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Review

Enhanced Accumulation of Vitamins, Nutraceuticals and Minerals in Lettuces Associated with Arbuscular Mycorrhizal Fungi (AMF): A Question of Interest for Both Vegetables and Humans

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Abstract: Lettuce (*Lactuca sativa* L.) is extensively grown and is the most widely used food crop for the called "Fourth Range" of vegetables. Lettuce exhibits healthy properties mainly due to the presence of antioxidant compounds (vitamins C and E, carotenoids, polyphenols) alongside significant fibre content and useful amounts of certain minerals. Lettuce can establish a mutualistic association with arbuscular mycorrhizal fungi (AMF). The establishment of the symbiosis involves a continuous cellular and molecular dialogue between both symbionts, which includes the activation of antioxidant, phenylpropanoid or carotenoid metabolic pathways. The presence of AMF colonizing roots of greenhouse-grown lettuces can induce an accumulation of secondary metabolites, vitamins and minerals in leaves that overcome the dilution effect due to the increased size of mycorrhizal plants. Therefore, AMF would allow the intake of minerals and compounds with antioxidant properties to be enhanced without increasing the consumption of lettuce in the diet. In addition, increased quantities of secondary metabolites may help lettuce plants to withstand biotic and abiotic stresses. Our review discusses the influence exerted by several environmental factors and agronomic practices on the ability of AMF for enhancing the levels of vitamins, nutraceuticals and minerals in leaves of green and red-leaf types of lettuces.

1. Introduction

Since plants are devoid of motility and an immune system, they have developed alternative defense strategies that include a huge variety of secondary metabolites (SMs) as tools to overcome stress constraints, adapt to the changing environment and survive. These SMs also play a major role in helping plants to defend themselves against herbivores (insects, mollusks and vertebrates), microbes, viruses and other competing plants [1]. While the biosynthesis of several SMs, such as carotenoids or lignin, may be mainly constitutive, the synthesis of numerous SMs, such as many phenolic compounds, can be induced and enhanced by both abiotic and biotic stresses. It is well known that the accumulation of SMs may increase when plants are undergoing high or low temperatures, high light intensity, UV radiation, drought, mineral deficiencies, salinity or pathogen infection [2]. The enhanced amount of biologically active SMs can be a consequence of either the activation of genes responsible for their synthesis, transport and storage or the hydrolysis of glycosides that are stored as "prodrugs" in plant cells. However, the production of these compounds (whose levels in tissues constitute less than 1% of the total dry weight) greatly depends on the physiological and developmental stage of the plant [1,2].

There is increasing evidence that several plant SMs can exert protective functions in the human body, and thus they are starting to be recognized as important integrants of a nutritious diet that promotes long-term health. In fact, some plant SMs can act as antioxidants by quenching free radicals thus reducing the risk of health problems related to the production of free-radicals, such as cancer and atherosclerosis [3]. The antioxidant effectiveness of carotenoids increases with the number of conjugated double bonds. Among carotenoids, lutein and lycopene may potentially prevent some types of cancer; β-carotene and lycopene have been shown to be inversely related to the risk of cardiovascular diseases and lutein and zeaxanthin can prevent disorders related to the eye, such as macular degeneration [4,5]. Phenolic compounds also have antioxidant properties; for example, anthocyanins have exhibited anti-carcinogenic effects in several cell culture systems including colon cancer cells, endothelial, liver and leukemic cells [6]. Therefore, the increasing interest in elucidating the mechanisms involved in the antioxidant activity of different compounds present in foods and beverages is justified.

Lettuce (*Lactuca sativa* L.), one of the most widely consumed vegetables in the human diet, can provide an important source of water, antioxidant compounds (including vitamins A, C and E, polyphenols and carotenoids) as well as fiber and useful amounts of certain minerals, such as Ca, Fe and K [7–9]. Lettuce, widely grown worldwide and especially in temperate and subtropical regions, is a major food crop within the European Union. According to FAOSTAT (FAO statistics Division) 2012, the world production of lettuce and chicory in 2011 reached 24,239,979 tons on about 1 million ha. The USDA (United States Department of Agriculture) Vegetables 2011 Summary (January 2012) estimates that the total production of lettuce in USA reached 3,889,120 tons in 2011. In addition,

lettuce is the most widely used food crop for the called "Fourth Range" of vegetables. The term originally meant fresh, cleaned, possibly chopped and mixed vegetables ready to be seasoned and eaten [10]. These vegetables are increasingly accepted by consumers because they are healthy and easy to prepare for eating.

The nutritional value of lettuce can vary greatly among different varieties or cultivars within a given species. Mou [11] found a wide range of genetic variability in the concentrations of carotenoids produced by different types (commercialized or wild) of lettuce, suggesting that it may be possible to achieve increased nutritional quality through crosses between different lettuces, at least one of them producing high levels of carotenoids. By using plant breeding and biotechnology approaches, it is also possible to develop genotypes with improved flavor and nutritional quality that can encourage consumers to eat more fruits and vegetables [12]. For example, plant breeders have been successful in obtaining a four- to seven-fold increase in vitamin C content in lettuce cv. Black Seeded Simpson, Grand Rapids and Prizehead after constitutive expression of the rat cDNA encoding L-gulono-1,4-lactone oxidase, the enzyme involved in the final step of the animal vitamin C biosynthesis [13]. Subsequently, Yabuta [14] reported that levels of tocopherol and activity of vitamin E increased in transplastomic lettuce plants compared with the wild-type and hypothesized that chloroplast genetic engineering can be useful for improving vitamin E content in lettuce. Goto *et al.* [15] produced transgenic lettuce with increased quantity of the iron storage protein ferritin via the integration and further expression of the ferritin gene in lettuce leaves. Despite these successful examples of genetically modified lettuces, the final commercialization of transgenic lettuce may depend not only on progress in transgene expression but also on public acceptance, economic and marketing challenges, intellectual property issues and risk assessment [11]. All these difficulties represent inducements to find alternatives to genetic engineering for improving the nutritional quality of lettuce. One of these alternatives could be arbuscular mycorrhizal (AM) symbiosis, which is the default situation for most crop plants in the field. The association of crop plants with arbuscular mycorrhizal fungi (AMF) may provide benefits to plants that include enhanced yield or improved P uptake and others unrelated to yield or P fertilizer use, such as tolerance to disease and drought, improved soil structure and C sequestration in soil [16]. Moreover, several studies have demonstrated the beneficial role of AMF in the increased production of health-related biomolecules by fruits or vegetables associated with them [17]. The establishment of AM symbiosis requires a high degree of coordination between the two partners based on a finely regulated molecular dialogue [18]. This communication begins in the rhizosphere with the production and exudation of signaling molecules by the host plants that are recognized by AMF and stimulate hyphal growth. Among these signal molecules strigolactones are supposed to be essential at early stages of the AM interaction [19] and recently they have been classified as a new class of plant hormones regulating above- and below-ground plant architecture, and reproductive development [20]. In lettuce, the production of strigolactones increases when plants are grown in saline soil as the establishment of AM symbiosis may be promoted in order to cope with salt stress [20]. Apart from the role of strigolactones at the early stages of symbiosis, other classes of apocarotenoids accumulate in large amounts in mycorrhizal roots at the later stages of AM association [19]. However, the molecular dialogue between mycorrhizal fungi and their host plants not only induces metabolic changes in plant roots, but also can induce the

activation of the antioxidant metabolism [21] and the accumulation of essential oils, carotenoids, total phenolics or anthocyanins in shoots, leaves or fruits [17].

In a very recent review, Vosátka *et al.* [22] discuss the current achievements and bottlenecks in the development of arbuscular mycorrhizal biotechnology and industry. According to these authors, advances in the scientific knowledge of AM symbiosis have increased the potential for implementation of mycorrhizal biotechnology in horticulture and agriculture. Moreover, new technologies (such as novel formulations for liquid applications or seed coating, compounds that stimulate the formation of mycorrhizal symbiosis or new application modes) have been developed for using AMF in greenhouses or in the field. However, the weak aspects of mycorrhizal biotechnology are still the lack of appropriate, cheap, highly reproducible and effective methods for inocula purity testing and quality control [22]. Quality control in terms of infectivity, absence of pathogens and growth promoting effects in relation to dosage is essential but effective guidelines for the expanding industry have not still been adopted [16]. Currently, relatively high production costs limit the application of mycorrhizal inocula mainly to nursery-scale inoculation before transplanting or to field-scale inoculation only of high-value crops [16]. Moreover, some agricultural practices, such as plowing of soil together with high fertilizer applications, disrupt mycelial networks, limit the range of AMF that can survive in agricultural soils and inhibit or reduce the colonization of high-value crop plants, especially cereals [23].

However, and despite all the aforementioned limitations, renowned scientists highlight the key role that AM symbioses can provide to horticulture and agriculture. In the words of Smith and Smith [16] there is "imperative for research that is aimed at increasing benefits of AM symbioses in the field at a time of increasing prices of P-fertilizer and increasing demands on agriculture to feed the world". Fitter *et al.* [23] suggest that "it would be possible to breed crop plants with reduced root systems, because the bulk of their nutrient requirements could be satisfied by mycelial transport" and support the "combination of AMF with no-till agriculture to achieve a genuinely sustainable agriculture". Gianinazzi *et al.* [17] ensure that "the appropriate management of ecosystem services rendered by AM will impact on natural resource conservation and utilization with an obvious net gain for human society".

2. Nutraceuticals, Vitamins and Minerals in Mycorrhizal Lettuce Plants

2.1. Chlorophylls (Chl)

Pigments are normal constituents of cells and tissues and perform physiological functions. Chlorophylls (Chl), vital for photosynthesis, are photoreceptors localized in the chloroplasts that allow plants to absorb energy from light. Chlorophyll a (Chl a) is the unique chlorophyll in higher plants that acts as primary electron donor in the reaction center of photosystems, although it also helps, together with chlorophyll b (Chl b) and carotenoids, to transfer energy in the antenna complex. In recent years, there has been a growing interest in natural and semi synthetic chlorophyll derivatives, not only as food colorants, but also as food supplements due to their potential effect against the development of several chronic diseases and to their anti-inflammatory activity measured *in vitro* [24].

Numerous researchers [21,25–28] have found increased concentrations of total Chl in leaves of several crops (onion, strawberry, pepper and chickpea) inoculated with different AM species belonging to the genus *Glomus* (*G. mosseae*, *G. versiforme*, *G. intraradices*, *G. etonicatum* or *G. deserticola*).

Similar results were obtained in Trifoliate Orange, a tree whose fruits are widely used in Oriental medicine as a remedy for allergic inflammation, when it was inoculated with *G. mosseae* [29]. Selvaraj *et al.* [30] also observed increased levels of Chl in *Pogostemon patchouli*, an aromatic herb belonging to the mint family and cultivated for its essential oil used in cosmetics, when it was inoculated with isolates of seven indigenous AMF (*Acaulospora scrobiculata*, *Gigaspora margarita*, *G. aggregatum*, *G. geosporum*, *G. mosseae*, *Sclerocystis pakistanika* and *Scutellospora heterogama*); however, Chl enhancements varied with the AMF species inoculated.

Similar to the aforementioned studies, Baslam *et al.* [31–33] reported that the symbiosis of greenhouse grown lettuce plants with AMF resulted in enhanced amounts of Chl in leaves. However, increases in the levels of Chl varied depending on the type of lettuce [31,32], the species of AMF associated with roots of lettuce [31,32], the source of P applied to plants [33] and the season of the year in which lettuces were cultivated [31–33]. For example, compared with the non-inoculated controls, the application of a mixture of *Glomus intraradices* and *G. mosseae* induced higher enhancements in the levels of both Chl a and Chl b in the red leaf lettuce Maravilla de Verano than in the green leaf lettuces Cogollos de Tudela and Batavia Rubia Munguía [32]. Moreover, the mixture of *G. intraradices* and *G. mosseae* was more effective than the application of *G. fasciculatum* for inducing the accumulation of Chl in leaves of the three aforementioned types of lettuces [32]. Furthermore, increases in the levels of total Chl were more evident when lettuces were fertilized with either modified Hewitt's nutrient solution or a water-insoluble (WI) source of P than when they received single superphosphate (SSP) as P source [33]. The degree of effectiveness of AMF for improving the contents of Chl in leaves of greenhouse-grown lettuces was also dependent on the season in which plants were cultivated: The highest contents of total Chl in leaves of mycorrhizal Maravilla de Verano and Batavia Rubia Munguía were found in autumn [34]. Unfortunately, when lettuce plants were cultivated under optimal irrigation, the greatest improvements in the levels of Chl due to mycorrhizal association were found in outer leaves, which are usually stripped-off before this vegetable is consumed. In contrast, when cultivated with restricted water supply, the highest increments in the amounts of Chl were observed in inner leaves of both green and red leaf mycorrhizal lettuces [34]. The increased levels of Chl in mycorrhizal lettuce plants presumably would have contributed to achieve higher photosynthetic rates in the whole plant, thus benefiting both growth of host plants (Figure 1) and the development and functionality of the symbiosis.

Figure 1. *Lactuca sativa* (**a**) var. Longifolia (Cogollos de Tudela); (**b**) var. Capitata cv. Batavia Rubia Munguía; and (**c**) var. Capitata cv. Maravilla de Verano, inoculated with either *Glomus fasciculatum* or a mixture of *Glomus intraradices* and *Glomus mosseae* or non-mycorrhizal. Photographs were taken at harvest, 7 weeks after transplanting. Growth conditions as described by Baslam *et al.* [31].

Glomus fasciculatum   *Glomus intraradices + Glomus mosseae*   **Non-mycorrhizal**

2.2. Carotenoids

Carotenoids are pigmented compounds that are uniquely synthesized by higher plants, algae, fungi and bacteria. During photosynthesis, carotenoids present in the thylakoid membrane of chloroplasts harvest light and transfer the absorbed energy to the reaction center of photosystems. Carotenoids also protect Chl from photo-damage by dissipating the excess of energy and quenching reactive oxygen species (ROS) produced under excess light [35]. Apart from their function in the assembly of photosynthesis, light capture and photoprotection, carotenoids play other essential functions in nature: They promote animal behavior, reproduction and survival, they improve nutrition and human health and they provide substrates for the biosynthesis of plant hormones (e.g., abscisic acid) and signaling molecules (e.g., mycorradicin in the root-mycorrhizal symbiosis) [36]. Carotenoid biosynthesis is regulated throughout the life cycle of a plant with dynamic changes in composition matched to prevailing developmental requirements and in response to external environmental stimuli [37]. Fruits and vegetables constitute the primary sources of carotenoid in human diet and are responsible for their yellow, orange and red colours of some plant organs [5]. Major dietary carotenoids include hydrocarbons, such as β-carotene, α-carotene and lycopene, as well as xanthophylls or oxygen-containing

carotenoids, including β-cryptoxanthin, lutein and zeaxanthin. Most carotenoids have provitamin A activity. Vitamin A deficiency is responsible for a number of disorders that range from impaired iron mobilization, growth retardation and blindness to a depressed immune response, as well as increased susceptibility to infectious disease [38]. Apart from their provitamin A activity, carotenoids are considered to have many other biological functions: They are proposed to be efficient scavengers of free radicals, protectors of low density lipoproteins (LDLs) against oxidation and they are believed to prevent cardiovascular diseases, cancer and other chronic diseases that affect human beings [39–41].

It is well-known that AMF can stimulate carotenoid metabolic pathways in plant roots [19,42,43]. AM symbiosis activates the plastidial methylerythritole phosphate (MEP) pathway by increasing transcript levels of MEP and carotenoid biosynthetic and cleavage genes [42], which stimulates the production of carotenoids and carotenoid cleavage products including C_{13} cyclohexenone derivatives (e.g., blumenol), C_{14} apocarotenoids (e.g., mycorradicin) and strigolactones. However, few analyses have targeted final crop products of the aerial part in mycorrhizal plants used for food or medicinal purposes. Some of these scarce studies are those performed by Ulrichs *et al.* [44], who measured increased quantity of lycopene and β-carotene in fruits of tomato inoculated with *Glomus* sp. and those carried out by Mena-Violante *et al.* [45], who reported enhanced carotenoid content in chile ancho (*Capsicum annuum* L. cv. San Luis) plants inoculated with *G. fasciculatum* and subjected to drought. In a recent study, Giovannetti *et al.* [46] found that *G. intraradices* can enhance nutritional and nutraceutical value of tomato fruits without production of mutagenic compounds. One explanation for the enhanced levels of carotenoids in fruits of mycorrhizal plants is that AMF can induce changes in the chloroplasts. It is known that chloroplasts of mycorrhizal plants can have higher and more numerous plastoglobuli [47], the subcellular compartment where carotenoids are mainly localized during the chloroplast to chromoplast transformation.

According to Baslam *et al.* [32], β-carotene was the carotenoid most accumulated in leaves of greenhouse-grown lettuces, although its distribution between outer and inner leaves differed among types of lettuce. Such a distribution was not related to the distinction between green and red leaf lettuces. In fact, the green leaf Cogollos de Tudela (var. Longifolia) and the red leaf Maravilla de Verano (var. Capitata) had higher levels of β-carotene in inner than in outer leaves. In contrast, the green leaf Batavia Rubia Munguía (another cultivar belonging to the var. Capitata) accumulated greater levels of β-carotene in the external than in internal leaves. The distribution of main xanthophylls between outer and inner leaves followed a similar pattern. Low levels of pigments in the internal leaves of Batavia Rubia Munguía may indicate an acclimation pattern [48] to the low light incidence on leaves that form a round and dense lettuce head. The levels of main carotenoids significantly increased after the inoculation of the aforementioned types of lettuce with AMF [32]. However, the most relevant enhancements in the levels of carotenoids were observed in outer leaves, with the exception of Cogollos de Tudela, the variety that formed a less dense head. After applying a mixture of *G. mosseae* and *G. intraradices* to the red leaf Maravilla de Verano levels of carotenoids in the outer leaves were four to eight times higher than those found in comparable leaves of their non-inoculated control plants. Zeaxanthin was the xanthophyll whose levels in mycorrhizal Maravilla de Verano were eight times higher than those measured in the non-inoculated plants, which indicates that lettuces associated with AMF improved their ability for absorbing blue light and quenching reactive oxygen species thus reducing the amount of light that induces oxidative damage [35]. On the

other hand, consumption of mycorrhizal Maravilla de Verano in the diet would provide increased levels of violaxanthin, the pigment whose deficiency provokes macular degeneration and irreversible loss of vision [49], enhanced contents of β-carotene, inversely related to the risk of cardiovascular diseases and certain cankers, and improved amount of lutein, which prevents disorders related to the eye [4]. Similar conclusions could be inferred from the results obtained when applied mycorrhizal inocula to the green leaf types of lettuce, Batavia Rubia Munguía and Cogollos de Tudela [32]. Increased contents of carotenoids in leaves of Maravilla de Verano after inoculating AMF contrast with decreased levels of neoxanthin, lutein and β-carotene observed in other types of red leaf lettuces after applying supplemental UV radiation in the greenhouse [50].

Nevertheless, the effect of mycorrhizal symbiosis on carotenoid accumulation may vary according to environmental parameters and cultural practices. For example, contents of total carotenoids in mycorrhizal lettuces increased when plants were subjected to different kinds and degrees of water deficit [34]. This additive effect between AMF and water restriction was mainly observed in the external leaves of the green leaf lettuce Batavia Rubia Munguía and in the internal leaves of the red leaf lettuce Maravilla de Verano. The source of phosphorus nutrition applied to lettuce plants also modified the effectiveness of AMF in improving the levels of carotenoids: the positive effect of AMF was more evident when plants were fertilized with either modified Hewitt's nutrient solution or a water insoluble (WI) source of P than when they received the water soluble single superphosphate (SSP) [33]. Although the benefits of carbon dioxide (CO_2) supplementation on plant growth and production within the greenhouse environment have been well understood for many years, the levels of total carotenoids in both external and internal leaves decreased when Batavia Rubia Munguía and Maravilla de Verano were grown under increased atmospheric CO_2 concentrations (700 ppm) in the greenhouse [51].

2.3. Phenolic Compounds

Phenolic compounds are SMs that constitute one of the most common and widespread groups of substances in plants [52]. They are characterized by the presence of at least one benzene ring, to which is directly attached at least one hydroxyl group, free or bound to another function (esters, methyl esters, glycosides, among others). As a general rule, the terms phenolic and polyphenol refers to all secondary natural metabolites arising biogenetically from the phenylpropanoid and flavonoid pathways. The key enzyme in this biosynthetic pathway is phenylalanine ammonia-lyase (PAL), which can be induced by different stresses [52]. Plants need phenolic compounds for pigmentation, growth, UV sunscreens, reproduction and resistance to pathogens [53]. These compounds protect plants against oxidative damage and perform the same function in the human body. A clear relationship has been found between antioxidant activity and presence of phenols in common vegetables and fruits [54–56].

AMF colonization can alter or enhance the production of phenolics within the host plant. Some studies have reported an improvement of phenolic content in olive trees inoculated with a mixture of three different AMF, *G. invermaium, Acaulospora laevis and Scutellospora calospora* [57], in mycorrhizal plants of "sweet" basil [58,59] as well as in strawberry inoculated with *G. intraradices* [60].

Our experiments with non-mycorrhizal and mycorrhizal lettuce plants corroborated these results. Baslam *et al.* [31] observed that greenhouse grown lettuces belonging to the variety Longifolia

(Cogollos de Tudela) had similar levels of total soluble phenolics in outer and inner leaves when they had not established symbiotic association with AMF. However, the symbiosis of Cogollos de Tudela with AMF induced the accumulation of soluble phenolic compounds in external leaves. In contrast, the application of AMF to lettuces belonging to the variety Capitata only improved the amount of soluble phenolics significantly when plants received restricted water supply [34] or when they were cultivated in winter [33]. Once more, the source of phosphorus nutrition applied to lettuces also influenced the levels of soluble phenolic compounds in leaves regardless plants were or not associated with AMF [33]. Insufflations of CO_2 in the greenhouse were effective at inducing the accumulation of soluble phenolics but only in non-mycorrhizal lettuces [51]. Baslam *et al.* [51] hypothesized that in mycorrhizal lettuces grown under elevated CO_2 a relevant quantity of photoassimilates was used for both enhancing shoot growth and spreading mycorrhizal colonization in detriment to the secondary metabolism. Apart from the nutritional point of view, increased levels of these C-based SMs may have improved the defense capacity of lettuce plants against herbivores and/or pathogenic organisms [61].

The relationship between AMF colonization and the phenolic compounds produced in plants remains poorly understood. However, as hypothesized by Toussaint *et al.* [58], higher N assimilation in mycorrhizal plants might contribute to the higher production of phenylalanine ammonia lyase (PAL), a key enzyme involved in the synthesis of many phenolic compounds. Moreover, plant phenolic compounds are potential candidates as signals during mycorrhizal formation. Some reports show that flavonoids, together with the aforementioned strigolactones, can exert a positive effect on hyphal growth during the establishment of the AMF symbiosis [62].

Anthocyanins belong to the large family of phenolic compounds collectively known as flavonoids. The molecule of anthocyanins consists of an anthocyanidin (the aglycone chromophore) bonded to one or more glycosides, which are produced with a biochemical cost to the plant. Anthocyanins are water-soluble pigments that can provide red to blue colors in flowers, fruits, leaves, and storage organs [63]. The synthesis of these phenolic compounds in plants may also be induced or modulated by several environmental factors, such as light quality and quantity, extreme temperatures, osmotic stress, mineral and pH imbalances, mechanical damage, herbivore and pathogen attacks, pollution and application of herbicides [64], and they are known to play a crucial role as inducers of antioxidant responses in plants against biotic and abiotic stresses [64,65]. Anthocyanins have also been recognized as beneficial compounds for human health. Many pharmacological benefits have derived from their antioxidant, antimutagenic, antiallergic, anti-inflammatory, antiviral and anticarcinogenic properties [66].

The influence of AMF on the levels of anthocyanins in host plants has been studied in different crops, such as Cherry tomato inoculated with *G. etunicatum* [67], different cultivars of the culinary herb commonly known as "basil" [59] and strawberry [60] associated with *G. intraradices*. In all these studies, results shown that mycorrhizal plants had increased concentrations of total anthocyanins in leaves, stems and/or fruits compared to their respective non-mycorrhizal controls. Likewise, red leaf lettuces, such as Maravilla de Verano, have higher amounts of anthocyanins in leaves than green leaf lettuces, such as Batavia Rubia Munguía and Cogollos de Tudela. However, when associated with AMF, leaves of some green leaf types of lettuces (e.g., Batavia Rubia Munguía) can accumulate similar levels of anthocyanins to those found in leaves of non-mycorrhizal red leaf types of lettuces (e.g., Maravilla de Verano) [31]. The levels of anthocyanins also increased significantly in the red leaf lettuce Maravilla de Verano when associated with AMF, with the increases being more relevant when

a mixture of *G. mosseae* and *G. intraradices* was applied than when *G. fasciculatum* was used [31]. The effectiveness of AMF for inducing the accumulation of anthocyanins in leaves of lettuce plants also depends on the season of the year when this vegetable crop is grown: Differences in the contents of anthocyanins between non-mycorrhizal and mycorrhizal lettuces were greater when plants were cultivated in summer [31] than when grown in winter [33]. Other cultural practices, such as the irrigation regime applied to greenhouse grown lettuce plants, can also affect the levels of anthocyanins. The imposition of a continuous and moderate drought stress to non-mycorrhizal lettuce plants induced the accumulation of anthocyanins in both outer and inner leaves of the green leaf lettuce Batavia Rubia Munguía but had no effect on the amounts of these phenolic compounds in leaves of the red leaf type Maravilla de Verano [34]. However, amounts of anthocyanins always increased in mycorrhizal plants cultivated with restricted water supply: In mycorrhizal Batavia Rubia Munguía enhancements were more evident in inner than in outer leaves; in mycorrhizal Maravilla de Verano the most relevant increases occurred in external leaves [34]. Insufflations of CO_2 in the greenhouse can modify the concentrations of anthocyanins in leaves of lettuce plants, but results may be different in green and red leaf types of lettuces and also in non-mycorrhizal and mycorrhizal plants [51]. Elevated CO_2 (700 ppm) induced the accumulation of these phenolic compounds in outer leaves of the green leaf lettuce Batavia Rubia Munguía but only when plants were not associated with AMF. In contrast, increased atmospheric CO_2 had no effect on the levels of anthocyanins in leaves of the red leaf lettuce Maravilla de Verano when not associated with AMF. In mycorrhizal Maravilla de Verano, elevated CO_2 clearly decreased the contents of anthocyanins in outer leaves and moderately enhanced the amount of anthocyanins in inner leaves.

2.4. Vitamins

L-Ascorbic acid (for which the generic term is vitamin C) is a multifunctional compound in both plants and animals. This metabolite is one of the most abundant in green leaves. In favorable conditions, it represents 10% of the total soluble carbohydrate pool [68]. Vitamin C influences mitosis and cell growth in plants. It is also an important cofactor for violaxanthin de-epoxidase and dioxygenase type enzymes involved in the synthesis of hydroxyproline, ethylene, gibberellic acid, anthocyanins and a number of other secondary metabolites. Furthermore, vitamin C is already known to be an antioxidant. It protects cells and organelles from oxidative damage by scavenging ROS produced by aerobic metabolic processes, such as photosynthesis and respiration, or by environmental stresses including drought, cold and excess of light [68–71]. Vitamin C is one of the most important vitamins in fruits and vegetables for human nutrition. More than 90% of the vitamin C in human beings, who are incapable of synthesizing ascorbic acid due to the lack of the enzyme L-gulono-1,4-lactone oxidase, is supplied by fruits and vegetables in the diet. Ascorbic acid (AA) is the principal biologically active form, but L-dehydroascorbic acid (DHA), an oxidation product, also exhibits biological activity. Since DHA can be easily converted into AA in the human body, it is important to measure both AA and DHA in fruits and vegetables for vitamin C activity [72]. Vitamin C might protect against oxidative stress and thereby decrease cancer risk [73,74]. For these reasons, there is considerable interest in enhancing the ascorbate content in fruits and vegetables consumed in the human diet.

In lettuce, the distribution of total ascorbate between outer and inner leaves of plants can be distinct in different varieties or cultivars. While levels of total ascorbate were similar in outer and inner leaves of lettuces belonging to the variety Capitata, in the variety Longifolia external leaves showed significantly higher concentrations of total ascorbate than internal leaves did [31]. The application of mycorrhizal inocula to lettuce plants cultivated under an optimal irrigation regime slightly increased the amount of total ascorbate in leaves, with *G. fasciculatum* being more effective than the mixture of *G. mosseae* and *G. intraradices* in enhancing the levels of vitamin C [31]. According to Qiang-Sheng *et al.* [75], AMF symbiosis induced the accumulation of ascorbate in leaves of both well watered and water stressed trifoliate orange seedlings. Although Oh *et al.* [76] concluded that the adaptation of lettuce plants to water stress was associated with the activation of the enzyme L-galactose dehydrogenase (L-GalDH) implied in the biosynthesis of ascorbic acid, Baslam and Goicoechea [34] observed no significant effects of different types and degrees of water deficit on the concentrations of total ascorbate (reduced ascorbate + dehydroascorbate) pool in the two cultivars Batavia Rubia Munguía and Maravilla de Verano, regardless of whether they were or not associated with AMF. However, ratio between reduced ascorbate (ASC) and dehydroascorbate (DHA) changed in plants undergoing restricted irrigation, with the changes being mainly dependent on both cultivar of lettuce and mycorrhizal symbiosis. Redox state can be defined as the ratio between reduced and oxidized molecules within a pool and the most abundant contributor to general redox metabolism in plant cells is ascorbate [77]. In non-mycorrhizal Maravilla de Verano the relative abundance of ASC (high ASC/DHA) in plants subjected to maintained water deficits suggested that these redox metabolites cooperated in scavenging harmful concentrations of ROS in cells of leaves by fueling the ASC-glutathione cycle [37]. However, as well as the "big three" antioxidants, ascorbate (ASC), glutathione (GSH) and the pyridine nucleotides NADH and NADPH, plants contain many distinct redox-active compounds, including phenolics and carotenoids, that actively contribute to the global cellular redox state [77]. Declines in the ratio ASC/DHA in non-mycorrhizal Batavia Rubia Munguía and, especially, in all mycorrhizal lettuce plants when subjected to different types and degrees of water deficits [34] could be partially overcome by enhancements in the concentrations of phenolics and carotenoids previously explained. Moreover, AM symbiosis can also increase the activity of antioxidant enzymes in lettuce plants, as described by Kohler *et al.* [78]. In any case, whereas at the outset of plant stress, ROS were only considered as damaging the cell components, the view nowadays has shifted to ROS being an integrative part of cell signaling metabolism modulated by the cellular redox state, leading to different responses related to programmed cell death, gene expression, plant defense or plant development [77].

Tocopherols and tocotrienols, collectively known as tocochromanols, are lipid-soluble molecules that belong to the group of vitamin E compounds and are essential in the human diet. Tocopherols may play important roles in plants beyond their antioxidant function in photosynthetic membranes. Plants deficient in tocopherols show alterations in germination, export of photoassimilates, leaf senescence and responses to abiotic stresses [79]. Tocochromanols can be only synthesized by plants and other oxygenic, photosynthetic organisms. Supplementation of the human diet with vitamin E contained in fruits and vegetables is considered to provide health benefits against cardiovascular diseases through its antioxidant activity, the prevention of lipoprotein oxidation and the inhibition of platelet

aggregation [80,81]. Moreover, Lira *et al.* [82] suggested that vitamin E supplementation could be effective for avoiding obesity.

According to results obtained by Baslam *et al.* [32], greenhouse grown lettuces had quite similar concentrations of tocopherol (α-tocopherol + (β-γ)-tocopherol) in outer and inner leaves when they were not associated with any AMF. In general terms, mycorrhizal symbiosis induced the accumulation of tocopherol in leaves. To our knowledge, there are no previous reports on the effect of AM symbiosis on the accumulation of tocopherol in plant tissues. According to our results, the most relevant increases in the amount of tocopherols were observed in external leaves of any variety or cultivar of lettuce after applying a mixture of *G. mosseae* and *G. intraradices*. However, among all mycorrhizal plants, the highest levels of tocopherols were found in external leaves of the red leaf lettuce Maravilla de Verano. Lizarazo *et al.* [83] concluded that increased levels of tocopherols with leaf ageing represent a general trait in plants. Since AMF can shorten the life cycle of their host plants [84], the possible different phenological stage of mycorrhizal and non-mycorrhizal lettuces could explain why the outer leaves of mycorrhizal lettuces had greater amounts of tocopherols than the external leaves of their non-mycorrhizal controls [32].

2.5. Minerals

The level of minerals in vegetables depends on a number of factors including genetic properties of the crop species, climatic conditions, soil characteristics and the degree of maturity of the plant at the moment of harvesting [85]. As vegetables constitute the main source of minerals in the human diet, one of the most important challenges for agriculture, besides enhancing food production, is to provide almost all the essential minerals and organic nutrients to humans for maintenance of health and proper organ function. Humans need more than 22 mineral elements. Some are required in large amounts while others, such as Fe, Zn, Cu, I and Se, are required in trace amounts [86] but their absence renders human life impossible. Concentrations of several essential elements in modern crops are insufficient for optimal human nutrition, thus contributing to the huge "hidden hunger" problem [87]. This is the case of iron (Fe), whose deficiency affects more than 3.5 billion people, mostly in the developing world and impairs the cognitive development of children, causes productivity and educational losses and increases morbidity and maternal mortality [88]. Other important examples are zinc (Zn), a micronutrient whose levels of intake in the diet may be inadequate for nearly half of the world's population [89] and copper (Cu), essential for human health but consumed in inadequate quantities in some developed countries, such as the United Kingdom or the Unites States [90].

It is well known that AMF can increase the uptake of micronutrients and other mineral nutrients with low mobility including the aforementioned Fe [91], Zn [92] and Cu [93]. In greenhouse grown lettuces cultivated with optimal irrigation, mycorrhizal symbiosis improved the levels of Cu and Fe [31] but the effect was dependent on lettuce cultivar and the source of P applied to plants [33]. Azcón *et al.* [94] described that mycorrhizal lettuce plants with high availability of N and P in the soil reduced the content of macro and micronutrients in their tissues. However, when plants were fertilized with a low level of both, P and N, nearly all the macro and micronutrients (P, K, Ca, Mg, Cu, Fe, Mn, Zn and S) were enhanced by AMF colonization. Our results showed that the higher size achieved by mycorrhizal lettuces (Figure 1) produced a dilution effect on the concentrations of some mineral

nutrients in leaf tissues. Goicoechea *et al.* [95] found that AMF may be more beneficial for the mineral nutrition of host plants under water deficit than when plants grown with adequate water supply. This same idea can be inferred from data included in Table 1: The association of the green leaf lettuce Batavia Rubia Munguía with AMF enhanced the concentrations of N, P, Cu and Fe in inner leaves when plants were cultivated under a water regime equivalent to 2/3 of field capacity; moreover, the levels of P in internal and Fe in external leaves of mycorrhizal plants were greater than those of non-mycorrhizal plants when lettuces received a water content equivalent to 1/2 of field capacity. Enhanced P nutrition is considered as one of the major mechanisms by which AMF can improve the water status of their host plants [96]. However, this behavior was not observed in the red leaf lettuce Maravilla de Verano (Table 2), which indicates that results are highly dependent on the type of lettuce. Similarly, under saline conditions, Zuccarini [97] observed that mycorrhizal symbiosis stimulated the absorption of K and P of lettuce Meraviglia d'Inverno.

In an opinion paper dealing with the topic of rising atmospheric CO_2, Loladze [98] linked high concentrations of atmospheric CO_2 with plant stoichiometry and quality of human nutrition and hypothesized that elevated CO_2 could both reduce the ratio between nutritional and caloric values of crops and intensify the already acute problem of micronutrient malnutrition in the developing world. In this context of rising atmospheric CO_2, AMF are predicted to be important in defining plant responses to elevated CO_2 concentrations. As elevated CO_2 affects photosynthetic rates, it would also influence C supply to AMF colonizing roots [99]. In turn, AMF may diminish the plant nutrient limitation associated with enhanced photosynthetic rates [100]. In fact, lower concentrations of P in tissues of plants when grown under elevated CO_2 can be alleviated by the formation of AMF and any improvements in plant N nutrition resulting from the formation of AMF may be also important in determining plant responses to atmospheric CO_2 enrichment [61]. However, despite this optimistic approach on the potential role of AMF in a context of rising atmospheric CO_2, data obtained by Baslam *et al.* [51] showed that the accumulation of some mineral nutrients induced by AMF in leaves of greenhouse grown lettuces cultivated at ambient CO_2 diminished or even disappeared under elevated CO_2 (700 ppm), as happened with some SMs. Moreover, plant stoichiometry also was modified under elevated CO_2; in general terms, the association of lettuces with AMF together with the exposure of plants to elevate CO_2 increased the ratio between C and other mineral nutrients.

Table 1. Concentrations of mineral nutrients in outer and inner leaves of lettuces cv. Batavia Rubia Munguía, non-inoculated (NM) or inoculated (M) with a mixture of *Glomus intraradices* and *Glomus mosseae* (AMF), and cultivated with a water regimen equivalent to either 2/3 (2/3 FC) or 1/2 (1/2 FC) of field capacity. Values are means (n = 3) ± SE. Data were subjected to a three-factor ANOVA and LSD *post hoc* test. Within each column, data followed by the same letter indicate that values did not differ significantly ($p \leq 0.05$). ns: not significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$; FW: Fresh weight; PL: Position of leaves.

Treatments			N	Ca	K	P	Mg	Cu	Zn	Fe	Mn
			-------------------------------- (mg·g^{-1} FW) -------------------------------					-------------------------------- (ppm) -------------------------------			
2/3 FC	NM	Outer leaves	2.6 ± 0.12 cd	0.67 ± 0.02 bc	6.5 ± 0.16 bc	0.08 ± 0.00 e	0.28 ± 0.01 c	0.32 ± 0.06 bc	4.4 ± 0.80 ab	8.3 ± 0.96 b	17.8 ± 1.02 b
		Inner leaves	2.5 ± 0.12 d	0.36 ± 0.03 d	4.6 ± 0.28 e	0.15 ± 0.01 c	0.14 ± 0.01 e	0.08 ± 0.02 e	2.8 ± 0.29 cd	4.1 ± 0.50 d	7.1 ± 0.60 e
	M	Outer leaves	3.0 ± 0.16 bc	0.65 ± 0.08 c	6.5 ± 0.47 bc	0.10 ± 0.00 e	0.30 ± 0.03 bc	0.41 ± 0.01 ab	2.3 ± 0.30 d	7.2 ± 0.42 bc	12.8 ± 1.31 c
		Inner leaves	3.2 ± 0.25 b	0.40 ± 0.03 d	4.9 ± 0.27 de	0.22 ± 0.01 a	0.19 ± 0.01 de	0.23 ± 0.03 cd	3.0 ± 0.16 cd	6.4 ± 0.21 c	6.6 ± 0.76 e
1/2 FC	NM	Outer leaves	3.7 ± 0.18 a	0.83 ± 0.02 a	8.7 ± 0.44 a	0.11 ± 0.00 d	0.36 ± 0.02 ab	0.51 ± 0.07 a	5.6 ± 0.32 a	9.5 ± 0.36 b	25.7 ± 1.31 a
		Inner leaves	3.4 ± 0.16 ab	0.46 ± 0.05 d	5.8 ± 0.38 cd	0.20 ± 0.00 b	0.17 ± 0.02 de	0.18 ± 0.04 de	3.7 ± 0.52 bc	5.5 ± 0.38 cd	11.4 ± 1.53 cd
	M	Outer leaves	3.4 ± 0.08 ab	0.77 ± 0.04 ab	7.9 ± 0.12 ab	0.13 ± 0.01 cd	0.38 ± 0.03 a	0.50 ± 0.07 a	3.7 ± 0.23 bc	14.9 ± 1.65 a	17.5 ± 0.43 b
		Inner leaves	3.5 ± 0.34 ab	0.42 ± 0.04 d	5.3 ± 0.64 de	0.22 ± 0.02 a	0.20 ± 0.02 de	0.15 ± 0.04 de	3.4 ± 0.40 bc	7.1 ± 0.35 bc	9.4 ± 0.85 de
		Drought (degree)	***	**	***	**	*	ns	**	ns	***
		AMF	ns	ns	ns	***	ns	ns	**	ns	***
		Position of leaves (PL)	ns	***	***	***	***	***	*	*	***
		Drought × AMF	*	ns	ns	ns	ns	ns	ns	ns	ns
		Drought × PL	ns	ns	ns	ns	ns	ns	ns	ns	ns
		AMF × PL	ns	ns	ns	*	ns	ns	**	ns	**
		Drought × AMF × PL	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 2. Concentrations of mineral nutrients in outer and inner leaves of lettuces cv. Maravilla de Verano (MV), non-inoculated (NM) or inoculated (M) with a mixture of *Glomus intraradices* and *G. mosseae* (AMF), and cultivated with a water regimen equivalent to either 2/3 (2/3 FC) or 1/2 (1/2 FC) of field capacity. Values are means ($n = 3$) ± SE. Data were subjected to a three-factor ANOVA and LSD *post hoc* test. Within each column, data followed by the same letter indicate that values did not differ significantly ($p \leq 0.05$). ns, not significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$; FW: Fresh weight; PL: Position of leaves.

	Treatments	N	Ca	K	P	Mg	Cu	Zn	Fe	Mn
		---------------------------------- (mg g^{-1} FW) -----------------------------					-------------------------------- (ppm) --------------------------			
2/3 FC	NM Outer leaves	2.5 ± 0.19 d	0.90 ± 0.02 ab	6.1 ± 0.12 a	0.08 ± 0.00 b	0.26 ± 0.01 b	0.20 ± 0.04 a	2.2 ± 0.12 c	4.9 ± 0.72 c	18.0 ± 0.95 b
	NM Inner leaves	2.9 ± 0.12 bc	0.55 ± 0.03 c	4.4 ± 0.15 b	0.19 ± 0.02 a	0.16 ± 0.01 d	0.18 ± 0.03 ab	3.0 ± 0.12 b	6.3 ± 0.31 bc	7.9 ± 0.06 d
	M Outer leaves	2.6 ± 0.29 cd	0.94 ± 0.02 ab	6.7 ± 0.41 a	0.10 ± 0.00 b	0.28 ± 0.02 ab	0.12 ± 0.04 b	2.0 ± 0.27 c	4.8 ± 0.41 c	13.8 ± 1.82 c
	M Inner leaves	2.8 ± 0.15 bcd	0.57 ± 0.03 c	4.3 ± 0.18 b	0.19 ± 0.01 a	0.19 ± 0.01 cd	0.17 ± 0.04 ab	3.0 ± 0.16 b	5.6 ± 0.33 bc	7.2 ± 0.35 d
1/2 FC	NM Outer leaves	2.8 ± 0.13 bcd	0.99 ± 0.09 a	6.4 ± 0.29 a	0.10 ± 0.01 b	0.34 ± 0.04 a	0.19 ± 0.04 a	3.4 ± 0.31 b	6.3 ± 0.83 bc	22.5 ± 1.58 a
	NM Inner leaves	3.8 ± 0.41 a	0.79 ± 0.15 b	5.9 ± 0.63 a	0.23 ± 0.04 a	0.26 ± 0.03 b	0.18 ± 0.03 ab	5.4 ± 0.65 a	7.5 ± 0.91 a	13.2 ± 1.26 c
	M Outer leaves	2.6 ± 0.04 cd	0.82 ± 0.01 b	6.5 ± 0.11 a	0.09 ± 0.00 b	0.28 ± 0.01 ab	0.16 ± 0.04 ab	2.0 ± 0.23 c	6.6 ± 0.72 abc	15.0 ± 0.87 c
	M Inner leaves	3.2 ± 0.51 ab	0.57 ± 0.06 c	4.3 ± 0.50 b	0.19 ± 0.02 a	0.19 ± 0.02 cd	0.19 ± 0.21 a	3.1 ± 0.28 b	6.9 ± 0.74 ab	8.5 ± 1.16 d
	Drought (degree)	ns	ns	ns	ns	ns	ns	**	*	**
	AMF	ns	ns	ns	ns	ns	ns	***	ns	***
	Position of leaves (PL)	*	***	***	***	**	ns	***	ns	***
	Drought × AMF	ns	*	ns	ns	*	ns	**	ns	*
	Drought × PL	ns	ns	ns	ns	ns	ns	ns	ns	ns
	AMF × PL	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Drought × AMF × PL	ns	ns	ns	ns	ns	ns	ns	ns	ns

3. Conclusions and Future Prospects

The application of AMF appears as a feasible alternative to genetic engineering and other methods (e.g., supplemental UV application in greenhouses) to improve growth and nutritional quality of lettuce. However, the beneficial effect of AMF on the production and quality of this crop is highly dependent on several factors: type of lettuce, species of AMF and several factors related to cultural practices, such as the water regime applied to lettuce plants, the season chosen for cultivating lettuces and carbon dioxide (CO_2) supplementation within the greenhouse. Another important aspect that remains to be elucidated is the assessment of the potential biological effects that the enhanced amounts of nutraceuticals, vitamins and minerals accumulated in leaves of mycorrhizal lettuces could exert.

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