

1 **Localized and non-localized effects of arbuscular mycorrhizal symbiosis on**
2 **accumulation of osmolytes and aquaporins and on antioxidant systems in maize**
3 **plants subjected to total or partial root drying**

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16 **Running title:** Localized effects of AM symbiosis under drought

1 **ABSTRACT**

2 **The arbuscular mycorrhizal (AM) symbiosis alters host plant physiology under**
3 **drought stress, but no information is available on whether or not the AM effects**
4 **respond to drought locally or systemically. A split root system was used to obtain AM**
5 **plants with total or only half root system colonized, as well as, to induce physiological**
6 **drought affecting the whole plant or non-physiological drought affecting only half root**
7 **system. We analysed the local and/or systemic nature of the AM effects on**
8 **accumulation of osmoregulatory compounds and aquaporins and on antioxidant**
9 **systems. Maize plants accumulated proline both, locally in roots affected by drought**
10 **and systemically when the drought affected the whole root system, being the last**
11 **effect ampler in AM plants. PIPs aquaporins were also differently regulated by drought**
12 **in AM and nonAM root compartments. When the drought affected only the AM root**
13 **compartment, the rise of lipid peroxidation was restricted to such compartment. On**
14 **the contrary, when the drought affected the nonAM root fraction, the rise of lipid**
15 **peroxidation was similar in both root compartments. Thus, the benefits of the AM**
16 **symbiosis not only rely in a lower oxidative stress in the host plant, it also restricts**
17 **locally such oxidative stress.**

18

19 *Key-words:* Antioxidant, aquaporins, arbuscular mycorrhiza, drought, osmoregulation

20

1 INTRODUCTION

2 Plants have developed an array of strategies to cope with environmental stresses (Dobra et
3 al. 2010). Under drought stress plants use osmotic adjustment and regulate their antioxidant
4 systems, as well as, the permeability of tissues to water movement. The first of these
5 processes is based in the modification of cell and tissue water potential in order to maintain
6 cell turgor, through the accumulation of compounds such as soluble sugars, proline, glycine
7 betaine, pinitol, mannitol, etc (Morgan 1984; Bheemareddy & Lakshman 2011), being proline
8 one of the most common osmolytes accumulated by plants under conditions of dehydration
9 (Yoshiba et al. 1997; Kishor & Sreenivasulu 2014). Proline is a non-protein amino acid that
10 accumulates in most tissues subjected to water stress and, together with sugars, it is readily
11 metabolized upon recovery from drought (Singh et al. 2000). In addition to acting as an
12 osmoprotectant, proline also serves as a sink for energy to regulate redox potentials, as a
13 hydroxyl radical scavenger, as a solute that protects macromolecules against denaturation,
14 and as a means of reducing acidity in the cell (Kishor et al. 1995; Kishor & Sreenivasulu
15 2014).

16 Antioxidant systems aim the elimination of reactive oxygen species (ROS) produced in
17 excess under stress conditions (Gill & Tuteja 2010). Indeed, during drought stress, different
18 metabolic pathways are uncoupled and electrons are transferred to molecular oxygen to form
19 ROS (Noctor et al. 2014). It has been estimated that under shortage of CO₂ up to 50% of the
20 entire photosynthetic electron flow may end up as O₂⁻ (Biehler & Fock 1996). ROS are toxic
21 molecules capable of causing oxidative damage to proteins, DNA and lipids (Miller et al.
22 2010). On the other hand, ROS can act as signalling molecules for stress responses and its
23 generation is an early event in plant response to stress (Singh et al. 2011).

24 The scavenging of ROS is achieved through the action of different enzymatic and non-
25 enzymatic compounds. Enzymatic antioxidants include superoxide dismutase (SOD),

1 glutathione reductase (GR), catalase (CAT), ascorbate- or thiol-dependent peroxidases, and
2 the enzymes of the ascorbate-glutathione pathway. Non-enzymatic mechanisms include
3 compounds able to scavenge directly several ROS as are ascorbic acid (AsA), glutathione
4 (GSH), or α -tocopherol (Scheibe & Beck 2011).

5 Besides the osmotic adjustment and regulation of antioxidant systems, plants also
6 regulate the water permeability in their tissues, a process in which aquaporins are involved
7 (Javot & Maurel 2002; Maurel et al. 2008; Chaumont & Tyerman 2014). Aquaporins are water
8 channel proteins that facilitate and regulate the passive movement of water molecules down
9 a water potential gradient (Maurel et al. 2008), affecting directly the radial water flow through
10 the cell-to-cell pathway. Such pathway is more important for water movement under
11 conditions of low transpiration such as under drought stress (Steudle & Peterson 1998).
12 Aquaporins belonging to the plasma membrane intrinsic proteins group (PIPs1 and PIPs2)
13 are keys for whole plant water transport (Javot et al. 2003; Katsuhara et al. 2008; Chaumont
14 & Tyerman 2014). The activity of PIPs must be controlled by regulation mechanisms that
15 allow a rapid response to the frequent environmental changes that plants undergo. Post-
16 translational modifications are keys to achieve such rapid regulation (Vandeleur et al. 2014).
17 The first post-translational regulation mechanism found in aquaporins was the
18 phosphorylation/de-phosphorylation of specific serine residues, which generates
19 conformational changes allowing the aquaporin gating (Maurel et al. 1995; Johansson et al.
20 1998) or modifying the subcellular localization of PIPs in the membrane (Prak et al. 2008).
21 The phosphorylation of serine 115 (S₁₁₅) in loop B or S₂₇₄ in the C-terminal region of a PIP2 in
22 spinach open the pore and enhances the water transport (Törnroth-Horsefield et al. 2006).
23 On the contrary, the de-phosphorylation of these residues occurs under drought stress
24 conditions (Johansson et al. 1996; 1998) and may be a mechanism to prevent water loss
25 (Johansson et al. 2000).

1 The arbuscular mycorrhizal (AM) symbiosis confers to the host plant enhanced tolerance
2 to abiotic stresses, including drought stress (Augé 2001; Ruiz-Lozano et al. 2006, 2012b).
3 Under drought stress, the AM symbiosis modifies plant metabolism and accumulation of
4 soluble sugars and other compatible solutes (Subramanian & Charest 1995; Bheemareddy &
5 Lakshman 2011; He et al. 2011). However, the investigations carried out so far on
6 osmoregulation in the AM symbiosis are somewhat contradictory. It is noticeable that in a
7 study with lettuce plants Ruiz-Lozano et al. (2011) found that under drought stress nonAM
8 plants accumulated more proline in shoots than AM plants. In contrast, in roots, AM plants
9 accumulated more proline than nonAM plants. This suggests that in root tissues AM plants
10 accumulate more proline in order to cope with the low water potential of drying soil and to
11 keep a water potential gradient favourable to water entrance into the roots, as was observed
12 in soybean plants (Porcel & Ruiz-Lozano 2004). Moreover, Sheng et al. (2011) found
13 enhanced reducing sugar accumulation in AM maize plants subjected to salt stress, while the
14 content of proline was lower than in nonAM plants. Authors proposed that the high levels of
15 sugars in AM plants may be the result of an increase in photosynthetic capacity of plants and
16 that these sugars contributed to the osmotic adjustment of the plants (Sheng et al. 2011).

17 There are also examples of studies showing the capacity of the AM symbiosis to regulate
18 the activity of several plant antioxidant enzymes (Porcel et al. 2003; Talaat & Shawky 2011;
19 Lee et al. 2012) or the accumulation of non-enzymatic antioxidant compounds (Ruiz-Sánchez
20 et al. 2010; 2011; Baslam et al. 2012). However, the results also vary depending on the plant
21 tissue analyzed and the symbiotic partners involved.

22 On the other hand, the AM symbiosis has been shown to alter the root hydraulic
23 properties of the host plant (Khalvati et al. 2005) and several studies have pointed out that
24 the control of water transport through aquaporins may be determinant for the total hydraulic
25 conductivity in mycorrhizal plants (Marjanovic et al. 2005; Lee et al. 2010). This may be even

1 more relevant under conditions of drought stress. In any case, the effects of the AM
2 symbiosis on aquaporin genes depends on the intrinsic properties of the osmotic stress, on
3 the plant species studied and on the specific aquaporin gene analyzed (Aroca et al. 2007;
4 Ruiz-Lozano & Aroca 2010). In addition, Uehlein et al. (2007) and, more recently, Bárzana et
5 al. (2014) have suggested that the role of aquaporins in the AM symbiosis could be more
6 complex than simply regulating plant water status. These authors consider that the plant
7 aquaporins altered by the AM symbiosis could be involved in the symbiotic exchange
8 processes between the fungus and the plant, in accordance with the multiple physiological
9 roles attributed to plant aquaporins (Li et al. 2014). The own fungal aquaporins (Aroca et al.
10 2009; Li et al. 2013; Xu et al. 2014) could be also implicated in these processes. Thus, and
11 effect of the symbiosis on the accumulation and/or post-translational regulation of PIPs
12 aquaporins is expected under water limiting conditions.

13 No information is available on whether or not the AM effects described above respond
14 to drought locally or systemically. In contrast, the existence of localized and systemic AM
15 effects on plant responses against biotic stresses is well known (Pozo & Azcón-Aguilar 2007;
16 Khaosaad et al. 2007; Vierheilig et al. 2008). Thus, the objective of this study was to analyse
17 the local and/or systemic nature of the AM effects on plant responses to drought stress,
18 including accumulation of osmoregulatory compounds and aquaporins and antioxidant
19 systems. For that, a split root system was used with maize plants, so that one or both root
20 compartments were or not inoculated with the AM fungus *Rhizophagus intraradices* and
21 subjected or not to drought stress. This design allowed having non mycorrhizal plants and
22 mycorrhizal plants with total or only half root system colonized by the AM fungus, as well as,
23 to induce a physiological drought affecting the whole plant or a non-physiological drought
24 affecting only a part of the root system. The non-physiological drought was used as a tool to

1 ascertain if the AM effects occur in a systemic way on the whole root system and shoot or are
2 restricted to the root fraction subjected to drought.

3

4 **MATERIALS AND METHODS**

5 *Experimental design*

6 In this study, maize plants were cultivated into a split-root system (Fig. 1). The experiment
7 consisted of a factorial design with two inoculation treatments: (1) non-inoculated control
8 plants (C) and (2) plants inoculated with the AM fungus *Rhizophagus intraradices*, strain EEZ
9 58 (Ri). In AM treatments, the AM inoculum was applied to either both root compartments or
10 to a single root compartment, obtaining AM plants with the whole root system colonized or
11 AM plants with only half root system colonized, respectively. In addition, plants were
12 cultivated under well-watered conditions (ww) throughout the entire experiment or were
13 subjected to drought stress (ds) for 12 days before harvest. The water regimes were also
14 applied in a combined way to the roots compartments, so that there were plants with both
15 root halves cultivated under well-watered conditions, plants with both root halves subjected to
16 drought stress (thus experiencing a physiological drought) and plants with only a root half
17 (either the non-inoculated or the AM-inoculated) subjected to drought (thus, experiencing non
18 physiological drought). The different combinations of these factors gave a total of 10
19 treatments:

20

21 **Treatments cultivated under well-watered conditions:**

22 Cww/Cww: Both root compartments non mycorrhizal and under well-watered conditions.

23 Cww/Riww: One root compartment non mycorrhizal and the other mycorrhizal; both under
24 well-watered conditions.

25 Riww/Riww: Both root compartments mycorrhizal and under well-watered conditions.

1

2 **Treatments subjected to physiological drought:**

3 Cds/Cds: Both root compartments non mycorrhizal and subjected to drought stress.

4 Cds/Rids: One root compartment non mycorrhizal and the other mycorrhizal; both subjected
5 to drought stress.

6 Rids/Rids: Both root compartments mycorrhizal and subjected to drought stress.

7

8 **Treatments subjected to non-physiological drought:**

9 Cww/Cds: Both root compartments non mycorrhizal. One compartment under well-watered
10 conditions and the other subjected to drought.

11 Cww/Rids: One root compartment non mycorrhizal and under well-watered conditions and
12 the other compartment mycorrhizal and subjected to drought.

13 Riww/Cds: One root compartment mycorrhizal and under well-watered conditions and the
14 other compartment non mycorrhizal and subjected to drought.

15 Riww/Rids: Both root compartments mycorrhizal. One compartment under well-watered
16 conditions and the other subjected to drought.

17

18 *Soil and biological materials*

19 A loamy soil was collected from Dúrcal (Granada, Spain). The soil had a pH of 8.2 (measured
20 in water, 1:5 w/v); 1.8% organic matter, nutrient concentrations (g kg⁻¹): N, 2.5; P, 6.2
21 (NaHCO₃-extractable P); K, 13.2. The soils was sieved (5 mm), diluted with quartz-sand (<2
22 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h on 3 consecutive days).

23 Maize (*Zea mays* L. cv. Potro) seedlings were pre-germinated on vermiculite for ten
24 days and then transferred to containers prepared *ad hoc* for this split-root assay. These
25 containers were constructed from two black 1L plastic pots fastened together, side by side,

1 with adhesive tape, as described by Neumann et al. (2009). Each root compartment was
2 filled with 1200 g of the soil/sand mixture described above and contained half root system
3 from maize seedlings.

4 Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted
5 of soil, spores, mycelia and infected root fragments. The AM fungus was *Rhizophagus*
6 *intraradices* (Schenck and Smith), strain EEZ 58. Ten grams of inoculum with about 60
7 infective propagules per gram (according to the most probable number test), were added to
8 appropriate pots at sowing time. Non inoculated control plants received the same amount of
9 autoclaved mycorrhizal inoculum together with a 3 ml aliquot of a filtrate (<20 µm) of the AM
10 inoculum in order to provide a general microbial population free of AM propagules.

11

12 *Growth conditions*

13 The experiments were carried out under greenhouse conditions with temperatures ranging
14 from 19 to 25°C, 16/8 light/dark period, a relative humidity of 50-60% and an average
15 photosynthetic photon flux density of 800 µmol m⁻² s⁻¹, as measured with a light meter
16 (LICOR, Lincoln, NE, USA, model LI-188B). Plants were cultivated for a total of 9 weeks.

17 Soil moisture was measured with the ML2 ThetaProbe (AT Delta-T Devices Ltd.,
18 Cambridge, UK). Water was supplied daily to maintain soil at 100% of field capacity during
19 the first 7 weeks after sowing. The 100% soil water holding capacity corresponds to 22%
20 volumetric soil moisture measured with the ThetaProbe, as determined experimentally in a
21 previous experiment using a pressure plate apparatus. Then, half of the plants were allowed
22 to dry until soil water content reached 55% of field capacity (two days needed), while the
23 other half were maintained at field capacity. The 55% of soil water holding capacity
24 corresponds to 8% volumetric soil moisture measured with the ThetaProbe (also determined
25 experimentally with a pressure plate apparatus in a previous assay). The soil water content

1 was daily measured with the ThetaProbe ML2 before rewatering (at the end of the afternoon),
2 reaching a minimum soil water content around 50% of field capacity. The amount of water
3 lost was added to each pot in order to keep the soil water content at the desired levels of 8%
4 of volumetric soil moisture (Porcel & Ruiz-Lozano 2004). Plants were maintained under such
5 conditions for 12 additional days before harvesting.

6

7 ***Parameters measured***

8

9 *Biomass production and symbiotic development*

10 At harvest (9 weeks after sowing) the shoot and root system of six replicates per treatment
11 were separated and the shoot dry weight (DW) measured after drying in a forced hot-air oven
12 at 70 °C for 2 days.

13 The percentage of mycorrhizal fungal colonization in maize plants was estimated by
14 visual observation according to Phillips & Hayman (1970). The extent of mycorrhizal
15 colonization was calculated according to the gridline intersect method (Giovannetti & Mosse
16 1980) in six replicates per treatment.

17

18 *Leaf water potential*

19 The mid-day leaf water potential (Ψ) was determined with a C-52 thermocouple psychrometer
20 chamber and a HR-33T dew point microvoltmeter (Wescor Inc, Logan, UT, USA). Leaf discs
21 (1 cm diameter) corresponding to the second youngest leaf were cut, placed inside the
22 psychrometer chamber and allowed to reach temperature and water vapour equilibrium for
23 30 min before measurements were made by the dew point method.

24

25

1 *Stomatal conductance*

2 Stomatal conductance was measured two hours after the onset of photoperiod with a
3 porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user
4 manual instructions. Stomatal conductance measurements were taken in the second
5 youngest leaf from four different plants from each treatment.

6

7 *Photosynthetic efficiency*

8 The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems
9 Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant
10 photosynthetic performance by measuring chlorophyll a fluorescence. FluorPen quantifies the
11 quantum yield of photosystem II as the ratio between the actual fluorescence yield in the
12 light-adapted state (FV') and the maximum fluorescence yield in the light-adapted state (FM'),
13 according to Oxborough & Baker (1997). Measurements were taken in the second youngest
14 leaf of four different plants of each treatment.

15

16 *PIP aquaporins accumulation*

17 Microsomes were isolate from maize roots and leaves harvested 9 weeks after sowing and
18 kept at -80 °C, as described by Hachez et al. (2006) with slight modifications. Briefly, tissues
19 (1 g FW) were homogenized in grinding buffer consisting of 250 mM sorbitol, 50 mM Tris-HCl
20 (pH 8), 2 mM EDTA, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 µg/ml
21 each of leupeptin, aprotinin, antipain, chymostatin, and pepstatin (Sigma, St. Louis, MO,
22 USA)]. The extract was pre-filtered through a single layer of cheesecloth and centrifuged at
23 15,000 g for 15 min. Then, the supernatant was centrifuged again at 100,000 g for 2 h and
24 the resulting pellet (microsomal fraction) was resuspended in 30-60 µl of buffer (5 mM

1 KH_2PO_4 , 330 mM sucrose, 3 mM KCl, pH 7.8) and sonicated twice for 5 s. Microsomes were
2 isolated from three different root samples for each treatment.

3 As primary antibodies, we used two antibodies that recognize several PIP1s and
4 PIP2s in maize (Marulanda et al. 2010), an antibody that recognize the phosphorylation of
5 PIP2 proteins in their C-terminal region and an antibody that recognize the phosphorylation of
6 PIP2 proteins in a serine residue in loop B. All the antibodies were designed against the most
7 conservative regions of these aquaporin groups (Calvo-Polanco et al. 2014). To detect PIP1
8 aquaporins, we used the first 26 amino acids of the N-terminal part of the PvPIP1;3 protein
9 (accession No. DQ855475; Aroca et al. 2007), raised as a peptide to immunize mice. To
10 detect PIP2 aquaporins, we used the last 12 amino acids of the C-terminal part of the
11 PvPIP2;1 protein (accession No. AY995195; Aroca et al. 2006), raised as a peptide to
12 immunize rabbits. To detect phosphorylated PIP2, we used the same protein PvPIP2;1 as the
13 amino acid sequence but with a serine group phosphorylated PIP2(Ser₂₈₃),
14 AIKALGSFR{pSER}NA and a sequence with a serine group phosphorylated in loop B
15 PIP2(Ser₁₂₆), RKV{pSER}LIRA (Abyntek Biofarma SL, BiotechSpain). These antibodies had
16 been successfully used in maize (Aroca et al. 2005; Marulanda et al. 2010).

17 Immunodetection method for aquaporin quantification in microsomal extracts was
18 done as described by Bárzana et al. (2014). Briefly, two micrograms of protein were loaded in
19 triplicate on ELISA plates and incubated overnight in presence of coating buffer (0.05 M
20 carbonate/bicarbonate pH 9.6). After that, plates were washed three times with Tris-buffered-
21 saline (TBS) containing 0.05% Tween 20 (TTBS) and were blocked during 1 h at room
22 temperature with 1% BSA (w/v) in TTBS, then washed again three times for 10 minutes with
23 TTBS. The plates were then incubated at room temperature with 1:2,000 dilutions of
24 antibodies previously mentioned. Ig coupled to horseradish peroxidase (Sigma) was used as
25 secondary antibody at a 1:20,000 dilution. The signal was developed using a TMB substrate

1 (Sigma) and the colorimetric reaction was stopped with H₂SO₄ 2M. The amount of each
2 aquaporin was quantified by measuring the intensity of signals into each well at 450 nm with
3 a spectrophotometer InfiniteR 200 PRO series (Tecan Trading AG, Switzerland). Protein
4 quantification was carried out on three microsomal samples of each treatment without
5 significant differences among them. The equal loading of proteins in the different treatments
6 was confirmed by staining a gel blot loaded with the same quantities used for the ELISA
7 measurement with Coomassie brilliant blue and also by Bradford quantification.

8

9 *Proline and total soluble sugars accumulation*

10 At harvest, free proline and total soluble sugars were extracted from 1 g fresh tissues in
11 sulfosalicylic acid 5% (w/v) for proline or in 100 mM potassium phosphate buffer for total
12 soluble sugars. Proline was estimated by spectrophotometric analysis at 515 nm of the
13 ninhydrin reaction, according to Bates et al. (1973). Soluble sugars were analyzed by 0.1 ml
14 of plant extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72%
15 (w:w) H₂SO₄) and placed in a boiling water bath for 10 min according to Irigoyen et al. (1992).
16 After cooling, the absorbance at 620 nm was determined in a spectrophotometer Hitachi U-
17 1900 (Hitachi Corporation, Japan). The calibration curve was made using glucose in the
18 range of 0.2 to 0.4 mg/ml.

19

20 *Hydrogen peroxide content*

21 Hydrogen peroxide content was determined by Patterson's method (Patterson et al. 1984),
22 with slight modifications as described previously by Aroca et al. (2003). Five hundred
23 milligrams of fresh tissues were homogenized in a cold mortar with 5 ml 5% (w/v) TCA
24 containing 0.1 g of activated charcoal and 1% (w/v) PVPP. The homogenate was centrifuged
25 at 18,000g for 10 min. The supernatant was filtered through a Millipore filter (0.22 μm). A

1 volume of 1.2 ml of 100 mM potassium phosphate buffer (pH = 8.4) and 0.6 ml of the
2 colorimetric reagent were added to 130 μ l of the supernatant. The colorimetric reagent was
3 freshly made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-
4 pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 °C for 1 h and the
5 absorbance at 508 nm was recorded. The blanks were made by replacing leaf extract by 5%
6 TCA.

7

8 *Oxidative damage to lipids*

9 Lipid peroxides were extracted by grinding 500 mg of fresh tissues with an ice-cold mortar
10 and 6 ml of 100 mM potassium phosphate buffer (pH 7). Homogenates were filtered through
11 one Miracloth layer and centrifuged at 15,000 g for 20 min. The chromogen was formed by
12 mixing 200 μ l of supernatants with 1 ml of a reaction mixture containing 15% (w/v)
13 Trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl
14 hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100 °C for 30 min (Minotti and
15 Aust, 1987). After cooling at room temperature, tubes were centrifuged at 800 g for 5 min and
16 the supernatant was used for spectrophotometric reading at 532 nm. Lipid peroxidation was
17 estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed
18 as equivalents of malondialdehyde (MDA) according to Halliwell & Gutteridge (1989). The
19 calibration curve was made using MDA in the range of 0.1-10 nmol. A blank for all samples
20 was prepared by replacing the sample with extraction medium, and controls for each sample
21 were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl
22 hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of 2-
23 thiobarbituric acid-reactive substances (TBARS) during the acid-heating step of the assay.

24

25

1 *Glutathione and ascorbate contents*

2 Glutathione content was measured as described by Smith (1985). Five hundred milligrams of
3 fresh tissues of each plant group were homogenized in a cold mortar with 5 ml 5% (w/v)
4 sulfosalicylic acid, the homogenate was filtered and centrifuged at 1000 g for 10 min. One
5 millilitre of supernatant was neutralized by 1.5 ml 0.5 M K-phosphate buffer (pH 7.5). The
6 standard incubation medium was a mixture of: 0.5 ml 0.1M sodium phosphate buffer (pH 7.5)
7 containing 5 mM EDTA, 0.2 ml 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 ml 2 mM
8 NADPH, and 0.1 ml (1 unit) glutathione reductase. The reaction was initiated by the addition
9 of 0.1 ml glutathione standard or of extract. The change in absorbance at 412 nm was
10 recorded for 9 min.

11 Ascorbate was assayed photometrically by the reduction of 2,6-
12 dichlorophenolindophenol (DCPIP) as described by Leipner et al. (1997). Five hundred
13 milligrams fresh tissues from each plant group were homogenized in 5 ml ice-cold 2% (w/v)
14 metaphosphoric acid in the presence of 1 g NaCl. The homogenate was filtered through a
15 filter paper. An aliquot of 300 μ l was mixed with 200 μ l 45% (w/v) K_2HPO_4 . After 15 min
16 incubation at 25 °C, 1 ml 2M citrate-phosphate buffer (pH 2.3) and 1 ml 0.003% (w/v) DCPIP
17 were added. The absorbance at 524 nm was measured immediately. The content of
18 ascorbate was calculated by reference to a standard curve made of ascorbate.

19

20 **Statistical Analysis**

21 Statistical analysis was performed using SPSS 19.0 program (SPSS Inc., Chicago, IL, USA).
22 All data were subjected to analysis of variance (ANOVA) with inoculation treatment (non-
23 inoculated, partially inoculated or totally inoculated) and water regime (well watered,
24 physiological drought and non-physiological drought) as sources of variation. Post Hoc
25 comparisons with the LSD test were used to find out differences between groups.

1 RESULTS

2

3 **Plant biomass and AM root colonization**

4 In this study nonAM plants received nutrient solution in order to equalize plant biomass
5 before starting the drought treatments. Thus, no differences in plant growth were observed
6 between AM and nonAM plants. Only physiological drought decreased the plant biomass
7 production in both AM and nonAM plants (data not shown).

8 Non AM plants did not show mycorrhizal root colonization. In the AM treatments, the
9 percentage of mycorrhizal root length ranged from 71% to 83%, with no significant
10 differences among treatments (Table 1).

11

12 **Leaf water potential, stomatal conductance and efficiency of photosystem II**

13 The lowest leaf water potential value was found in nonAM plants subjected to drought
14 (Cds/Cds), reaching -1.06 MPa, while the highest value (-0.75 MPa) was found in AM plants
15 (Riww/Riww) cultivated under well watered conditions (Fig. 2A). It is remarkable that under
16 non physiological drought (one root compartment was well watered) the leaf water potential
17 was significantly higher when the root compartment subjected to drought was inoculated with
18 *R. intraradices* than when remained uninoculated (Cww/Rids>Cww/Cds). However, under
19 drought no significant differences were found between AM and nonAM plants.

20 Under well watered conditions, only AM plants in both roots compartments enhanced
21 significantly the stomatal conductance as compared to nonAM plants (Fig. 2B). Plants
22 subjected to physiological drought reduced the stomatal conductance by 80%. However, the
23 AM plants (Rids/Rids) maintained higher values of stomatal conductance than nonAM ones
24 or those with only half root colonized. Again, under non physiological drought, the stomatal

1 conductance was significantly higher when the root compartment subjected to drought was
2 inoculated with *R. intraradices* than when remained uninoculated (Cww/Rids>Cww/Cds).

3 The efficiency of photosystem II was enhanced by mycorrhization (half or whole root)
4 both under well watered and under physiological drought conditions (Fig. 2C). Indeed, AM
5 plants (half or whole root) exhibited values similar to well watered conditions, while nonAM
6 plants reduced further this parameter as a consequence of drought.

7

8 **Accumulation of PIPs in maize plants**

9 We analyzed the accumulation of PIP1s, PIP2s and PIP2s phosphorylated aquaporins at
10 position S₂₈₃ of C-terminal end (abbreviated P₂₈₃ from now on) and at position S₁₂₆ of loop B
11 (abbreviated P₁₂₆ from now on). Results showed that all these proteins followed a similar
12 trend and resulted regulated differently by drought in AM and in nonAM plants.

13 In roots, nonAM plants reduced the accumulation of all the PIPs analyzed when
14 subjected to physiological drought (Cds/Cds, Fig. 3, 4, 5 and 6). This reduction ranged from
15 33% for PIP1s and P₂₈₃ to 43% for PIP2s and P₁₂₆. AM plants (whole root colonized)
16 exhibited always lower levels of PIPs than nonAM plants (a reduction by about 70%), but
17 these levels were not further affected by drought.

18 Under well-watered conditions, all PIPs reduced gradually their accumulation level as
19 the degree of root colonization increased from half root system to whole root system,
20 affecting equally at both root compartments. However, in mycorrhizal plants with a non-
21 colonized root fraction (Cww/Riww), the accumulation of PIP1s was reduced (by about 40%)
22 only in the AM root compartment, without affecting the nonAM root fraction. Under
23 physiological drought stress conditions the accumulation of all PIPs was lower in the AM root
24 fraction than in the nonAM fraction (Cds/Rids). This reduction ranged from 55 to 60%
25 depending on the PIP analyzed.

1 Under non physiological drought, nonAM plants and AM plants having the whole root
2 system inoculated with *R. intraradices* showed similar levels of PIPs as their corresponding
3 well-watered counterparts. However, in the case of AM plants with only half of the root
4 system colonized by *R. intraradices* (Cww/Rids and Riww/Cds) we observed some
5 remarkable results. Indeed, these plants exhibited enhanced accumulation of PIP1s, P₂₈₃ and
6 P₁₂₆ in the root compartments maintained under well-watered conditions as compared to the
7 root compartment subjected to drought (Fig. 3, 5 and 6). Another remarkable result was that
8 when the drought stress affected the AM root compartment (Cww/Rids), PIP1s and P₂₈₃
9 enhanced their accumulation level by 71% in the nonAM root fraction maintained under well
10 watered conditions, while this enhancement was not observed in the AM root fraction
11 (Riww/Rids) (Fig. 3).

12 In the case of leaves, nonAM plants did not show significant changes in PIPs
13 accumulation by the water treatments applied (Fig. 3, 4, 5 and 6). PIP1s accumulated more
14 in leaves of AM plants both under well-watered and under non physiological drought
15 conditions.

16 Under physiological drought conditions, PIP1s and P₂₈₃ enhanced their accumulation
17 in leaves of plants with only half root systems colonized by the AM fungus. However, when
18 the AM fungus colonized both roots compartments these proteins showed levels similar to
19 nonAM plants.

20 Under non physiological drought we observed an effect of AM symbiosis on PIPs
21 accumulation in leaves when the AM fungus was present in the root compartment subjected
22 to drought (Cww/Rids and Riww/Rids), especially in plants with the whole root system
23 inoculated (Riww/Rids). Thus, PIP1s, PIP2s, P₂₈₃ and P₁₂₆ exhibited the highest content in
24 leaves of these plants.

25

1 **Accumulation of osmoregulatory compounds**

2 Under well-watered conditions no significant differences were observed among treatments in
3 the accumulation of proline in roots, while in leaves, nonAM plants accumulated 20% more
4 proline than AM plants (Fig. 7). When the plants were subjected to non-physiological drought,
5 only the roots of the compartment subjected to drought accumulated proline, regardless of
6 the presence of the AM fungus. However, these plants did not accumulate more proline in
7 leaves. Under physiological drought conditions, both roots compartments of AM plants
8 accumulated proline, even if only one of these compartments was colonized by the AM
9 fungus. In contrast, nonAM plants did not enhanced their proline levels in roots. In leaves, the
10 proline enhanced in all treatments subjected to physiological drought. The accumulation of
11 proline in leaves was higher in AM plants (45% more than well watered plants) than in
12 nonAM plants (20% of increase).

13 Total soluble sugars accumulated more in all the treatments subjected to physiological
14 drought, both in roots and in leaves (Fig. 8). In roots of nonAM plants or of AM plants with
15 only half of the root system colonized the enhancement of proline accumulation was about
16 45% as compared to well watered conditions. In AM plants with the whole root system
17 colonized the enhancement was about 70% in average. In leaves, total soluble sugars
18 increased in droughted plants, especially in nonAM ones or in those with only half root
19 system colonized. Non physiological drought treatment had no significant effect on soluble
20 sugars accumulation either in leaves or in roots.

21

22 **Accumulation of hydrogen peroxide**

23 Under well watered conditions the levels of hydrogen peroxide were similar in AM and
24 nonAM plants (Fig. 9). When plants were subjected to physiological drought, the
25 accumulation of hydrogen peroxide increased only in the nonAM root fractions of the

1 different treatments, increasing by 49% on average. On the contrary, in the AM root
2 fractions the levels of hydrogen peroxide were maintained similar to well-watered conditions.
3 In plants subjected to non-physiological drought, the level of hydrogen peroxide increased
4 locally in the root compartment subjected to drought of nonAM plants (Cww/Cds). All the AM
5 plants maintained lower hydrogen peroxide levels in both roots compartments.

6 Few differences in hydrogen peroxide accumulation were observed in leaves.

7

8 **Oxidative damage to lipids**

9 Under well watered conditions there were no significant differences in the accumulation of
10 lipid peroxides between AM and nonAM plants (Fig. 10). Under physiological drought
11 conditions, there was an increase of lipid peroxidation in roots. In nonAM plants the increase
12 was by 184% on average, in AM plants with only half root system colonized the increase was
13 by 118% on average and in AM plants with the whole root system colonized the increase was
14 not significant (36% on average), as compared to the corresponding well watered treatment.

15 Under non-physiological drought the behaviour of AM and nonAM plants was different.
16 Thus, when the drought was imposed in the AM root compartment (Cww/Rids and
17 Riww/Rids), the lipid peroxidation increased only locally in such compartment. In contrast, in
18 nonAM plants, where the drought was imposed in a nonAM root compartment (Cww/Cds),
19 the lipid peroxidation increased similarly in both root compartments. This indicates that the
20 AM symbiosis restricted locally the oxidative damage to lipids induced by drought.

21 No significant differences in lipid peroxidation were observed in leaves.

22

23 **Accumulation of ascorbate**

24 The levels of ascorbate were high in nonAM roots cultivated under well watered conditions,
25 while in AM roots the ascorbate levels were 20% lower in average (Fig. 11). When the plants

1 were subjected to drought, the levels of ascorbate decreased in all plants. The same
2 decrease was observed in nonAM plants subjected to non-physiological drought, while in AM
3 plants the reduction of ascorbate occurred only in the nonAM root half subjected to drought
4 (Riww/Cds). When the drought was imposed in the AM root half the levels of ascorbate were
5 similar to those under well-watered conditions.

6 In leaves, the levels of ascorbate under well-watered conditions increased in AM
7 plants as compared to nonAM ones. Drought stress also enhanced the levels of ascorbate,
8 especially in AM plants (either half or whole root colonized). Under non-physiological drought
9 the levels of ascorbate were similar to well-watered condition, except in AM plants with only
10 half root system colonized and subjected to drought in the AM root compartment, where the
11 ascorbate levels increased.

12

13 **Accumulation of glutathione**

14 Under well-watered conditions, the total glutathione content in roots was not affected by the
15 AM fungal presence (Fig. 12). The glutathione content increased considerably when the
16 plants were subjected to drought stress, especially in AM root compartments. Under non-
17 physiological drought, the glutathione content increased only locally in the AM root half, when
18 the drought was imposed in such root half (Cww/Rids and Riww/Rids). The increases were of
19 about 53% and 62% as compared to the well-watered root half, respectively.

20 No significant differences in glutathione content were observed in leaves.

21

22 **DISCUSSION**

23 *Plant water status, osmoregulation and physiology*

24 Plants in nature are constantly confronted to environmental limitations, being drought the
25 most common abiotic stress affecting plant development (Bray 2004; Farooq et al. 2014).

1 Drought induces morphological, physiological, biochemical and molecular changes, which
2 affect negatively the plant growth and development (Wang et al. 2001; Gollback et al. 2014).
3 Thus, most plant processes are affected by the water limitation directly or indirectly (Akinci &
4 Losel 2012). Drought causes plant tissue dehydration due to the imbalance between root
5 water uptake and leaf transpiration (Aroca et al. 2012). By that reason, many of the
6 physiological adaptations of plants to drought stress are directed toward the control of
7 transpiration rate (Akinci & Losel 2012), of root hydraulic conductivity (Aroca et al., 2012) and
8 of osmotic adjustment (Kishor & Sreenivasulu 2014). All together, these modifications allow
9 the plant to maintain cellular turgor and an adequate physiology in order to continue plant
10 growth and development (Nayyar & Gupta, 2006). In this study AM plants had a better
11 physiological response to the drought stress imposed than nonAM plants, as suggested by
12 their higher stomatal conductance, efficiency of photosystem II or accumulation of proline and
13 soluble sugars under drought. NonAM plants had low leaf water potential and reduced
14 stomatal conductance even under non-physiological drought.

15 Maintaining a high stomatal conductance allows the plant a higher CO₂ uptake for
16 photosynthesis (Davies et al. 1993; Sheng et al. 2008). In this study results showed that one
17 of the main benefits for AM plants was the maintenance of the photosynthetic efficiency as
18 compared to nonAM plants, both under well-watered and under drought stress conditions.
19 Despite the reduction of water potential in the leaves, under physiological drought AM plants
20 maintained the same levels of photosynthetic efficiency than under well-watered conditions,
21 concomitantly with higher stomatal conductance. This suggests that the benefit of AM
22 symbiosis goes beyond the simply supply of water. Indeed, Augé et al. (2008) observed that
23 under amply watered conditions the fungus *Glomus intraradices* enhanced by 27% the
24 stomatal conductance of squash plants, but leaf hydraulic conductance did not increase in
25 these plants. These changes have been linked to hormonal alterations in host plants (Augé

1 2000) or to a higher capacity for CO₂ fixation. For instance, Valentine et al. (2006) found that
2 grapevines inoculated with one AM fungus showed higher Rubisco activity than nonAM ones
3 during drought episodes.

4 When the soil water potential decreases due to drought the gradient of water potential
5 favourable to water entrance into roots also decreases, reducing the water flow toward roots.
6 Thus, plants subjected to drought tend to decrease their tissue water potential by
7 accumulating compatible solutes (such as proline or soluble sugars) in order to maintain the
8 root water uptake (Porcel & Ruiz-Lozano 2004; Flowers & Colmer 2008). Under well-watered
9 conditions AM plants accumulated less proline since these plants had higher shoot water
10 potential and did not need osmotic adjustment in their leaves. In such way these plants avoid
11 the expense of energy to synthesize proline. On the contrary, under physiological drought,
12 AM plants exhibited the capacity to accumulate more proline in leaves and roots, in spite of
13 the energy expenses, since it was necessary for osmotic adjustment (Talaat & Shawky 2011).
14 NonAM plants did not increase proline in roots when subjected to drought, which suggest a
15 lower osmotic adjustment capacity than in roots of AM plants. A similar response was found
16 also in soybean (Porcel & Ruiz-Lozano 2004) and lettuce (Ruiz-Lozano et al. 2011). However,
17 when only half root system was subjected to drought (non-physiological drought), data show
18 that proline accumulated only locally in droughted roots, regardless of AMF inoculation. This
19 suggests that the accumulation of proline in roots may be a mechanism operating at local
20 level to maintain the water flow toward the root fraction subjected to drought and could
21 explain the contradictory results found so far on proline accumulation in AM plants (Ruiz-
22 Lozano et al. 2012a). Thus, we observed that maize plants responded accumulating proline
23 both, locally in roots when the drought affected only to half root system and systemically
24 when the drought affected the whole root system, being the last effect ampler in AM plants.

1 The accumulation of soluble sugars under drought was, contrarily to proline, systemic,
2 affecting both roots compartments and leaves. In any case, AM plants accumulated a lower
3 amount of soluble sugars in leaves than nonAM plants. This effect has been previously
4 related to a higher carbon requirements and mobilization in AM plants (Subramanian &
5 Charest 1995; Subramanian et al. 1997). Indeed, data of stomatal conductance and
6 photosynthetic efficiency in AM plants suggest that these plants maintained the carbon
7 assimilation processes enhanced as compared to nonAM ones, as previously reported
8 (Fester et al. 2005; Lendenmann et al. 2011). In addition, AM plants seem to maintain a
9 higher metabolic activity and can be using the soluble sugars in order to form complex sugars.

10 A fine control of water transport is of key importance for plant survival under drought
11 stress conditions. Drought decreases the root hydraulic conductivity (Aroca et al. 2012), a
12 process in which PIPs play a fundamental role (Javot & Maurel 2002; Katsuhara et al. 2008).
13 In addition, AM fungi can affect the root hydraulic conductivity of host plants through
14 regulation of plant aquaporins (Aroca et al. 2007, 2008; Ruiz-Lozano et al. 2009; Bárzana et
15 al. 2014), being this effect considered as an important factor in the regulation of water
16 relations in mycorrhizal plants (Marjanovic et al. 2005; Lee et al. 2010; Ruiz-Lozano et al.
17 2012b).

18 When we analyzed the content of different PIPs in roots of plants cultivated under well-
19 watered conditions, we found a decrease of PIP amounts as a consequence of
20 mycorrhization. Indeed, the highest levels of PIPs were found in roots of nonAM plants under
21 well-watered or under non-physiological drought. At the same time, AM plants exhibited
22 higher leaf water potential than nonAM plants. This would suggest that under these
23 conditions AM plants regulated better their cellular water content. To this effect, it may have
24 contributed an also better control of the switching between cell-to-cell and apoplastic water
25 transport pathways (Bárzana et al. 2012). Moreover, we need to consider the role of AM

1 fungal aquaporins in transporting water (Li et al. 2013; Xu et al. 2014), which could regulate
2 also the aquaporin accumulation of the host plant (Aroca et al. 2009).

3 When plants were subjected to a physiological drought, we found that the levels of
4 different PIPs were reduced in nonAM roots fractions, as compared to well-watered
5 conditions. This has been interpreted as a mechanism to prevent the water loss from cells,
6 once the plant is suffering a drought stress (Yamada et al. 1997; Smart et al. 2001; Porcel et
7 al. 2006). However, the levels of PIPs were kept low in the mycorrhizal root fractions,
8 maintaining similar levels as under well-watered conditions. This result suggest that a lower
9 PIP content when plants grow under optimal conditions can be advantageous when the
10 environmental conditions change, since plants with a low PIP content would be intrinsically
11 prepared to conserve water, improving their water use efficiency (Hanba et al. 2004; Sade et
12 al. 2010; Belko et al. 2012, 2013).

13 Under non-physiological drought, we found a local regulation by the AM symbiosis of
14 PIP1s and phosphorylated PIP2s. Indeed, in AM plants having only half root system
15 colonized by *R. intraradices* (Cww/Rids and Riww/Cds) the accumulation of PIP1s, P₂₈₃ and
16 P₁₂₆ in the well-watered root compartment was higher than in the root compartment subjected
17 to drought. More precisely, when the root compartment kept under well-watered conditions
18 was the AM one, the amount of phosphorylated PIP2s (P₂₈₂ and P₁₂₆) increased in such root
19 fraction. When the root compartment kept under well-watered conditions was the nonAM one,
20 it increased more the amount of PIP1s. This could serve to maintain the water movement via
21 cell-to-cell pathway in those roots maintained under well-watered conditions, contributing to
22 the better water status in these plants. In any case, data show that there was local effect of
23 AM symbiosis on the accumulation of aquaporins in the roots.

24 It is known that PIPs can have a crucial role in the control of stomatal movements and
25 in mesophyll conductance, controlling the transport of both water and CO₂, with subsequent

1 effects on photosynthesis (Uehlein et al. 2003; Flexas et al. 2006; 2012; López et al. 2013;
2 Heinen et al. 2014). NonAM plants did not show changes in the levels of PIPs in leaves
3 under the different conditions assayed in this study. AM plants, in contrast, showed a more
4 fine regulation of PIPs amounts in leaves. PIP1s have been related directly with the transport
5 of CO₂ (Uehlein et al. 2003; Otto et al. 2010). We found a higher content of PIP1s in leaves of
6 AM plants (half or whole root system colonized) under well-watered or under non-
7 physiological drought, and this correlated with and elevated stomatal conductance and
8 efficiency of photosystem II in these plants. A correlation between CO₂ conductance in leaf
9 mesophyll, mediated by NtAQP1, and plant photosynthesis and growth has also been
10 observed in tomato mutant plants expressing NtAQP1 and a hexokinase gene (Kelly et al.
11 2014).

12 In this study we have observed that under physiological drought PIP1s accumulate in
13 leaves to higher levels in plants with only half root system colonized by the AMF than in
14 plants with whole root system colonized. This effect may be related to the production of more
15 ABA by the nonAM root fraction in these plants. Such ABA may act as a signal in leaves
16 inducing the accumulation of PIP1s in this specific treatment. Indeed, higher accumulation of
17 ABA under drought stress in nonAM roots than in AM ones has been described (Estrada-
18 Luna & Davies 2003). Also, modulation of PIP aquaporins by ABA has been described (Ruiz-
19 Lozano et al. 2009).

20 We also observed that all PIP2s analyzed increased in leaves of fully-colonized AM
21 plants under non-physiological drought or in the root fraction not subjected to drought. The
22 application of a drought stress to only a part of the fully-colonized root system may induce a
23 chemical signal acting systemically and inducing the accumulation of PIP2s in leaves or in
24 the other root fraction. This only happened when the whole root system was colonized by the
25 AM fungus, while in plants with only half root system colonized this signal was not induced.

1 The signal is likely to be a plant hormone, i.e. jasmonic acid, which has been shown to affect
2 several plant aquaporins in tomato, including PIP2s accumulation (Sánchez-Romera et al.
3 2014).

4 Under drought stress, AM plants with only half root system colonized exhibited
5 enhanced accumulation of PIP1s and P₂₈₃ in their leaves. In contrast, when the whole root
6 system was colonized by *R. intraradices*, these proteins were maintained at the same level
7 than nonAM plants. This may involve an increase of cell-to-cell water transport pathway in
8 the leaves of AM plants with only half root system colonized. Moreover, this plant group
9 maintained a similar efficiency of photosystem II than plants with the whole root system
10 colonized by AMF, but a lower stomatal conductance. This would suggest that enhanced
11 mesophyll conductivity is necessary in these plants to increase the permeability to water and
12 CO₂ under conditions of limited transpiration and to maintain the integrity of photosynthetic
13 system. Indeed, Morillón & Chrispeels (2001) found that attenuation of the transpiration
14 stream was required for the up-regulation of osmotic water permeability in leaf cells, and that
15 this up-regulation was mediated by the activation of aquaporins in the plasma membrane.

16

17 *Antioxidant defence*

18 Plants subjected to water deficit accumulate ROS that can generate a secondary oxidative
19 stress in their tissues (Miller et al. 2010; Noctor et al. 2014). Hydrogen peroxide is one of the
20 most abundant ROS, with a relatively high half-life, as compared to other ROS. At low
21 concentrations, hydrogen peroxide can function as molecular signal to activate plant stress
22 responses, but at high concentrations it is harmful as other ROS (Quan et al. 2008).

23 In the present study the level of oxidative damage to lipids did not increase
24 significantly in roots of AM plants having the whole root system colonized and in those with
25 only half root system colonized the increase was lower (118%) than in the nonAM ones

1 (184%). Moreover, the roots of nonAM plants exhibited the highest lipid peroxidation under
2 drought and it was also increased by a non-physiological drought even in the non-stressed
3 root fraction. Thus, the antioxidant defences were more effective in AM plants than in the
4 nonAM plants, as previously found (Porcel et al. 2003; Talaat & Shawky 2011, 2014; Lee et
5 al. 2012). Indeed, data showed that under drought stress conditions the mycorrhizal roots
6 accumulated less hydrogen peroxide than nonAM roots, even locally in the AM plants with
7 only half root system colonized.

8 Ascorbate is an important non enzymatic antioxidant compound involved in the
9 removal of H_2O_2 by ascorbate peroxidases, which use ascorbate as electron donor (Foyer &
10 Noctor 2011). Results on ascorbate accumulation suggest that under drought, this compound
11 could be being used by plants to scavenge hydrogen peroxide, since its concentration
12 decreased significantly as compared to well-watered conditions. However, in roots of nonAM
13 plants the accumulation of H_2O_2 was not counteracted by the use of ascorbate, and this lead
14 to higher lipid peroxidation in these plants. In contrast, in AM roots, the use of ascorbate
15 would be enough to maintain low levels of H_2O_2 , as was found in citrus (Wu & Zou 2009) or
16 rice (Ruiz-Sánchez et al. 2011) and this could be also related to an increased level of
17 glutathione in AM compartments subjected to drought. Indeed, when drought affected the
18 mycorrhizal root half (Cww/Rids and Riww/Rids), the lipid peroxidation was restricted locally
19 to these roots and the levels of glutathione were also locally enhanced in such root fraction.
20 Glutathione has not only the functions of scavenging peroxides or regenerating ascorbate
21 pool, but it also may keep the cell pools of reducing power (NADPH) under the necessary
22 conditions for plant cells (Noctor et al. 2012).

23 For most of the oxidative parameters measured in this study, nonAM plants subjected
24 to non-physiological drought (Cww/Cds) responded to partial root stress similarly as plants
25 subject to physiological drought. Indeed, these plants accumulated an important amount of

1 H₂O₂ in the root compartment subjected to drought, while in the corresponding AM treatment
2 (Riww/Cds) this enhancement of H₂O₂ was not significant. Moreover, plants from treatment
3 Riww/Rids were the only ones avoiding completely the partial root drying effects, maintaining
4 all their physiological parameters at similar levels as well watered plants. Thus, all the above-
5 discussed results suggest that AM plants cope better with the oxidative stress induced by
6 drought, allowing them to continue with their physiological processes.

7 Summarizing, maize plants responded to drought by accumulating proline both, locally
8 in roots affected by drought and systemically when the drought affected the two root
9 compartments, being the last effect ampler in AM plants. In addition, the accumulation of
10 PIPs aquaporins was also differently regulated by the water treatment imposed in AM and
11 nonAM root compartments, which could serve to maintain the water movement via cell-to-cell
12 pathway or to favour the switching between cell-to-cell and apoplastic pathways in AM roots,
13 contributing to the better water status in these plants. The antioxidant systems in AM plants
14 were also more efficient to reduce the hydrogen peroxide generated under drought stress.
15 These systems acted locally, affecting specially the root fractions subjected to drought and
16 with a better regulation in AM root compartments. Indeed, when the drought affected only the
17 AM root compartment, the rise of lipid peroxidation was restricted to such compartment,
18 being low in the rest of the plant. On the contrary, in nonAM plants (Cww/Cds), the rise of
19 lipid peroxidation was similar in all root compartments. Thus, the benefits of mycorrhizal
20 symbiosis not only rely in a lower oxidative stress in the host plant, the symbiosis also
21 restricts locally such oxidative stress, allowing the plants to continue with their physiological
22 processes.

23

24

25

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4

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14

Table 1. Percentage of mycorrhizal root length in maize plants cultivated in a split root system. Plants remained as uninoculated controls in both roots compartments (C/C) or were inoculated with the AM fungus *Rhizophagus intraradices* (Ri) either in one or in both root compartments (C/Ri or Ri/Ri, respectively). Plants were cultivated under well-watered conditions (ww/ww), subjected to physiological drought (ds/ds) or subjected to non-physiological drought, affecting only one root fraction (ww/ds).

<u>Treatment</u>	<u>Root compartment</u>							
	<u>C</u>	<u>C</u>	<u>Ri</u>	<u>Ri</u>	<u>C</u>	<u>Ri</u>	<u>Ri</u>	<u>C</u>
ww/ww	0b	0b	76a	81a	0b	79a	-	-
ds/ds	0b	0b	79a	81a	0b	71a	-	-
ww/ds	0b	0b	80a	83a	0b	73a	83a	0b

Means followed by different letters are significantly different ($P < 0,05$) as determined by LSD tests ($n = 6$).

Note: For treatments Ww/Ww or Ds/Ds the combination Ri/C does not exist (-) since it is the same as C/Ri. In contrast, in the treatment Ww/Ds the combinations C/Ri and Ri/C are used in order to distinguish drought stress application either in the AM root half or in the nonAM root half.

1 **Figure 1.** Schematic representation of the split root system used. Each root compartment
2 remained as uninoculated control (C) or was inoculated with the AM fungus *Rhizophagus*
3 *intraradices* (Ri), giving thus, nonAM plants (C/C), AM plants with only half root system
4 colonized (C/Ri) or AM plants with whole root system colonized (Ri/Ri). Plants were cultivated
5 under well-watered conditions (ww/ww), subjected to physiological drought (ds/ds) or
6 subjected to non-physiological drought affecting only one root fraction (ww/ds).

7
8 **Figure 2.** (A) Leaf water potential, (B) stomatal conductance and (C) efficiency of
9 photosystem II in maize plants cultivated in a split root system. Plants remained as
10 uninoculated controls in both roots compartments (C/C) or were inoculated with the AM
11 fungus *Rhizophagus intraradices* (Ri) either in one or in both root compartments (C/Ri or
12 Ri/Ri, respectively). Plants were cultivated under well-watered conditions (ww/ww, grey
13 columns), subjected to physiological drought (ds/ds, black columns) or subjected to a non-
14 physiological drought, affecting only one root fraction (ww/ds, dark-grey columns).

15
16 **Figure 3.** Accumulation of PIP1s in roots and leaves of maize plants cultivated in a split root
17 system. Plants remained as uninoculated controls in both roots compartments (C/C) or were
18 inoculated with the AM fungus *Rhizophagus intraradices* (Ri) either in one or in both root
19 compartments (C/Ri or Ri/Ri, respectively). Non-mycorrhizal root fractions are represented in
20 white columns and mycorrhizal root fractions are represented in black columns. Plants were
21 cultivated under well-watered conditions (ww/ww), subjected to physiological drought (ds/ds)
22 or subjected to non-physiological drought affecting only one root fraction (ww/ds).

23
24 **Figure 4.** Accumulation of non-phosphorylated PIP2s in roots and leaves of maize plants
25 cultivated in a split root system. See legend for Figure 3.

1

2 **Figure 5.** Accumulation of PIP2s phosphorylated at Ser₂₈₃ (P₂₈₃) in roots and leaves of maize
3 plants cultivated in a split root system. See legend for Figure 3.

4

5 **Figure 6.** Accumulation of PIP2s phosphorylated at Ser₁₂₆ (P₁₂₆) in roots and leaves of maize
6 plants cultivated in a split root system. See legend for Figure 3.

7

8 **Figure 7.** Accumulation of proline in roots and leaves of maize plants cultivated in a split root
9 system. See legend for Figure 3.

10

11 **Figure 8.** Accumulation of total soluble sugars in roots and leaves of maize plants cultivated
12 in a split root system. See legend for Figure 3.

13

14 **Figure 9.** Accumulation of hydrogen peroxide in roots and leaves of maize plants cultivated
15 in a split root system. See legend for Figure 3.

16

17 **Figure 10.** Oxidative damage to lipids in roots and leaves of maize plants cultivated in a split
18 root system. See legend for Figure 3.

19

20 **Figure 11.** Accumulation of ascorbate in roots and leaves of maize plants cultivated in a split
21 root system. See legend for Figure 3.

22

23 **Figure 12.** Accumulation of glutathione in roots and leaves of maize plants cultivated in a
24 split root system. See legend for Figure 3.

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1 Figure 1

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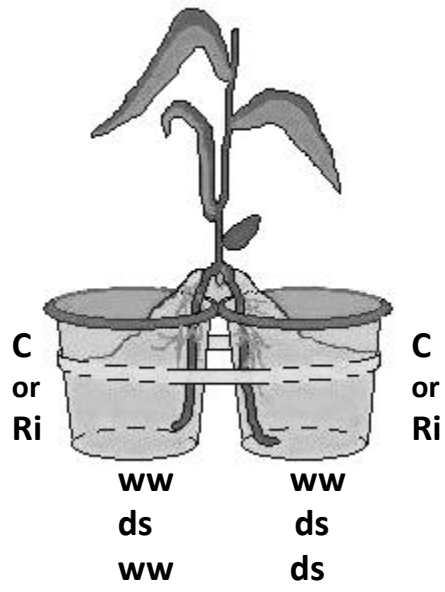
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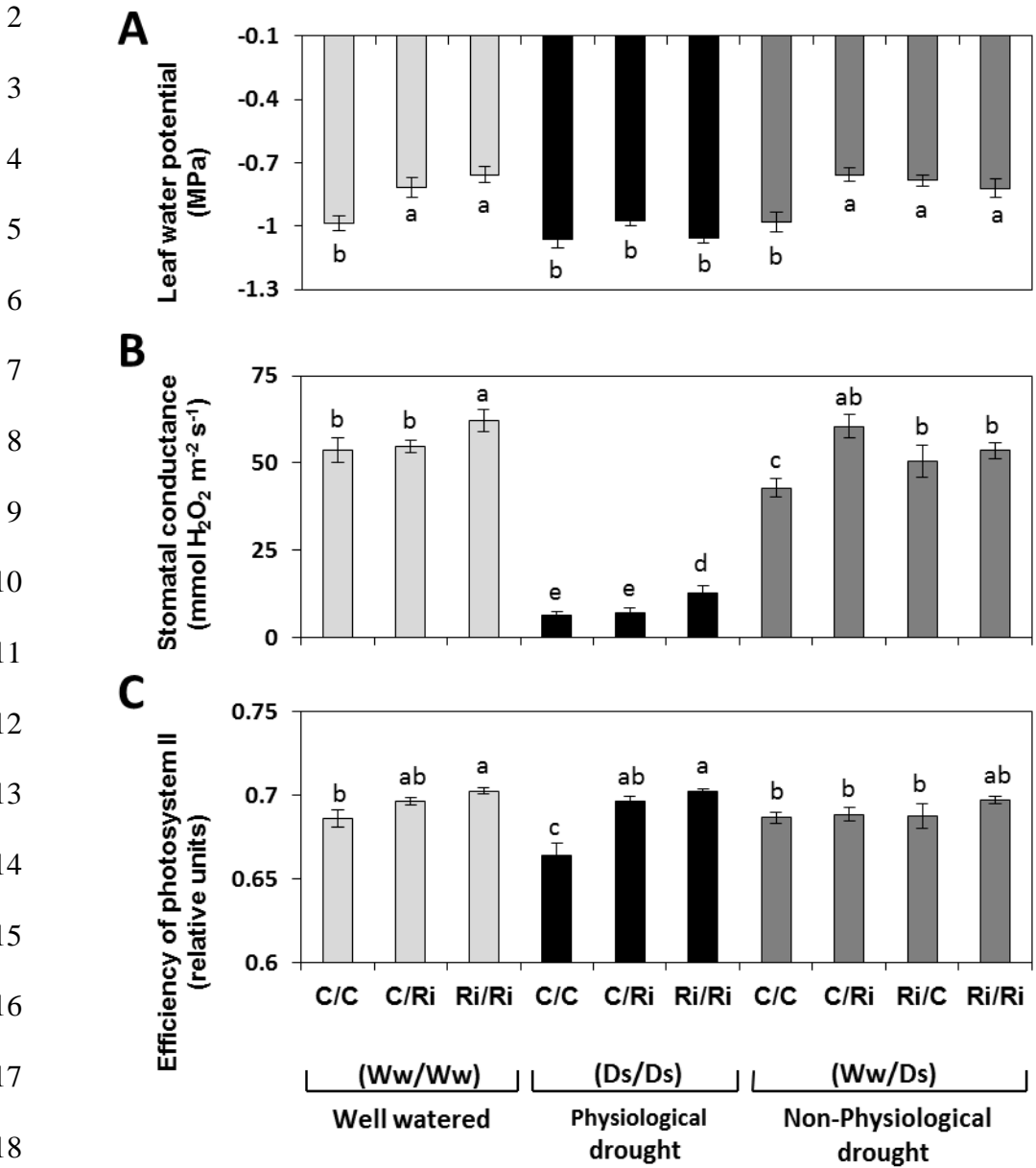
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1 Figure 2



1 Figure 3

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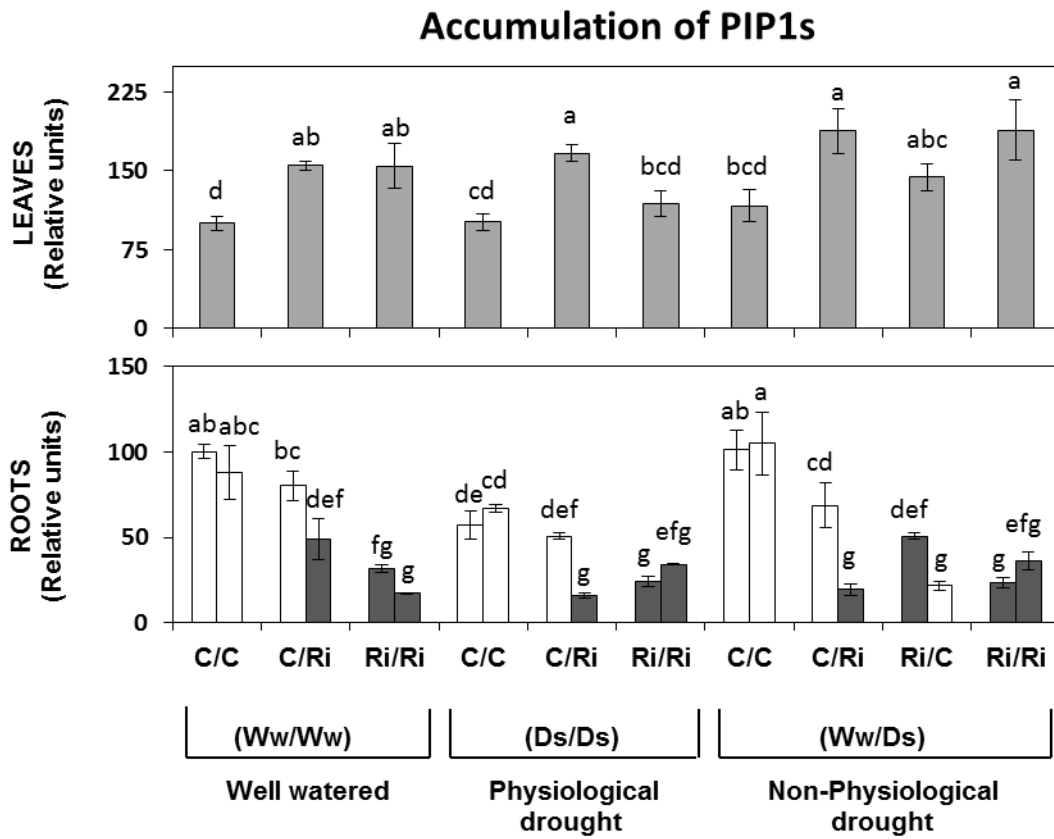
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1 Figure 4

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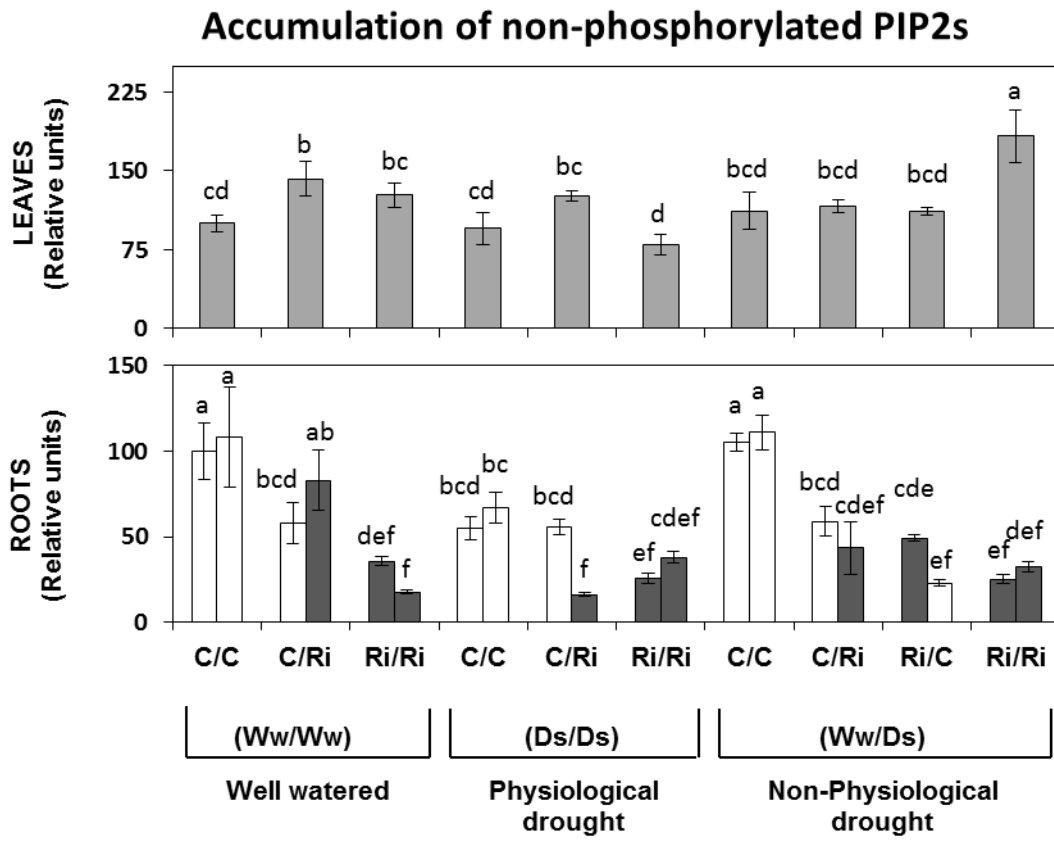
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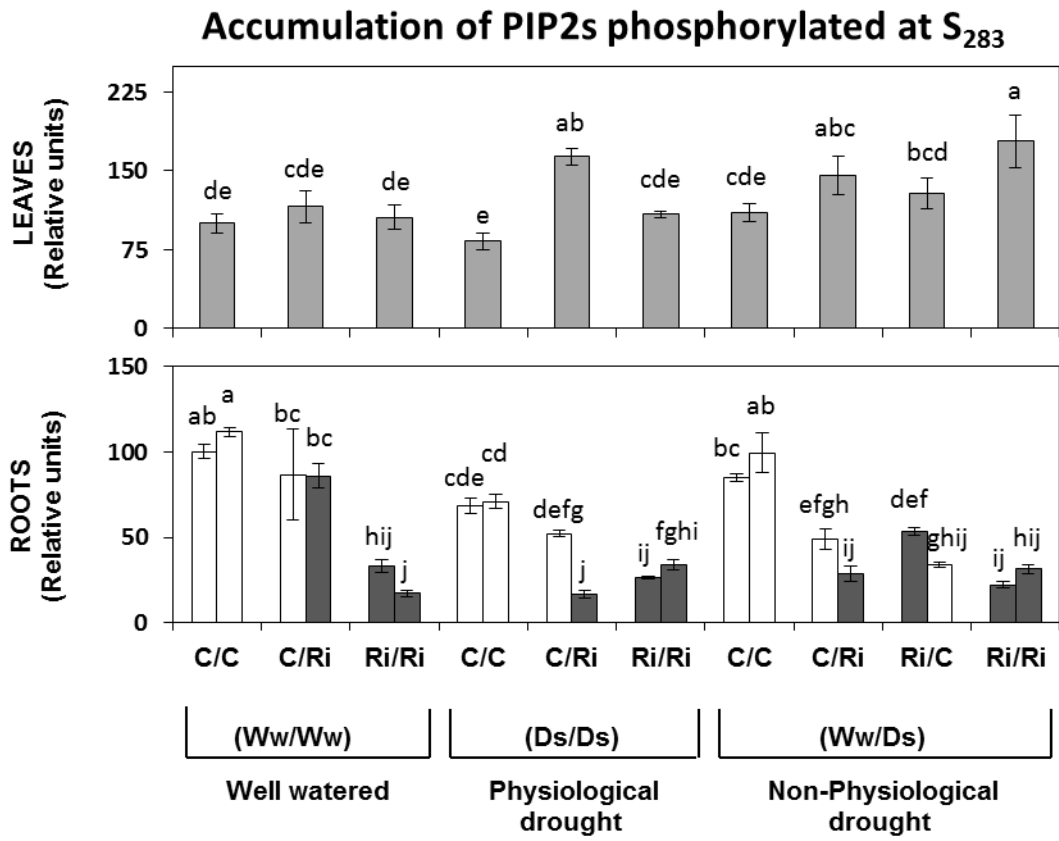
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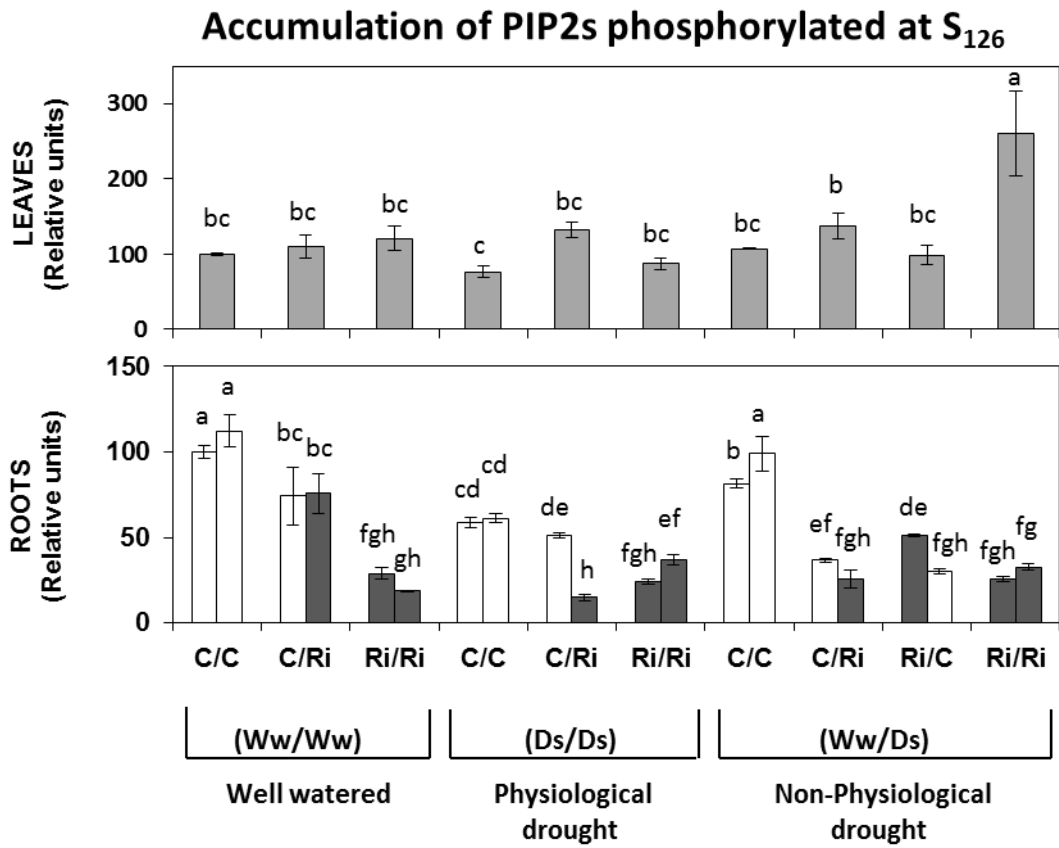
1 Figure 5

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1 Figure 6

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1 Figure 7

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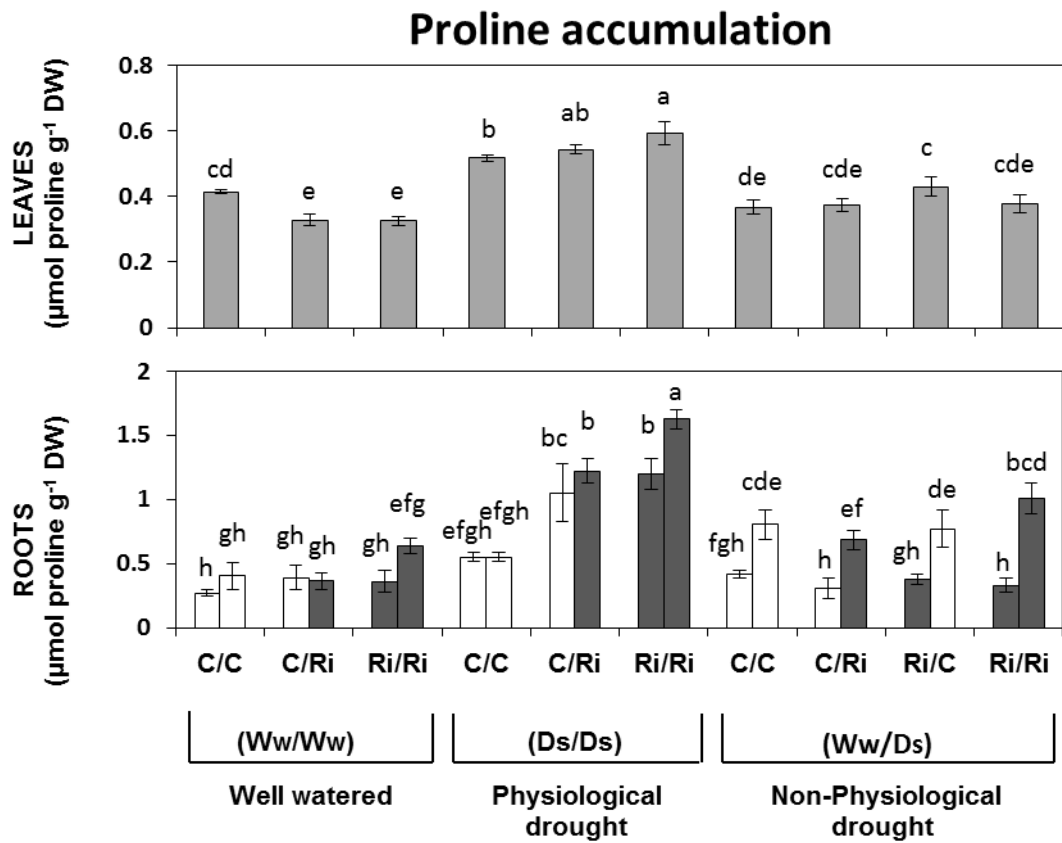
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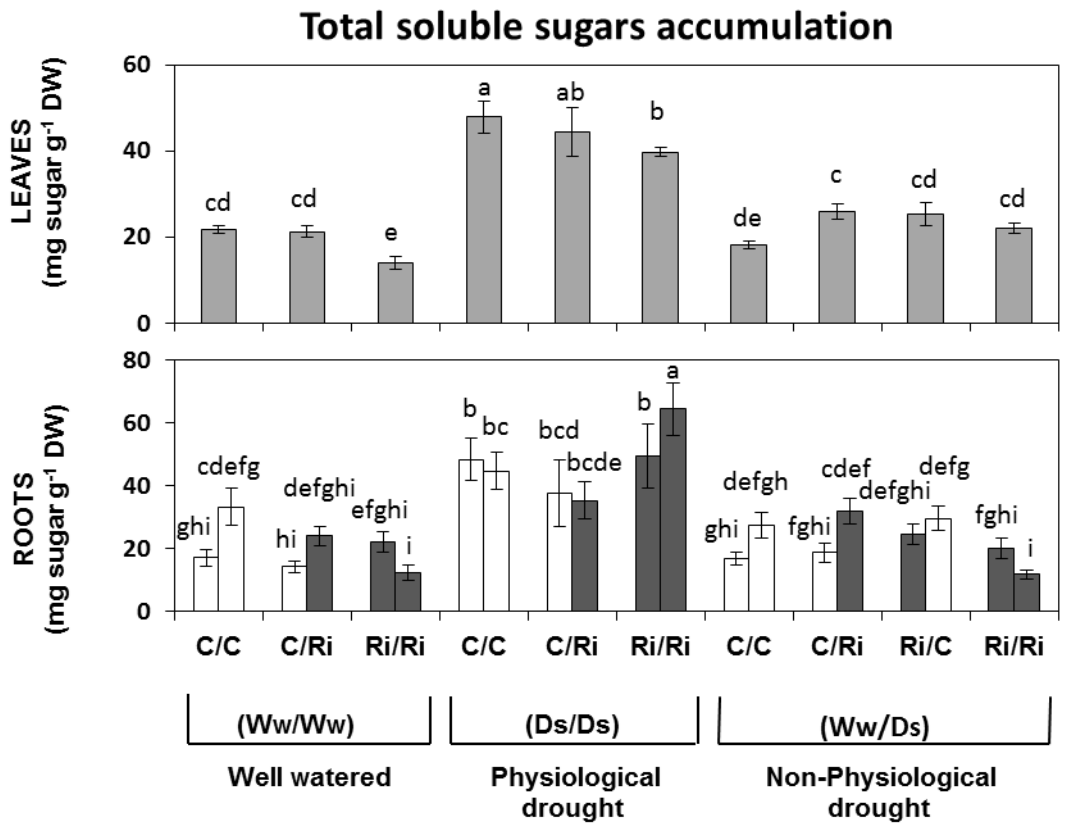
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1 Figure 8

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1 Figure 9

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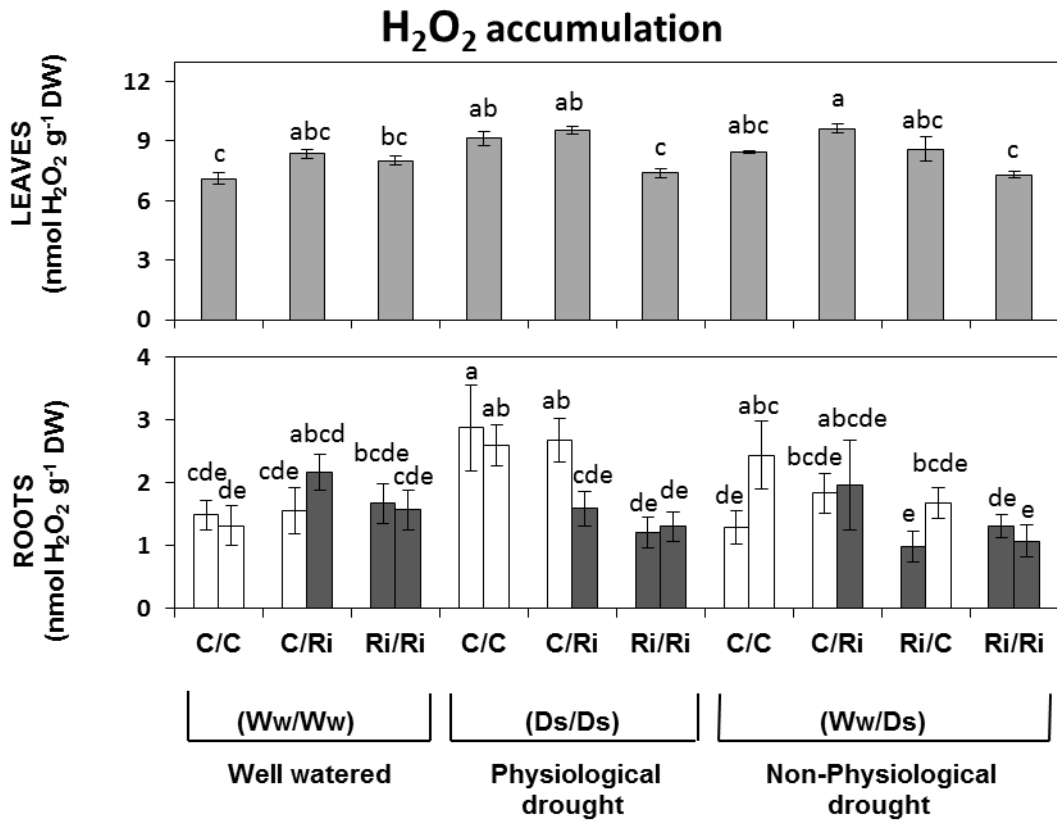
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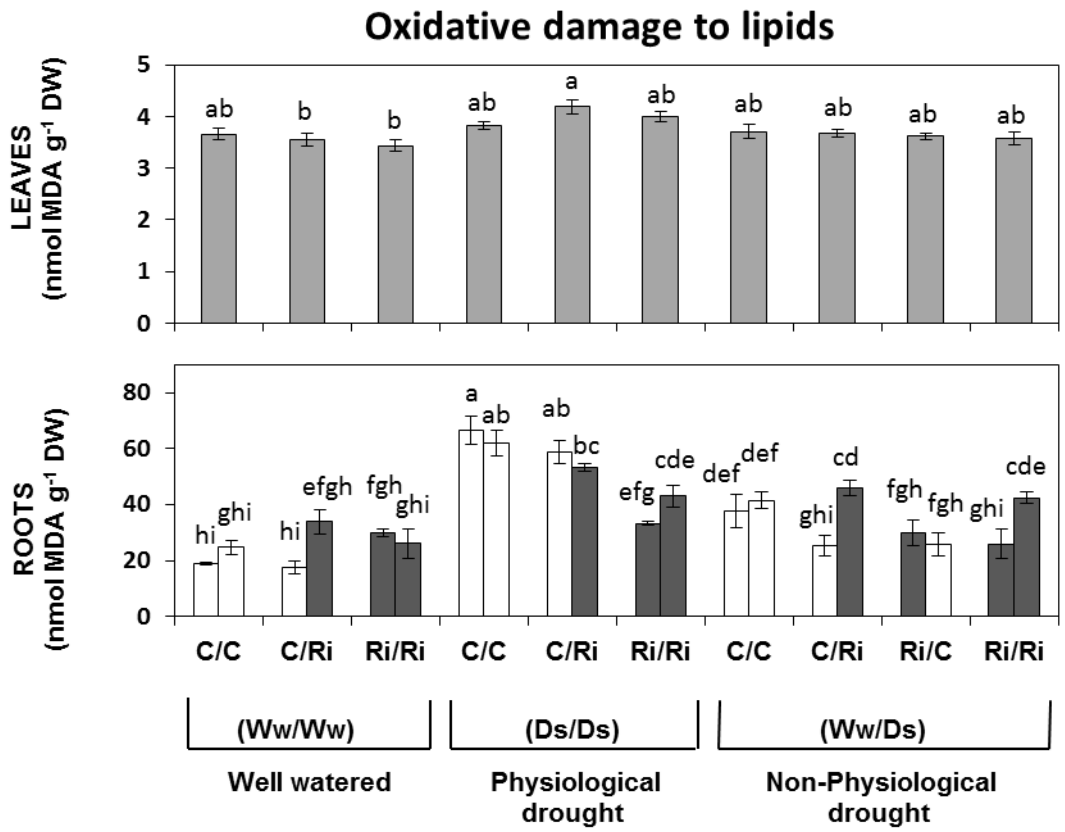
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1 Figure 10

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1 Figure 11

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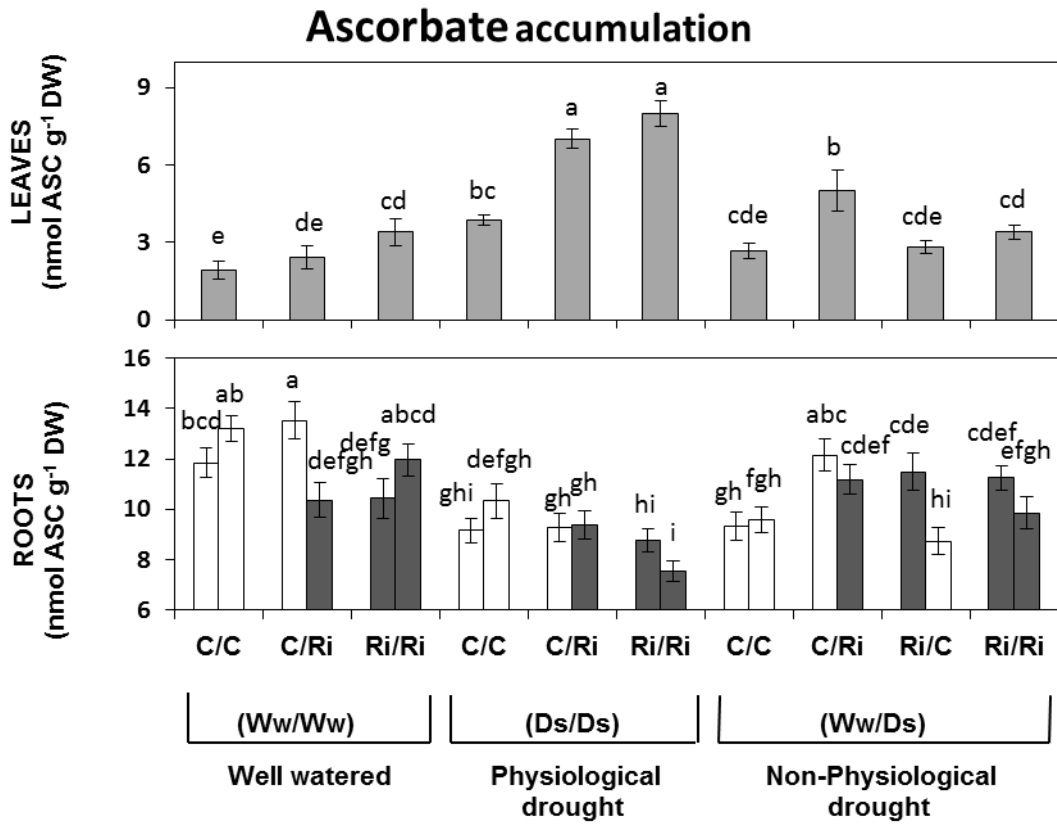
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1 Figure 12

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