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Improvement of Nutritional Quality of Greenhouse-Grown Lettuce by Arbuscular Mycorrhizal Fungi Is Conditioned by the Source of Phosphorus Nutrition

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Supporting Information

ABSTRACT: The improvement of the nutritional quality of lettuce by its association with arbuscular mycorrhizal fungi (AMF) has been recently reported in a previous study. The aim of this research was to evaluate if the fertilization with three P sources differing in water solubility affects the effectiveness of AMF for improving lettuce growth and nutritional quality. The application of either water-soluble P sources (Hewitt's solution and single superphosphate) or the water-insoluble (WI) fraction of a "rhizosphere-controlled fertilizer" did not exert negative effects on the establishment of the mycorrhizal symbiosis. AMF improved lettuce growth and nutritional quality. Nevertheless, the effect was dependent on the source of P and cultivar. Batavia Rubia Munguía (green cultivar) benefited more than Maravilla de Verano (red cultivar) in terms of mineral nutrients, total soluble sugars, and ascorbate contents. The association of lettuce with AMF resulted in greater quantities of anthocyanins in plants fertilized with WI, carotenoids when plants received either Hewitt's solution or WI, and phenolics regardless of the P fertilizer applied.

KEYWORDS: arbuscular mycorrhizal fungi, growth, lettuce, nutritional quality, plant phosphorus nutrition

INTRODUCTION

Plants are considered as sources of human health. The main goal of food production is to provide adequate nutritional factors to allow human beings to reach their full intellectual and physical potential. There is growing evidence that specific dietary components, the so-called "nutraceutical" metabolites often found in plant-based food, may prevent or control particular diseases and disorders.[1] The human body contains an elaborate antioxidant defense system that is usually influenced by lifestyle, dietary intake of minerals and antioxidant compounds (e.g., polyphenols, vitamins C and E, carotenoids), and the endogenous production of antioxidant compounds.[2]

Lettuce (*Lactuca sativa* L.) is a major food crop within the European Union. According to FAOSTAT (FAO Statistics Division; FAO = Food and Agriculture Organization of the United Nations), 2011, the productions 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 is the most widely 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.[3] These vegetables are widely accepted by consumers because they are easy to prepare for eating. Moreover, lettuce exhibits healthy properties mainly due to the presence of antioxidant compounds together with high fiber content and useful amounts of some minerals [4−6] in its tissues.

Mycorrhizal fungi colonize the roots of over 90% of plant species mostly to the mutual benefit of both plant host and fungus.[7] The most common are the arbuscular mycorrhizas (AMs), which are formed by the majority of crop and horticultural plants, including lettuce. The establishment of this mutualistic association involves a continuous cellular and molecular dialogue between the mycorrhizal fungus and the host plant[8] that includes the activation of the antioxidant,[9] phenylpropanoid,[10] or carotenoid metabolic pathways.[11] It is becoming evident that the AM symbiosis can stimulate the synthesis of secondary metabolites and enhance the accumulation of antioxidant compounds which are potentially beneficial to human health in plant tissues,[12] including greenhouse-grown lettuce.[13] Mycorrhizal lettuce plants have been found to accumulate higher concentrations (on a wet basis) of Cu, Fe, anthocyanins, carotenoids, and, to a lesser extent, phenolics than nonmycorrhizal plants.[13] In this study, lettuce plants were fertilized with a modified Hewitt's nutrient solution and, therefore, with a water-soluble source of P. Water-soluble fertilizers as P sources for plants, however, can exhibit rather low efficiency when applied in acidic and calcareous soils as a consequence of the precipitation of water-insoluble iron, aluminum, or calcium phosphates.[14,15] Therefore, recent research[16−18] has been conducted to produce fertilizers that include P sources of limited water solubility but high solubility in solutions containing the main organic acids released

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to the rhizosphere by plant roots in response to nutrient-limiting conditions. In addition, it has been found that, when dicalcium phosphate ($CaHPO_4$), a water-insoluble and citrate-soluble source of P, is added to the medicinal plant "sweet basil", arbuscular mycorrhizal fungi (AMF) induce the production of phytochemicals irrespective of the dose of $CaHPO_4$ applied.[19]

Two objectives were pursued in this study: (i) to evaluate if the application of P fertilizers differing in water solubility affected the establishment of the mycorrhizal symbiosis in roots of two cultivars of lettuce and (ii) to assess if the application of P sources differing in water solubility influenced the effectiveness of a commercial formulation of AMF for improving both growth and nutrient quality of two cultivars of lettuce (the green cv. Batavia Rubia Munguía and the red cv. Maravilla de Verano).

As the discussion and conclusions are mainly focused on the nutritional quality of two types of lettuce consumed as salads, results have been expressed and discussed on a fresh basis.

MATERIALS AND METHODS

Biological Material and Experimental Design. Batavia Rubia Munguía (*L. sativa* L. var. Capitata) and Maravilla de Verano (*L. sativa* L. var. Capitata) were the two types of lettuce chosen for this study. They are extensively cultivated in greenhouses, are highly commercialized, and are greatly appreciated for consumption in salads in Europe. Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV) are two cultivars of Batavia characterized by 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 a good-sized, firm head.

For each cultivar of lettuce (BRM or MV), the experiment was conducted as a factorial design with two factors and 5-fold replication. The first factor had two levels: nonmycorrhizal (NM) plants and plants inoculated with AM fungi (M). The second factor included three types of phosphorus (P) sources: modified Hewitt's nutrient solution (H), single superphosphate (SSP), and a water-insoluble (WI) source of P.

Seeds of BRM and MV were surface sterilized by 10% bleach for 10 min and sown (on Jan 4) in a mixture of light peat and sand (1:1, v/v). When seedlings had 2−3 fully developed leaves, they were transferred (on Jan 25) to 1.5 L pots (1 plant per pot, 30 pots with BRM and 30 pots with MV, thus making a total of 60 pots) and filled with a mixture of vermiculite−sand−light peat (2.5:2.5:1, v/v/v). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) had a pH of 5.2−6.0, 70−150 mg L^{-1} nitrogen, 80−180 mg L^{-1} P_2O_5, and 140−220 mg L^{-1} K_2O, and it was previously sterilized at 100 °C for 1 h on three consecutive days. At transplantation, 15 pots with BRM and 15 pots with MV were inoculated with the commercial inoculum AEGIS Endo Gránulo and another 15 plants of each cultivar of lettuce were not inoculated and kept as nonmycorrhizal controls. 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 was provided and commercialized by Atens (Tarragona, Spain). The application of this commercial inoculum was found to improve the quality of greenhouse lettuce in previous studies.[13] A total of 9.5 g of the commercial mycorrhizal formulation was added to each pot (around 950 spores). Mycorrhizal inoculum was mixed into the top 10 cm of substrate in each pot just before transplantation of the seedlings to facilitate the early contact between AMF and growing roots of young lettuce plants. Eleven days after transplantation, all plants received distilled water until field capacity (FC).

On day 12 after transplantation (and thus 12 days after application of the mycorrhizal inoculum), five nonmycorrhizal and five mycorrhizal plants from each cultivar of lettuce received 360 mg of the water-soluble source of P, SSP,[18] and another five nonmycorrhizal and five mycorrhizal plants from each cultivar of lettuce received 340 mg of the WI fraction of a "rhizosphere-controlled fertilizer" (RCF).[18] All these plants also received 300 mL per week of a modified Hewitt's nutrient solution without P to avoid deficiencies in other mineral nutrients and 300 mL of distilled water twice a week to maintain optimal irrigation. From day 12 after transplantation, another five nonmycorrhizal and five mycorrhizal plants from each cultivar of lettuce were fertilized with 300 mL of complete Hewitt's nutrient solution with some modifications[13] and also received 300 mL of distilled water twice a week to maintain an optimal water supply. Doses of P were previously calculated to apply the same amount of total P to each plant, independently of the type of fertilizer. Therefore, from day 12 after transplantation, we had 12 different treatments depending on the cultivar of lettuce (BRM and MV), presence or absence of mycorrhization, and type of P fertilization (SSP, WI, or H). For each treatment, we had five pots.

Lettuce plants were grown in a greenhouse at 25 °C/15 °C day/night temperatures and 50%/85% day/night relative humidity (RH) and received natural daylight supplemented with irradiation from Son-T-Agro high-pressure sodium lamps (Philips Nederland B.V., Eindhoven, The Netherlands) that provided a minimum photosynthetic photon flux (PPF) of around 300−400 μmol m^{-2} s^{-1} during a 14 h photoperiod. All lettuce plants were harvested seven weeks after transplantation.

Samples for analytical determinations were collected from both the inner (internal zone) and outer (external zone) leaves of the 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 located midway between the center of the head and the outer portion. The outer leaves exhibited a darker color and larger size than the inner leaves and were not compact in the lettuce head.

Growth Parameters and Leaf Relative Water Content. At harvest, five plants of each cultivar of lettuce and treatment were randomly selected for determination of the fresh weight (FW) of the aerial part and biomass production of both shoots and roots. Dry matter (DM) of the leaves and roots was determined after the plant material was dried at 80 °C for 2 days. Before introduction of those plants 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 the FW. These leaf samples were then fully hydrated in darkness at 4 °C for 24 h to calculate the turgid weight (TW). Subsequently, the disk samples were dried at 80 °C for 2 days to calculate the DM. One square centimeter was approximately equivalent to 30 mg of FW in BRM and to 40 mg of FW in MV. The relative water content (RWC) was estimated by a modification of the method of Weatherley[20] and calculated as RWC = 100(FW − DM)/(TW − DM), and the results were expressed as percentages.

Mycorrhizal Analyses. 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) under the microscope. The results are expressed as the 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 the RMD allows the response of crop plants to mycorrhizal fungi to be ascertained. In addition, mycorrhizal colonization was characterized by assessing the presence or absence of arbuscules.

Mineral Analyses and Nitrate Concentration in Leaves. To determine the mineral nutrient content, samples (0.2 g of DM of either outer or inner leaves) of three lettuce plants per treatment were

Table 1. Growth Parameters of Lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), Nonmycorrhizal (NM) or Mycorrhizal (M), and Fertilized with Hewitt's Nutrient Solution (H), Single Superphosphate (SSP), or the Water-Insoluble (WI) Fraction of a Rhizosphere-Controlled Fertilizer (RCF)[a]

		shoot FW (g plant^{-1})	root FW (g plant^{-1})	shoot DM (g plant^{-1})	root DM (g plant^{-1})	root DM/shoot DM
				BRM		
NM	H	151.56 e	63.31 b	11.86 c	15.18 b	1.30 a
	SSP	178.56 cd	65.30 b	12.73 b	12.02 b	0.95 b
	WI	156.14 e	39.84 c	10.91 d	6.83 c	0.63 c
M	H	172.56 d	68.31 b	13.74 a	19.71 a	1.44 a
	SSP	201.90 a	82.87 a	14.37 a	22.88 a	1.59 a
	WI	182.36 bc	66.25 b	12.59 bc	7.44 c	0.59 c
				MV		
NM	H	154.66 d	65.38 b	12.92 cd	16.49 b	1.28 a
	SSP	176.60 c	69.21 b	13.46 c	16.37 b	1.21 a
	WI	152.58 d	57.34 c	12.66 d	14.90 b	1.18 a
M	H	181.28 b	57.86 c	15.44 b	14.90 b	1.00 a
	SSP	207.52 a	97.98 a	16.57 a	21.03 a	1.30 a
	WI	188.74 b	54.54 c	15.53 b	11.48 c	0.74 b

[a] FW = fresh weight; DM = dry matter. Values are means (n = 5). Within each parameter and cultivar of lettuce, the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

homogenized in a mill and then attacked with 6 mL of HNO_3 (65%) and 2 mL of H_2O_2 (33%) and digested in a microwave (Milestone, Ethos One). The digested samples were analyzed by induced coupled plasma optical emission spectrometry (ICP-OES; Thermo Elemental Co. Iris Intrepid II XDL, Waltham, MA). Shoot and root NO_3^- concentrations were evaluated from aqueous extraction as described by Lowe and Hamilton.[24] Briefly, 0.1 g of dry leaf was extracted with 10 mL of deionized water at 80 °C for 10 min. NO_3^- was determined by ion exchange chromatography (Dionex Corp., Sunnyvale, CA). The results are expressed on a wet basis.

Starch, Total Soluble Sugars, and Total Soluble Proteins in Leaves. Starch, total soluble sugars (TSSs), and total soluble proteins were quantified in potassium phosphate buffer (KPB; 50 mM, pH 7.5) extracts of fresh leaves (1 g of either outer or inner leaves per plant, five plants per treatment). These extracts were filtered through four cheesecloth layers and centrifuged at 38720*g* for 10 min at 4 °C. The pellet was used for starch determination.[25] The supernatant was collected and stored at 4 °C for TSSs and protein determinations. TSSs were analyzed with the anthrone reagent in a Spectronic 2000 (Bausch and Lomb, Rochester, NY).[26] Leaf soluble proteins were measured by the protein dye-binding method of Bradford[27] using bovine serum albumin (BSA) as the standard. The results are expressed as milligrams of starch, TSSs, or total soluble proteins per gram of FW. Outer and inner leaves were analyzed separately.

Chlorophylls and Carotenoids. Chlorophyll (Chl *a* + Chl *b*) and total carotenoid contents were determined according to Séstak et al.[28] One sample (1 cm^2) of fresh outer leaves and one sample (1 cm^2) of fresh inner leaves per plant (five plants per treatment) were immersed in 5 mL of 96% ethanol at 80 °C for 10 min to extract the pigments. The absorbance of the extracts was measured at 470, 649, 665, and 750 nm using a Spectronic 2000 (Bausch and Lomb, Rochester, NY). Chl *a* and Chl *b* and total carotenoids in the same extract solution were determined using the extinction coefficients and equations established by Lichtenthaler.[29] The results are expressed as milligrams of total chlorophylls (*a* + *b*) or carotenoids per gram of FW. Outer and inner leaves were analyzed separately.

Total Phenolics and Anthocyanins. Total phenolic compounds were extracted according to Chapuis-Lardy et al.[30] with some modifications. One sample (0.5 g of FW) of outer leaves and one sample (0.5 g of FW) of inner leaves per plant (three 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. The total phenolic content was determined from aqueous solutions by spectrophotometric analysis at 760 nm with Folin−Ciocalteu reagent.[31] 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,[32] the Folin−Ciocalteu method is commonly used to measure phenolic content. The results are expressed as milligrams of gallic acid per gram of FW. Outer and inner leaves were analyzed separately.

Anthocyanins were analyzed according to Cevahir et al.[33] with some modifications.[34] One sample (1 cm^2) of fresh outer leaves and one sample (1 cm^2) of fresh inner leaves (five 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 darkness to avoid degradation of the chlorophylls. After addition of 665 μL of distilled water, the chlorophylls were separated with 1.6 mL of chloroform. Particulates were removed by centrifugation at 26890*g* 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. The relative amount of anthocyanins was calculated as the optical density (OD) per gram of FW as described by Mancinelli.[35] Outer and inner leaves were analyzed separately.

Ascorbate. Ascorbate (ASC) and dehydroascorbate (DHA) contents were assayed photometrically by reduction of 2,6-dichlorophenolindophenol (DCPIP) according to Leipner et al.[36] Leaves (0.5 g of FW) 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 DHA to ASC and determine the total ASC pool (ASC + DHA). For the determination of ASC, the homocysteine solution was replaced by the same volume of water. After 15 min of incubation at 25 °C, 1 mL of citrate−phosphate buffer (2 M, 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. The content of ASC was calculated by reference to a standard curve. The amount of DHA resulted from the subtraction of the total ASC pool (ASC + DHA) and ASC.

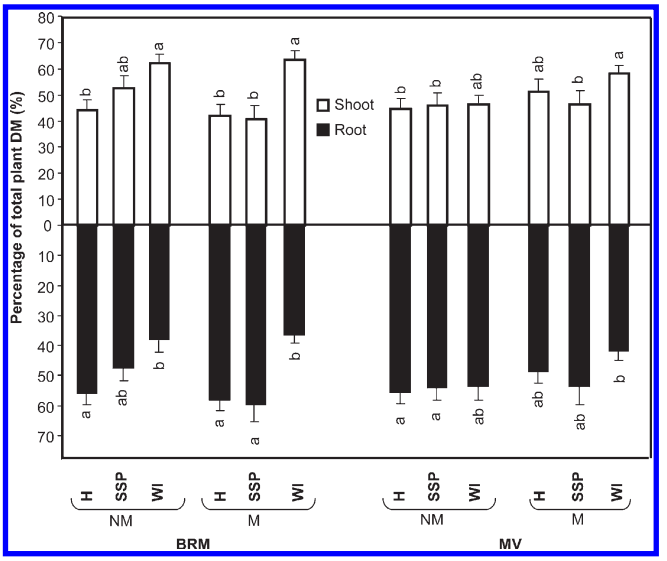

Figure 1. Total plant dry matter (DM) partitioning into different organs (shoots and roots) expressed as a percentage in lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means (n = 5) ± SE. Within each cultivar of lettuce and plant organ (shoot or root), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

Statistical Analysis. Within each cultivar of lettuce, data were subjected to a three-factor analysis of variance (ANOVA; factorial 2 × 3 × 2; SPSS version 15.0) to partition the variance into the main effects and the interaction between them. Inoculation or not with AMF and the P source were used as the first and second factors, respectively, as mentioned in the experimental design. The type of leaf (outer or inner leaf) in each lettuce plant was included as the third factor. Significant differences between factors were calculated at 5%. When interaction between factors was significant according to the ANOVA analysis, the least significant differences were evaluated using the least significant difference (LSD) *post hoc* test ($p < 0.05$).

RESULTS

Growth Parameters. In nonmycorrhizal BRM, the use of water-soluble sources of P (H or SSP) improved the shoot and root biomass in comparison with those of lettuces that received the WI fraction of the RCF (Table 1). However, these effects attributed to the distinct sources of P applied to plants were less evident in nonmycorrhizal MV.

Mycorrhizal symbiosis always enhanced growth of plants, regardless of the cultivar of lettuce and the type of fertilizer applied, with the aerial part benefiting more than roots from the mycorrhizal association. In fact, both the FW and DM of the shoots were always greater in mycorrhizal plants than in their respective nonmycorrhizal controls (Table 1).

When we compared growth of mycorrhizal lettuce plants, we found that water-soluble P fertilizers (H and SSP) were more effective than the WI fertilizer in improving the shoot and root DM of BRM (Table 1). In the cultivar MV, the greatest production of shoot and root biomass was observed in plants fertilized with SSP.

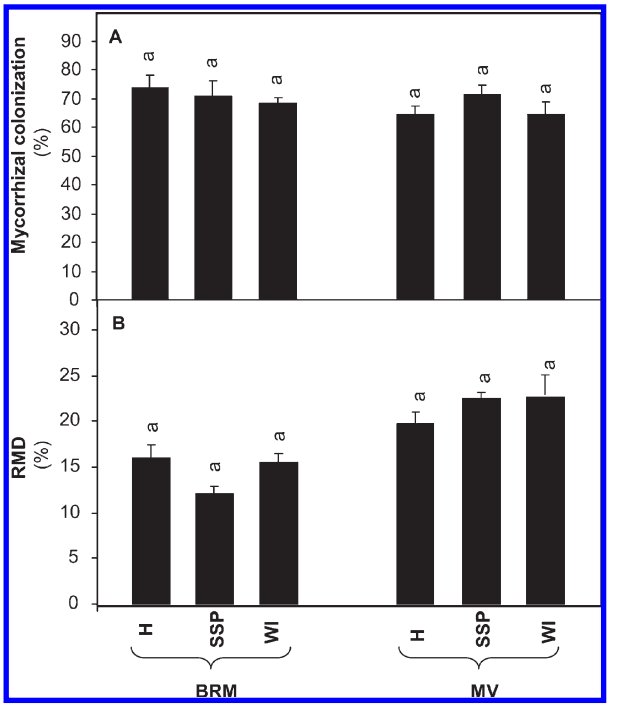

Figure 2. Percentage of mycorrhizal colonization (%) (A) and relative mycorrhizal dependency (RMD) (%) (B) in lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means (n = 5) ± SE. Within each cultivar of lettuce and parameter (mycorrhizal colonization or RMD), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

The ratio between the root DM and shoot DM always decreased in BRM after application of the WI fertilizer, regardless of whethere the plants were or were not associated with AMF (Table 1). In fact, while DM partitioning to roots in BRM fertilized with water-soluble sources of P (H or SSP) achieved values of around 50−60% of the total plant biomass (Figure 1), DM partitioning to the roots in plants that received the WI fertilizer was slightly lower than 40% of the total plant biomass. In MV, the use of WI only reduced the root to shoot DM ratio in mycorrhizal plants (Table 1). Data shown in Figure 1 demonstrate that the application of WI to MV associated with AMF induced the use of a larger amount of energy and resources for producing aerial parts, so that DM partitioning to the shoots achieved approximately 60% of the total plant biomass in detriment of root development.

Mycorrhizal Analyses. Percentages of mycorrhizal colonization reached values ranging between 64% and 74% after the application of the commercial inoculum (Figure 2A). We did not observe detrimental effects of any type of P fertilization on the establishment of the mycorrhizal symbiosis in either cultivar of lettuce. In addition, percentages of mycorrhizal colonization were similar in plants that received either water-soluble or water-insoluble sources of P. Lettuce plants that did not receive the commercial inoculum of AMF did not show mycorrhizal structures in the roots (data not shown). Therefore, we will refer indistinctly to these plants as "noninoculated" or "nonmycorrhizal" here.

When we calculated the RMD, the values were lower in BRM (around 15%) than those observed in MV (20−23%)

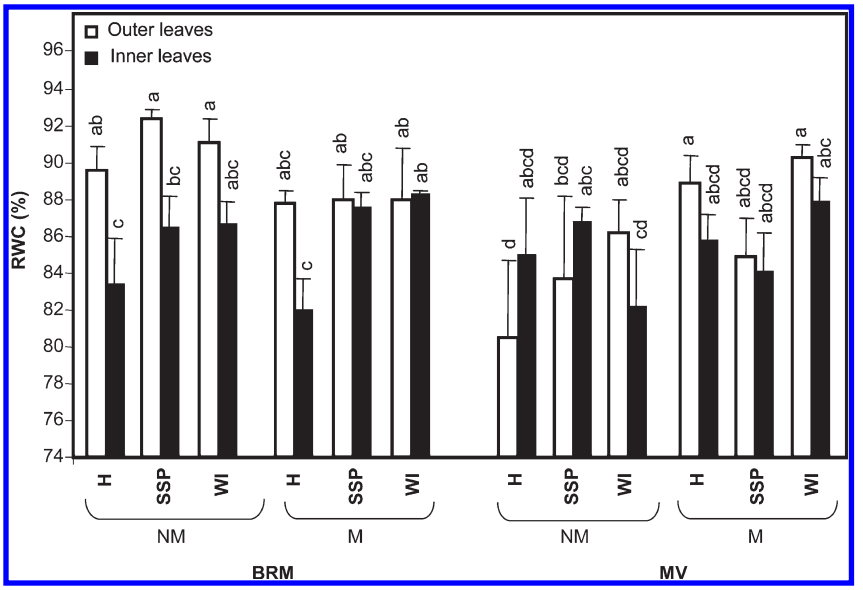

Figure 3. Relative water content (RWC) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means (n = 5) ± SE. Within each cultivar of lettuce, the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

(Figure 2B). Within each cultivar of lettuce, RMD was quite similar between plants fertilized with P sources differing in water solubility.

In BRM, the percentage of root fragments showing arbuscules varied from 68.9% when plants received the WI source of P to 71.1% when plants were fertilized with SSP. Similarly, in MV the highest incidence of arbuscules (75.6%) was found in roots of plants fertilized with SSP. In MV, the lowest percentage of root fragments showing arbuscules (65.2%) corresponded to plants that received modified Hewitt's nutrient solution (data not shown).

Leaf Relative Water Content. The leaf RWC always reached values above 82% because all plants were cultivated under optimal irrigation (Figure 3). When compared with nonmycorrhizal plants, we found that the cultivar BRM had more hydrated leaves than MV, independently of the P fertilizer applied. Moreover, the highest amount of water in BRM was measured in the outer leaves.

The association of lettuce plants with AMF had no significant effect on the RWC of leaves in BRM. In contrast, mycorrhizal MV accumulated higher amounts of water than nonmycorrhizal plants, with these increases being more evident when plants were fertilized with either modified Hewitt's nutrient solution or WI (Figure 3).

Mineral Analyses and Nitrate Concentration in Leaves. In general terms, external leaves accumulated higher concentrations of macronutrients (Ca, K, Mg, S, N) than internal leaves, with the exception of P (Table 2). When we compared the levels of several macronutrients in nonmycorrhizal and mycorrhizal plants, the concentrations were quite similar or even lower in lettuce associated with AMF presumably due to the dilution effect caused by the higher size of mycorrhizal plants. There were, however, some exceptions: outer leaves of mycorrhizal BRM accumulated greater contents of Ca, K, Mg, S, and N than their respective nonmycorrhizal controls when plants received the WI source of P nutrition. Moreover, when fertilized with any water-soluble P nutrition (H or SSP), the quantity of P in the inner leaves of BRM was greater in mycorrhizal than in nonmycorrhizal plants. In MV, the application of the WI source of P was the most efficient fertilization for nonmycorrhizal plants. However, in mycorrhizal MV, the greatest amounts of macronutrients were reached when plants were fertilized with either H or the WI source of P.

In general terms, the concentrations of the micronutrients B, Mn, Mo, and Zn were higher in outer than in inner leaves, regardless of the cultivar, the presence or absence of AMF in the roots, and the source of P applied to the plants (Table 2). In BRM, the association of plants with AMF clearly enhanced the levels of Cu and Fe in the inner leaves when the plants were fertilized with either of the water-soluble sources of P (H or SSP). Mycorrhizal BRM also showed higher levels of Mo than nonmycorrhizal plants in external leaves after application of either SSP or WI.

Nitrate concentrations were always greater in external than in internal leaves, independently of the cultivar, presence or absence of mycorrhizal association, and source of P nutrition applied to the plants (Figure 4). Mycorrhization increased the levels of NO_3^- in the outer leaves of BRM fertilized with either modified Hewitt's nutrient solution or the WI source of P. The highest value of NO_3^- reached 30 ppm on a wet basis in plants that received the aforementioned water-soluble source of P. In contrast, the association of lettuce MV with AMF decreased the levels of nitrates both in outer and inner leaves of plants fertilized with either the water-soluble SSP or the water-insoluble WI source of P.

Significant interactions among inoculation with AMF, P source, and type of leaf were observed for many mineral nutrients (Ca, K, Mg, P, S, Si, N, Cu, Fe, and Mn) and nitrate concentrations in both cultivars of lettuce (BRM and MV) (Table 1 in the Supporting Information).

Starch, Total Soluble Sugars, and Total Soluble Proteins in Leaves. The concentrations of starch in the leaves of the cultivar

Table 2. Concentrations of Mineral Nutrients in the Outer and Inner Leaves of Lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV)[a]

treatment			[Ca] (mg g^{-1} of FW)	[K] (mg g^{-1} of FW)	[Mg] (mg g^{-1} of FW)	[P] (mg g^{-1} of FW)	[S] (mg g^{-1} of FW)	[N] (mg g^{-1} of FW)	[B] (ppm)	[Cu] (ppm)	[Fe] (ppm)	[Mn] (ppm)	[Mo] (ppm)	[Zn] (ppm)
							BRM							
NM	H	outer leaves	0.84 d	6.42 c	0.39 e	0.13 h	0.17 c	3.16 b	2.97 c	0.55 d	5.75 h	27.04 c	0.21 b	7.57 dc
		inner leaves	0.38 ef	4.34 ef	0.21 f	0.19 d	0.16 c	2.31 d	1.25 ef	0.57 d	5.00 h	8.97 e	0.21 b	3.95 f
	SSP	outer leaves	0.98 c	7.68 b	0.49 c	0.16 f	0.19 b	2.78 c	3.43 bc	0.62 cd	17.41 de	31.58 b	0.19 b	8.24 d
		inner leaves	0.30 f	4.01 f	0.20 fg	0.20 cd	0.14 d	2.08 e	1.26 ef	0.63 cd	9.01 g	7.45 f	0.10 d	4.09 ef
	WI	outer leaves	1.04 b	7.69 b	0.54 b	0.16 f	0.18 b	2.82 c	3.21 b	0.76 bc	22.94 c	33.57 ab	0.23 b	18.30 a
		inner leaves	0.35 ef	4.64 e	0.24 f	0.24 b	0.16 c	1.87 f	1.38 de	2.25 a	75.13 a	8.61 ef	0.19 b	9.85 c
M	H	outer leaves	0.84 d	7.65 b	0.45 d	0.15 g	0.18 b	3.36 a	2.95 c	0.53 d	4.94 h	22.18 d	0.22 b	7.31 dc
		inner leaves	0.39 e	4.69 de	0.25 f	0.21 c	0.17 c	2.05 e	1.42 de	0.75 b	18.66 d	8.35 ef	0.14 c	4.34 ef
	SSP	outer leaves	0.82 d	7.62 b	0.44 d	0.17 ef	0.18 b	2.64 c	2.97 c	0.56 d	12.49 f	22.33 d	0.24 b	6.90 c
		inner leaves	0.34 f	5.07 d	0.24 f	0.26 a	0.19 b	2.17 e	1.56 d	0.78 b	17.53 de	7.21 f	0.10 d	5.05 e
	WI	outer leaves	1.11 a	8.54 a	0.58 a	0.17 e	0.21 a	3.33 a	3.98 a	0.75 bc	25.50 b	33.85 a	0.32 a	14.35 b
		inner leaves	0.25 g	3.49 g	0.18 g	0.17 e	0.12 e	1.47 f	1.03 f	0.61 d	16.19 e	5.75 g	0.07 d	3.81 f
							MV							
NM	H	outer leaves	1.05 b	6.52 c	0.37 b	0.13 d	0.15 c	2.21 c	2.27 b	0.56 abc	8.98 bc	27.08 a	0.15 abc	5.38 cd
		inner leaves	0.60 f	3.77 f	0.24 de	0.19 b	0.18 b	1.95 d	1.36 d	0.66 ab	5.48 d	13.36 f	0.10 d	5.33 cd
	SSP	outer leaves	0.82 d	5.39 d	0.33 b	0.12 d	0.14 c	1.58 c	1.78 c	0.42 d	14.09 a	22.38 c	0.13 bcd	3.59 g
		inner leaves	0.56 g	3.96 ef	0.23 e	0.22 a	0.19 a	2.12 d	1.25 d	0.61 abc	9.47 bc	10.60 g	0.10 cd	3.85 fg
	WI	outer leaves	1.27 a	7.89 a	0.43 a	0.15 c	0.18 b	2.56 a	2.57 a	0.68 a	16.15 a	30.15 a	0.19 a	8.44 a
		inner leaves	0.66 e	4.00 ef	0.23 e	0.21 a	0.19 a	1.96 d	1.37 d	0.49 cd	8.11 cd	10.81 g	0.09 d	4.72 d
M	H	outer leaves	0.98 c	6.85 b	0.38 b	0.14 c	0.18 b	2.31 b	2.34 ab	0.64 ab	10.50 b	23.97 b	0.17 ab	5.01 de
		inner leaves	0.54 g	4.06 ef	0.24 de	0.21 a	0.20 a	2.20 c	1.28 d	0.54 abc	7.64 cd	10.45 g	0.14 bcd	4.85 def
	SSP	outer leaves	0.67 e	5.18 e	0.26 d	0.11 d	0.13 d	1.32 f	1.77 c	0.38 d	6.13 d	15.59 e	0.11 cd	4.03 efg
		inner leaves	0.57 fg	4.11 ef	0.22 e	0.21 a	0.18 b	1.88 e	1.36 d	0.48 cd	5.78 d	10.19 g	0.09 d	4.18 efg
	WI	outer leaves	0.99 c	6.99 b	0.38 b	0.15 c	0.18 b	2.00 d	2.46 ab	0.52 b	10.51 b	20.22 d	0.18 ab	6.83 b
		inner leaves	0.61 f	4.23 e	0.25 de	0.21 a	0.20 a	2.03 d	1.38 d	0.56 abc	8.23 bc	10.08 g	0.12 cd	6.14 bc

[a] Values are means of nine observations (three samples, three measurements per sample). Within each column and cultivar, data followed by the same letter indicate that the values did not differ significantly ($p \leq 0.05$). NM = nonmycorrhizal, M = plants inoculated with the commercial inoculum, H = modified Hewitt's nutrient solution, SSP = single superphosphate, and WI = water-insoluble fraction of a rhizosphere-controlled fertilizer.

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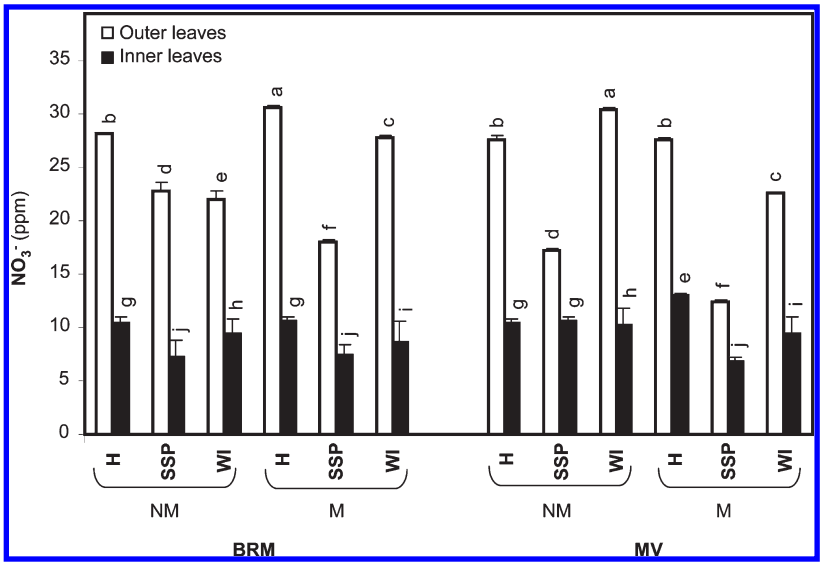

Figure 4. Nitrate (NO_3^-) concentrations (ppm) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means ($n = 3$) ± SE. Within each cultivar of lettuce, the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$). Values are expressed on a wet basis.

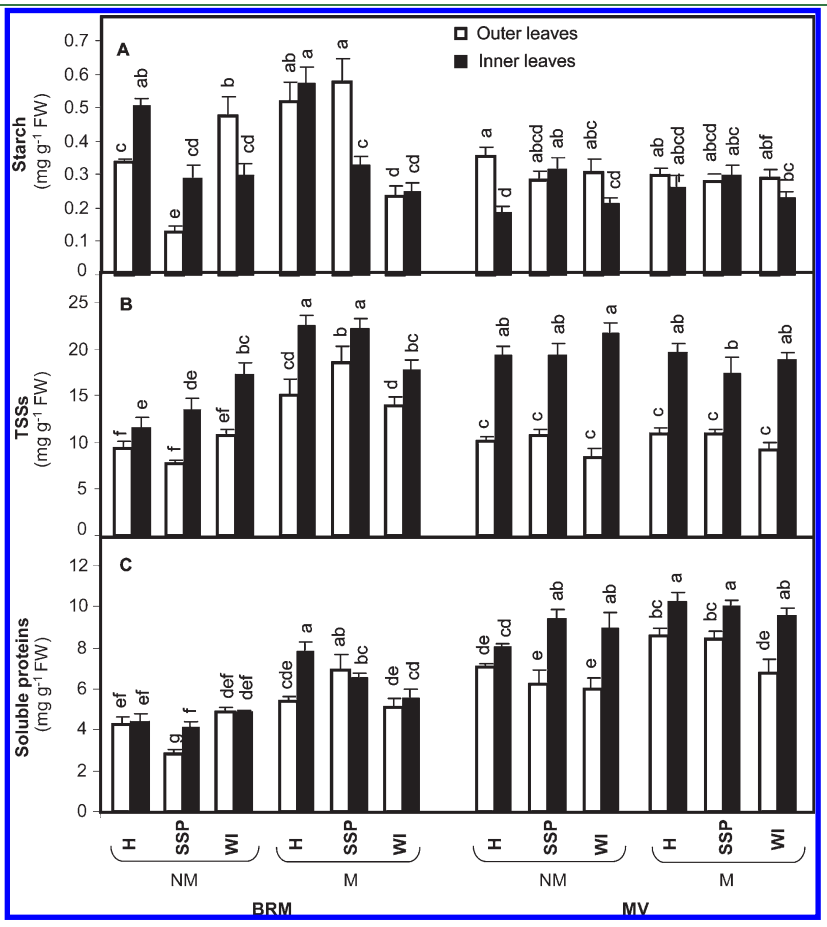

Figure 5. Concentrations (mg g^{-1} of FW) of starch (A), total soluble sugars (TSSs) (B), and total soluble proteins (C) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means of five observations (five samples per treatment, one measurement per sample) ± SE for starch. Values are means of ten observations (five samples per treatment, two measurements per sample) ± SE for TSSs and soluble proteins. Within each cultivar of lettuce and parameter (starch, TSSs, or total soluble proteins), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

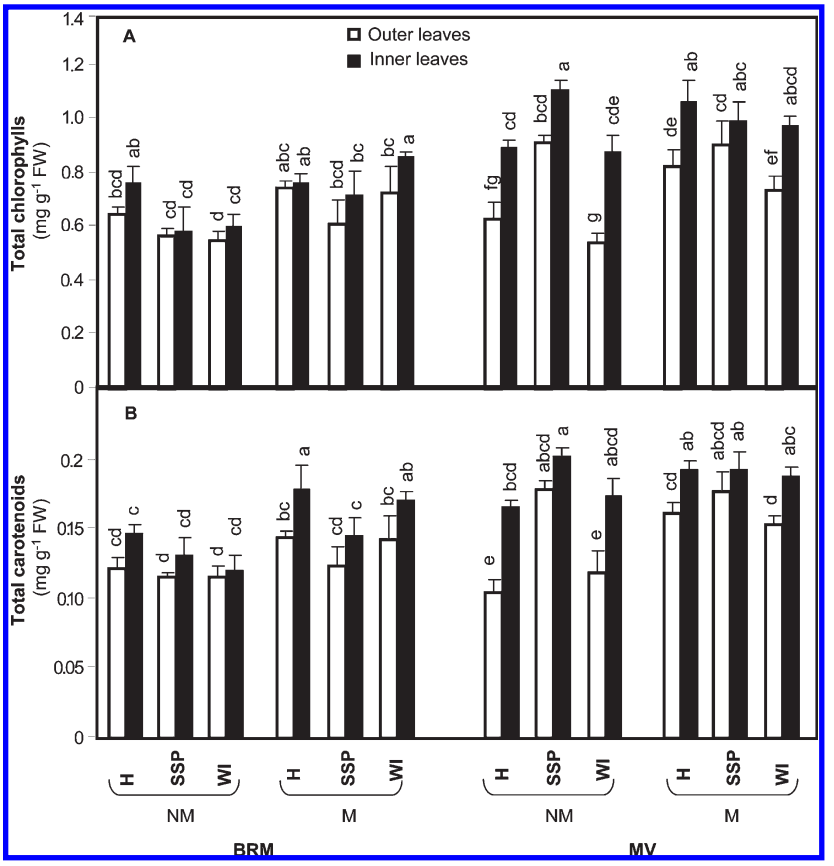

Figure 6. Concentrations (mg g^{-1} of FW) of total clorophylls ($a + b$) (A) and total carotenoids (B) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means ($n = 5$) ± SE. Within each cultivar of lettuce and parameter (total clorophylls or total carotenoids), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

BRM depended on both the source of P applied and the presence or absence of AMF associated with the roots (Figure 5A). In nonmycorrhizal plants, fertilization with SSP reduced the starch content, with such decreases being more evident in outer than in inner leaves. On the other hand, while the use of Hewitt's solution increased the accumulation of starch in inner leaves, the application of WI enhanced the quantity of starch in external leaves. Mycorrhizal symbiosis induced the accumulation of starch in the leaves of plants that received water-soluble P sources. In contrast, mycorrhizal plants had lower concentrations of starch in the leaves than their respective nonmycorrhizal controls when the plants were fertilized with the WI source of P. In the cultivar MV the starch content of the leaves was quite comparable in nonmycorrhizal and mycorrhizal plants. Moreover, in this cultivar the starch levels were not dependent on the type of fertilizer applied.

Internal leaves of lettuce always accumulated higher amounts of TSSs than external leaves, especially in the cultivar MV (Figure 5B). The application of the WI source of P fertilizer slightly increased the concentrations of TSSs in BRM compared with plants that received water-soluble sources of P. The association of BRM with AMF produced significant enhancements in the TSSs content in both outer and inner leaves in comparison with their respective nonmycorrhizal controls, with such increases being greater when the plants were fertilized with water-soluble P sources. As was the case for the pattern observed for starch, the TSSs content in the leaves of MV were comparable in nonmycorrhizal and mycorrhizal plants and the concentrations of TSSs did not differ when different sources of P were applied.

The highest quantities of proteins in the leaves were measured in the cultivar MV, and those contents increased when the plants were associated with AMF and fertilized with water-soluble P sources (Figure 5C). The beneficial effect of mycorrhization on protein levels was also observed in the leaves of plants belonging to the cultivar BRM when they received water-soluble P fertilization (H or SSP).

Significant interactions among inoculation with AMF, P source, and type of leaf were found for concentrations of starch, TSSs, and proteins in BRM (Table 1 in the Supporting Information).

Chlorophylls and Carotenoids. When nonmycorrhizal plants were compared, MV had higher concentrations of total chlorophylls than BRM in both outer and inner leaves when the plants were fertilized with SSP and in internal leaves when the plants received the WI source of P (Figure 6A). Mycorrhizal colonization increased the levels of chlorophylls in BRM fertilized with the WI source of P.

Outer and inner leaves of nonmycorrhizal BRM accumulated similar amounts of total carotenoids independently of the type of P fertilizer (Figure 6B). In contrast, the application of the SSP increased the concentrations of carotenoids in the leaves of nonmycorrhizal MV compared with plants fertilized with either Hewitt's solution or the WI source of P, with this enhancement being more significant in external than in internal leaves. In

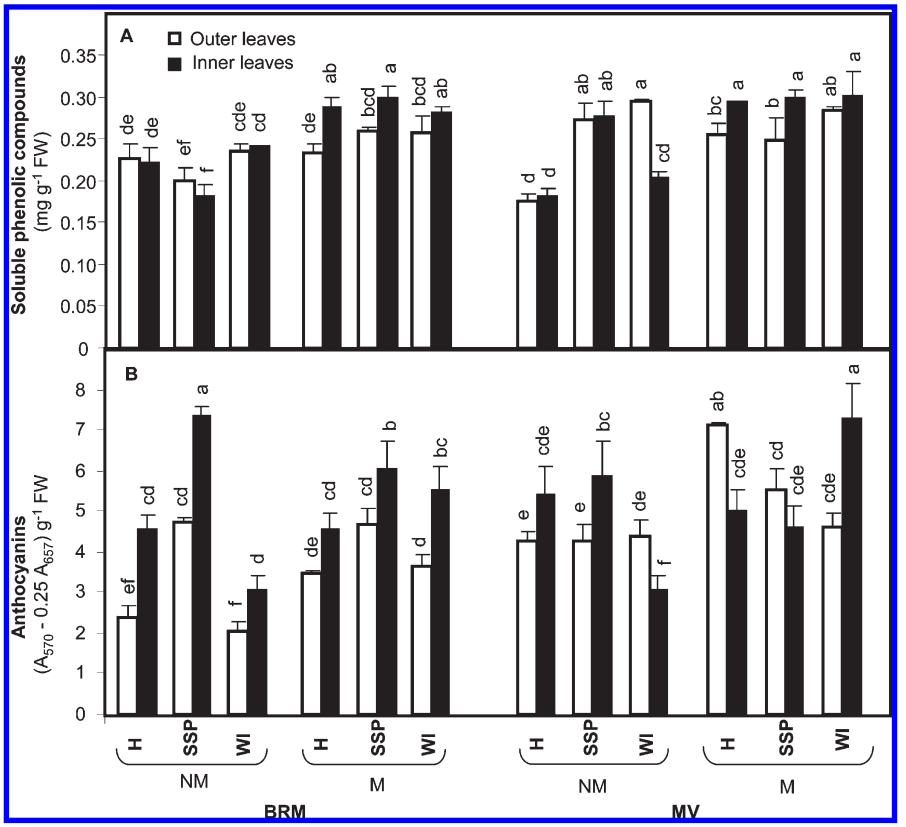

Figure 7. Concentrations of soluble phenolic compounds (A) and anthocyanins (B) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Concentrations of phenolic compounds are expressed in milligrams per gram of FW. Concentrations of anthocyanins are expressed as optical density (OD) ($A_{530} - 0.25A_{657}$) per gram of FW. Values are means ($n = 3$ for phenolics and $n = 5$ for anthocyanins) ± SE. Within each cultivar of lettuce and parameter (soluble phenolic compounds or anthocyanins), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

general terms, the association of lettuce with AMF resulted in increased quantities of total carotenoids in the leaves in comparison with nonmycorrhizal controls, and these increases were more evident when the plants were fertilized with either Hewitt's nutrient solution or the WI source of P. However, while in BRM mycorrhizal symbiosis enhanced the carotenoid content in the inner leaves, in MV mycorrhizal symbiosis increased the concentrations of carotenoids in the outer leaves.

Total Phenolics and Anthocyanins. The source of P applied to plants had no significant influence on the concentrations of phenolic compounds in the leaves of nonmycorrhizal BRM (Figure 7A). In contrast, in MV, the highest contents of phenolics were measured in both outer and inner leaves of plants fertilized with SSP and in external leaves of plants that received the WI source of P. The association of lettuce with AMF resulted in a greater quantity of phenolic compounds in the inner leaves of BRM, regardless of the type of P source applied, as well as in both outer and inner leaves of MV fertilized with Hewitt's nutrient solution and in internal leaves of MV that received the WI source of P compared with their respective nonmycorrhizal controls.

In nonmycorrhizal plants the highest concentrations of anthocyanins were found in the leaves of the red cultivar MV (Figure 7B), with the only exception being the inner leaves of BRM fertilized with SSP. Mycorrhizal symbiosis clearly enhanced the levels of anthocyanins in the inner leaves of plants fertilized with the WI source of P, regardless of the cultivar of lettuce.

The interaction among inoculation with AMF, P source, and type of leaf was significant for total phenolics in BRM and anthocyanins in MV (Table 1 in the Supporting Information).

Ascorbate. No significant effect of P source was observed on the total ASC concentrations in both outer and inner leaves of the two varieties of lettuce regardless of their association or not with AMF (Figure 8A). However, mycorrhizal symbiosis enhanced the amount of total ascorbate in BRM. In contrast, the levels of reduced ASC in the leaves of BRM were not affected by either the source of P applied or the presence or absence of mycorrhizal symbiosis (Figure 8B). In MV, the association of lettuce with AMF decreased the quantity of reduced ASC in external leaves of plants that received modified Hewitt's nutrient solution and in internal leaves of plants fertilized with SSP. Contrariwise, mycorrhizal symbiosis slightly increased the levels of reduced ascorbate in internal leaves of MV fertilized with the WI source of P.

Significant interaction among inoculation with AMF, P source, and type of leaf was observed for the concentration of reduced ASC in the MV cultivar (Table 1 in the Supporting Information).

DISCUSSION

High levels of available P in soil can negatively affect mycorrhizal symbiosis by delaying the establishment of mycorrhizal associations, decreasing the rate of infection along the root

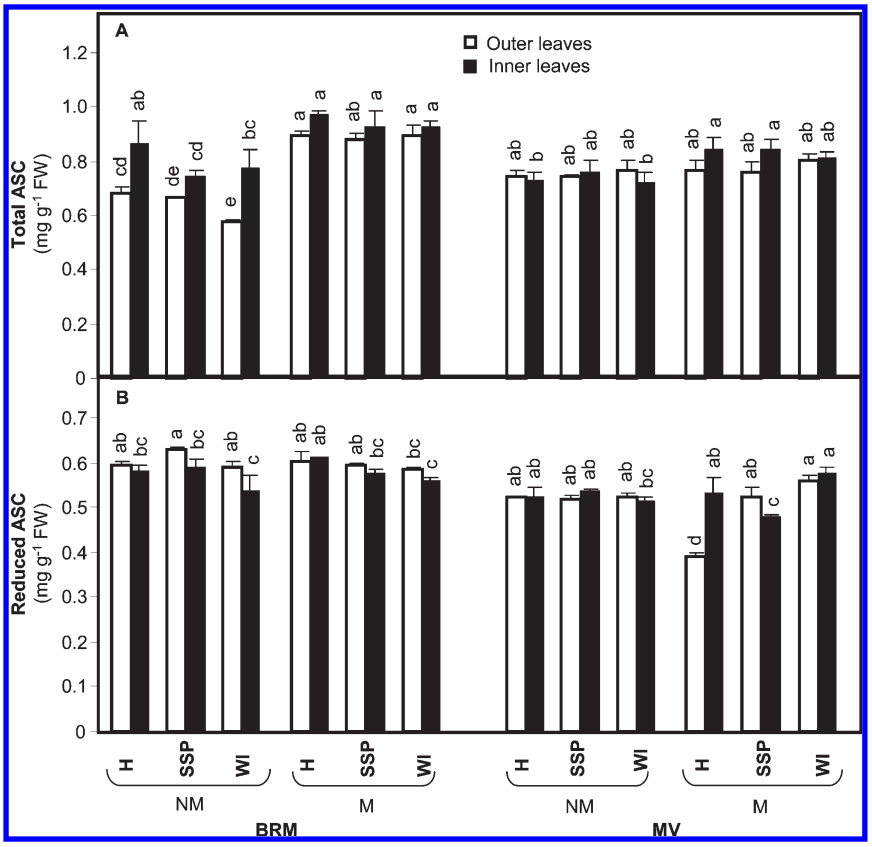

Figure 8. Concentrations (mg g^{-1} of FW) of total (A) and reduced (B) ascorbate (ASC) in outer (white histograms) and inner (black histograms) leaves of lettuces Batavia Rubia Munguía (BRM) and Maravilla de Verano (MV), nonmycorrhizal (NM) or mycorrhizal (M), and fertilized with modified Hewitt's nutrient solution (H), single superphosphate (SSP), or the water-insoluble (WI) fraction of a rhizosphere-controlled fertilizer (RCF). Values are means ($n = 3$) ± SE. Within each cultivar of lettuce and parameter (total or reduced ASC), the coincidence of letters indicates that the values are not significantly different ($p \leq 0.05$).

cortex, and/or reducing the intensity of internal colonization.[37] In our study, however, rates of mycorrhizal colonization were high and similar in all lettuce plants after inoculatation with the commercial formulation of AMF, regardless of the cultivar of lettuce and the source of P applied. In addition, although Ryan and Graham[38] concluded that highly available soil P often causes the carbon costs to the host plant to outweigh any benefits from mycorrhizal colonization, mycorrhizal lettuce plants (BRM and MV) fertilized with either water-soluble source of P (modified Hewitt's solution or SSP) or the WI fraction of RCF (with high solubility in solutions containing the main organic acids released to the rhizosphere by plant roots) achieved significantly greater size than their respective noninoculated controls.

Mycorrhizal symbiosis can benefit plant growth due to uptake of nutrients with low mobility, such as P.[39] In a study in which plants were fertilized with modified Hewitt's solution, Baslam et al.[13] found that lettuce associated with AMF achieved greater size than nonmycorrhizal plants. Results of the present study confirm the previous findings and demonstrate that the association of lettuce with AMF also enhances growth of lettuce when WI P sources are applied. Moreover, the improved plant development took place despite the lower P concentrations in the shoots of mycorrhizal BRM as compared with nonmycorrhizal plants when fertilized with WI and also occurred regardless of the similar P concentrations achieved in shoot tissues of mycorrhizal and nonmycorrhizal MV that received WI. These findings are in agreement with those of Toussaint et al.[19] working with sweet basil plants associated or not with AMF and fertilized with the water-insoluble dicalcium phosphate ($CaHPO_4$). Mycorrhizal symbiosis also allowed MV lettuce to significantly reduce the root to shoot ratio compared with their nonmycorrhizal controls when both types of plants were fertilized with WI, which could be related to the high RMD observed in the red cultivar of lettuce.

Mycorrhizal BRM showed higher levels of nonstructural carbohydrates (starch and TSSs) in the shoots than nonmycorrhizal plants, suggesting greater photosynthetic rates in the leaves of BRM associated with AMF.[13,40] The enhanced production of sugars could explain the increased concentrations of some secondary metabolites (carotenoids, phenolics, or anthocyanins) in the leaves of mycorrhizal BRM. In fact, in mycorrhizal BRM, soluble sugars could be precursors of carotenoids accumulated in the leaves of plants fertilized with either modified Hewitt's solution or WI, phenolics in inner leaves of this cultivar (irrespective of the P source), and anthocyanins in both outer and inner leaves of plants that received WI. In contrast, in the red cultivar MV, the enhanced levels of some phytochemicals in mycorrhizal plants cannot be explained by increased concentrations of soluble sugars. In MV the association of plants with AMF favored the accumulation of soluble proteins. Some of these soluble proteins could presumably be key enzymes of secondary metabolite pathways. When fertilized with modified Hewitt's solution, mycorrhizal MV plants showed greater N concentrations in the shoots than nonmycorrhizal plants, suggesting higher N assimilation in plants associated with

AMF.[7,19] As hypothesized by Toussaint et al.,[19] higher N assimilation in mycorrhizal plants might contribute to higher production of phenylalanine ammonia lyase (PAL), a key enzyme involved in the synthesis of many phenolic compounds, which could explain the increased concentrations of soluble phenolic compounds in both outer and inner leaves and anthocyanins in external leaves of mycorrhizal MV. In contrast, when MV lettuces were fertilized with either SSP or WI, mycorrhizal plants did not accumulate greater amounts of N in the shoots than nonmycorrhizal plants. However, in these cases, mycorrhizal plants had significantly lower levels of nitrate in the leaves than their respective noninoculated controls, indicating that the greater part of N may have been used to synthesize organic compounds, including enzymes involved in the production of secondary metabolites.

The dilution effect (especially significant in the cultivar MV) due to the greater size reached by mycorrhizal lettuce could explain the similar or lower concentrations of many macro- and micronutrients in leaves of plants associated with AMF when compared with their respective noninoculated controls. There were, however, several cases demonstrating that AMF can increase mineral nutrient content in lettuce, although the results depended on both the cultivar of lettuce and type of P fertilization applied. Similar to previous findings of our research group,[13] when the water-soluble sources of P (modified Hewitt's nutrient solution or SSP) were applied, Cu and Fe were in greater concentrations in the inner leaves of mycorrhizal BRM than in their respective noninoculated controls. When lettuce plants were fertilized with the WI source of P, BRM was the cultivar that benefited most from the mycorrhizal symbiosis: levels of Ca, Mg, K, S, N, B, Fe, and Mo in external leaves were enhanced by AMF. In MV, the presence of AMF only improved Zn concentrations in inner leaves of plants that received the WI source of P. Enhancements of K, S, Cu, Fe, Mo, or Zn are very interesting for human health. However, increases in the levels of these minerals by AMF mainly occurred in external leaves of lettuce, those usually stripped off at harvest. Consequently, according to our results, the preferential accumulation of minerals in outer leaves should be taken into account when lettuce is consumed as salads or used as food crop for the fourth range of vegetables.

In summary, the application of either water-soluble or water-insoluble sources of P did not exert any negative effect on the establishment of mycorrhizal symbiosis in the roots of lettuce (BRM and MV), and the growth of plants was significantly improved by AMF, regardless of the cultivar of lettuce and type of P fertilizer applied. Moreover, mycorrhizal symbiosis improved the nutritional quality of lettuce plants, although the effect of AMF was dependent on both the cultivar of lettuce and source of P nutrition. When SSP was applied, BRM was the cultivar which benefited most from mycorrhizal symbiosis, with the levels of Cu, Fe, TSSs, starch, proteins, phenolics, and total ASC being most enhanced when compared with those of nonmycorrhizal plants. When modified Hewitt's nutrient solution was used, mycorrhizal symbiosis improved the contents of nonstructural sugars (soluble sugars and starch) and total ASC in BRM, increased the levels of chlorophylls and anthocyanins in MV, and enhanced the accumulation of proteins, carotenoids, and phenolics in both BRM and MV. Finally, when the WI was the source of P, mycorrhizal BRM showed increased quantities of K, S, N, Fe, Mo, and total ASC, mycorrhizal MV accumulated enhanced amounts of Zn, and both cultivars had increased contents of chlorophylls, carotenoids, phenolics, and anthocyanins when associated with AMF. The possible presence of some microorganisms accompanying the mycorrhizal population could also contribute to enhancement of the beneficial effect of AMF on the nutritional quality of greenhouse-grown lettuces.[41]

■ ASSOCIATED CONTENT

Supporting Information. Table listing the interactions among factors (AMF, P source, and type of leaf) for parameters related to the nutritional quality of lettuces cv. Batavia Rubia Munguía and cv. Maravilla de Verano. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AMF, arbuscular mycorrhizal fungi; ASC, ascorbate; BRM, Batavia Rubia Munguía; Chl, chlorophyll; DM, dry matter; FW, fresh weight; H, modified Hewitt's nutrient solution; MV, Maravilla de Verano; NM, nonmycorrhizal; RCF, rhizosphere-controlled fertilizer; RMD, relative mycorrhizal dependency; RWC, relative water content; SSP, single superphosphate; TSSs, total soluble sugars; TW, turgid weight; WI, water insoluble fraction of a "rhizosphere-controlled fertilizer"

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