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Mycorrhizal colonization and drought stress affect $\delta^{13}C$ in $^{13}CO_2$-labeled lettuce plants

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We studied the role of different arbuscular-mycorrhizal (AM) fungi on lettuce (*Lactuca sativa* L.) plant carbon metabolism under drought stress. Plants were grown in pots maintained at two levels of soil moisture and labeled during photosynthesis with $^{13}CO_2$. P-fertilized plants were used as a non-mycorrhizal control. Well-watered mycorrhizal plants showed similar growth to that of P-fertilized plants. The level of mycorrhizal root infection was not significantly affected by fungal species or by water treatment. In contrast, important differences in $\delta^{13}C$ between P-fertilized and AM plants were found in shoot and root tissues as a consequence of both water limitation and fungal presence. $\delta^{13}C$ in shoots and roots increased in non-mycorrhizal treatment as compared with the well-watered plants, whereas this parameter decreased significantly in mycorrhizal plants. Photosynthetic activity was increased in AM plants in well-watered and droughted plants. *G. deserticola* was the most beneficial endophyte for water use efficiency in both water treatments. Transpiration rate was not affected by any of the treatments. On the basis of total ^{13}C in plant tissues, in AM plants the newly fixed C seemed to be preferentially utilized for fungal activity rather than being stored in roots.

Introduction

Efficient water use confers drought tolerance to plants and is the most important of all yield components (Martin and Thorstenson 1988). Plant production is highly dependent on carbon fixation, although the growth of plants is the result of integrated processes of assimilation, translocation, storage and utilization of photoassimilated carbon (Kouchi and Yoneyama 1984a,b, Jordan and Habib 1996). Utilization of tracers is one of the most straightforward methods to understand these integrated processes (Kouchi and Yoneyama 1984a). In recent years, the use of stable C isotopes has become frequent for agricultural and ecological studies (Tieszen and Boutton 1988, Acevedo 1993). The two stable isotopes of C are ^{12}C and ^{13}C, which represent 98.9 and 1.1%, respectively, of all C in nature (Farquhar et al. 1989). In addition to C isotopic studies based on natural $^{13}C/^{12}C$ variation, plants can also be labeled with $^{13}CO_2$ during photosynthesis to assess C allocation (Svejcar et al. 1990). Kouchi and Yoneyama (1984a,b) used long-term ^{13}C labeling to study accumulation, translocation and metabolism of photosynthetically assimilated C, whereas Mordacq et al. (1986) studied C flow to roots and respiratory losses in a chestnut coppice using $^{13}CO_2$. Boutton et al. (1987) labeled rice to obtain grains enriched in ^{13}C for nutrition studies.

Fungi are a sink for photosynthetic products in the mycorrhizal symbiosis and it has been estimated that about 4–20% of the total C fixed is used by the fungal partner in the root (Pang and Paul 1980, Kucey and Paul 1982, Harris et al. 1985, Wang et al. 1989, Wright et al. 1998a). While the fungal C demand can be seen as a 'cost' of symbiosis, it has been postulated that mycorrhizas can also stimulate C assimilation in order to compensate the cost on the plant's C economy and thus contribute to the overall benefit derived from association with mycorrhizal fungi (Fitter 1991, Tinker et al. 1994, Wright et al. 1998a). However, fungal consump-

Abbreviations – AM, arbuscular mycorrhizas; PDB, PeeDee Belemnite; PPFD, photosynthetic photon flux density; WUE, water use efficiency.

tion of carbohydrates can be critical for plant growth when environmental conditions are limiting, as happens under drought stress. Plants growing under water stress normally reduce stomatal apertures to decrease the rate of water loss. Consequently, intercellular CO_2 concentration and net photosynthesis decline, while the ratio $^{13}C/^{12}C$ increases (Martin and Thorstenson 1988, Farquhar et al. 1989, Stewart et al. 1995, Araus et al. 1997). Hence, it would be expected that both drought and mycorrhizal symbiosis can affect the process of CO_2 assimilation and C allocation. Drought should decrease sub-stomatal CO_2 concentration, leading to reduced photosynthesis, while it is expected that mycorrhizal symbiosis will help in maintenance of optimal CO_2 concentrations for photosynthesis. On the other hand, fungal activity will be a sink for photosynthetic products in roots and drought could decrease the rate of C allocation to mycorrhizal roots.

In previous studies on drought stress, we demonstrated the existence of non-nutritional effects on mycorrhizal plants due to the presence of the fungus: direct hyphal water uptake (Ruiz-Lozano and Azcón 1995), increased leaf-gas exchange parameters (Ruiz-Lozano et al. 1995a,b) or enhanced superoxide dismutase and nitrate reductase activities (Ruiz-Lozano et al. 1996, Ruiz-Lozano and Azcón 1996). We expand this information by testing whether or not mycorrhizal symbiosis can compensate for the detrimental effect of drought stress on CO_2 assimilation and also testing if drought can modify the pattern of C allocation to mycorrhizal roots. The study is based on measurements of growth, nutrition, leaf-gas exchange parameters, $^{13}C/^{12}C$ discrimination and total ^{13}C content in $^{13}CO_2$-labeled mycorrhizal and droughted plants.

Materials and methods

Experimental design and statistical analysis

The experiment consisted of a two-factor randomized complete block design of: (1) mycorrhizal treatment consisting of 3 *Glomus* species or a P-fertilized non-arbuscular mycorrhizas (AM) treatment and (2) two water treatments (well-watered or drought stressed), with 5 replications per treatment, totaling 40 pots (one plant per pot).

Data were subjected to analysis of variance (ANOVA) with AM treatment, water supply and AM treatment-water supply interaction as sources of variation and followed by Duncan's multiple range test (Duncan 1955). Percentage values were arcsin transformed before statistical analysis.

Soil and biological materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (< 1 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100°C for 1 h on 3 days). The soil had a pH of 8.1; 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay. Pots were filled with 500 g of the sterilized soil/sand mixture (1:1, v/v).

Mycorrhizal inoculum for each endophyte was bulked in an open-pot culture of *Allium cepa* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species, from the Zaidin Experimental Station collection (Ruiz-Lozano et al. 1995a), were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, *Glomus fasciculatum* (Thax. sensu Gerd.) Gerd and Trappe and *Glomus deserticola* (Trappe, Bloss and Menge). Five grams of inoculum possessing similar characteristics (an average of 30 spores g^{-1} and 75% of root infected) of the 3 *Glomus* isolates were added to each pot at sowing time just below *Lactuca sativa* L. cv. Romana seeds. P-fertilized pots received the same amount of autoclaved inoculum together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in order to provide a general microbial population free of AM propagules. Four seeds were sown in each pot and thinned after emergence to leave one seedling per pot.

Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% relative humidity, day/night temperatures of 25/15°C and a photoperiod of 16 h at a photosynthetic photon flux density (PPFD) of 460–500 μmol m^{-2} s^{-1} (Li-Cor, Lincoln, NE, USA, model LI-188B).

Water was supplied daily to maintain constant soil water potential close to -0.04 MPa during the first 4 weeks of plant growth. At this stage, half of the plants were allowed to dry until soil water potential reached -0.17 MPa for a further 4 weeks. Soil water potential was determined by a pressure plate apparatus (Soilmoisture Equipment Corp., Santa Barbara, CA, USA. 15 Bar Ceramic Plate Extractor, Cat. No 1500) and soil water content was measured by weighing the soil before and after drying at 110°C for 24 h (Bethlenfalvay et al. 1990).

Throughout the experiment, plants received 10 ml of Hewitt's nutrient solution lacking P (Hewitt 1952) each week. Non-mycorrhizal P-fertilized plants received P as KH_2PO_4 (7 mg P pot^{-1} $week^{-1}$). This P application was selected from a previous experiment to match growth and P concentration of AM plants.

The plants were labeled with ^{13}C for 1 h in a tight plexiglas chamber, 24 h before the end the experiment according to Svejcar et al. (1990). Briefly, a gas-tight syringe was used to inject 47 ml of 99% atom $^{13}CO_2$. The total CO_2 concentration following injection was determined in the chamber as 865 μmol mol^{-1}. One 8-cm diameter fan was used to mix the air during the labeling period. During labeling, photosynthetically active radiation increased and ranged from 1060 to 1240 μmol m^{-2} s^{-1} and chamber temperature averaged 2°C above ambient temperature which was from 21 to 23°C.

Parameters measured

The CO_2 exchange rate, the transpiration rate, sub-stomatal cavity CO_2 concentration and instantaneous water use efficiency (WUE) were measured on the fourth leaf from each plant, just before labeling. Atmospheric CO_2 was measured

Table 1. Shoot and root $\delta^{13}C$ (‰ vs PDB) in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. * $P\leq0.05$; ** $P\leq0.01$; *** $P\leq0.001$.

Treatment	$\delta^{13}C$ (shoot)		$\delta^{13}C$ (root)	
	Well-watered	Droughted	Well-watered	Droughted
P-fertilized	379b	447a	263ab	306a
G. mosseae	349b	277c	254b	124c
G. fasciculatum	337b	265c	219b	172c
G. deserticola	337b	231c	217b	170c
Significance of sources of variation				
AM treatment (A)	0.0000***		0.0067**	
Water regime (W)	0.0009***		0.0326*	
A × W	0.0002***		0.0474*	

5 m above ground level. PPFD was 1180 μmol m^{-2} s^{-1}, which ensured that no limitation in photon irradiance occurred (Long and Hällgren 1987). Light was provided by a halogen lamp (General Electric 300 PAR 56/WFL). A model LCA-3 portable, integrated infrared CO_2 analyzer (Analytical Development Co., Hoddesdon, UK) was used for these determinations. Measurements were made 2 h after the light was turned on.

To measure the $^{13}C/^{12}C$ ratio, we used an Elemental Analyzer (Fison NA 1500 NC) coupled to a continuous flow-isotope ratio mass spectrometer (Finnigan MAT 251) system. Stable C isotope ratios were determined and correction factors applied according to Craig (1957), and the results are expressed as $\delta^{13}C$ (‰ vs PeeDee Belemnite [PDB]) as indicated by Svejcar et al. (1990). For calculation of total ^{13}C in plant tissues $\delta^{13}C$ values were first converted into absolute ratios (R) and then into fractional abundances (F) according to the following relationships:

$$R = [(\delta^{13}C/1000) + 1] \times R_{PDB}$$

$$F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R + 1)$$

where $\delta^{13}C$ values are expressed relative to the PDB standard and R_{PDB} is the absolute ratio ($^{13}C/^{12}C$) of the PDB standard with a value of 0.0112372 (Craig 1957, Hayes 1982). The amount of ^{13}C in each tissue was calculated as the product of its corresponding fractional abundance (F) and the total C content (mg) in such tissue. Finally, allocation to a given tissue (shoot or root) was calculated from total ^{13}C values as:

(total ^{13}C in tissue/total ^{13}C in whole plant) × 100

At harvest (8 weeks after planting), the root system was separated from the shoot and weights were measured after drying in a forced draught oven at 70°C for 2 days. Shoot contents (mg $plant^{-1}$) of N (micro-Kjeldahl) and P (Olsen and Dean 1965) were determined.

The percentage of mycorrhizal root infection was estimated by visual observation after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). Quantification was performed using the grid-line intersect method (Giovannetti and Mosse 1980).

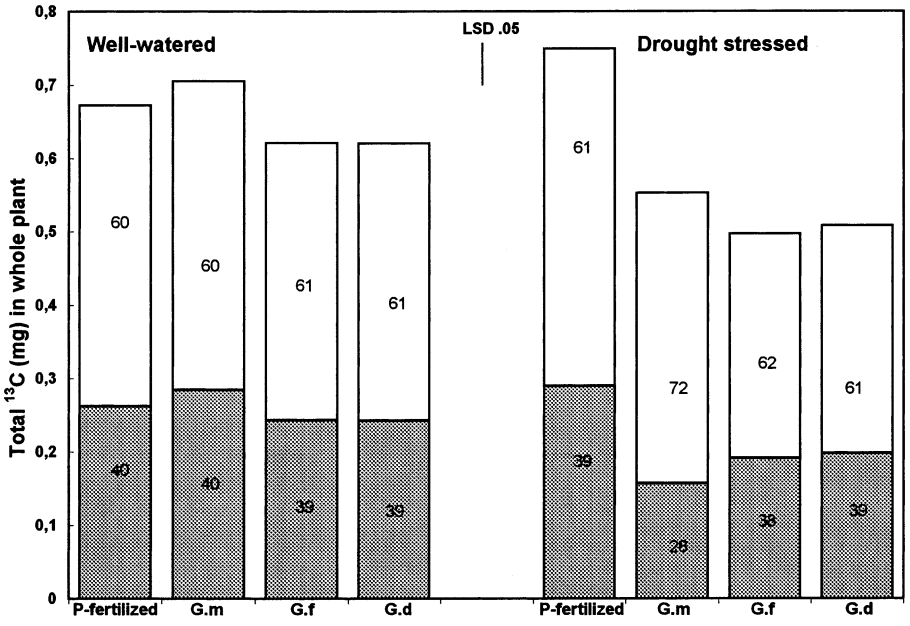

Fig. 1. Total ^{13}C content (mg, complete bars) and relative distribution of ^{13}C (%, values inside bars) between shoot (□) and root (▩) tissues in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. G.m, *Glomus mosseae*; G.f, *Glomus fasciculatum*; G.d, *Glomus deserticola*.

Table 2. Shoot and root dry weights (mg plant^{-1}) and mycorrhizal root infection (%) in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. * $P\leq0.05$; ** $P\leq0.01$; NS, not significant.

Treatment	Shoot DW		Root DW		Mycorrhizal infection	
	Well-watered	Droughted	Well-watered	Droughted	Well-watered	Droughted
P-fertilized	1 660ab	1 200c	1 215ab	957c	0b	0b
G. mosseae	1 660ab	1 600ab	1 450a	1 330ab	45a	42a
G. fasciculatum	1 570ab	1 477b	1 222ab	845c	47a	44a
G. deserticola	1 740a	1 662ab	980c	1 018bc	44a	50a
Significance of sources of variation						
AM treatment (A)	0.0715NS		0.0346*		0.3528NS	
Water regime	0.0024**		0.0760NS		0.0932NS	
A × W	0.0265*		NS		NS	

Results

Mycorrhizal inoculation did not affect $\delta^{13}C$ in well-watered plants (Table 1). By contrast, water limitation lead to important differences in $\delta^{13}C$ of shoots and roots between P-fertilized and AM plants (Table 1). An enhancement of $\delta^{13}C$ was observed in non-mycorrhizal droughted treatment as compared with the respective well-watered plants. In addition, $\delta^{13}C$ decreased significantly in mycorrhizal plants, both in shoots and roots, so that the 3 fungal treatments showed values below those of P-fertilized plants and also below those of mycorrhizal plants grown at well-watered conditions.

The total ^{13}C values for whole plant and the relative distribution between root and shoot tissues are shown in Fig. 1. In mycorrhizal plants, total ^{13}C was negatively affected by drought stress by 22, 20 and 18% in *G. mosseae*, *G. fasciculatum* and *G. deserticola*-colonized plants, respectively. Total ^{13}C increased in P-fertilized plants as consequence of drought, but this increase was not significant. Drought stress generally did not affect the ratio of carbon which is allocated to the root (about 40%) or which remains in shoot (about 60%). Only plants colonized by *G. mosseae* showed a decreased amount of C allocated to the roots from 40 to 28% as consequence of drought stress.

Shoot weight in mycorrhizal lettuce plants grown under well-watered conditions was similar to that of P-fertilized plants (Table 2). It was decreased by drought, and the decrease was significant in P-fertilized non-mycorrhizal plants (28%). A non significant decrease between 4 and 10% was observed in mycorrhizal plants. Similar trends in growth were observed for root and shoot (Table 2). Only well-watered *G. deserticola*-plants showed a lower root weight compared with the other well-watered treatments. No mycorrhizal colonization was found in uninoculated P-fertilized plants. Mycorrhizal colonization percentages in plants colonized by the three fungal isolates were similar and not affected by water availability (Table 2).

Table 3 shows that P-fertilized plants had the same N content as mycorrhizal plants, except in the case of *G. deserticola* plants under well-watered conditions. Nitrogen content was negatively affected by decrease in soil water status only in the case of *G. deserticola*-colonized plants.

Photosynthetic rates and WUE (Table 4) were higher in AM plants than in P-fertilized controls under both water regimes applied, with *G. deserticola*-colonized plants showing the highest values. Leaves of *G. deserticola*-infected plants showed a 64% enhancement of photosynthesis under both water regimes over non-mycorrhizal controls. The positive effect of the other two mycorrhizal fungi was also significant, but not as strong as that observed with *G. deserticola*. Increases in WUE under well-watered conditions were of 66% (*G. deserticola*), 44% (*G. mosseae*) and 21% (*G. fasciculatum*). Water limitation did not affect differences in WUE between mycorrhizal and control plants.

Transpiration rate (Table 5) was not affected in any of the treatments. The sub-stomatal cavity CO_2 concentration (Table 5) was increased in mycorrhizal plants in comparison with P-fertilized plants, particularly those colonized by *G. deserticola*. Watering regime affected sub-stomatal cavity CO_2 concentration only in plants inoculated with *G. fasciculatum*.

Table 3. Shoot N and P contents (mg plant^{-1}) in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. * $P\leq0.05$; ** $P\leq0.01$.

Treatment	N content		P content	
	Well-watered	Droughted	Well-watered	Droughted
P-fertilized	35.5bc	30.7c	3.0ab	1.9c
G. mosseae	41.2ab	35.7bc	3.0ab	2.5bc
G. fasciculatum	38.9ab	35.9bc	2.4bc	2.0bc
G. deserticola	43.3a	31.1c	3.6a	2.6b
Significance of sources of variation				
AM treatment (A)	0.0213*		0.0198*	
Water regime (W)	0.0084**		0.0092**	
A × W	0.0102*		0.0153*	

Table 4. Photosynthetic activity (μmol CO_2 m^{-2} s^{-1}) and instantaneous WUE (μmol CO_2 $mmol^{-1}$ H_2O) in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. *** $P \leq 0.001$; NS, not significant.

Treatment	Photosynthesis		WUE	
	Well-watered	Droughted	Well-watered	Droughted
P-fertilized	2.5de	2.2e	1.49e	1.44e
G. mosseae	3.7b	3.5b	2.14c	2.25bc
G. fasciculatum	3.0c	2.7cd	1.81d	1.72d
G. deserticola	4.1a	3.6b	2.47a	2.38ab
Significance of sources of variation				
AM treatment (A)	0.0000***		0.0000***	
Water regime (W)	0.0000***		NS	
A × W	0.3872 NS		NS	

Discussion

We have found previously, using the same test plant and experimental conditions, that AM fungi can enable lettuce plants to maintain growth under drought stress (Ruiz-Lozano et al. 1995a,b). The present results confirm and extend these findings.

Plants labeled with $^{13}CO_2$ for 1 h during photosynthesis showed a considerable increase in $\delta^{13}C$ relative to baseline values due to natural ^{13}C abundance (Farquhar et al. 1989). We expected that AM colonization would affect the $\delta^{13}C$ values when plants were growing under drought conditions. Water limitation decreased $\delta^{13}C$ values in mycorrhizal plants but in the control P-fertilized plants the behavior was the opposite. Plants exposed to drought stress normally show reduced stomatal aperture, decreasing the rate of water loss, so that the intercellular concentration of CO_2 goes down and, therefore, $\delta^{13}C$ goes up (Martin and Thorstenson 1988, Stewart et al. 1995, Araus et al. 1997). This appears to have happened in P-fertilized plants during the labeling period. Uninoculated plants also showed the lowest levels of sub-stomatal cavity CO_2 concentration. In contrast, mycorrhizal plants had significantly decreased $\delta^{13}C$ values, probably by maintaining high values of intercellular concentration of CO_2 during the labeling period. High intercellular concentration of CO_2 maintained photosynthesis at values similar to well-watered mycorrhizal plants, as was evidenced when determining photosynthetic activity and sub-stomatal CO_2 concentration just before labeling. In agreement with this hypothesis, mycorrhizal plants were also able to maintain WUE under drought stress conditions to values similar to those under well-watered conditions, which were considerably higher than in P-fertilized plants. As transpiration rates were not significantly affected, photosynthesis must have been maintained at similar levels between well-watered and drought stressed mycorrhizal plants, as also found by Morison (1985). Wright et al. (1998a,b) postulated that, in mycorrhizal plants, the stimulation of these physiological parameters may be caused by an increased sink strength arising from the additional C requirement of the mycorrhizal fungus colonizing the root.

The total ^{13}C values found in shoots were higher than those from root tissues. The likely explanation for this is that shoots are the source organ, whereas roots are the sink organ. Large amounts of currently fixed carbon in leaves are transported to roots or are lost by respiration (Jordan and Habib 1996). The decreased amounts of labeled carbon determined in droughted AM plants when compared with droughted controls suggest that either respiration is increased in AM plants or the fungal C requirement decreased the total amount of fixed C in mycorrhizal tissues. AM fungi are highly dependent on currently photosynthate (Kucey and Paul 1982, Harris et al. 1985, Wang et al. 1989). In agreement with our suggestion is the observation that the C sink of the fungus and respiration should have been higher than expected, since photosynthetic rate in mycorrhizal plants was greatly increased but not translated into enhanced total ^{13}C. We do not expect that the increase in respiration by mycorrhizal tissues was so high to justify the low ^{13}C values obtained in AM plants. Hence, we hypothe-

Table 5. Transpiration rate (mol H_2O m^{-2} s^{-1}) and sub-stomatal cavity CO_2 concentration (μmol CO_2 m^{-2} s^{-1}) in P-fertilized non-mycorrhizal or mycorrhizal lettuce plants grown under well-watered or drought stress conditions. Means followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan's multiple-range test (n = 5). Significance of the sources of variation is also displayed. *** $P \leq 0.001$; NS, not significant.

Treatment	Transpiration		Sub-stomatal CO_2 concentration	
	Well-watered	Droughted	Well-watered	Droughted
P-fertilized	1.7a	1.5a	277d	269d
G. mosseae	1.8a	1.6a	302b	302b
G. fasciculatum	1.7a	1.6a	290c	303b
G. deserticola	1.7a	1.5a	318a	308ab
Significance of sources of variation				
AM treatment (A)	0.3617 NS		0.0000***	
Water regime (W)	0.2102 NS		0.0716 NS	
A × W	NS		0.2269 NS	

size that the newly fixed C, instead of C stored in roots, seems to be preferentially utilized for fungal activity, as found by Wright et al. (1998a). Drought stress only modified the ratio of carbon allocated to the roots in *G. mosseae*-colonized plants, which decreased carbon allocation to roots from 40 to 28%. This may be related to lower C requirements of this fungus compared with *G. fasciculatum* (Kucey and Paul 1982) and, probably, with respect to *G. deserticola*. This effect can be important for maintenance of plant growth under such limiting conditions, as evidenced by growth data.

In conclusion, we show that combination of both mycorrhizal colonization and drought stress decreased $\delta^{13}C$ values in lettuce plants, as compared with uninoculated P-fertilized plants. This was related to improved leaf-gas exchange and WUE in AM plants. Our data also suggest that the newly fixed C is being preferentially utilized by the fungal partner, although this did not have a detrimental effect on the final plant production. Hence, C cost must be outweighed by other compensatory mechanisms which lead to an overall positive effect on the host plant.

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