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Effect of Calcium Application on the Tolerance of Mycorrhizal Lettuce Plants to Polyethylene Glycol-Induced Water Stress

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Abstract

Although the effect of arbuscular-mycorrhizal (AM) fungi on drought alleviation has been studied, there is little information on the role of Ca as a drought stress reliever in mycorrhizal symbiosis. *Lactuca sativa* plants were grown in a soil/sand mixture (1/9 v/v) with application of a nutrient solution containing increasing amounts of Ca. The water stress was imposed by dipping the plant root systems in the appropriate polyethylene glycol (PEG) solution for 22 h. The calcium increase in the medium decreased the colonizing ability of the fungi (*Glomus mosseae* and *G. fasciculatum*) assayed, but did not affect the growth of the mycorrhizal plants. In contrast, the control plants decreased their growth when the calcium reached 8 mM. Under both severe and moderate stress, mycorrhizal plants had decreased proline accumulation in response to the increase in Ca availability, indicating that mycorrhizal plants suffered less from the detrimental effect of PEG-induced water stress as a consequence of the Ca increase in the medium. Results of treatments on relative water content (RWC) paralleled those of proline. With 4 mM Ca in the medium, the mycorrhizal plants were more negatively affected by PEG application than the control plants, but above this calcium level the proline content decreased and the RWC increased in mycorrhizal plants. In the control plants the behaviour was reversed. This response could be due to the higher membranous surface area in

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mycorrhizal plants than in nonmycorrhizal plants. In conclusion, the application and uptake of increasing amounts of Ca enhances the tolerance of mycorrhizal plants to PEG-induced water stress.

Keywords: Calcium, mycorrhizal plants, polyethylene glycol, water stress

1. Introduction

AM fungi are important in sustainable agriculture because they increase mineral uptake, which reduces the use of fertilizers (Bethlenfalvay, 1992), they improve disease control (Graham and Menge, 1982) and they enhance the drought resistance of host plants (Allen and Allen, 1986; Ruiz-Lozano et al., 1995a; Ruiz-Lozano et al., 1995b).

Although the effect of AM fungi on drought alleviation has been studied (Sanchez-Diaz and Honrubia, 1994; Ruiz-Lozano et al., 1995a; Ruiz-Lozano et al., 1995b), there is little information on the role of Ca as a drought stress reliever in mycorrhizal symbiosis. In fact, calcium has an important role in plant metabolism, since it is required for numerous physiological processes (Ramalho et al., 1995). In addition to its role as a cellular messenger (Poovaiah, 1993), effects of Ca on integrity and stability of membranes (Allan and Rubery, 1991), rigidity of cell wall , maintenance of cell-to-cell contact, cell division and elongation (Ginzberg, 1961), polymerisation of proteins and enzyme regulation (Hepler and Wayne, 1985) have been reported.

Calcium is a nutrient of great interest in plants exposed to stress (Lynch and Läuchli, 1985). Optimal Ca content and proper distribution in individual organs prevents the incidence and severity of physiological disorders that are caused in many cases by unfavorable external conditions (Poovaiah, 1993). Therefore, it is suggested that the beneficial effect of supplemental Ca may, at least in part, be due to the maintenance of the integrity and function of the plasma membranes, both in roots and shoots, and also to a faster cellular stress recovery (Starch et al., 1994). In this sense, there is much information on the positive effect of supplemental Ca on plant tolerance to several stresses, mainly salinity (Ehret et al., 1990; Cachorro et al., 1994). Considerable evidence also shows that calcium acts as signal transducer in the expression of specific genes and in new protein synthesis under water stress conditions, resulting in an increase in the tolerance to water deficits by the plant (Roberts and Harmon, 1992).

The objective of this study was to determine, in mycorrhizal plants, the importance of Ca as a possible factor contributing to the mechanisms of plant protection against drought stress induced by a polyethylene glycol (PEG) solution.

2. Materials and Methods

The experiment used a randomized complete block design. The factors were: AM colonization (two AM isolates, one P-fertilized non-AM control, and one unfertilized non-AM control) and level of Ca applied in the nutrient solution (4 levels). Nine replicates per treatment were carried out, making a total of 144 experimental units.

The loamy soil used was collected from the grounds of the Estación Experimental del Zaidín (Granada), sieved (2 mm), diluted with quartz sand (1/9 v/v) and autoclaved (100°C, 1 h on 3 consecutive days). The soil had: pH 8.1; 1.8% organic matter; nutrient concentrations (mg kg^{-1}): N, 2.5; P ($NaHCO_3$-extractable P), 6.24; K, 132; and 41.5 mEq 100 g^{-1} of extractable Ca. The texture was made up of 35.8% sand, 46.3% silt and 20.5% clay. Pots were filled with 300 g of sterilized soil:sand (1/9, v/v) mixture.

Mycorrhizal fungal inoculum from each endophyte was multiplied in an open pot culture of *Lactuca sativa* L. and consisted of soil, spores, hyphae and AM root fragments. The AM species, belonging to the collection of the Estación Experimental del Zaidín (Ruiz-Lozano et al., 1995a) were *G. fasciculatum* (Thaxter Sensu Gerd.) and *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe. Five grams of each inoculum, having similar characteristics (an average of 30 spores g^{-1} and 75% mycorrhization), were placed directly below the seeds of *Lactuca sativa* L. cv. Romana. Nonmycorrhizal treatments received the same amount of autoclaved inoculum together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in an attempt to provide a general microbial population free of AM propagules. Four seeds were sown and thinned after emergence to one seedling per pot.

Plants were grown in a controlled environment chamber under conditions of 70–80% relative humidity (RH), day/night temperatures of 25/15°C, and a photoperiod of 16 h. Photosynthetic photon flux (PPF) was 500 μmol m^{-2} s^{-1}, as measured with a lightmeter (model LI-188B, LICOR, Lincoln, NE, USA).

Each week throughout the experiment, plants received 50 ml pot^{-1} of a nutrient solution described by Hepper and O'Shea (1984) and modified by Ruiz-Lozano and Azcón (1996). Four levels of Ca as ($CaSO_4$) were applied along the experiment: 2 mM, 4 mM, 6 mM and 8 mM. Nonmycorrhizal P-fertilized plants received P as KH_2PO_4 (7 mg pot^{-1} $week^{-1}$) to equalize size and tissue P concentration between mycorrhizal and nonmycorrhizal plants. The growth medium used in this experiment (similar to a hydroponic system) allowed plants to be similar in growth and nutritive status before the drought stress application.

A polyethylene glycol solution (purified PEG 6000 Merck, molecular weight 5000–7000) was used as osmoticum to induce moderate (10% PEG w/v) or severe

(20% PEG w/v) stress. After ten weeks of planting, mycorrhizal and non-mycorrhizal plants were carefully extracted from pots in order to maintain the radical system intact. The water stress was imposed by dipping for a period of 22 h the plant root systems in the appropriate PEG solution (Robin et al., 1989). A control without PEG was also tested.

Proline content in leaves was evaluated after the water stress imposition (by PEG, 10% and 20%, solutions). Proline was determined by colorimetry (Bates et al., 1973). The relative water content (RWC) was determined following the Barrs and Weatherley (1962) method. Five leaf discs (1 cm diameter) of five leaves per plant were weighed (fresh weight, FW) immediately after harvesting from the plant. They were placed in a Petri dish containing distilled water for 3 h at 25°C under fluorescent light of 40 W, and then their turgid weights (TW) were determined. The samples were then dried in an oven at 60°C for 24 h to obtain their dry weights (DW). The RWC was calculated as:

$$RWC = \frac{FW - DW}{TW - DW}$$

At harvest (10 weeks after planting), the root system was separated from the shoot and dry weight was determined of the shoots.

Concentrations of N (micro-Kjeldahl) and P (Olsen and Dean, 1965) were measured. Concentration of Ca was also determined by atomic absorption spectrometry (Lachica et al., 1973) using a Perkin-Elmer 5000 spectrophotometer.

The percentage of mycorrhizal root infection was estimated by visual observation of mycorrhizal infection 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).

Data were subjected to analysis of variance (ANOVA) with AM treatments and calcium level as factors. When the main effects were significant ($P<0.05$), differences among means were evaluated for significance by Duncan's multiple range test (Duncan, 1955) in an orthogonal design. For the percentage values an Arc Sin transformation was made before the statistical analysis.

3. Results

The Ca increase in the medium did not affect the growth of mycorrhizal plants (Table 1). In contrast, growth of control plants decreased when the Ca reached 8 mM. Non-inoculated plants fertilized with P showed the highest shoot dry weight regardless of the Ca level.

Table 1. Shoot dry weight (g) and radical colonization (%) in mycorrhizal (*Glomus mosseae* or *G. fasciculatum*) or non inoculated (Control and P-fertilized) lettuce plants grown in medium with four levels of Ca (2 mM, 4 mM, 6 mM or 8 mM).

	Shoot dry weight				% radical colonization			
Treatments	2 mM	4 mM	6 mM	8 mM	2 mM	4 mM	6 mM	8 mM
Control	2.8cd	2.8cd	2.8cd	2.2e	0d	0d	0d	0d
P-fertilized	3.4a	3.2ab	3.5a	2.9bc	0d	0d	0d	0d
G. mosseae	2.5de	2.6de	2.8cd	2.4dc	87a	81a	72bc	71bc
G. fasciculatum	2.9bc	2.8cd	2.6de	2.8cd	83a	74b	71b	66c

Within each parameter (four columns), values followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test ($n = 9$).

Table 2. N, P and Ca content (mg/plant) in mycorrhizal (*Glomus mosseae* or *G. fasciculatum*) or non inoculated (Control and P-fertilized) lettuce plants grown in medium with four levels of Ca (2 mM, 4 mM, 6 mM or 8 mM).

	N				P			
Treatments	2 mM	4 mM	6 mM	8 mM	2 mM	4 mM	6 mM	8 mM
Control	28.2f	31.1ef	49.2bc	53.8b	2.9de	3.0de	2.9de	2.5e
P-fertilized	37.7de	32.4ef	52.6b	59.8b	4.2a	3.9ab	3.5bc	3.3cd
G. mosseae	34.4ef	32.6ef	43.9cd	55.7b	3.5bc	3.3cd	2.9de	2.8e
G. fasciculatum	46.8c	43.1cd	53.7b	70.4a	3.3cd	3.3cd	3.1de	3.7bc

	Ca			
Treatments	2 mM	4 mM	6 mM	8 mM
Control	15.9f	20.5e	22.5de	22.7de
P-fertilized	21.3e	21.8de	24.8cd	26.2bc
G. mosseae	19.8e	20.1e	23.9d	27.0b
G. fasciculatum	23.4d	24.6cd	27.0b	29.5a

Within each parameter (four columns), values followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test ($n = 5$).

The percentage of root colonized by *G. mosseae* and *G. fasciculatum* decreased when the Ca applied reached 6 mM and 4 mM, respectively. In the case of *G. fasciculatum* this parameter showed the lowest value at 8 mM Ca (Table 1).

In relation to nutrient uptake (Table 2), the increased level of Ca in the medium increased the N uptake in all the treatments. *G. fasciculatum*-colonized plants showed the highest N content. Non-inoculated P-fertilized plants had more P than the other treatments. In general, the plant P content decreased in response to an increase in Ca in the medium (except in the case of plants colonized by *G. fasciculatum*).

All the treatments increased the Ca content in tissues in response to the higher availability of Ca in the medium. Plants inoculated with *G. fasciculatum* showed the highest Ca content at whatever level of Ca applied, while control plants showed the lowest.

In the absence of PEG the plant proline content was similar and very low for all the treatments, while the PEG-induced water stress during 22 h resulted in a considerable increase in proline (Fig. 1). The maximum amount of proline was reached at the treatment with 20% PEG solution (severe stress). However, under both severe and moderate stress (10% PEG solution) the proline accumulation of mycorrhizal plants decreased in response to the increase in Ca availability. This effect was more evident in plants colonized by *G. fasciculatum* than in those colonized by *G. mosseae*, particularly under moderate stress (10% PEG). Thus, under these conditions and at a Ca level of 8 mM, control plants had 304% more proline than *G. fasciculatum*-colonized plants.

Curiously, at 2 mM and 4 mM Ca in the medium and at both stress levels, mycorrhizal plants accumulated more proline than control plants, but over 4 mM Ca the proline content decreased in mycorrhizal plants and increased in control plants.

The relative water content in plants was also negatively affected by PEG exposure (Fig. 2). So plants not subjected to PEG solution showed a RWC varying between 83 and 90%, but in plants exposed to PEG, the RWC varied between 60 and 75%.

The results of RWC are parallel to those of proline, since mycorrhizal plants had enhanced RWC when the calcium availability increased. This happened even in plants not subjected to PEG. In contrast, in control treatments exposed to PEG, the RWC decreased in response to the increased Ca in the medium.

The RWC of mycorrhizal plants grown with less than 4 mM Ca in the medium was lower than in control plants, but over 4 mM Ca, the RWC increased in mycorrhizal plants and decreased in control ones as a consequence of the increased Ca availability.

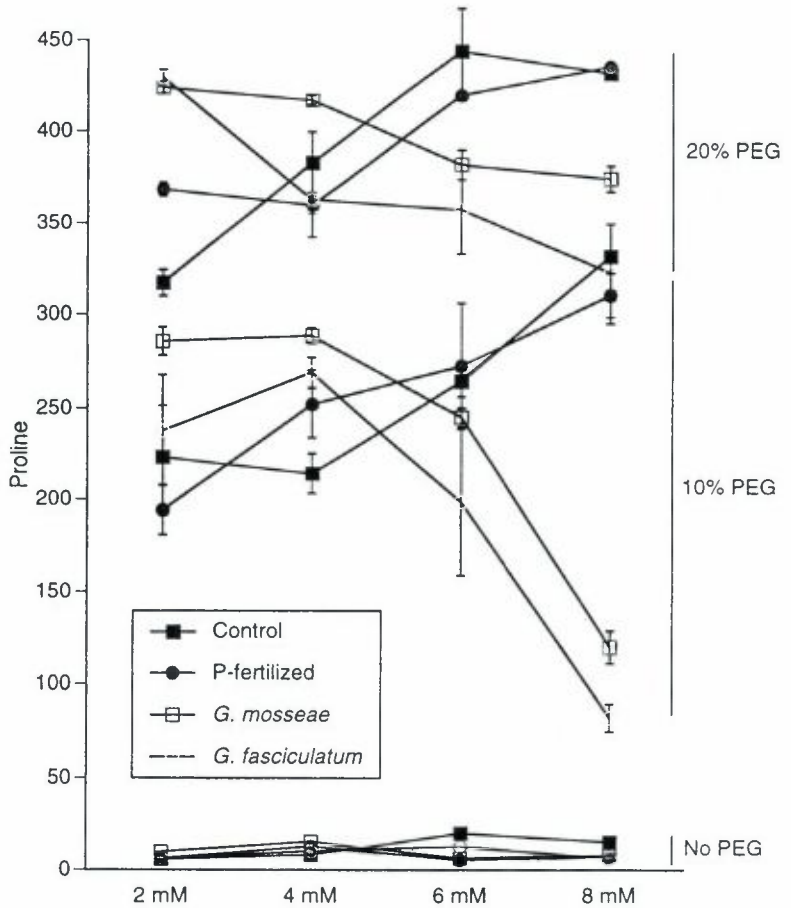

Figure 1. Proline content (nmol/g F.W.) in mycorrhizal (*Glomus mosseae* or *G. fasciculatum*) or non inoculated (Control and P-fertilized) lettuce plants grown in medium with four levels of Ca (2 mM, 4 mM, 6 mM or 8 mM) and subjected or not to PEG-induced water stress. Bars indicate the standard deviation (n = 3).

4. Discussion

The beneficial effect of mycorrhization on plant growth and nutrition is absent in this study (except N and Ca in *G. fasciculatum*-colonized plants). This fact can be attributed to the growth conditions of the experiment (similar to a hydroponic system), with a limited soil substrate (only 30 g from the total 300 g pots capacity) and a nutrient solution for plant growth (Azcon et al., 1996).

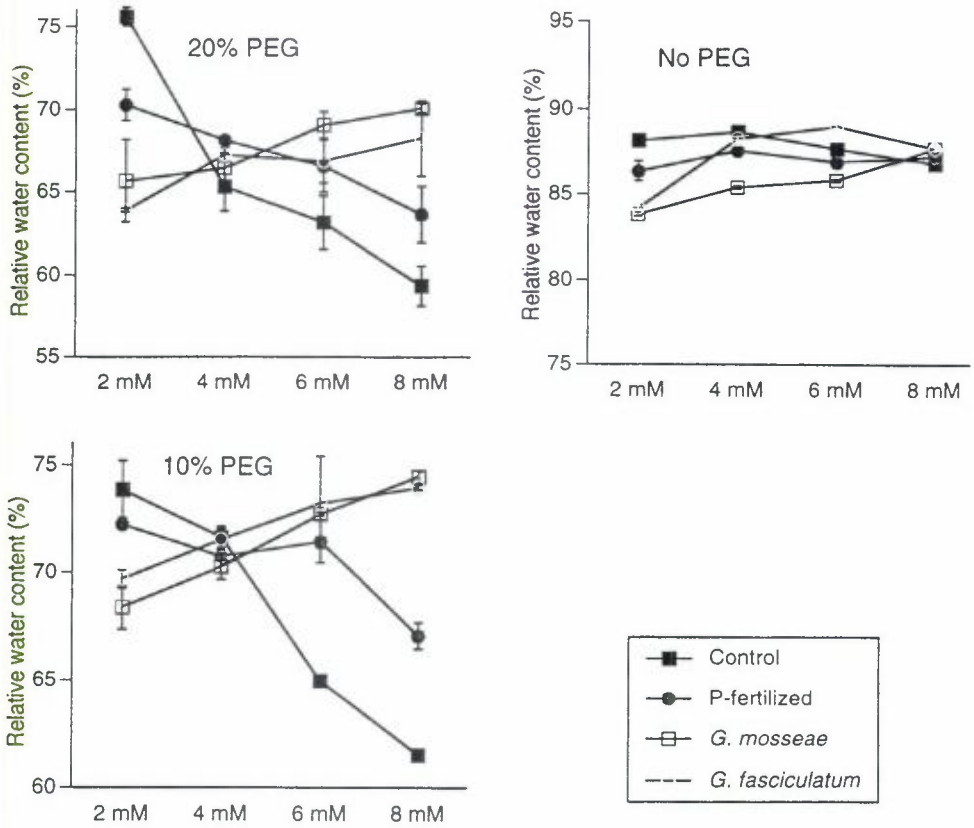

Figure 2. Relative water content (%) in mycorrhizal (*Glomus mosseae* or *G. fasciculatum*) or non inoculated (Control and P-fertilized) lettuce plants grown in medium with four levels of Ca (2 mM, 4 mM, 6 mM or 8 mM) and subjected or not to PEG-induced water stress. Bars indicate the standard deviation (n = 3).

P-fertilized plants showed the highest growth values. However, plants from both nonmycorrhizal treatments decreased their growth when the Ca applied reached 8 mM. It has been proposed that Ca may be a cytotoxin (Hepler and Wayne, 1985). In fact, at elevated levels it can react with inorganic phosphate forming an insoluble precipitate. Under these conditions the phosphate-based energy metabolism would be severely inhibited if the Ca concentration approached the millimolar quantities found outside the cell, thus causing growth reduction. The growth reduction could also be attributed to the known role of Ca in cell division and expansion, possibly due to interferences of Ca with auxin transport (Allan and Rubery, 1991).

In the case of mycorrhizal plants growth was not affected by the Ca increase perhaps because mycorrhizal plants may need more Ca than nonmycorrhizal plants in order to maintain the stability and proper function of the greater membranous surfaces existing in the symbiotic plants (Bonfante-Fasolo, 1994).

The nutrient status of the host can influence the amount of colonization in terms of the total length of infected root, the density and the anatomy of the infection (Hepper, 1983). In this study the colonizing ability from both AM fungi was decreased by increasing the Ca in the growth medium. This effect could be due to mechanisms operating via the host (Hepper and O'Shea, 1984). In fact, high levels of Ca can inhibit the activity of polygalacturonase (Bateman, 1964), an enzyme known to be involved in the colonization of plants by AM fungi (Garcia-Romera et al., 1991). Ca is also known to increase the rigidity of plant cell walls (Ginzberg, 1961; Wyn Jones and Lunt, 1967), thus slowing root infection by AM fungi. In leaves receiving high level of CA, CA-pectate is formed, which makes the tissue highly resistant to degradation by polygalacturonase (Cassells and Barlass, 1976). This aspect is of importance not only for determining the susceptibility of the tissue to fungal infections but also by delaying or even preventing leaf senescense caused by any stress condition (as drought).

It is well known that Ca as well as phytohormones (cytokinins) is involved in the regulation of senescence being additive to the effects of both substances (Poovaiah and Leopold, 1973). Recently Goicoechea et al. (1995) reported that mycorrhizal plants maintained leaf cytokinin levels during drought, while non-symbiotic plants showed a large drop in cytokinins as well as an increased degree of leaf senescence, emphasizing the protective effects of Ca against drought in mycorrhizal plants.

Increased Ca in the medium increased the N uptake in all the treatments as Fenn and Taylor (1990) and Fenn et al. (1991) also found (for radish and onion, respectively). In contrast, the plant P content of P-fertilized and *G. mosseae*-colonized plants decreased in response to an increase in Ca in the medium, which are the opposite of results obtained by Robson et al. (1970). However, these authors also found that the uptake of Ca and P by plants can be interdependent. In fact, major ions can influence each other's absorption by competitive interactions or by affecting ion selectivity of membranes (Ramalho et al., 1995).

The Ca-mediated linkages respond to changes in environmental conditions as drought and are part of the mechanisms for controlling developmental and growth processes. Ca has essential structural functions as the regulation of membrane permeability and related processes. The membrane-protecting effects of Ca are most prominent under stress conditions (Zsoldos and Karvaly, 1978). Ca is an activator of enzymes such as ATPases (Wyn Jones and Lunt, 1967)

involved in cell transport and nutrient exchange processes highly increased in AM plants.

PEG can cause some damage to the plant if it is applied for a long time (up to 24 hours), thus it is necessary to use PEG for a short time. For this reason we applied PEG for only 22 h in order to ensure that it did not affect the plant's physiology or nutritional status (Azcon et al., 1996). These two factors are normally affected when water stress is imposed by decreasing the soil water availability (Turner, 1986).

Under drought stress the adjustment of leaf osmotic potential requires intracellular osmotic balance. Proline accumulates in the leaves and enhances osmotic adjustment (Ruiz-Lozano et al., 1995a). In this study, the application of PEG resulted in a considerable increase of plant proline content. The testing of high concentrations of this compound on isolated enzyme systems has shown that it protects various enzyme systems against dehydration produced by a range of perturbing effects including salt or drought stress (Paleg et al., 1984). However, in our study, mycorrhizal plants decreased the proline accumulation in response to the increase in Ca availability. This fact indicates that mycorrhizal plants suffered less from the detrimental effect of PEG-induced water stress as a consequence of the Ca increase in the medium, since a lower proline content is an indication of reduced stress from drought (Ruiz-Lozano et al., 1995a). In contrast, the nonmycorrhizal plants had more proline when the Ca increased.

Results of RWC are parallel to those of proline, since mycorrhizal plants increased the water content with Ca, but control plants decreased their RWC as a consequence of the increased Ca in the medium.

It is curious that under 4 mM Ca in the medium the mycorrhizal plants suffered more from the detrimental effect of PEG application (higher proline accumulation and lower RWC) than the nonmycorrhizal plants. Above that Ca level the proline content decreased and the RWC increased in mycorrhizal plants while in control plants the behaviour was just the opposite. This fact could be attributed to a higher Ca requirement of mycorrhizal plants due to the bigger membranous surface area in these plants than in nonmycorrhizal plants (Bonfante-Fasolo, 1994). In this sense, the concept of Ca action in regulation of plant processes is based on its effect on membrane integrity, stability and function (Starch et al., 1994). Thus, the absence of a sufficient level of Ca in the growth medium could result in a general deterioration and loss of selectivity of the plasma membrane (Whittington and Smith, 1992) which can affect the general cell metabolism (Ramalho et al., 1995). This aggravates the drought effects, as has also been found for salt stress (Ehret et al., 1990).

In conclusion, the application of increasing amounts of Ca enhances the tolerance of mycorrhizal plants to PEG-induced water stress, possibly because

these plants have higher Ca requirement than nonmycorrhizal ones. The additional Ca availability would allow the maintenance of stability, integrity and function of the big membrane surface existing in the mycorrhizal roots, increasing in this way the plant resistance under such stress conditions. Different drought response between symbiotic and non-symbiotic plants appears in the present study to be mediated by this nutrient.

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