Differential efficiency of two strains of the arbuscular mycorrhizal fungus Rhizophagus irregularis *on olive (*Olea europaea*) plants under two water regimes*

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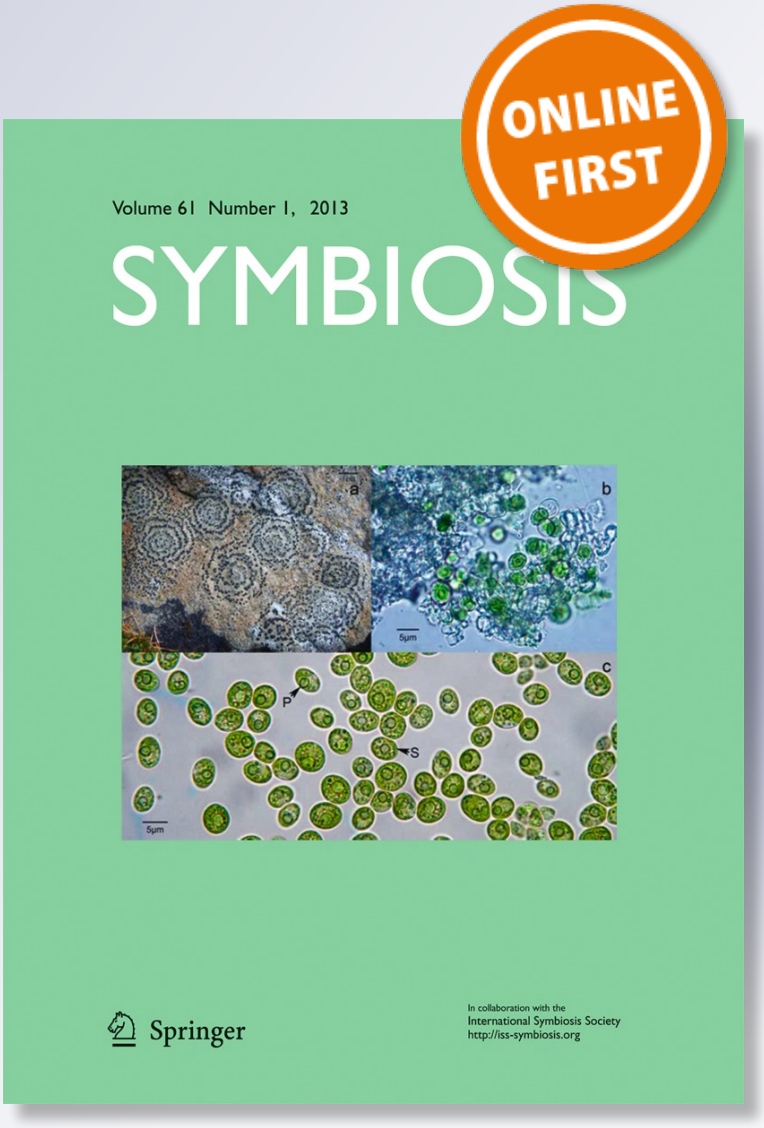

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Differential efficiency of two strains of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* on olive (*Olea europaea*) plants under two water regimes

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Abstract The water regime affects a wide variety of physiological and biochemical processes in plants including an increased production of reactive oxygen species (ROS) capable of causing oxidative damage to proteins, DNA and lipids. Arbuscular mycorrhizal fungi (AMF) colonize a wide range of plant species though the ability of different AMF species to promote host growth or contribute to plant water deficit resistance varies. The first phase of olive tree cultivation takes place in a nursery where plants usually suffer stress by drying. Currently, olive production systems do not use of AMF to counteract this problem. To study the colonization strategies of two AMF strains and their efficiency with respect to growth and their effect on enzymatic activities, we inoculated them individually and co-inoculated then on olive plants under nursery growing conditions. The results showed the benefits generated by these fungi in terms of growth and survival rate. Co-inoculation, particularly, improved growth and reduced the damage due to water stress, partly as a result of the activation of the antioxidant defenses in the olive plant host.

Keywords Arbuscular mycorrhizal fungim · Nursery · Catalase · Superoxide dismutase · Ascorbate peroxidase · Glutathione reductase

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1 Introduction

Olive (*Olea europaea*), native to the Mediterranean basin, is a long-lived tree that reaches 15 m in height and has a broad crown and a thick, twisted, and often very short trunk. Olive trees can be propagated by layers, cuttings, grafting and seeds. They grow in very diverse environments but their best performance occurs in areas with rainfall ranging between 600–800 mm/year (Parodi 1978). More than 75 % of the world production of olive oil is concentrated in Spain, Italy and Greece (FAO 2004). In Argentina, since the 1990s, La Rioja and Catamarca has been the main olive producing provinces with 67 % (49.011 ha) of their areas being devoted to olive oil production, and the rest (23.761 ha) to olives fruit (SENASA 2006; Calvelo 2011).

Water stress can be a problem during the growth, flowering and fruiting phases of olive trees but especially during propagation which takes place in a nursery where the young plants suffer from drought during the day (Marín 2005). Water stress affects a wide variety of physiological and biochemical processes in plants (Sánchez-Díaz and Aguirreolea 2002). Low water availability is the main cause of reduction in primary root elongation, which is needed for water uptake (Franco et al. 2011). Abiotic stresses in plants increase

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production of reactive oxygen species (ROS) such as $^1O_2$, H_2O_2, $O_2^{\cdot -}$ and HO·. These are toxic molecules capable of causing oxidative damage to proteins, DNA and lipids (Miller et al. 2010). Pathogens can also trigger the production of ROS (Mittler 2002). However, ROS-scavenging enzyme including catalase (CAT) found in peroxisomes, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) found in cytosol, chloroplasts, mitochondria and peroxisomes help to protect plants (Miller et al. 2010).

AMF are obligate symbionts grouped in the Glomerales (Schüβler et al. 2001). They can colonize a wide range of plants and the same strain can colonize more than one species. The ability of different AMF species to promote host growth varies (Trappe 1986; Porcel et al. 2003; Janoušková et al. 2009). AMF establishes an extensive hyphal network in the soil, mobilizing soil nutrients. During symbiosis, AMF gains carbohydrates from root cells, while the plant receive waters and diverse nutrients, mainly phosphates and nitrogen (Bonfante and Genre 2010). The potential of AMF to enhance plant tolerance to abiotic stress conditions has long been recognized (Al-Karaki 1998; Wu et al. 2006a; Smith and Read 2008; Ruíz-Lozano et al. 2012). There is evidence that AMF symbiosis increases plant resistance by improving water absorption from soils with low water availability (Augé 2001). Other indirect positive effects include the formation of stable soil aggregates via glomalin (Wu and Zou 2009) and protection against the oxidative damage by enhancing antioxidant enzyme activities (Porcel et al. 2003; Ruíz-Lozano et al. 1996; Wu et al. 2010).

The focus of this paper was to study how the colonization strategies of two *Rhizophagus irregularis* (Krüger et al. 2012) strains inoculated individually and co-inoculated on olive plants under nursery growing conditions improved the ability of the plants to respond to adverse environmental conditions.

2 Materials and methods

2.1 Plants

A vigorous and young tree of *Olea europaea* cv. Manzanillo 4 m in height was selected by the Departamento de Producción Vegetal (Facultad de Agronomía, FAUBA, Argentina) in order to obtain healthy and vigorous cuttings. Young branches 7 cm length with 2 shoots were cut from the tree to make a total of 150 cuttings and by means eliminated any genetic variability. The cut base of the cutting was dipped into a hormone rooting powder made up of 2,500 ppm of indole butiric acid (IBA) disolved in ethanol and adsorbed onto talcum powder (Knight et al. 2005).

The cuttings were rooted in perlite (1 m wide by 10 m long and 10 cm deep) on a raised table to 1 m at a 1,000 cuttings per m^2 density in order to maintain a constant humidity, reduce temperature and create appropiate microclimate conditions for olive rooting, under intermittent irrigation over a period of 60 days in nursery conditions. A 70 % rooting was obtained and provided sufficient plants for the study.

2.2 Mycorrhizal inoculation

The raised rooting tables were divided into 4 separated parts with plastic panels to prevent mycorrhizae or roots from moving from between the treatments. After a rooting period of 30 days, the cuttings were inoculated with 2 strains of *Rhizophagus irregularis* (formerly *Glomus intraradices*).

The two strains GC2 and GA5, have different strategies of colonization in vitro and in the soil conditions tested in our laboratory (Silvani 2011). The strains were provided by the Banco de Glomeromycota In Vitro (BGIV) (http://www.bgiv.com.ar/strains/Rhizophagus-intraradices/gc2; http://www.bgiv.com.ar/strains/Rhizophagus-intraradices/ga5). The GC2 strain has a high density of external mycelium, is slow growing at the beginning of in vitro culture, but increases with as the mycelium ramifies and it forms a few large spores (160.52±19.8 cm^2; 87.4±0.4 μm) that are limited to the vicinity of the roots where the infection takes place (Silvani 2011). In contrast, GA5 forms little external mycelium at the beginning of culture but this increases forming a mycelium that is little branched but has a higher growth rate higher than the GC2 strain. The spores of GA5 are smaller but more abundant than the GC2 (293.4±81.8 cm^2; 70.8±0.5 μm) (Silvani 2011).

The two strain lines were propagated using *Trifolium repens* as the host in 1.5 L pots with a tindalized mixture (100 °C for 1 h, three consecutive days) of perlite:soil (3:1). The soil characteristics are: pH 7.1; total C 12.08 g kg^{-1} and N 1.1 g kg^{-1}; P 34.2 mg kg^{-1}; K 0.9 cmol kg^{-1}; Ca 7.5 cmol kg^{-1}; Mg 1.7 cmol kg^{-1} and Na 0.2 cmol kg^{-1}. They were maintained for 4 months under greenhouse conditions (450 μE. m^{-2} s^{-2}, 400–700 nm; 16/8 light-darkness; 25/18 °C day/night; 60–70 % relative humidity). All plants were watered with Hewitt (1952) solution without phosphorous addition every 15 days. After this, they were unwatered to dryness to obtain a dry mycorrhizal inoculum.

Cuttings were inoculated as follows: control (C) without AMF; inoculation with *R. irregularis* strain GC2; inoculation with *R. irregularis* strain GA5 and inoculation of a 1:1 mixture of GA5 and GC2 strains. For inoculation, furrows were made between groups of cuttings that were 3 cm deep. A total of 10 g of dry inoculum of the appropriate strain was then added to the furrow for each treatment. It was estimated that there were for GA5: 1,161±13 spores/100 g dry soil; and for GC2: 851±5 spores/100 g dry soil. The control treatment received 10 g of autoclaved mixture inoculum supplemented with a filtrate (<20 μm) of mycorrhizal inoculum to provide a similar microbial population.

2.3 Experimental design

After 30 days of inoculation on the raised rooting table, all plants (a total of 96 plants for all the treatments were transplanted to 1 L pots with a tindalized mixture of perlite:vermiculite:soil (2:1:1) (see above for the soil characteristics and tindalization) and maintained 30 days at 80 % of field capacity, at an ideal humidity for olive cultivation under the same nursery conditions as above. Thereafter, half of the experiment (48 plants) were maintained at field capacity (FC), while the other half (48 plants) were kept at 50 % FC, a 50 % reduction in water availability. This was for a further 60 days the standard nursery condition (see above). The field capacity was calculated by weighing samples of the substrate before and after drying at 105 °C for 24 h. All the plants were watered with Hewitt (1952) solution without phosphorous addition after 30 days from the beginning of the study.

2.4 Growing parameters

Mycorrhization was tested 30 days after inoculation but before the water stress treatments were started. A root was taken from each treatment and stained according to Phillips and Hayman (1970). Mycorrhizal (MI%), arbuscules (A%) and vesicles (V%) percentages were calculated (Giovanetti and Mosse 1980). The survival percentage, shoots and roots fresh and dry weight were also quantified (70 °C until constant weight). Water content was calculated as the difference between fresh and dry weight, also evaluated shoot/root ratio plant biomass. The mycorrhizal dependency (MD%) was calculated as (mycorrhizal plant biomass/non-mycorrhizal plant biomass average) * 100 (Menge et al. 1978). At the end of the experiment the number of internodes per plant was measured. The internodes lengths frequency was classified into 0.5 cm, 1 cm, 1.5 cm and 2 cm long respectively to observe growing behavior.

Table 1 Mycorrhizal colonization, arbuscules and vesicles percentages on olive roots

Treatment	MI%	A%	V%
C	n.d. a	n.d. a	n.d. a
GA5	66.21±3.82 bc	32.59±5.22 b	55.64±4.44 b
GC2	63.27±4.45 b	43.90±6.86 b	35.61±4.00 c
GA5+GC2	79.13±3.86 c	47.16±6.34 b	60.29±4.19 b

Treatments: C (control without inoculation); GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). Mycorrhizal percentage (MI%), arbuscules percentage (A%), vesicles percentage (V%). Not detectable (n.d.). Data were analyzed with ANOVA; comparisons were made using the Tukey test. Different letters in the same column indicate significant differences at $P<0.05$. Data represent mean of fourtheen replicates ± standard error

2.5 Biochemical parameters

2.5.1 Enzyme extraction

A gram of fresh material (shoot and roots) was weighed and put in liquid nitrogen to maintain the integrity of the tissue until use, and each sample was pulverized in a mortar with liquid nitrogen. Six millilitre of extraction buffer (KH_2PO_4–K_2HPO_4 50 mM pH 7.8 plus 0.1 mM EDTA) and polyvinylpolypyrrolidone (PVPP, 0.06 g/6 ml extraction buffer) were added. The samples were filtered through a nylon membrane to remove cell debris, and centrifuged at 13, 000 rpm for 20 min. Supernatant aliquots were put into Eppendorf tubes and stored at −70 °C until use (Gogorcena et al. 1995).

2.5.2 Enzymatic measuring and MDA content

The following intracellular enzyme activities associated to oxidative stress were measured. Catalase (CAT) (EC 1.16.1.6): following a method based on absorbance diminishing measure at 240 nm occasioned by H2O2 disappearance (Aebi 1984). Ascorbate peroxidase (APX) (EC 1.11.1.11): method based on 290 nm measure of ascorbic acid oxidation (Hossain and Asada 1984), ascorbic acid (4 mM) was added in order to preserve enzyme activity (Moran et al. 1994). Superoxide dismutase (SOD) (EC 1.15.1.1): determination based on superoxide dismutase capacity to inhibit nitroblue tetrazolium (NBT) reduction to superoxide radicals generated photochemically (Beyer and Fridovich 1987). Glutathione reductase (GR) (EC 1.6.4.2): it was estimated as the necessary amount to transform oxidized glutathione (GSSG) into reduced glutathione (GSH) through the measurement of the rate of oxidation of NADPH (Calberg and Mannervik 1985), β-mercaptoethanol (10 mM) was added in order to maintain the reducing environment (Moran et al. 1994). The total protein content (PROT) was measured according to Bradford (1976) protocol. Shoots and roots of enzymatic extracts were used to measure the malon dialdehyde (MDA) reaction to thiobarbituric acid (TBA) according to Hodges et al. (1999). All enzymes activities and MDA content were standardized by protein determination.

2.6 Statistical analysis

The experiment was arranged in a completely randomized design with equal replications for each treatment. Six pots were established per treatment. All data were subjected to analysis of variance (factorial ANOVA). Homogeneity of

Table 2 Olive growth: shoot and root fresh, dry and water content, shoot to root ratio

Treatment	Fresh weight (g)				Dry weight (g)				Shoot to root ratio[2]		Water content (g H_2O)			
	Shoots[1]		Roots[2]		Shoots[1]		Roots[2]				Shoots[1]		Roots[2]	
	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC
C	1.57±0.14 ab	1.40±0.30 ab	1.18±0.13 a	0.98±0.13 ab	0.56±0.14 ab	0.52±0.11 ab	0.19±0.02 ac	0.19±0.02 ac	2.91±0.61 ac	2.94±0.61 ac	1.01±0.21 ab	0.88±0.18 ab	0.99±0.10 ac	0.79±0.10 ac
GA5	1.16±0.22 a	1.27±0.17 a	0.76±0.13 b	0.99±0.12 ab	0.42±0.08 a	0.49±0.07 a	0.15±0.02 a	0.24±0.02 bc	2.95±0.69 ac	2.09±0.30 a	0.74±0.13 a	0.79±0.10 a	0.61±0.11 ac	0.75±0.09 ac
GC2	1.29±0.20 a	1.14±0.30 a	1.17±0.15 a	0.68±0.25 ab	0.45±0.07 a	0.44±0.11 a	0.23±0.03 bc	0.16±0.06 ab	2.09±0.33 a	4.12±0.98 bc	0.84±0.13 a	0.69±0.19 a	0.94±0.11 a	0.52±0.19 bc
GA5+GC2	2.17±0.41 b	2.10±0.38 b	1.51±0.32 a	1.20±0.30 ab	0.79±0.14 b	0.80±0.13 b	0.28±0.05 bc	0.29±0.08 bc	3.14±0.33 bc	3.67±0.86 bc	1.38±0.26 b	1.30±0.25 b	1.23±0.26 ac	0.91±0.21 ac
ANOVA														
AMF	**		*		**		n.s.		n.s.		**		n.s.	
FC	n.s.		n.s.		n.s.		**		*		n.s.		*	
AMF x FC	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	

Treatments: C (control without inoculation); GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). FC (Field capacity); ½ FC (50 % field capacity). Data were analyzed with ANOVA, comparisons were made using [1]Tukey test, [2]Fisher test. Different letters in the same column indicate significant differences at $*P<0.05$, $**P<0.01$, not significant (n.s.). Data represent mean of six replicates ± standard error

variance and normal distribution were checked. Comparisons among mean values in each treatment were made using the Tukey test (honest significant difference HSD) and the LSD Fisher test ($P<0.05$) (Clewer and Scarisbrick 2001). Statistical procedures were carried out with the software package STATISTICA 6.0 for Windows XP.

3 Results

3.1 Root colonization

Mycorrhizal percentage was significantly lower in plants inoculated with the GC2 strain, while plants co-inoculated with GA5 and GC2 showed significantly higher percentages. Plants inoculated with GA5 exhibited intermediate but not significantly different colonization values from those inoculated with GC2. The percentage of vesicles was significantly lower in plants inoculated with GC2 strain (Table 1).

3.2 Olive growth

A 100 % survival rate was observed in treatments that involved AMF inoculation, whereas only 94.8 % of the control plants survived (data not shown). There was no interaction between treatments and water regime in terms of fresh weight, biomass and water content in shoots and roots, or in the shoot/root ratios. There was a significant improvement in shoots fresh and dry weight and in water content of co-inoculated plants. This was independently of the water regime employed (Table 2).

The GA5 inoculated plants were found to have a decrease in fresh root weight at FC condition in comparison with the control treatment, but at ½ FC they exhibited similar values to the rest of the treatments. A similar effect was observed on root biomass. The GC2 strain inoculation increased shoot to root ratio and decreased root water content at ½ FC (Table 2). Co-inoculation had a synergic effect on mycorrhizal dependency (MD%) compared with individual strain inoculations regardless of the water regime employed (Fig. 1a). In spite of no statistical interaction between treatments and water regime on MD% of olive roots, a synergic effect on MD% was observed in co-inoculated plants grown at ½ FC. Furthermore, in roots, at FC there was a significant improvement in MD% in GC2 inoculated plants, however at ½ FC this significantly decreased (Fig. 1b). The frequency of internodes length was not affected by inoculation at either water regime (data not shown). However, the number of internodes was significantly higher in the co-inoculation treatment at FC condition, but when ½ FC was applied the internode number drastically decreased, though still higher than in control plants (Fig. 2)

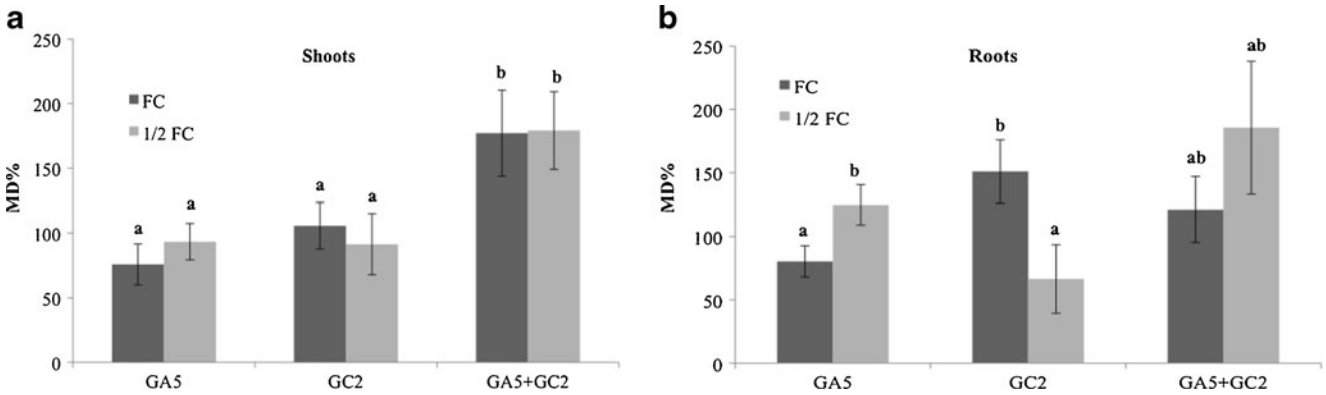

Fig. 1 Mycorrhizal dependency (MD%) on olive **a** shoots; **b** roots. Treatments: GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). FC (Field capacity); ½ FC (50 % field capacity). Data were analyzed with ANOVA; comparisons were made using the Tukey test. Different letters indicate significant differences at $P < 0.05$. Data represent mean values of six replicates ± standard error

3.3 Antioxidant responses

Shoot PROT significantly decreased at ½ FC in single inoculated plants compared with controls and co-inoculation treatments. An increase in CAT activity was observed in inoculated and non-inoculated shoots when ½ FC was applied except for co-inoculated plants. SOD enzyme activity was significantly higher for all treatments subjected to ½ FC; moreover it was more markedly in single-inoculated plants. An increase in the APX enzyme activity at ½ FC was only observed for co-inoculated plants; moreover, in control plants there was a significant decreased in the APX enzyme activity. On the other hand, in control plants there was a significant increase in the GR enzyme activity under ½ FC condition, unlike inoculated plants where the activity decreased. At FC and ½ FC conditions, the MDA content was significantly lower in inoculated plants. Under ½ FC only GC2-inoculated plants showed a decrease MDA content (Table 3).

CAT activity decreased in GC2 and co-inoculated roots when ½ FC conditions were applied, and at FC, co-inoculated plants had significantly higher CAT enzyme activity. No differences were observed on SOD activity. The GC2 inoculated, co-inoculated and control exhibited significantly increased APX enzyme activity at ½ FC, although the values for plants inoculated with GC2 strain were lower than for the other treatments (Table 4). The GR enzyme activity was constant in GA5 and control plants regardless of the water regime employed and this was low for GC2 strain inoculations. Co-inoculated plants showed significantly decreased GR at ½ FC in comparison with control plants.

Inoculated plants exhibited a significantly increased MDA content in comparison to control plants regardless of the water regime applied. GA5 inoculated plants had the highest MDA content on ½ FC treatment. GC2 and co-inoculation significantly decreased the MDA content at ½ FC but for GC2 strain inoculation there was no difference at FC (Table 4).

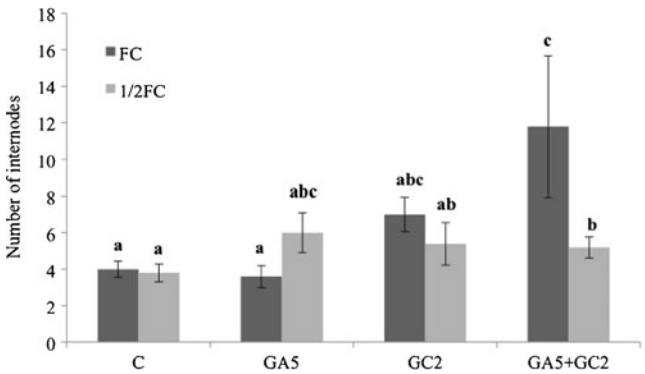

Fig. 2 Number of internodes of olive plants. Treatments: C (control without inoculation); GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GC2 (*Rhizophagus irregularis* inoculation strain); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). FC (Field capacity); ½ FC (50 % field capacity). Data were analyzed with ANOVA; comparisons were made using the Tukey test. Different letters indicate significant differences at $P < 0.05$. Data represent mean values of five replicates ± standard error

4 Discussion

In this study we found that a combination of two different strains of mycorrhizal fungi generated a benefit in terms of growth of inoculated olive plants. Inoculated plants showed a higher survival rate under conditions of water stress as has been observed previously (Porcel et al. 2003; Al-Karaki et al. 2004; Wu et al. 2008). Our results showed that co-inoculation had a synergic effect compared with a single strain. Shoots increased regardless of the water regime. Other authors have found differences related to the ability of different strains to relieve water stress. For example, *Funneliformis monosporus* isolates have proved to be less effective on wheat plants in water deficit condition in comparison with *Funneliformis*

Table 3 Protein, MDA content and enzymes specific activities in olive shoots

	PROT[1]		CAT[2]		SOD[2]		APX[2]		GR[2]		MDA[2]	
Treatment	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC
C	0.18±0.03 a	0.17±0.02 a	157±14 a	201±13 c	155±15 a	269±35 a	1.16±0.17 a	0.48±0.10 a	0.31±0.12 a	1.13±0.25 a	544±14 a	527±44 a
GA5	0.13±0.02 ab	0.11±0.006 b	99±11 b	257±14 e	190±8 ab	413±58 ab	1.33±0.18 ab	1.72±0.34 ab	0.76±0.27 a	0.65±0.22 a	430±24 ab	465±18 ab
GC2	0.12±0.03 ab	0.10±0.004 b	199±7 c	216±19 c	252±17 b	424±53 b	1.72±0.50 b	2.16±0.43 b	0.71±0.26 a	0.46±0.14 a	456±33 ab	256±62 c
GA5+GC2	0.17±0.01 a	0.17±0.01 a	134±5 d	134±4 d	162±14 a	291±48 a	0.58±0.18 a	1.16±0.17 a	0.63±0.14 a	0.47±0.16 a	422±14 b	421±54 b
ANOVA												
AMF	**		***		**		**		n.s.		***	
FC	n.s.		***		***		n.s.		n.s.		n.s.	
AMF x FC	n.s.		***		n.s.		n.s.		*		*	

Protein content (PROT, mg ml^{-1}), catalase (CAT, μmol min^{-1} mg^{-1} prot), superoxide dismutase (SOD, Units of SOD min^{-1} mg^{-1} prot), ascorbate peroxidase (APX, μmol min^{-1} mg^{-1} prot), glutathione reductase (GR, μmol min^{-1} mg^{-1} prot), malon dialdehyde content (nmol MDA mg^{-1} prot). Treatments: C (control without inoculation); GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). Different letters in each variable measured indicate significant differences at $P < 0.05$. FC (Field capacity); ½ FC (50 % field capacity). Data were analyzed with ANOVA, comparisons were made using [1] Fisher test, [2] Tukey test

*$P<0.05$

**$P<0.01$

***$P<0.001$

not significant (n.s.)

Data represent mean of six replicates ± standard error

mosseae (Al-Karaki 1998). Furthermore, Wu et al. (2006a) found that inoculated plants exhibited improved root biomass even in well-watered conditions. In our experiment we found that the FC condition was detrimental in plants inoculated with GA5 and GC2, although with co-inoculation, there was no effect.

Calvente et al. (2004) studied the effect of native strains of AMF on olive plants, and showed the benefit provided by native AMF on two varieties. In out studies, it is important to emphasize that when olive plants were inoculated with the GC2 strain, they showed the lowest colonization percentage and a low percentage of vesicles, while co-inoculation

Table 4 Protein, MDA content and enzymes specific activities on olive roots

	PROT		CAT		SOD		APX		GR		MDA	
Treatment	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC	FC	1/2FC
C	0.10±0.02 a	0.09±0.01 b	55±4 a	50±3 a	84±7.6 a	75±13.8 a	0.28±0.05 c	0.59±0.1 a	0.17±0.04 a	0.20±0.01 a	55±8 a	45±27 a
GA5	0.08±0.01 b	0.07±0.01 b	51±2 a	56±4 a	81±14.6 a	84±10.3 a	0.40±0.07 c	0.60±0.1 a	0.20±0.03 a	0.20±0.01 a	139±29 b	205±11 c
GC2	0.09±0.005 b	0.09±0.01 b	60±2 a	42±3 a	64±15.2 a	60±8.6 a	0.09±0.02 b	0.29±0.05 c	0.05±0.01 b	0.05±0.02 b	122±22 b	107±6 b
GA5+GC2	0.10±0.01 a	0.10±0.01 a	71±3 a	42±6 a	58±6.8 a	61±8.5 a	0.38±0.05 c	0.55±0.03 a	0.18±0.01 a	0.12±0.01 a	180±17 c	113±8 b
ANOVA												
AMF	***		n.s.		n.s.		**		***		***	
FC	n.s.		n.s.		n.s.		***		n.s.		n.s.	
AMF x FC	n.s.		n.s.		n.s.		n.s.		n.s.		**	

Protein content (*PROT*, mg ml^{-1}), catalase (*CAT*, μmol min^{-1} mg^{-1} prot), superoxide dismutase (*SOD*, Units of SOD min^{-1} mg^{-1} prot), ascorbate peroxidase (*APX*, μmol min^{-1} mg^{-1} prot), glutathione reductase (*GR*, μmol min^{-1} mg^{-1} prot), malon dialdehyde content (nmol MDA mg^{-1} prot). Treatments: C (control without inoculation); GA5 (*Rhizophagus irregularis* GA5 strain inoculation); GC2 (*Rhizophagus irregularis* GC2 strain inoculation); GA5 + GC2 (mixture 1:1 of GA5 and GC2 strains). Different letters into each variable measured indicate significant differences. *FC* (Field capacity); ½ FC (50 % field capacity). Data were analyzed with ANOVA; comparisons were made using the Tukey test

**$P<0.01$

***$P<0.001$

Not significant (n.s.)

Data represent mean of six replicates ± standard error

resulted in the highest colonization and vesicles percentages. When roots were inoculated with GA5 strain, a more aggressive colonization was established although the functionality as shown by the number of arbuscules was not different. However co-inoculation and GA5 seems to be more efficient under low water conditions. On the other hand, Wu et al. (2006b) found that seeds of *Citrus tangerine* inoculated with *Glomus versiforme* showed no difference in growth between low-watered plants inoculated and non-inoculated from well-watered conditions. However more recently, they found that high efficiency strains did have an impact (Wu et al. 2008). Mycorrhizal dependency is defined by the degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth (Gerdemann 1975) In olive plants, co-inoculation improved olive growth through an increased biomass and the development of numerous internodes.

Our results showed that under a low water regime (½ FC), MDA content was low in shoots of GC2 and co-inoculated treatments. ROS causes important lipid damage, so if the enzymatic response is active the MDA content diminishes and the plant stress is alleviated. An increase of the CAT and SOD activities was observed in GC2 inoculated shoots. Additionally, co-inoculation increased the APX activity. Control shoots increased the CAT, SOD and GR enzyme activities and decreased the APX enzyme activity. Several authors have observed such changes. Porcel and Ruíz-Lozano (2004) and Ruíz-Lozano et al. (1996) found high SOD activity in inoculated soybean and *Latuca sativa* plants respectively while Roldán et al. (2008) found a diminished SOD activity in inoculated *Juniperus oxycedrus*.

AMF symbiosis improved shoot GR activity in our study and Wu et al. (2006b) also found higher GR activity in *C. tangerine* plants inoculated with *G. versiforme*. The antioxidative response in olive shoots was rapidly activated in GC2 and in co-inoculated plants under ½ FC condition. We also found that olive plants that were inoculated with GA5 presented a significantly high MDA content in roots with low water conditions (½ FC) in contrast to shoots. Nevertheless, when co-inoculated with GC2, the MDA content significantly decreased. These results were likely due to the aggressive colonization observed in GA5 inoculated plants, so that when plants were inoculated with GC2 as well, a control mechanisms may be triggered that involve antioxidative responses in olive roots. A decrease in malon dialdehyde content has previously been observed (Ruíz-Lozano et al. 1996; Roldán et al. 2008; Wu et al. 2006a; Wu and Zou 2009). When ROS is induced it results in a lipid peroxidation and production of MDA, so a decrease in MDA content in mycorrhizal plants could be attributed to stress tolerance protection. In terms of antioxidative enzymes, GC2 and co-inoculation increased APX and decreased CAT and GR in olive roots. Control plant roots had only increased the APX enzyme activity. Thus, in olive roots, the antioxidative mechanisms involved in GC2 strain and co-inoculated plants were alleviated and less oxidative damage was observed in comparison to shoots.

The beneficial effects produced by the AMF symbiosis is less evident in nursery practices due to well-watered regimes and well-controlled environmental conditions that are normal in them. Under these conditions, AMF do not increase growth in the nursery but can promote transplant success when plants are transferred outside (Calvente et al. 2004). Inoculation with two fungi does not always bring additional benefit to the host plants in comparison to single inoculation (Janoušková et al. 2009). However, our study with olive plants, shows that a combination of mycorrhizal strains can improve plant growth in the nursery and that the benefits depend on the water regimes and on activation of the antioxidant defenses.

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