JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Arbuscular Mycorrhizal Fungi (AMF) Improved Growth and Nutritional Quality of Greenhouse-Grown Lettuce

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Supporting Information

ABSTRACT: Lettuce can be associated with arbuscular mycorrhizal fungi (AMF). This symbiosis involves a molecular dialogue between fungus and plant that includes the activation of antioxidant, phenylpropanoid, or carotenoid pathways. The objective of this study was to test if the association of lettuce with AMF benefited plant growth and increased the contents of compounds potentially beneficial for human health. Results showed that AMF improved growth of lettuce, thus producing a dilution effect on the concentrations of some mineral nutrients (e.g., Ca and Mn). However, Cu, Fe, anthocyanins, carotenoids, and, to a lesser extent, phenolics appeared in higher concentrations (on a wet basis) in mycorrhizal than in nonmycorrhizal plants.

KEYWORDS: antioxidant compounds, lettuce, mycorrhizal symbiosis, proteins, sugars, water content

INTRODUCTION

Plants are traditionally part of the human diet containing bioactive components that may exert physiological effects beyond nutrition, promoting human health and well-being.[1] Regular consumption of fruits and vegetables is associated with reduced risks of chronic diseases such as cancer, cardiovascular disease, stroke, Alzheimer's disease, cataract, and age-related functional decline.[2−4] Lettuce (*Lactuca sativa* L.), a crop widely grown on all continents, is a major food crop within the European Union. According to FAOSTAT (FAO Statistics Division) 2011, the production quantities of lettuce and chicory in Spain, France, Germany, and Greece were, respectively, 1,000,000, 430,000, 320,000, and 80,000 tons in 2009. Lettuce exhibits healthy properties mainly due to the presence of antioxidant compounds (e.g., vitamins C and E, carotenoids, and polyphenols) together with a large fiber content and useful amount of some minerals[5−7] in its tissues. Lettuce is also the most used food crop for the so-called "Fourth Range" of vegetables. The term originally meant fresh, cleaned, possibly chopped, and mixed vegetables ready to be seasoned and eaten.[8] These vegetables are widely accepted by consumers because they are easy to prepare for eating. In addition, there is an increasing demand by consumers for safe and nutritious foods that improve physical performance, reduce risks of diseases, and increase the life span.

Enhancing the nutritional levels of vegetables would improve nutrient intake without requiring an increase in consumption. In a recent paper, Mou[9] discusses the problems derived from the improvement of nutritional quality in lettuce by applying conventional plant breeding or biotechnology. According to this author, the complicated genotype and environment interactions present great challenges for plant breeding programs that often lack capacities for nutritional analysis. On the other hand, genetic engineering offers opportunities to significantly elevate the nutritional levels of lettuce. However, the commercialization of transgenic lettuce awaits progress in transgene expression, public acceptance, economic and marketing challenges, intellectual property issues, and risk assessment.

Mycorrhizal fungi colonize the roots of >90% of plant species to the mutual benefit of both the plant host and fungus. The most common are the arbuscular mycorrhizas, which are formed by the majority of crop and horticultural plants, including lettuce. The association between arbuscular mycorrhizal fungi (AMF) and plant roots develops in two functional phases:[10] the extraradical phase extending from the root into the soil and the intraradical phase with intercellular hyphae and specialized intracellular structures called "arbuscules". Arbuscules are the structures where exchanges of carbon to the fungus and nutrients to the host plant take place. The establishment of this mutualistic association involves a continuous cellular and molecular dialogue between both symbionts (mycorrhizal fungus and plant)[11,12] that includes the activation of the antioxidant,[13] phenylpropanoid,[14] or carotenoid metabolic pathways.[15] Colonization by AMF induces dramatic changes in the shape and number of organelles of root cortical cells, and the nucleus of arbusculated cells undergoes hypertrophy, which reflects increased transcriptional activity of the plant genome in colonized cells.[12,16] It is becoming evident that the AM symbiosis can stimulate the synthesis of plant secondary metabolites, which are important for increased plant tolerance to abiotic and biotic stresses or beneficial to human health through their antioxidant activity.[17] However, only a few analyses have targeted final crop products (leaves, roots, or fruits) of mycorrhizal plants used in food or in medicinal remedies.[18] On the other hand, cell processes modified by symbiosis-related plant

Received: February 4, 2011
Revised: March 31, 2011
Accepted: April 19, 2011
Published: April 19, 2011

genes affect root interactions by directly modulating the activity of AMF.[19]

We hypothesized that the association of lettuce plants with AMF could increase the levels of some antioxidant compounds in leaves, thus improving the nutritional quality of this vegetable. If so, the application of AMF would represent an alternative to genetic engineering for the improvement of nutritional quality in lettuce. This hypothetical enhancement of the nutritional quality would be more beneficial for human health when lettuce plants are consumed as salads, avoiding processes, such as boiling, that could alter the nutritional properties. Therefore, the main objective of our study was to test if the association of three cultivars of lettuce consumed as salads with AMF benefited plant growth and increased the contents of some compounds potentially beneficial for human health (e.g., proteins, sugars, minerals, carotenoids, phenolics, anthocyanins, and ascorbate) in leaves.

MATERIALS AND METHODS

Biological Material and Experimental Design. Batavia Rubia Munguía (*L. sativa* L. var. Capitata) (BRM), Maravilla de Verano (*L. sativa* L. var. Capitata) (MV), and Cogollos de Tudela (*L. sativa* L. var. Longifolia) (CT) were the three types of lettuce chosen for this study. They are extensively cultivated in greenhouses, highly commercialized, and very appreciated for consumption in salads in Spain. BRM and MV are two cultivars of Batavia characterized for an excellent shelf life that allows maintenance of their crispness from the time they are harvested until the time they are consumed. BRM has yellow-green leaves, with very ruffled borders and a consistent, crisp texture. It develops a round, dense head. MV has leaves with green color and red pigmentation, especially in the borders of the most ruffled leaves. It develops good size and a firm head. CT is a delicious variety of fan-leafed lettuce, which has received its designation because it is mainly grown in the city of Tudela and surrounding municipalities in Navarra, a region in northern Spain. It is around 10 cm long and is characterized by its long, thick, strong leaves, ranging in color from the bright green of the outer leaves to the yellow of the inner ones. Its head is dense and erect.

Seeds of BRM, MV, and CT were surface sterilized by 10% bleach for 10 min and sown (on June 22) in a mixture of peat and sand (1:1, v/v). When seedlings had two to three fully developed leaves, they were transferred (on July 13) to 1.5 L pots (one plant per pot and 30 pots per each type of lettuce) filled with a mixture of vermiculite, sand, and peat (2.5:2.5:1, v/v/v). Peat was previously sterilized at 100 °C for 1 h on three consecutive days. At transplanting, 10 pots of each cultivar of lettuce were inoculated with the mycorrhizal fungus *Glomus fasciculatum* (*Taxter sensu* Gerd.) Gerdemann and Trappe, another 10 pots were inoculated with the commercial inoculum AEGIS Endo Gránulo, and another 10 pots were not inoculated and kept as nonmycorrhizal controls. *G. fasciculatum* came from a pot culture with alfalfa (*Medicago sativa* L.) as host plant, and inoculum (Gf) consisted of 20.0 g of soil with alfalfa root fragments, spores, and hyphae per 2.0 L of total substrate. The commercial inoculum (CI) was a mixture of *Glomus intraradices* (Schenck and Smith) and *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe that contained around 100 spores and other infective propagules per gram of product and provided and commercialized by Atens (Tarragona, Spain). A total of 9.5 g of the commercial mycorrhizal formulation was added to each pot.

Lettuce plants were grown in a greenhouse at 25/15 °C day/night temperatures and 40/80% day/night relative humidity (RH) and received natural daylight supplemented with irradiation from fluorescent lamps (Sylvania DECOR 183, Professional-58 W, Erlangen, Germany) that provided a minimum photosynthetic photon flux (PPF) of around 300−400 μmol m^{-2} s^{-1} during a 14 h photoperiod. All plants were fertilized once a week with 300 mL of the following nutrient solution:[20] 6 mM $Ca(NO_3)_2$, 6 mM $CaCl_2$, 3 mM KNO_3, 2.3 mM K_2SO_4,1.5 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 68 μM EDTA-Fe, 13 μM $MnSO_4$, 9 μM H_3BO_4, 1 μM $CuSO_4$, 1 μM $ZnSO_4$, and 0.2 μM Na_2MoO_4. All plants also received 300 mL of distilled water twice a week. Plants were harvested 7 weeks after they had been transferred to pots.

Samples for analytical determinations were collected from both inner (internal zone) and outer (external zone) leaves of lettuces. Both zones were visually delimited, and each had a mean of approximately 15 leaves. The internal zone was quite close to the meristematic tip of the shoot and included light green leaves. The harvested inner leaves were placed midway between the center of the head and the outer portion. Outer leaves exhibited darker color and larger size than inner leaves and were not compact in the lettuce head.

Growth Parameters and Leaf Water Content (WC). At harvest, three plants of each cultivar of lettuce and treatment were randomly selected for determining fresh weight (FW) of the aerial part and biomass production of both shoots and roots. Dry matter (DM) of leaves and roots was determined after the plant material had been dried at 80 °C for 2 days. Before those plants were introduced into the oven, disk samples (1 cm^2) of fully developed outer (one sample per plant) and inner (one sample per plant) leaves were collected to determine FW. Then those leaf samples were dried at 80 °C for 2 days to calculate DM. Disk samples were also collected from outer and inner leaves of the other seven plants of each treatment thus, making a total of 10 observations for every cultivar of lettuce, treatment, and type of leaves. One square centimeter was approximately equivalent to 30 mg of FW in CT and BRM and to 40 mg of FW in MV. Leaf WC was calculated as (FW − DM)/ DM × 100, and results were expressed as percentages.

Mycorrhizal Analysis. Root samples of lettuce plants were cleared and stained as described by Phillips and Hayman,[21] and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per each type of lettuce and type of mycorrhizal inoculum) under the microscope. Results are expressed as percentage of infection.[22] The relative mycorrhizal dependency (RMD) was estimated according to Bagyaraj:[23] RMD = DM of inoculated plant − DM of noninoculated plant × 100/ DM of noninoculated plant. Determination of RMD allows ascertaining the response of crop plants to mycorrhizal fungi.

Mineral Analyses. For mineral analyses, samples (0.5 g DM) of three plants per treatment were dry-ashed and dissolved in HCl according to the method of Duque.[24] Phosphorus, potassium, magnesium, calcium, manganese, iron, zinc, copper, and sodium were determined using a Perkin-Elmer Optima 4300 inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin-Elmer, USA). The operating parameters of the ICP-OES were as follows: radio frequency power, 1300 W; nebulizer flow, 0.85 L min^{-1}; nebulizer pressure, 30 psi; auxiliary gas flow, 0.2 L min^{-1}; sample introduction, 1 mL min^{-1}; with 3 replicates. Total nitrogen and sulfur were quantified after combustion (950 °C) of leaf DM with pure oxygen by an elemental analyzer (Carlo Erba CHNS-O EA1108, Carlo Erba Instruments, Milan, Italy). Three measurements per sample were performed. Results are expressed both on a wet basis and per plant.

Total Soluble Sugars (TSS) and Total Soluble Proteins in Leaves. TSS and total soluble proteins were quantified in potassium phosphate buffer (KPB) (50 mM, pH 7.5) extracts of fresh leaves (1 g of outer leaves and 1 g of inner leaves per plant, 7 plants per treatment). These extracts were filtered through four cheesecloth layers and centrifuged at 38720g for 10 min at 4 °C. The supernatant was collected and stored at 4 °C for TSS and protein determinations. TSS were analyzed with the anthrone reagent in a Spectronic 2000 (Bausch and Lomb, Rochester, NY).[25] Leaf soluble proteins were measured by the protein dye-binding method of Bradford[26] using bovine serum albumin (BSA) as standard. Every sample was measured twice. Results were expressed as milligrams of TSS or total soluble proteins per gram of FW or per plant. Outer and inner leaves were separately analyzed.

Table 1. Mycorrhizal (AM) Colonization and Growth Parameters of Lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV): Fresh Weight (FW) of Leaves, Dry Matter (DM) of Leaves and Roots, Root to Shoot Ratio, and Number of Leaves per Plant[a]

	AM colonization (%)	leaf FW (g plant^{-1})	leaf DM (g plant^{-1})	root DM (g plant^{-1})	root DM/shoot DM	leaves per plant
CT						
NM	ND	93.6 c	8.0 b	7.3 b	1.02 b	34.7 b
Gf	62.8 a	111.7 b	9.7 a	8.9 b	0.91 b	39.0 ab
CI	61.2 a	129.0 a	10.4 a	16.4 a	1.56 a	42.3 a
BRM						
NM	ND	87.8 b	6.5 c	7.7 a	1.18 a	31.2 b
Gf	66.6 a	99.2 ab	7.7 b	8.1 a	1.04 a	33.7 ab
CI	57.4 b	102.6 a	9.2 a	10.6 a	1.09 a	36.3 a
MV						
NM	ND	118.9 c	9.2 b	8.4 a	0.90 a	36.0 a
Gf	64.1 a	132.9 b	11.7 a	11.0 a	0.92 a	40.0 a
CI	56.3 b	153.7 a	13.5 a	11.4 a	0.91 a	39.7 a

[a] Values are means of 10 observations (10 samples from 10 plants) for AM colonization or 3 observations (3 plants) for growth parameters. Within each column and cultivar, data followed by the same letter indicate that values did not differ significantly ($p \leq 0.05$). ND, not detected; NM, nonmycorrhizal; Gf, plants inoculated with *Glomus fasciculatum*; CI, plants inoculated with the commercial inoculum.

Chlorophylls and Carotenoids. Contents of chlorophylls (chl a + chl b) and total carotenoids were determined according to the method of Séstak et al.[27] One sample (1 cm^2) of fresh outer leaves and one sample (1 cm^2) of fresh inner leaves per plant (7 plants per treatment) were immersed in 5 mL of 96% ethanol at 80 °C during 10 min to extract the pigments. The absorbance of every extract was measured once at 470, 649, 665, and 750 nm using a Spectronic 2000 (Bausch and Lomb). Estimation of chl a and chl b and total carotenoids in the same extract solution was performed by using the extinction coefficients and equations determined by Lichtenthaler.[28] Results were expressed as milligrams of total chlorophylls ($a + b$) or carotenoids per gram of FW or per plant. Outer and inner leaves were separately analyzed.

Total Phenolics and Anthocyanins. Total phenolic compounds were extracted according to the method of Chapuis-Lardy et al.[29] with some modifications. One sample (0.5 g FW) of outer leaves and one sample (0.5 g FW) of inner leaves per plant (7 plants per treatment) were pulverized in liquid nitrogen, mixed with 20 mL of 80% methanol, and homogenized at room temperature for 1 min. After filtration, 0.5 mL of each sample was mixed with 10 mL of distilled water. Total phenolic content was determined from aqueous solutions by spectrophotometric analysis at 760 nm with Folin−Ciocalteu reagent[30] (one measurement per extract). Although it is not completely specific for phenolic compounds (e.g., it is affected by other constituents) and not all phenolic compounds exhibit the same level of activity in the assay,[31] the Folin−Ciocalteu method is commonly used to measure phenolic content. Results were expressed as milligrams of gallic acid per gram of FW or per plant. Outer and inner leaves were separately analyzed.

Anthocyanins were analyzed according to the method of Cevahir et al.[32] with some modifications.[33] One sample (1 cm^2) of fresh outer leaves and one sample (1 cm^2) of fresh inner leaves per plant (7 plants per treatment) were collected and homogenized in 1 mL of acidified methanol (2.27 mL of HCl 37% + 97.73 mL of methanol) and maintained at 4 °C overnight in the dark to avoid degradation of chlorophylls. After the addition of 665 μL of distilled water, chlorophylls were separated with 1.6 mL of chloroform. Particulates were removed by centrifugation at 26890g for 10 min, and the supernatant was passed through four cheesecloth layers. Total anthocyanins were determined by measuring A_{530} and A_{657} of the aqueous phase (one measurement per every sample). The relative amount of anthocyanins was calculated as the optical density (OD) per gram of FW or per plant as described by Mancinelli.[34] Outer and inner leaves were separately analyzed.

Ascorbate. Ascorbate (ASC) content was assayed photometrically by reduction of 2,6-dichlorophenolindophenol (DCPIP) according to the method of Leipner et al.[35] One sample (0.5 g FW) of outer leaves and one sample (0.5 g FW) of inner leaves per plant (7 plants per treatment) were homogenized in liquid nitrogen in the presence of 1 g of NaCl and extracted in 5 mL of ice-cold 2% (w/v) metaphosphoric acid. The homogenate was filtered. An aliquot of 0.3 mL was mixed with 0.2 mL of 45% (w/v) K_2HPO_4 and 0.1 mL of 0.1% (w/v) homocysteine to reduce dehydroascorbate (DHA) to ASC and determine the total ASC pool (ASC + DHA). After 15 min of incubation at 25 °C, 1 mL of 2 M citrate−phosphate buffer (pH 2−3) and 1 mL of 0.003% (w/v) DCPIP were added. The absorbance at 524 nm was measured immediately using a spectrophotometer (one measurement per every sample). The content of ASC was calculated by reference to a standard curve. Results were expressed as milligrams of total ASC per gram of FW and per plant of either outer or inner leaves.

Statistical Analysis. Analysis of variance was performed for each parameter. Means ± standard errors (SE) were calculated and, when the F ratio was significant ($p \leq 0.05$), a least difference (LSD) test was applied as available in the SPSS statistical package version 15.0 programs for Windows XP.

RESULTS

Growth Parameters and Mycorrhizal Analysis. The application of either *G. fasciculatum* or the commercial inoculum significantly improved growth of the aerial part in CT, BRM, and MV (Table 1). Leaves of mycorrhizal plants showed higher fresh weight (FW) and dry matter (DM) than their respective noninoculated controls. The improvement of both FW and DM of the aerial part in CT and BRM when inoculated with the commercial inoculum was in part due to the higher number of leaves per plant when compared with their respective nonmycorrhizal controls. The application of the commercial inoculum to the variety CT also stimulated biomass (DM) production in roots.

Lettuce plants not inoculated with any of the mycorrhizal inocula did not show mycorrhizal structures in roots (data not shown). Therefore, we will refer indistinctly to these plants as "noninoculated" or "nonmycorrhizal" here. When inoculated with *G. fasciculatum* (Gf), lettuce plants showed percentages of root

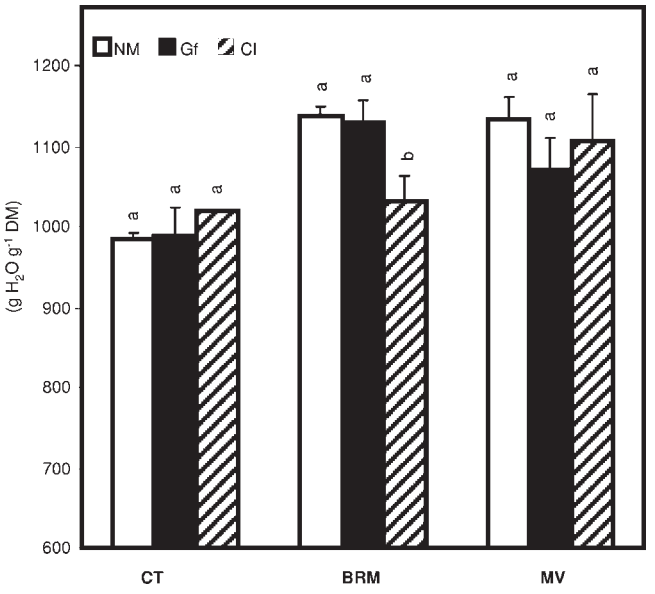

Figure 1. Water content (WC) (g of H_2O g^{-1} DM) in leaves of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM) (white histograms), inoculated with *Glomus fasciculatum* (Gf) (black histograms), or inoculated with the commercial inoculum (CI) (slashed histograms). Values are the mean of 10 observations (10 samples, 1 measurement per sample) ± SE. Within each cultivar of lettuce the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$).

colonization of around 65%, regardless the variety of lettuce (Table 1). When inoculated with the commercial inoculum (CI), percentages of colonization ranged from 56% in MV to 61% in CT (Table 1). Relative mycorrhizal dependency (RMD) in lettuce associated with *G. fasciculatum* reached values of 15.44, 16.30, and 20.31% in CT, BRM, and MV, respectively. When the commercial inoculum was applied, RMD achieved higher values than when *G. fasciculatum* was applied (22.93, 29.18, and 31.34% in CT, BRM, and MV, respectively), the difference being significant for BRM and MV.

Leaf Water Content (WC). CT was the variety with the lowest amount of water in leaf tissues (near 1000 g of H_2O g^{-1} DM) (Figure 1). Only the application of the commercial inoculum slightly increased WC in leaves of this variety. When not inoculated with any AMF, leaves of BRM and MV exhibited higher WC (around 1100 g of H_2O g^{-1} DM) than CT. The association of these two last types of lettuce with *G. fasciculatum* had no effect on WC. In contrast, the application of the commercial inoculum caused a decline in the water content in leaves of BRM.

Mineral Analyses. When nonmycorrhizal lettuce plants were compared, CT showed higher concentrations of mineral nutrients in leaves than BRM and MV (Table 2 in the Supporting Information). The greater amount of water in tissues of these two last cultivars of lettuce (Figure 1) would have produced a dilution effect when expressed as data of mineral nutrients on a wet basis. Similarly, the higher production of shoot biomass in lettuce associated with AM than in nonmycorrhizal plants (Table 1) would have contributed to a dilution effect in mycorrhizal plants. For example, in CT, the concentrations of K in both outer and inner leaves were lower in plants inoculated with the commercial inoculum than in the nonmycorrhizal controls (Table 2 in the Supporting Information). However, when the content of K in the whole plant was compared (Table 3 in the Supporting Information), CT inoculated with the commercial inoculum showed a greater amount of K than nonmycorrhizal controls. A similar observation can be applied to the concentrations (Table 2 in the Supporting Information) and total contents (Table 3 in the Supporting Information) of Fe in BRM associated with *G. fasciculatum*. There were, however, several mineral nutrients that appeared in higher concentrations in leaf tissues of mycorrhizal plants on a fresh weight basis (Table 2 in the Supporting Information). This is the case of Cu, which was in greater concentrations in both outer and inner leaves of BRM when compared with their respective nonmycorrhizal controls. Likewise, concentrations of Fe in both outer and inner leaves and concentrations of Zn in external leaves of CT were significantly higher in plants inoculated with either *G. fasciculatum* or the commercial inoculum than in the nonmycorrhizal plants. Similarly, concentrations of Fe in external leaves of MV inoculated with the commercial inoculum were greater than those of nonmycorrhizal plants (Table 2 in the Supporting Information).

TSS and Total Soluble Proteins in Leaves. The concentration of TSS on a wet basis in the external leaves of nonmycorrhizal lettuce plants reached values of around 3−4 mg g^{-1} FW in the three cultivars (Figure 2A). In contrast, the internal leaves of MV had lower amount of TSS (near 4 mg g^{-1} FW) than the inner leaves of CT (around 6.4 mg g^{-1} FW) and BRM (around 6 mg g^{-1} FW). The effect of AMF on the concentrations of TSS strongly differed between cultivars of lettuce (Figure 2A). In CT, the application of either *G. fasciculatum* or the commercial inoculum had no effect on the amount of TSS in external leaves and decreased the levels in the internal leaves when data were expressed on a wet basis. Concentrations of TSS were also similar in the outer leaves of BRM and MV when plants inoculated with *G. fasciculatum* were compared with their respective nonmycorrhizal controls. However, the application of the commercial inoculum enhanced the levels of TSS in the inner leaves of these two last types of lettuce in comparison with their respective noninoculated controls, reaching values of around 7.5 mg g^{-1} FW in BRM and 5.1 mg g^{-1} FW in MV (Figure 2A). The total content of TSS per plant (Figure 2B) always increased in the internal leaves when plants were inoculated with the commercial inoculum. In MV, *G. fasciculatum* also enhanced the total content of TSS in the inner leaves. In external leaves, inoculation of lettuce with AMF slightly increased the total content of TSS per plant, the increase being significant in BRM and MV after application of the commercial inoculum.

In noninoculated plants, the highest concentration of soluble proteins on a wet basis was found in the internal leaves of CT (around 6 mg g^{-1} FW). In this variety of lettuce, the application of either *G. fasciculatum* or the commercial inoculum did not affect the levels of proteins (on a wet basis) in the outer leaves and decreased the amount of proteins in the inner leaves. Compared with their respective nonmycorrhizal controls, mycorrhizal BRM and MV showed similar concentrations of soluble proteins in both external and internal leaves, with values ranging from 2.5 to 3 mg g^{-1} FW in outer leaves and from 3 to 4 mg g^{-1} FW in inner leaves. Contrarily, the application of mycorrhizal inocula increased the total content of proteins per plant in the three cultivars of lettuce. In CT, the commercial inoculum significantly enhanced the content of proteins in the inner leaves (from 250 mg $plant^{-1}$ in noninoculated plants to 370 mg $plant^{-1}$ in inoculated ones). In BRM both types of mycorrhizal inocula, *G. fasciculatum* and

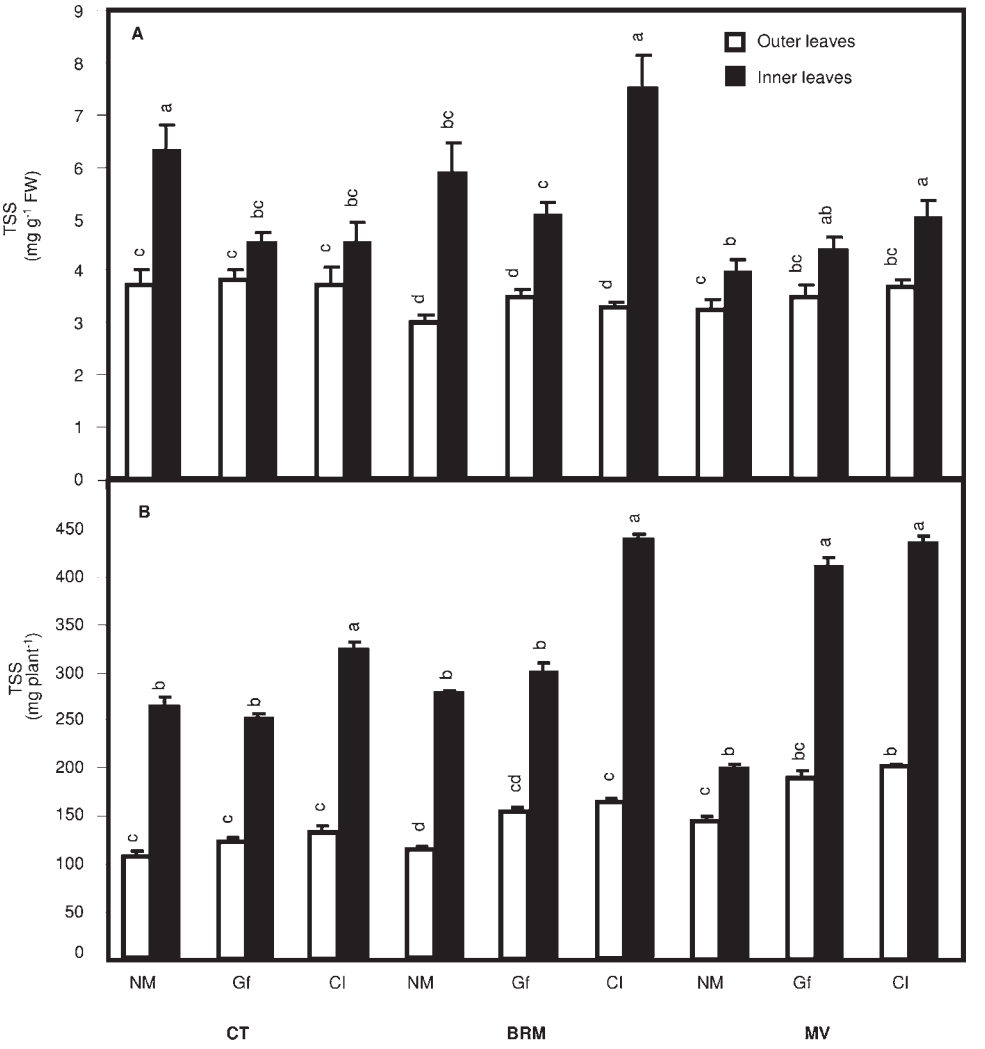

Figure 2. Concentration (mg g^{-1} FW) (A) and total content (mg $plant^{-1}$) (B) of total soluble sugars (TSS) in outer (white histograms) and inner leaves (black histograms) of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM), inoculated with *Glomus fasciculatum* (Gf), or inoculated with the commercial inoculum (CI). Values are the mean of 14 observations (7 samples, 2 measurements per sample) ± SE. Within each panel and cultivar of lettuce the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$).

the commercial inoculum, increased the protein content in the inner leaves (from 125 mg $plant^{-1}$ in noninoculated plants to 250 and 200 mg $plant^{-1}$ in plants inoculated, respectively, with *G. fasciculatum* or the commercial inoculum). In MV, *G. fasciculatum* favored the accumulation of proteins in both outer and inner leaves (around 220−250 mg $plant^{-1}$ in both types of leaves), whereas the commercial inoculum enhanced protein content only in the internal leaves (from 150 mg $plant^{-1}$ in nonmycorrhizal controls to 275 mg $plant^{-1}$ in mycorrhizal plants).

Chlorophylls and Carotenoids. When not inoculated with any AMF, BRM was the variety with the lowest levels of chlorophylls in both outer and inner leaves (around 0.70 mg g^{-1} FW). The association of this type of lettuce with AMF increased the amount of chlorophylls on a wet basis, the enhancement being especially evident when plants were inoculated with the commercial inoculum (around 0.90−0.95 mg g^{-1} FW in outer and inner leaves, respectively). *G. fasciculatum* also increased the amount of chlorophylls on a wet basis in the external leaves of CT, achieving values of 1.30 mg g^{-1} FW. In contrast, the concentrations of chlorophylls were similar in nonmycorrhizal and mycorrhizal lettuce MV (0.83−0.98 mg g^{-1} FW in external leaves and 0.72−0.90 mg g^{-1} FW in inner leaves). When values were expressed per plant, inoculation with either *G. fasciculatum* or the commercial inoculum enhanced the chlorophyll contents in the three cultivars of lettuce. In CT and MV such increases were equally evident in external and internal leaves. In BRM the increases were higher in outer than in inner leaves (data not shown).

Nonmycorrhizal BRM had the lowest concentrations of total carotenoids in both outer (around 0.06 mg g^{-1} FW) and inner (around 0.07 mg g^{-1} FW) leaves on a wet basis (Figure 3A). However, this was the variety of lettuce most benefited by its association with AMF: the concentrations of carotenoids increased in both external and internal leaves as a consequence of the inoculation with either *G. fasciculatum* or the commercial inoculum,

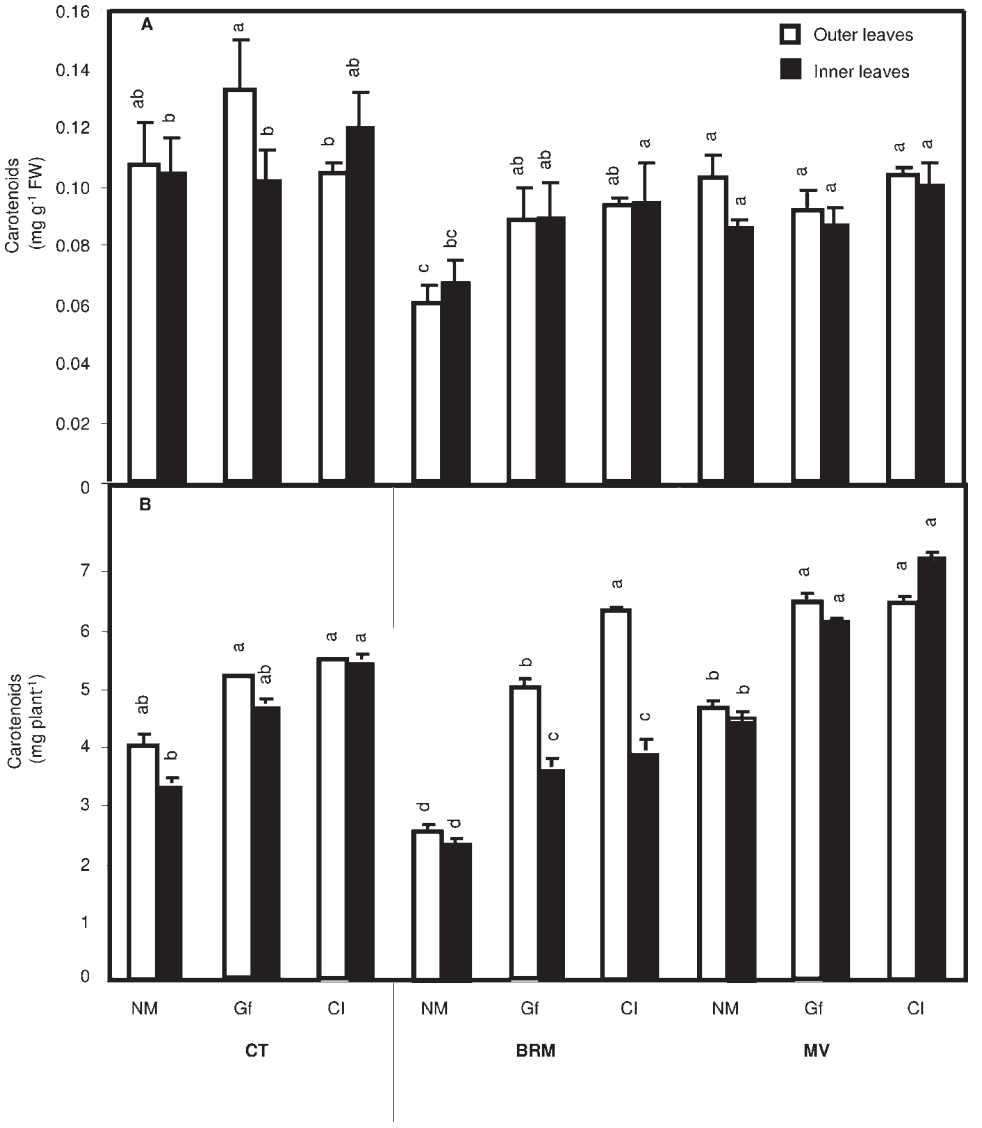

Figure 3. Concentration (mg g^{-1} FW) (A) and total content (mg plant^{-1}) (B) of total carotenoids in outer (white histograms) and inner leaves (black histograms) of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM), inoculated with *Glomus fasciculatum* (Gf), or inoculated with the commercial inoculum (CI). Values are the mean of 7 observations (7 samples, 1 measurement per sample) ± SE. Within each panel and cultivar of lettuce, the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$).

reaching values of around 0.09 mg of total carotenoids per gram of FW. When data were expressed per plant (Figure 3B), the content of total carotenoids was enhanced in both outer and inner leaves of the three cultivars of lettuce after inoculation with any of the mycorrhizal inocula. However, the greatest increases occurred in external leaves of BRM and internal leaves of MV inoculated with the commercial inoculum.

Total Phenolics and Anthocyanins. On a wet basis, in non-inoculated CT and BRM the concentrations of phenolics were similar in outer and inner leaves (Figure 4A). In contrast, non-mycorrhizal MV accumulated a greater content of phenolic compounds in outer than in inner leaves. The association of lettuce with AMF had no significant effects on the amount of phenolics in BRM and MV. However, the levels of these compounds increased in the external leaves of CT after application of either *G. fasciculatum* or the commercial inoculum. The beneficial effects of AMF on the accumulation of soluble phenolic compounds were more evident when data were expressed per plant (Figure 4B). The total contents of phenolics in both external and internal leaves of CT and MV clearly increased in mycorrhizal plants regardless of the type of mycorrhizal inocula applied. Both types of mycorrhizal inocula also enhanced the total content of phenolics in outer leaves of BRM.

Both external and internal leaves of MV accumulated higher concentrations of anthocyanins on a wet basis than leaves from CT and BRM regardless of whether plants were or not inoculated with AMF (Figure 5A). In CT, the application of mycorrhizal inocula increased concentrations of anthocyanins only in the inner leaves. On the other hand, inoculation with either *G. fasciculatum* or the commercial inocula enhanced the levels of anthocyanins in both outer and inner leaves in BRM and MV. Similar results were observed concerning the total contents of anthocyanins per plant (Figure 5B).

Ascorbate. The association of lettuce with *G. fasciculatum* enhanced the concentrations of total ASC in both outer and

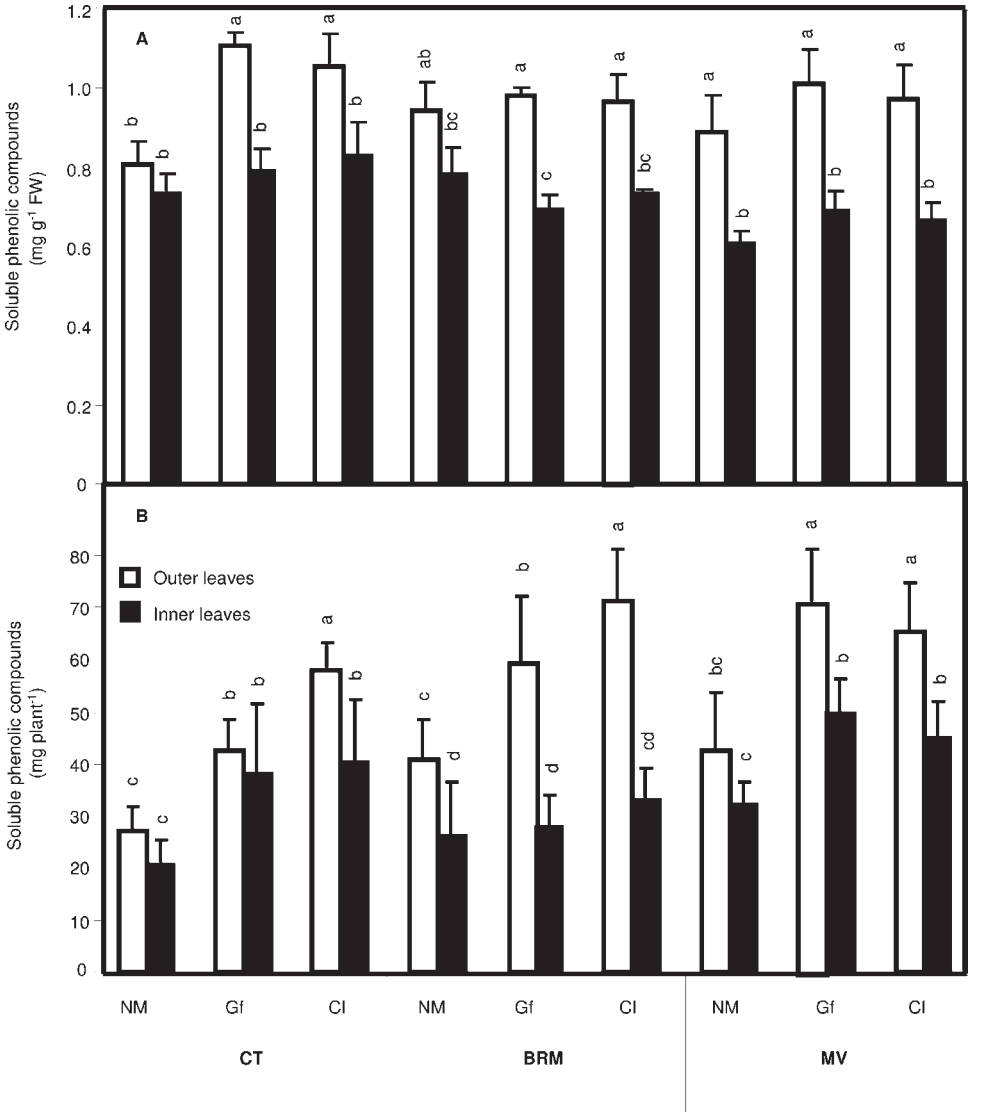

Figure 4. Concentration (mg g^{-1} FW) (A) and total content (mg plant^{-1}) (B) of soluble phenolic compounds in outer (white histograms) and inner leaves (black histograms) of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM), inoculated with *Glomus fasciculatum* (Gf), or inoculated with the commercial inoculum (CI). Values are the mean of 7 observations (7 samples, 1 measurement per sample) ± SE. Within each panel and cultivar of lettuce, the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$).

inner leaves of the three studied cultivars (Figure 6A), but only in the inside leaves of CT was the increase significant in comparison with the respective noninoculated controls. When the total contents of ASC per plant in CT and BRM were analyzed (Figure 6B), lettuce inoculated with the commercial inoculum had the greatest amount of ASC in leaves. The effects of *G. fasciculatum* and the commercial inoculum on the total contents of ASC in MV were comparable: in both cases mycorrhizal plants accumulated higher quantities of ASC in outer and inner leaves than their respective noninoculated controls.

DISCUSSION

It is well-known that mycorrhizal symbiosis can benefit plant growth due mostly to uptake of nutrients with low mobility, such as P, by external hyphae.[36] In many cases, improved P uptake is the primary cause of growth enhancement in plants associated with AMF.[37] In our study, concentrations of P on a wet basis were quite similar in mycorrhizal and nonmycorrhizal lettuce plants due to the dilution effect caused by the improved size of plants associated with AMF. However, compared with their respective nonmycorrhizal controls, the total content of P was higher in inner leaves of CT inoculated with either *G. fasciculatum* or the commercial inoculum as well as in both outer and inner leaves of BRM and in inner leaves of MV after application of the commercial inoculum. Improved P nutrition is not always sufficient to explain the effects of AMF on the host plant's physiology.[37] For example, when nonmycorrhizal alfalfa plants received supplemental P fertilization to achieve similar levels of P in tissues of nonmycorrhizal and mycorrhizal plants, Goicoechea et al.[38] concluded that hormonal factors were implied in the physiological differences observed between both types of plants.

Percentages of root cortex colonized by mycorrhizal fungi in our study achieved values comparable to those found by Ruiz-Lozano

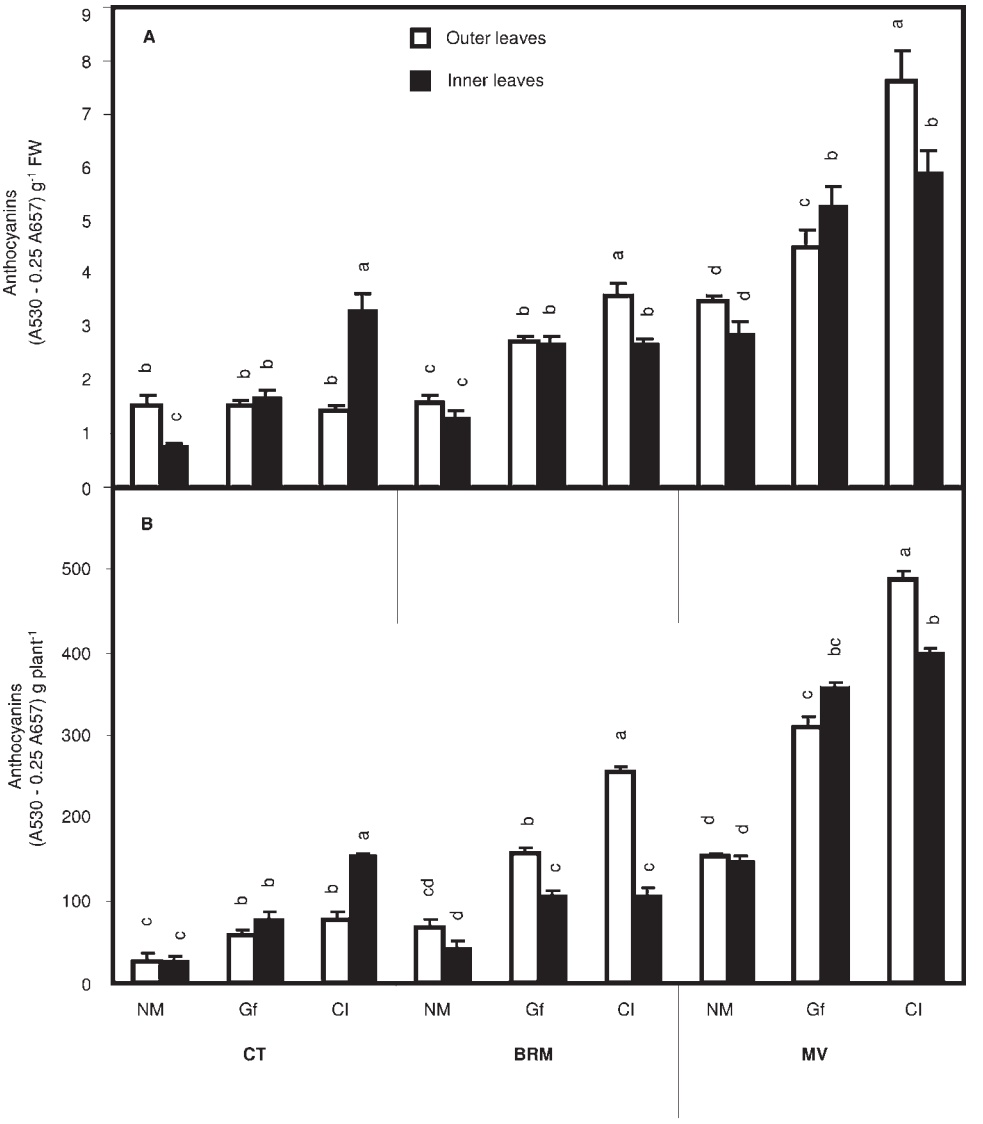

Figure 5. Concentration (A) and total content (B) of anthocyanins in outer (white histograms) and inner leaves (black histograms) of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM), inoculated with *Glomus fasciculatum* (Gf), or inoculated with the commercial inoculum (CI). Values are the mean of 7 observations (7 samples, 1 measurement per sample) ± SE. Within each panel and cultivar of lettuce, the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$). Data on anthocyanins are expressed as optical density (OD) ($A_{530} - 0.25\ A_{657}$) g^{-1} FW (A) or as OD $plant^{-1}$ (B).

et al.[39] in lettuce plants inoculated with *G. fasciculatum*, *G. mosseae*, or *G. etunicatum*. Root colonization development exhibits a curvilinear response to the inoculum density, but the maximum level achieved can vary between fungi and with different environmental conditions.[40] The concept of effectiveness can be defined as the ability of a given AMF to increase plant growth and nutrient uptake.[40] According to this definition, our values of RMD suggest that the commercial inoculum (a mixture of *G. mosseae* and *G. intraradices*) was more effective than *G. fasciculatum* in improving growth of lettuce plants (especially for BRM and MV), despite the slightly lower percentages of mycorrhizal colonization achieved. Similarly, other authors[41] have also observed a lack of close relationship between the level of root colonization by AMF and the effect on plant growth.

As the present research is mainly focused on the nutritional quality of three types of lettuce consumed as salads, concentrations of different compounds have been expressed on a fresh basis. In CT and BRM not inoculated with AMF, the internal leaves accumulated higher levels of TSS than the external leaves on a fresh weight basis. Because the contents of chlorophylls in outer leaves of these cultivars of lettuce were similar to those found in inner leaves, the lower quantity of soluble sugars in external leaves cannot be attributed to a senescence process. It is possible that inside leaves would be still acting as a strong sink of sugars synthesized in outer leaves. The presence of *G. fasciculatum* in roots of CT and BRM caused a decline in the concentrations of TSS (on a fresh weight basis) in inner leaves perhaps due to the translocation of sugars from shoots to roots to support both the mycorrhizal structures and the functionality of the symbiosis.[10] In contrast, the application of the commercial inoculum to the cultivars BRM and MV increased the accumulation of TSS in the internal leaves compared with their respective nonmycorrhizal control plants, suggesting an enhancement of the photosynthesis in plants associated with the mixture of *G. mosseae* and *G. intraradices*.[42]

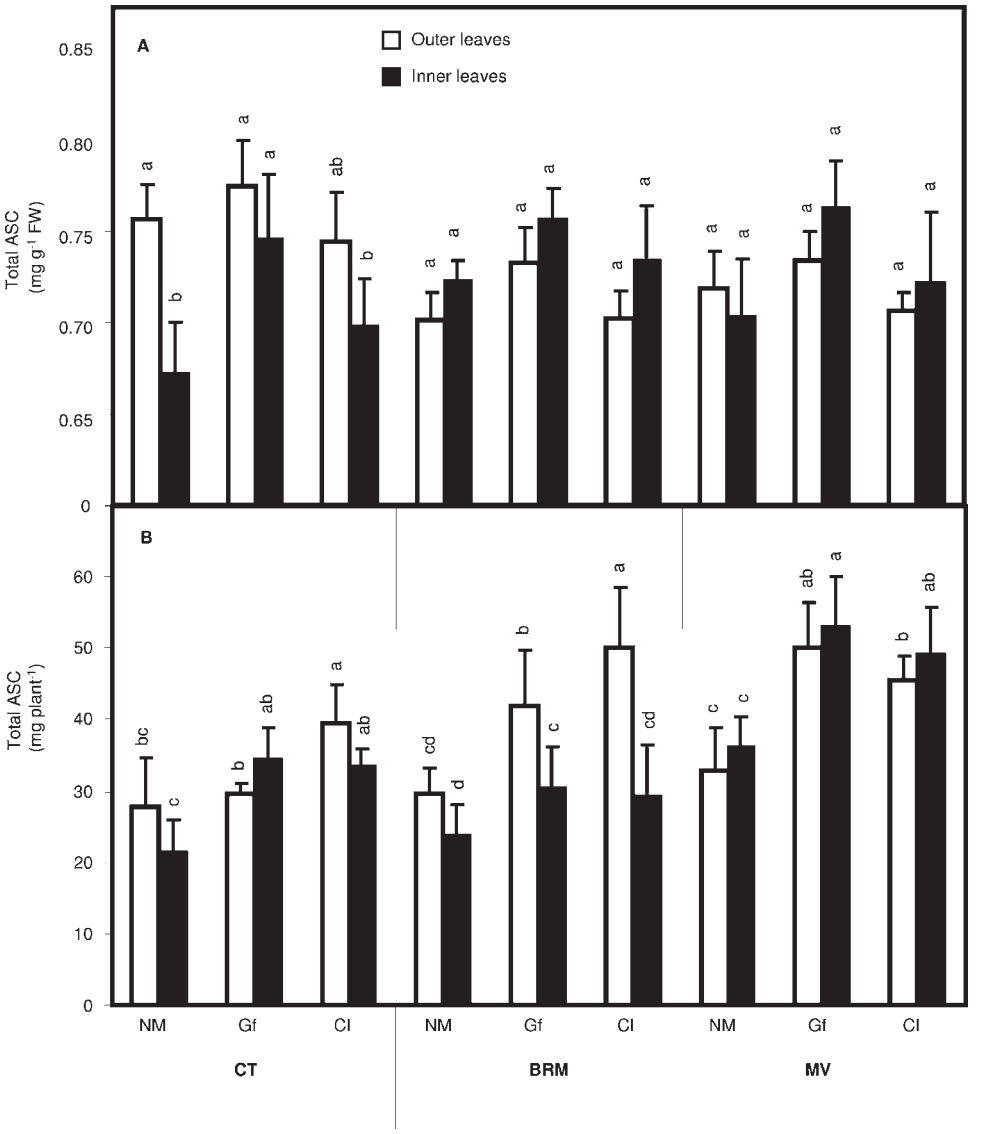

Figure 6. Concentration (mg g^{-1} FW) (A) and total content (mg $plant^{-1}$) (B) of total ascorbate (ASC) in outer (white histograms) and inner leaves (black histograms) of lettuces Cogollos de Tudela (CT), Batavia Rubia Munguía (BRM), and Maravilla de Verano (MV) not inoculated with mycorrhizal fungi (NM), inoculated with *Glomus fasciculatum* (Gf), or inoculated with the commercial inoculum (CI). Values are the mean of 7 observations (7 samples, 1 measurement per sample) ± SE. Within each panel and cultivar of lettuce, the coincidence of letters indicates that values are not significantly different ($p \leq 0.05$).

Different species of *Glomus* can follow distinct dynamics when establishing a functional symbiotic association with the same type of host plant.[43]

When nonmycorrhizal plants were compared, CT was the variety of lettuce that accumulated the highest amount of total nitrogen on a fresh weight basis, especially in outer leaves (4.8 g kg^{-1}, equivalent to around 4800 ppm). Reinink and Eenink,[44] working with several lettuce cultivars, estimated that nitrates represented from 20 to 36% of the total nitrogen measured in leaves. Consequently, nitrate amount in leaves of CT (and therefore in BRM and MV) would be far from the maximum levels of nitrates allowed for consumption of lettuce in salads: around 4000 ppm, restricted to 3500 ppm in some countries such as Germany and Switzerland. The application of *G. fasciculatum* and, especially, the commercial inoculum diminished the concentration of total N in outer leaves of CT without causing a decrease in the protein levels, which means that the application of mycorrhizal inocula reduced the quantity of inorganic nitrogen in outer leaves of the aforementioned variety of lettuce.

Mycorrhizal fungi can increase the levels of some essential micronutrients for human health in crop products. For example, Cavagnaro et al.[45] observed a significant enhancement of Zn in fruits of mycorrhizal tomato plants. In our study, the dilution effect produced by increased size contributed to the similar concentrations of Zn in leaves of mycorrhizal lettuce plants BRM and MV compared with their respective noninoculated controls. However, the application of AMF stimulated the accumulation of Zn in outer leaves of CT and sharply enhanced the concentrations of Cu in both outer and inner leaves of BRM. In CT and MV only *G. fasciculatum* was clearly effective in enhancing Cu levels in the inner leaves. Copper is essential to human health. However, modern research has shown that many people in developed

countries (e.g., the United Kingdom or the United States) do not consume adequate quantities of copper.[46]

According to the results obtained in nonmycorrhizal plants, BRM was the variety with the lowest concentrations of both total chlorophylls and carotenoids in outer and inner leaves. However, it was the variety that most benefited from the application of mycorrhizal inocula for enhancing the amount of those pigments, which can contribute to improve the quality of this variety of lettuce for human consumption. In recent years, there has been a growing interest in natural and semisynthetic chlorophyll derivatives, not only as food colorants but as food supplements due to their potential effect against the development of several chronic diseases[47] and their anti-inflammatory activity in vitro.[48] Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing human diseases including cardiovascular diseases, cancer, and other chronic diseases.[49]

Natural phenolic compounds are secondary metabolites and a major class of antioxidants in plants.[50] In lettuce, two main classes of phenols and polyphenols have been identified: caffeic acid derivatives and flavonols.[51] Caffeic acid derivatives seem to be the main phenolics in green varieties, whereas flavonols have been detected in higher quantities in red varieties. Moreover, red-leafed varieties of lettuce also accumulate anthocyanins.[7] The application of AMF increased the total levels of soluble phenolic compounds per plant in the three studied cultivars of lettuce. However, only the outer leaves of CT showed higher concentrations of these compounds than their respective nonmycorrhizal controls on a wet basis. The effect of AMF on the content of total phenols is contradictory in the literature. Whereas Toussaint et al.[52] showed the potential of three AMF to enhance the production of phenolic compounds with antioxidant activity in sweet basil leaves, Geneva et al.[53] observed decreased concentrations of total phenols in leaves of *Salvia officinalis* associated with *G. intraradices*.

Mycorrhizal symbiosis enhanced the concentrations of anthocyanins in the inner leaves of the three cultivars of lettuce and also in the outer leaves of BRM and MV. Anthocyanins are the most important group of water-soluble pigments in plants, and they are regarded as important components in human nutrition due to their antioxidant capacities.[1] In addition, they have exhibited anticarcinogenic effects in several cell culture systems including cancer cells of the colon, endothelial, liver, and leukemic.[50] Because outer leaves of head lettuce are usually stripped off during harvest, the relevant increases of anthocyanins in the internal leaves of mycorrhizal lettuce plants can be especially interesting for the human diet.

Leafy vegetables, especially when consumed fresh, are a valuable source of vitamin C for the human diet,[54] and this is particularly true for lettuce, a major constituent of fresh salads and widely used for the so-called "Fourth Range" of vegetables. The concentrations of total ascorbate (vitamin C) in nonmycorrhizal plants were very similar to those measured by Konstantopoulou et al.[55] in greenhouse lettuce (cv. Parris Island) at harvest. Although the total content of ascorbate in plants increased after the inoculation of the three cultivars of lettuce with AMF, on a wet basis the concentrations of ascorbate were very similar between nonmycorrhizal and mycorrhizal plants as a consequence of the dilution effect due to the greater size of lettuce associated with AMF. The only exception was the enhancement of the concentration of total ascorbate in the inner leaves of CT after inoculation with *G. fasciculatum*. Recently, Geneva et al.[53] have found a favorable effect of mycorrhizal infection with *G. intraradices* for increasing the ascorbate content in leaves of *S. officinalis*.

Finally, we will include some considerations concerning food safety of mycorrhizal lettuce. When crops are attacked by pathogenic fungi, food safety may be affected due to either the accumulation of mycotoxins in edible plant organs or the synthesis of antifungal metabolites known as phytoalexins in plant tissues. The most significant mycotoxins, such as aflatoxins, trichothecenes, and fumonisins, are not normally a problem in fresh fruits and vegetables. However, the accumulation of patulin and ochratoxin A in both fresh fruits and vegetables, which is mainly induced by fungi from the genera *Aspergillus* and *Penicillium*, can negatively affect human health. In the context of fresh vegetables for human consumption *Alternaria alternata* (the causal agent of leaf rot on lettuce) must be taken into account due to its capacity for producing tenuazonic acid. However, there is no clear evidence implicating this mycotoxin in human illness.[56] To the best of our knowledge, no mycotoxins produced by AMF have been currently described. On the other hand, phytoalexin production by plants may also have health implications for humans. This is the case of ipomeamarone and 4-hydroxymyoporone and their subproduct 4-ipomenol produced by the sweet potato, which have antifungal activity and can also act as hepatotoxins or cause edema of the lung.[56] Accumulation of flavonoid compounds with a hypothetical role of phytoalexins in mycorrhizal infected roots has been widely investigated. However, it is not clear if these products stimulate the growth of AMF or act as antimicrobial phytoalexins.[12] Moreover, the differential activation of defense-related genes in host plants induced by AMF may reflect a partial elicitation of a general plant defense response to early stages of fungal invasion.[12] This defense response in plant−mycorrhizal association is probably more uncoordinated, weaker, slower, more localized, and more transient than either compatible or incompatible plant−pathogen interactions once the symbiosis becomes established.[16,57] In our experimental design, plant harvesting was carried out 7 weeks after mycorrhizal inoculation.

In summary, growth of lettuce clearly benefited from the association of plants with AMF. The greater size achieved by mycorrhizal lettuce plants compared with nonmycorrhizal ones can have a subsequent dilution effect on the concentrations of some mineral nutrients of interest for human diet. Nevertheless, the levels of Cu, Fe, and other antioxidant compounds, such as anthocyanins, carotenoids, and, to a lesser extent, phenolics, appeared in higher concentrations (on a wet basis) in mycorrhizal than in nonmycorrhizal lettuce plants. This would mean that cultivation of mycorrhizal lettuce would allow improving the intake of those compounds without requiring an increase in consumption. However, the beneficial effect of AMF on nutrient quality of lettuce varied among types of mycorrhizal inocula applied and cultivars of lettuce and between outer and inner leaves. We do not know if it would be possible to enhance the nutritional quality of lettuce just by increasing P fertilization to nonmycorrhizal plants. Anyway, the use of AMF as biofertilizers to counteract the application of extra fertilization could promote the agriculture of the future, based on the implementation of practices that maintain resilience of ecosystem services.[18,58] Moreover, in the context of modern agriculture, "unravelling the contribution of mycorrhizal fungi to the nutritional quality of edible plant organs becomes a priority".[58]

■ ASSOCIATED CONTENT

Supporting Information. Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

M.B. is the recipient of a grant from Asociación de Amigos de la Universidad de Navarra (ADA).

ACKNOWLEDGMENT

We are very grateful to Adriana Hernández, from Atens, Tarragona, Spain, for kindly providing the commercial inoculum.

ABBREVIATIONS USED

AMF, arbuscular mycorrhizal fungi; ASC, ascorbate; BRM, Batavia Rubia Munguía; chl, chlorophyll; CT, Cogollos de Tudela; DM, dry matter; FW, fresh weight; MV, Maravilla de Verano; RMD, relative mycorrhizal dependency; TSS, total soluble sugars; WC, water content.

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