Thomashow, 1994; Glick, 1995; Probanza et al., 1996), which is a free-living genus generally established worldwide and employed in horticulture (Consentino et al., 2022b). The inoculation of PGPB increases the yield of vegetables due to its capacity in improving NUE, stimulating mineral absorption and use proficiency (Consentino et al., 2022b). Even though copious studies have

been conducted to study the combinatorial effects of grafting and of other agronomical practices (Ioannou, 2001; Ozturk and Ozer, 2019; Joshi et al., 2021), no specified study has been performed to investigate the synergistic influences between wild and allied eggplant relatives' rootstocks and PGPB inoculation in enhancing the eggplant yield and quality. Starting from the aforementioned consideration, the aim of the current study was to evaluate the interactive action of eggplant wild/allied relatives' rootstocks and *A. Brasiliense* PGPB application on plant growth, yield and yield-related traits, fruit quality and NUE of 'Gloria' F1 eggplant cultivated in a protected environment. The findings of the current research should deliver precious data on new potential eggplant rootstocks and on their responses to PGPB inoculation.

Materials and Methods

Experimental Location and Type of Plants

The trial was conducted for two consecutive years (2020/2021 and 2021/2022 during the fall-spring periods) at the experimental farm of the University of Palermo (SAAF), near Marsala (Trapani Province). The research was performed in a polyethylene-coated greenhouse. Five plant types (T) represented by the solanaceous species *S. melongena* 'Gloria' F_1 not grafted, self-grafted or grafted onto *S. torvum*, *S. aethiopicum* gr. gilo or *S. macrocarpon* rootstocks, were studied. The self-grafted plant type was comprised, because there are proofs that self-grafted plants need to be included in grafting experiments to better understand the real effects of grafting (Sabatino et al., 2018 and 2019; Khan et al., 2006; Gisbert et al., 2010 and 2011). On 12 October 2020 (first year) and on 11 October 2021 (second year), eggplants were transplanted with a plant density of 2 plants m^{-2} (1 m × 0.5 m). A data logger was installed in the greenhouse to record the maximum and minimum temperatures (Figure 1).

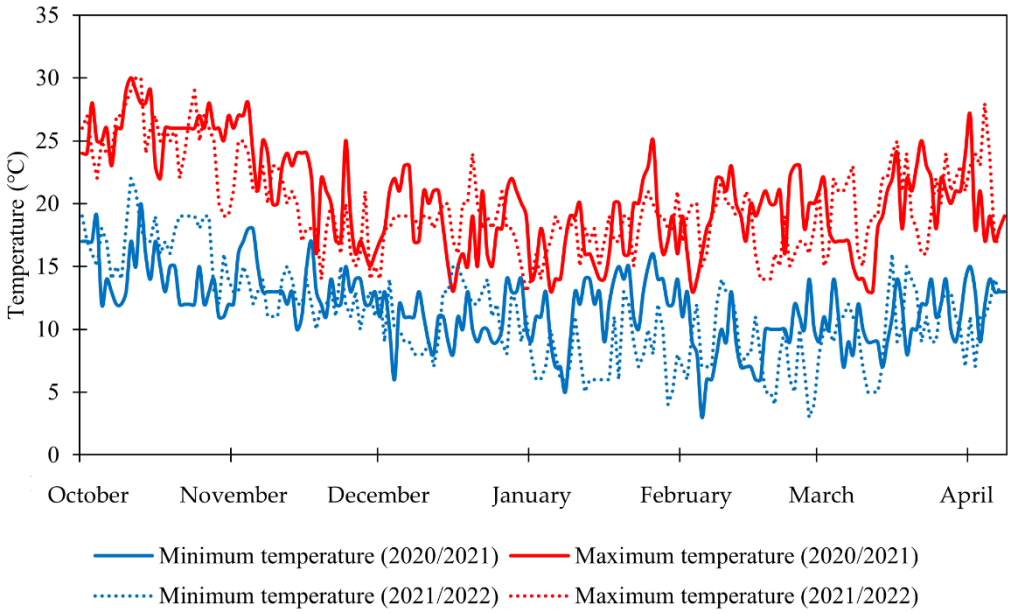


Figure 1. Maximum and minimum temperatures recorded during the two cultivation cycles.

The soil hosting the experiment was a medium-textured soil characterised by pH 7.0, 1.6% total N and 2.9% organic matter. Throughout the growing cycles, all eggplants were managed according to Tesi (2010).

Azospirillum brasilense Culture Preparation and Inoculation

The *A. brasilense* DSM 2298 microorganism strain employed for this research is conserved in the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). As reported by Keswani et al. (2019), *A. brasilense* belongs to the biosafety level-1 (BSL-1, low individual and low community risk), including organisms that are non-pathogenic and declared safe for laboratory personnel and the environment. *A. brasilense* was cultivated on an agar plate. The colony was inoculated in 100-mL Erlenmeyer flasks at a temperature of 28 °C with 10 mL of nutrient broth (NB), containing 5.0 g L^{-1} of peptone and 3.0 g L^{-1} of meat extract spinning at 150 rpm in a centrifuge for 24 h. After the incubation period, the bacterial culture was centrifuged (4000 rpm for 10 min); then, the supernatant was eliminated, and the pellet was mixed with NaCl (0.8%). The final optical density was about 109 CFU/mL. Two millilitres of this suspension were inoculated with 120 mL of NB. The growth was established optically at 600 nm (Beckman DU730 spectrophotometer). The plants were inoculated with *A. brasilense* in two phases:

during the transplanting phase (2 min of roots soaking) and 15 days after transplanting (100 mL of solution per plant). The control plants received only water.

Plant Development and Productive Traits

The plant growth and productive parameters were assessed for all plants. Plant heights 50 days after transplant (DAT) and the number of leaves 50 DAT were recorded. Fruits were harvested only at the commercial maturity stage, as reported by Sabatino et al. (2018), and six harvests in total were accomplished. After harvest, the production was divided into marketable and unmarketable. Hence, the total production (kg plant^{-1}), marketable yield (kg plant^{-1}) and number of marketable fruits (n. plant^{-1}) were recorded. Furthermore, the mean mass of marketable fruits (g) was determined.

Fruit Nutritional Traits, Element Concentration, Functional Compounds and Nitrogen Use Efficiency

All analyses on the fruit quality and functional features were accomplished on fruits from the 2nd–4th harvests; in particular, 6 fruits, randomly chosen from each replicate, were used. Fruits firmness (Newton, N) was assessed using a penetrometer (Trsnc, Milan, Italy). The soluble solids content (SSC), expressed as °Brix, was measured in 120 g of fruit pulp. The pulp was blended and filtered; then, the analysis was realised using an optical refractometer. The fruit dry matter percentage was achieved by drying 200 g of fruit at 102 °C. The Kjeldahl method was employed to measure the fruit nitrogen concentration; then, the value was transformed into the protein content (N content × 6.25). The values were reported as g 100 g^{-1} dry weight (dw). The phosphorous concentration in fruits was assessed by the methos described by Fogg and Wilkinson (1958), whereas the potassium concentration was measured via atomic absorption spectroscopy (SavantAA, 200 ERRECI, Milan, Italy). For Ca and Mg measurements, atomic absorption spectroscopy was employed as described by Morand and Gullo (1970). The mineral determinations were reported as mg 100 g^{-1} dw. To determine the fruit ascorbic acid concentrations, a reflectometer (Merck RQflex® 10) and ascorbic acid test strips were used (Merck, Darmstadt, Germany). Distilled H_2O was utilised to dissolve the

fruit pulp sample. Water was added and mixed until the solution was filtered. Then, the test strips were immersed in the prepared samples and placed into the reflectometer. The values were presented as mg 100 g^{-1} fresh weight (fw). Fruit chlorogenic acid content (mg 100 g^{-1} dw) was measured following the procedure of Stommel and Whitaker (2003). Briefly, a binary mobile phase gradient of methanol (Sigma-Aldrich, St. Louis, MO, USA) in 0.01% aqueous phosphoric acid (Titolchimica spa, Rovigo, Italy) was used. The quantification of chlorogenic acid (CA), conducted after a RP-HPLC separation, was based on absorbance at 325 nm relative to the sesamol internal standard and an external standard of authentic CA (Sigma-Aldrich, St. Louis, MO, USA). The fruit glycoalkaloids concentration (mg 100 g^{-1} dw) was assessed as reported by Sabatino et al. (2018) using liquid chromatography (HPLC) (Agilent, 6546 Quadrupole Time of Flight LC/MS, Santa Clara, CA, USA). Partially purified solasonine and solamargine (Sigma-Aldrich, St. Louis, MO, USA) were used as the external standard. The total anthocyanins determination was performed via HPLC, as reported by Mennella et al. (2012). Purified delphinidin-3-rutinoside (D3R, Polyphenols Laboratories AS, Sandnes, Norway) was used as the external standard. The nitrogen use efficiency (NUE) was calculated on all plants using the following formula: NUE = yield (t)/nitrogen application rate (kg).

Experimental Layout and Statistics

To evaluate the effect of the year, a preliminary three-way ANOVA (type of plant (T) × biostimulant (B) × year (Y)) was accomplished. The treatments were identified by a factorial combination of five plant types (not grafted, self-grafted or grafted onto *S. torvum*, *S. aethiopicum* or *S. macrocarpon*) and two biostimulant treatments (control or inoculation with *A. brasilense* DSM 2298). The study was organised in a completely randomised block design with 3 replicates (30 plants per treatments), obtaining a total of 300 plants. All recorded data were analysed by a two-way ANOVA (type of plant × biostimulant). A separation of means was accomplished via Tukey's HSD test at $p \leq 0.05$.

Results

Plant Growth and Yield

The research was repeated over two consecutive years applying the same experimental set-up and achieving comparable data (Table 1).

Table 1. Significance of three-way ANOVA analysis (type of plant (T) × biostimulant (B) × year(Y)).

Variables	T	B	Y	T × B	T × Y	B × Y	T × B × Y
Plant height 50 DAT	***	***	NS	NS	NS	NS	NS
N. leaves 50 DAT	***	***	NS	*	NS	NS	NS
Total yield	***	*	NS	NS	NS	NS	NS
Marketable yield	***	***	NS	NS	NS	NS	NS
Number of marketable fruits	***	**	NS	NS	NS	NS	NS
Mean mass of marketable fruits	***	***	NS	NS	NS	NS	NS
Fruit dry matter	NS	*	NS	NS	NS	NS	NS
Firmness	NS	***	NS	NS	NS	NS	NS
SSC	***	***	NS	NS	NS	NS	NS
P	***	NS	NS	NS	NS	NS	NS
K	***	NS	NS	NS	NS	NS	NS
Ca	***	NS	NS	NS	NS	NS	NS
Mg	***	***	NS	NS	NS	NS	NS
Proteins	***	***	NS	*	NS	NS	NS
NUE	***	*	NS	NS	NS	NS	NS
Ascorbic acid	***	***	NS	***	NS	NS	NS
Chlorogenic acid	***	***	NS	NS	NS	NS	NS
Total anthocyanins	***	***	NS	NS	NS	NS	NS
Glycoalkaloids	***	***	NS	**	NS	NS	NS

Consequently, the results from the 2020-2021 trial are presented. The ANOVA for plant heights 50 DAT did not show a significant effect of the interaction between T and B (Figure 2a), whereas, for the N of leaves 50 DAT, a significant interaction was found (Figure 2b).

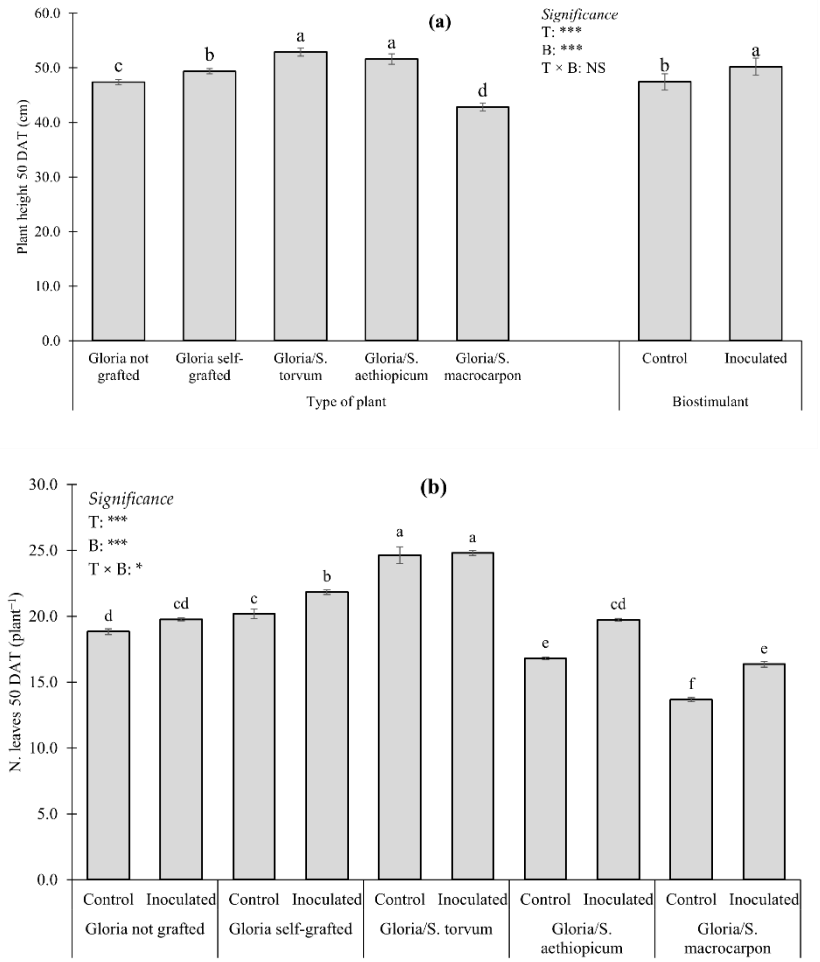


Figure 2. Influences of type of plant and biostimulant application on plant height 50 days after transplanting (DAT) (a) and number of leaves 50 DAT (b) of eggplant cultivated in a protected environment. Means with different letters are significantly dissimilar at $p \leq 0.05$ according to Tuckey test. NS=not significant; *=significant at $p \leq 0.05$; ***=significant at $p \leq 0.001$. Bars indicate means ± standard error.

The 'Gloria' F_1 plant grafted onto *S. torvum* and *S. aethiopicum* rootstocks showed the highest plant height 50 DAT, followed by self-grafted plants. The lowest height values were measured in plants grafted onto *S. macrocarpon*. On the other hand, the biostimulant enhanced the plant height compared to the control. Plants grafted onto *S. torvum*, inoculated with *A. brasilense* DSM 2298 or not, gave the highest number of leaves 50 DAT, followed by those self-grafted and treated with the biostimulant. The lowest values were in the control plants grafted onto *S. macrocarpon*. The ANOVA for eggplant production traits (total yield, marketable yield, number of marketable fruits and mean mass of marketable fruits) revealed no significant effect of the interaction of T × B (Table 2).

Table 2. Influences of type of plant and biostimulant application on total yield, marketable yield, number of marketable fruits and mean mass of marketable fruits of eggplant cultivated in a protected environment.

Treatments		Total yield (kg plant^{-1})	Marketable yield (kg plant^{-1})	Number of marketable fruits (no. plant^{-1})	Mean mass marketable fruits (g fruit^{-1})
Type of plant (T)	Gloria not grafted	3.8 c	3.5 c	9.8 b	355.3 b
	Gloria self-grafted	4.1 c	3.8 c	10.7 b	355.9 b
	Gloria/*S. torvum*	4.8 a	4.6 a	12.5 a	365.9 a
	Gloria/*S. aethiopicum*	4.4 b	4.2 b	11.9 a	349.0 b
	Gloria/*S. macrocarpon*	1.7 d	1.4 d	5.1 c	275.0 c
Biostimulant (B)	Control	3.7 b	3.3 b	9.6 b	336.0 b
	Inoculated	3.9 a	3.6 a	10.3 a	344.4 a
Significance					
	T	***	***	***	***
	B	*	***	**	***
	T × B	NS	NS	NS	NS

Values in the same column and with the same letter did not significantly differ at $p \leq 0.05$ according to Tuckey test. NS=not significant; *=significant at $p \leq 0.05$; **=significant at $p \leq 0.01$; ***=significant at $p \leq 0.001$.

Plants grafted onto *S. torvum* had the highest total yield (+26% compared to the not grafted and +18% compared to the self-grafted). Plants grafted onto *S. macrocarpon* showed the lowest total yield. The biostimulant application significantly increased the total yield by 5% compared to the control. Data on the marketable yield followed the trend recognised for the total yield; in particular, plants grafted onto *S. torvum* had marketable yields higher by 31% and 21% compared to the not grafted and self-grafted plants, respectively, whereas the biostimulant treatment increased the marketable yield by 9% compared with the untreated plants. The highest number of marketable fruits was recorded in plants grafted onto *S. torvum* and in those grafted onto *S. aethiopicum*, while the lowest values were assessed in *S. macrocarpon*-grafted plants. Not considering the type of plant, the biostimulant significantly increased the number of marketable fruits. When the plants were grafted onto *S. torvum*, they revealed the highest values in terms of the mean mass of marketable fruits, followed by those grafted onto *S. aethiopicum*. The lowest values were recorded in plants grafted onto *S. macrocarpon*. Averaged from the

type of plant, the biostimulant significantly enhanced the mean mass of marketable fruits.

Regardless of the biostimulant treatment, the type of plant did not influence the fruit dry matter, whereas, disregarding of the type of plant, the biostimulant significantly increased the fruit dry matter by 3% compared to the control (Table 3).

Table 3. Influences of type of plant and biostimulant application on fruit dry matter, firmness and soluble solids content (SSC) of eggplant cultivated in a protected environment.

Treatments		Fruit dry matter (%)	Firmness (N)	SSC (°Brix)
Type of plant (T)	Gloria not grafted	7.1 a	-46.8 a	4.6 c
	Gloria self-grafted	7.1 a	-46.6 a	4.3 d
	Gloria/*S. torvum*	7.1 a	-46.6 a	4.1 e
	Gloria/*S. aethiopicum*	7.1 a	-46.8 a	4.9 b
	Gloria/*S. macrocarpon*	7.2 a	-46.7 a	5.2 a
Biostimulant (B)	Control	7.0 b	-46.0 a	4.5 b
	Inoculated	7.2 a	-47.4 b	4.7 a
Significance				
T		NS	NS	***
B		*	***	***
T × B		NS	NS	NS

Values in the same column and with the same letter did not significantly differ at $p \leq 0.05$ according to Tuckey test. NS=not significant; *=significant at $p \leq 0.05$; ***=significant at $p \leq 0.001$.

The type of plant had no significant effect on the fruit firmness; conversely, the biostimulant application significantly reduced this parameter. When averaged over the biostimulant treatment, *S. macrocarpon* grafted plants had the highest SSC values, followed by those grafted onto *S. aethiopicum*. The lowest SSC values were noted in *S. torvum*-grafted plants. Disregarding the type of plant, the inoculation with *A. brasilense* DSM 2298 meaningfully boosted the fruits' SSC.

Minerals, Proteins and NUE

The ANOVA for the mineral profile did not reveal a significant effect of the interaction between T and B (Table 4).

Table 4. Influences of type of plant and biostimulant application on mineral profile (phosphorous, potassium, calcium and magnesium) of eggplant cultivated in a protected environment.

Treatments		P (mg 100 g^{-1} dw)	K (mg 100 g^{-1} dw)	Ca (mg 100 g^{-1} dw)	Mg (mg 100 g^{-1} dw)
Type of plant	Gloria not grafted	543.3 a	339.7 b	108.7 a	19.1 b
	Gloria self-grafted	544.0 a	340.0 b	109.3 a	18.8 b
	Gloria/*S. torvum*	541.7 a	343.6 b	109.0 a	14.5 c
	Gloria/*S. aethiopicum*	522.9 b	301.9 c	98.8 c	20.6 a
	Gloria/*S. macrocarpon*	499.9 c	348.2 a	106.3 b	19.2 b
Biostimulant	Control	530.6 a	334.2 a	106.3 a	17.4 b
	Inoculated	530.1 a	335.1 a	106.5 a	19.5 a
Significance					
T		***	***	***	***
B		NS	NS	NS	***
T × B		NS	NS	NS	NS

Values in the same column and with the same letter did not significantly differ at $p \leq 0.05$ according to Tuckey test. NS=not significant; ***=significant at $p \leq 0.001$.

The highest P fruit concentration was recorded in the not grafted plants, self-grafted and in *S. torvum*-grafted plants, whereas the lowest values were found in *S. macrocarpon* grafted plants. Conversely, the biostimulant application had no effect on the P fruit concentration. The highest K values were observed in fruits from plants grafted onto *S. macrocarpon*, with the values higher by 2.5%, 2.4% and 1.5% compared to those observed in the not grafted, self-grafted and *S. torvum*-grafted plants, respectively. Regardless of the type of plant, the biostimulant inoculation did not significantly influence the K concentration in eggplant fruits. Not grafted, self-grafted and *S. torvum*-grafted plants had the highest fruit Ca concentrations, whereas the lowest amounts were appraised in plants grafted onto *S. aethiopicum*. The inoculation with *A. brasilense* DSM 2298 did not affect the Ca concentration. Regardless of the biostimulant application, the highest fruit Mg concentration was shown by plants grafted onto *S. aethiopicum*, whereas the lowest values were assessed in those grafted onto *S. torvum*. Contrariwise, not considering the type of plant, the biostimulant inoculation meaningfully boosted the fruit Mg concentration. The statistics on proteins underlined a significant influence of the

interaction between T and B (Figure 3a), whereas, for NUE, no significant interactive effect was found (Figure 3b).

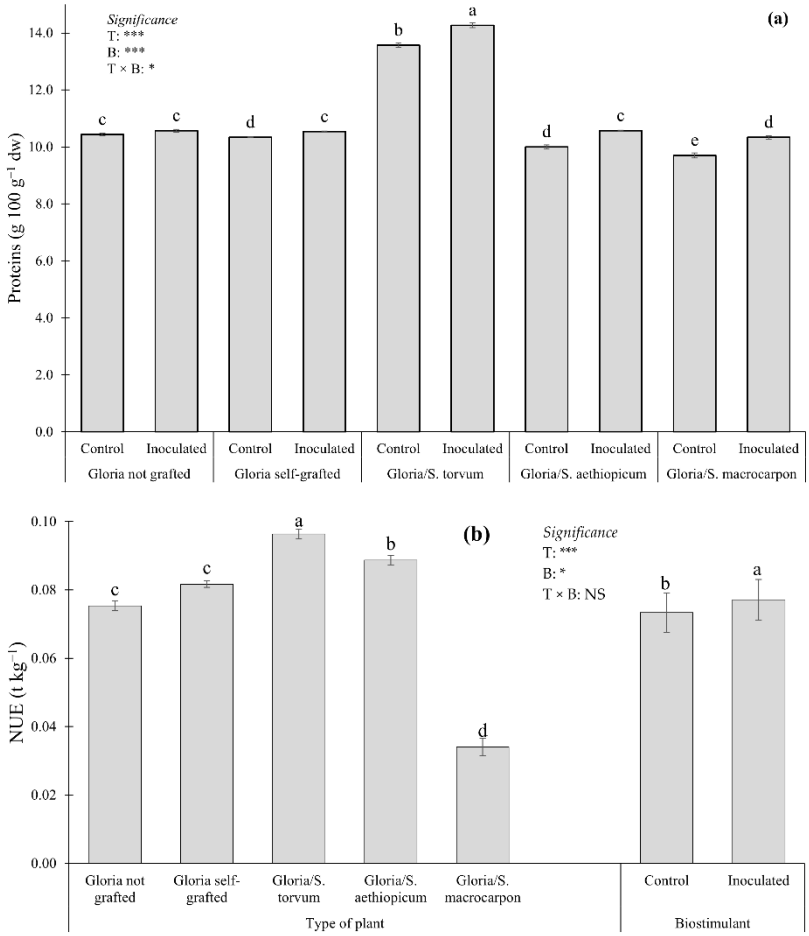
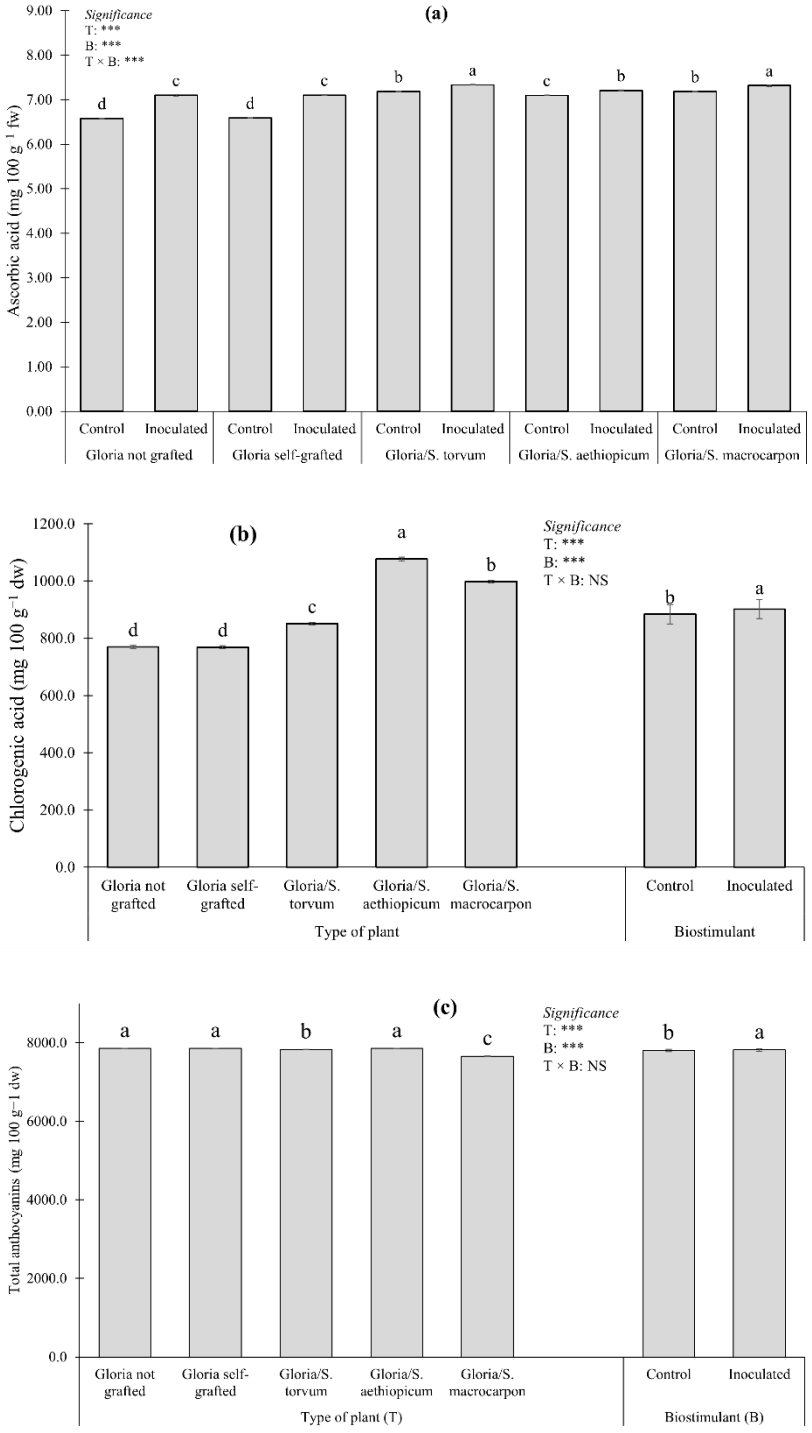


Figure 3. Influences of type of plant and biostimulant application on proteins (a) and nitrogen use efficiency (NUE) (b) of eggplant cultivated in a protected environment. Means with different letters are significantly dissimilar at $p \leq 0.05$ according to Tuckey test. NS=not significant; *=significant at $p \leq 0.05$; ***=significant at $p \leq 0.001$. Bars indicate means ± standard error.

Fruits from *S. torvum*-grafted plants treated with *A. brasilense* revealed the highest protein concentration, followed by those from plants grafted on the same rootstock but not inoculated. The combination Gloria/*S. macrocarpon* × control had the lowest value. Regardless of the biostimulant, plants grafted onto *S. torvum* had the highest NUE index values, followed by those grafted onto *S. aethiopicum*, which, in turn, highlighted a higher value than the self-grafted or not grafted plants. Conversely, not considering the type of plant, the biostimulant inoculation meaningfully boosted the NUE index.

Fruit Functional Features

The ANOVA for ascorbic acid (Figure 4a) and glycoalkaloids (Figure 4d) showed a significant effect of the interaction of T × B, while, for chlorogenic acid (Figure 4b) and total anthocyanins (Figure 4c), no interactive effect was found.



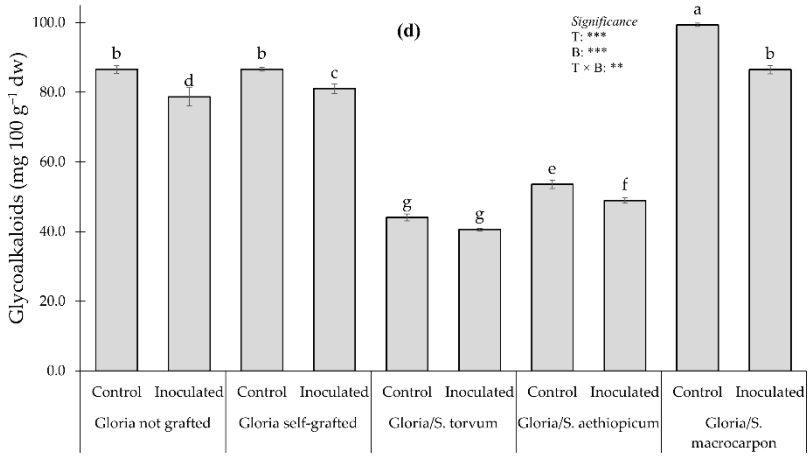


Figure 4. Influences of type of plant and biostimulant application on ascorbic acid (a), chlorogenic acid (b), total anthocyanins (c) and glycoalkaloids (d), and fruit concentrations of eggplant cultivated in a protected environment. Means with different letters are significantly dissimilar at $p \leq 0.05$ according to Tuckey test. NS=not significant; **=significant at $p \leq 0.01$; ***=significant at $p \leq 0.001$. Bars indicate means ± standard error.

Plants grafted onto *S. torvum* and those grafted onto *S. macrocarpon* and inoculated with the PGPB had the highest ascorbic acid concentrations, followed by the combinations Gloria/S. *torvum* × control, Gloria/*S. aethiopicum* × inoculated and Gloria/*S. macrocarpon* × control. The lowest values were appraised in the not grafted or self-grafted control plants. Regardless of the biostimulant application, fruits from plants grafted onto *S. aethiopicum* had the highest chlorogenic acid concentrations, followed by those from plants grafted onto *S. macrocarpon*. The lowest values were recorded on the not grafted or self-grafted plants. Contrariwise, not considering the type of plants, the inoculation with the biostimulant significantly increased the chlorogenic acid concentration compared to the control. When averaged over the biostimulant treatments, fruits from plants not grafted, self-grafted or grafted onto *S. aethiopicum* had the highest total anthocyanins contents, whereas the lowest values were recorded in fruits from *S. macrocarpon*-grafted plants. The biostimulant treatments significantly enhanced the total anthocyanins fruits concentration. Fruits from the combination Gloria/*S. macrocarpon* × control had the highest values, followed by those from the combinations Gloria not grafted × control, Gloria self-grafted × control and Gloria/*S. macrocarpon* × inoculated. The lowest values were appraised in plants grafted onto *S. torvum*, either inoculated or not.

Discussions

In the current research, we appraised the use of different potential rootstocks for eggplant and the inoculation of *A. brasilense* DSM 2298, alone or in combination, to improve the overall plant fitness, plant productivity and fruit quality of 'Gloria' eggplant grown in a greenhouse. The results showed that *S. torvum*- and *S. aethiopicum*-grafted plants had the highest plant heights 50 DAT. Furthermore, our findings also showed that *S. torvum*-grafted plants (inoculated or not) had the highest number of leaves 50 DAT. Our data are coherent with those stated by Sabatino et al. (2019), who, studying the potential use of *S. aethiopicum* and the interspecific hybrid of *S. melongena* × *S. aethiopicum* as potential rootstocks for eggplant, found that *S. torvum* and *S. aethiopicum* (accession 1) are the most vigorous rootstocks. Simultaneously, the outcomes revealed that *A. brasilense* DSM 2298 application significantly elicited plant vigour traits. These records entirely agree with those by Consentino et al. (2022b), who, studying the combinatorial effects of PGPBs and different N-application rates, found that lettuce plants colonised by *A. brasilense* and supplied with suboptimal N rates have the best performances in terms of plant vigour traits. The results are also in agreement with those reported by Gravel et al. (2007), who, investigating the influence of diverse PGPB on the growth and yield features of tomatoes, found that plants inoculated with PGPB showed a higher performance in terms of the plant vigour parameters. Our findings highlighted that *S. torvum*-grafted plants had the highest yield and yield related traits. Contextually, the *A. brasilense* inoculation proved to be a valid agronomic practice for increasing the yield traits. In this regard, the data are totally in agreement with that of Sabatino et al. (2020), who, examining the interactive effect between rootstock and arbuscular mycorrhiza on plant production and fruit features of 'Birgah' F_1 eggplant, found that-irrespective of the AM application- *S. torvum*-grafted plants revealed the highest yield. However, considering that plants grafted onto *S. aethiopicum* performed well in terms of yield (higher than 4.0 kg plant^{-1}), it seems that the combinatorial use of *S. aethiopicum* rootstock and *A. brasilense* DSM 2298 application could be a feasible alternative to the most common *S. torvum* rootstock. Although the strict actions by which PGPB promote the overall plant growth and yield are not completely

acknowledged, Parewa et al. (2014) stated that PGPB have an influence on plant growth through a number of actions, such as N fixation, inorganic phosphate solubilisation and the biosynthesis of key hormones. The current study showed that the type of plant did not significantly influence the fruit dry matter percentage, whereas the *A. brasilense* application improved it. These findings tie in well with those of Bhattacharyya and Jha (2012), who described that PGPB inoculation significantly boosts dry matter production in different solanaceous crops (e.g., tomatoes and potatoes). Our results also showed that, although the type of plants did not significantly affect the fruit firmness, an *A. brasilense* application significantly augmented the fruit firmness. Palmer et al. (2010) reported that the dry matter concentration is positively related to fruit firmness; therefore, we may assume that our outcomes could be linked to the higher dry matter content recorded in fruits from inoculated plants. In line with a previous study (Sabatino et al., 2018), the results underlined that fruits from plants grafted onto *S. macrocarpon* rootstock had the highest SSC, whereas those from plants grafted onto *S. torvum* rootstock had the lowest, remarking that the SSC trait is also influenced by genotype. Concurrently, our outcomes also displayed that SSC was enhanced by the application of PGPB. Overall, this was in accordance with those reported by Consentino et al. (2022b), who found that the application of *A. brasilense* exerted a positive action on lettuce SSC. Our outcomes were also coherent with those of Ordookhani and Zare (2011), who, studying the effect of PGPBs on the tomato fruit quality, recognised an encouraging influence of the microorganism on fruit SSC. The fruit mineral profile exhibited that plants grafted onto *S. torvum* had the highest P and Ca concentrations. Plants grafted onto S. macrocarpon had the highest K concentration, whereas *S. aethiopicum*-grafted plants revealed the highest Mg concentration. These findings underline the imperative action of the scion–rootstock combination on the nutritional fruit quality in grafted plants. Furthermore, the current research highlighted that the *A. brasilense* DSM 2298 inoculation did not significantly affect the fruit P concentration. In this respect, our results are in line with those of Consentino et al. (2022b) on lettuce and with those of Hungria et al. (2010) on maize and wheat. The findings also pointed out that the K and Ca concentrations were not affected by the biostimulant treatment. These findings are

partially in line with those of Sabatino et al. (2022), who found that *A. brasilense* DSM 2298 improved the K concentration in leaf tissues; however, it did not influence the Ca concentration in lettuce plants. Our data are in contrast with those of Radhakrishnan and Lee (2016), who connected the leaf lettuce K concentration increase to a catalysed metabolism of proteins, enzymes, lipids and nucleic acids. However, these dissimilar findings could be linked to a different plant organ accumulation/distribution. The data revealed that the biostimulant application emphasised the fruit Mg concentration. These outcomes are totally in agreement with those described by different authors (Consentino et al., 2022b; Hungria et al., 2010). The data showed that *S. torvum* rootstock and *A. brasilense* inoculation synergistically enhanced the protein concentrations in eggplant fruits. The findings concur with previous findings reported by Sabatino et al. (2020), who found that 'Birgah' eggplant grafted onto *S. torvum* and treated with a microbial biostimulant (arbuscular mycorrhiza fungi) had the highest fruit protein concentrations. Moreover, the outcomes revealed that plants colonised by *A. brasilense* DSM 2298 had higher fruit protein concentrations. As reported by Radhakrishnan and Lee (2016), the upsurge in plant N concentration through PGPB action is widely described. de Santi Ferrara et al. (2012) reported that nitrogen fixation via microorganisms in soil is the chief motivation for this increase. Specifically, Hungria et al. (2010) established that *A. brasilense*, strains Ab-V5 and Ab-V6, had comparable nif and fix genes that prompted the aptitude to fix atmospheric N. Although plants grafted onto *S. torvum* rootstock revealed the highest fruit protein concentrations, they also showed the highest NUE index, whereas *S. macrocarpon*-grafted plants had the lowest one. This is in accordance with the higher production of *S. torvum* grafted plants (4.8 kg plant^{-1}). Averaged from the type of plant, plants treated with *A. brasilense* showed a higher NUE than the control plants. These results agreed with those of Consentino et al. (2022b) and with those of Zeffa et al. (2019), who found that *A. brasilense* improves the NUE of maize via an augmented plants growth. Antioxidant compounds, such as polyphenols and ascorbic acid, are recognised as promoters of human health, particularly as they relate to noncommunicable diseases (NCDs) (Hollman et al., 2010; Hooper et al., 2008). In contrast to other nutrients, the plant polyphenol intake deficiency is not associated

with precise illnesses, making it problematic to delineate the proper reference intake values for these foodstuff constituents. Our findings showed that the biostimulant application and grafting onto *S. torvum*, *S. aethiopicum* or *S. macrocarpon* improved the fruit ascorbic acid and chlorogenic acid concentrations. Correspondingly, Sabatino et al. (2020) detected that *S. torvum*-grafted plants promoted the fruit ascorbic acid content in eggplant. However, Oztekin et al. (2013) underlined that grafting does not significantly influence the fruit ascorbic acid concentration in tomatoes. These conflicting outcomes could be ascribed to the genetic diversity of the employed rootstocks (Kyriacou et al., 2017). Our findings are also sustained by those of Parewa et al. (2014), who reported that the application of PGPB elicits secondary metabolism. Equally, Cappellari et al. (2017) found that three PGPB genera triggered phenolic biosynthesis in *Mentha piperita*. There are reports (Cassidy, 2018; Van Loon, 2007) that various PGPB activate plant tolerance to phytopathogens through alteration of the secondary metabolism, producing phenolic components. Overall, the increased ascorbic acid and chlorogenic acid concentrations in fruits from grafted plants inoculated with *A. brasilense* are most likely related not only to a modified secondary metabolism but also to a changed plant nutritional status of grafted plants. Anthocyanins are flavonoids largely found in red and blue fruits and vegetables. As reported by Babalola (2010), anthocyanins, such as other polyphenols, are metabolised by the host and the microbiome to form active metabolites that have anti-inflammatory properties and produce positive vascular effects. In line with other research (Consentino et al., 2022a; Sabatino et al., 2020), our data indicated that the use of *S. torvum* as a rootstock for eggplant reduced the fruit total anthocyanins concentration. Since - as reported by Manach et al. (2004) and Awad et al. (2001) - anthocyanins biosynthesis is connected to light exposure and considering that *S. torvum* is one of the most vigorous rootstocks for eggplant, we may assume that the total anthocyanins decline in *S. torvum*-grafted plants is related to a higher leaf shading effect on fruits. Furthermore, our results showed that *A. brasilense* inoculation enhanced the fruit total anthocyanins. These data are in agreement with those of Badar et al. (2022), who, studying the effects of different PGPBs on three strawberry cultivars, found that the application of PGPB significantly increased the total anthocyanins concentration. Similar

results were found by Lingua et al. (2013), who reported an increase in the total anthocyanins concentration in fruits from plants treated with arbuscular mycorrhizal fungi in combination with *Pseudomonas* strains. The results revealed that specific rootstocks, such as *S. torvum* and *S. aethiopicum*, especially when combined with *A. brasilense* colonisation, significantly decreased the glycoalkaloids concentration in eggplant fruits. The results are in accordance with former findings (Sabatino et al., 2022), who showed that microbial biostimulant application and grafting reduced the fruit glycoalkaloids concentration in eggplant. Accordingly, Mystkowska (2019) reported that potato plants treated with biostimulants had a lower glycoalkaloids concentration than the control plants. Since suboptimal cultivation conditions increased the glycoalkaloids concentration in potatoes (1999), we may suppose that *S. torvum*- and *S. aethiopicum*-grafted plants and those colonised by *A. brasilense* DSM 2298 were less stressed. These assumptions are totally in line with the results of Sabatino et al. (2020), who reported that grafting and AM fungi synergistically decreased the fruit glycoalkaloids concentration in eggplant.

Conclusions

The combined use of sustainable agronomic practices, such as the herbaceous grafting onto specific rootstocks and the biostimulant application, is attaining a reputation in the horticulture sector due to its capacity to boost the yield and quality of vegetable crops. *S. torvum* and *S. aethiopicum* rootstocks and *A. brasilense* DSM 2298 inoculation considerably acted together, enhancing the plant growth, yield and yield components, as well as nutritional and functional parameters of 'Gloria' F_1 eggplant. However, regardless of the type of plant, the microbial biostimulant tested significantly improved the overall plant fitness. The current research could be valuable information for the vegetable plug plant production sector involved in the use of new eggplant rootstocks that effectively react when combined with the microbial biostimulant.

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4.0. Concluding remarks

Eco-friendly tools are needed to reduce the environmental impact of cultivations and to increase the yield of crops for nourishing an upsurging the worldwide population. During the last years, the application of eco-friendly means of production such as biostimulant and herbaceous grafting has been useful for increasing yield and some quality aspects of horticultural crops. However, recent scientific findings pose a threat to the quality of the food in terms of micronutrients content, underlining that mineral malnutrition is one of the most important global health issues. In this scenario, the use of biofortification significantly may improve the nutritional and nutraceutical values of crops and decrease the micronutrient deficiency symptoms. The present thesis wants to study the possible interactive effects among these production means. For this purpose, several experiments on leafy a fruiting vegetable were carried out. From my data emerged that some qualitative and productive traits of vegetables could be affected by the mutual application of these tools.

From the experiment 1 emerged that rootstocks and arbuscular mycorrhizal fungi significantly interacted each other increasing crop performance and quality of eggplant. From the experiment 2 appeared that the combination of plant protein hydrolysates and Mo biofortification meaningfully boosts plant nutritional and functional features. The experiment 3 pointed out that the combination of seaweed extracts and Mo biofortification significantly increased yield and quality parameters as well as functional traits. The experiment 4 revealed that the mutual use of grafting and Se on tomato can be useful to increase productive and quality traits of tomato. In the experiment 5, the combination between grafting and I biofortification increased some qualitative and productive traits of eggplant. Finally, in the experiment 6 the combination of grafting and different growth promoting bacteria enhanced plant growth, yield, and functional traits of eggplant.

From all these studies emerged that biostimulant products, biofortification, and herbaceous grafting are capable of increasing yield and quality of vegetables. Interestingly, the combination of biostimulant or grafting with the biofortification

had a buffer effect on the microelement toxicity level, ensuring a higher accumulation in plant tissues and, consequently, a higher biofortification efficiency. Moreover, the interaction between grafting and biostimulant revealed interesting findings since it significantly boosted several yield and qualitative parameters of the vegetable crops tested. In conclusion, the combined use of these eco-friendly tools was successful in increasing yield and quality of vegetables and this information could be very useful for farmers. However, since the thesis study approach was exquisitely agronomic, further studies (e.g., with omics sciences) are necessary to better understand the mechanisms behind the crop responses.