1   **Running Title:** Drought and AM symbiosis induce strigolactones

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3   **Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and**

4   **improves drought tolerance in lettuce and tomato**

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1 **SUMMARY**

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3 Arbuscular mycorrhizal (AM) symbiosis alleviates drought stress in plants. However the

4 intimate mechanisms involved, as well as its effect on the production of signalling molecules

5 associated to the host plant-AM fungus interaction remains largely unknown. In the present

6 work, the effects of drought on lettuce and tomato plant performance and hormone levels

7 were investigated in non-AM and AM plants. Three different water regimes were applied and

8 their effects analysed over time. AM plants showed an improved growth rate and efficiency of

9 photosystem II than non-AM plants under drought from very early stages of plant

10 colonization. The levels of the phytohormone abscisic acid, as well as the expression of the

11 corresponding marker genes, were influenced by drought stress in non-AM and AM plants.

12 The levels of strigolactones and the expression of corresponding marker genes were affected

13 by both AM symbiosis and drought. The results suggest that AM symbiosis alleviates drought

14 stress by altering the hormonal profiles and affecting plant physiology in the host plant. In

15 addition, a correlation between AM root colonization, strigolactone levels and drought

16 severity is shown, suggesting that under these unfavourable conditions plants might increase

17 strigolactone production in order to promote symbiosis establishment to cope with the stress.

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20 **KEY WORDS** Abscisic Acid; Arbuscular Mycorrhiza; Drought Stress; Lettuce;

21 Phytohormones; Strigolactones; Tomato

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INTRODUCTION

In natural environments, plants are continuously exposed to adverse environmental conditions of both biotic and abiotic origin such as pathogens, extreme temperatures, nutrient imbalance, salinity and drought, which have a negative impact on plant survival, development and productivity. In recent years, harmful effects of water-related stresses, including salinity and drought are increasing dangerously (Albacete *et al*., 2014, Golldack *et al*., 2014). In addition, global climate change is contributing to spread these problems worldwide (Chaves & Oliveira, 2004, Trenberth *et al*., 2014). Drought is considered the most important abiotic factor limiting plant growth and yield in many areas (Bray, 2004, Trenberth *et al.*, 2014). The severity of drought depends on many different factors including rainfall levels, evaporative demands and moisture storing capacity of soils (Farooq *et al*, 2009, Farooq *et al*, 2014). In plants, drought induces morphological, physiological, biochemical and molecular changes. Thus, most plant processes are affected directly or indirectly by the water limitation (Bárzana *et al*., 2015). Plant responses to water deficiency are complex and, although different plant species vary in their sensitivity and response, it is assumed that all plants have encoded capability for stress perception, signalling and response (Bohnert *et al*., 1995, Golldack *et al.*, 2014). Plants have developed several mechanisms to cope with drought stress such as morphological adaptations, osmotic adjustment, optimization of water resources, improvement of antioxidant system, reduction of growth and photosynthesis rate, and stomatal closure, all aimed to optimize water use (Farooq *et al.*, 2009, Osakabe *et al*., 2014, Ruiz-Lozano *et al*., 2012, Shinozaki & Yamaguchi-Shinozaki, 2006). The different plant responses to cope with environmental stresses are regulated by a crosstalk between hormones and signal molecules, being abscisic acid (ABA) the phytohormone most studied in the response of plants to abiotic stress, specially water-related stresses (Bray, 2004, Peleg & Blumwald, 2011). Indeed, ABA is

3

considered the 'stress hormone' as its biosynthesis is rapidly promoted under this type of stresses (Hong *et al.*, 2013, Osakabe *et al.*, 2014). ABA has an important signalling role in the regulation of plant growth and development, but also in the promotion of plant defence responses (Christmann *et al.*, 2006, Ton *et al.*, 2009).

In addition to the intrinsic protective systems against environmental stresses, plants can establish beneficial associations with a number of microorganisms present in the rhizosphere that can alleviate the stress symptoms (Badri *et al.*, 2009, Mendes et al., 2013). One of the most studied and widespread mutualistic plant-microorganism associations is that established with arbuscular mycorrhizal (AM) fungi. About 80% of terrestrial plants, including most agricultural and horticultural crop species, are able to establish this type of symbiosis with fungi from the phylum Glomeromycota (Barea *et al.*, 2005, Smith & Read, 2008). Through this mutualistic beneficial association, the AM fungus obtains photoassimilates from the host plant to complete its lifecycle and, in turn, it helps the plant in the acquisition of water and mineral nutrients. Thus, AM plants generally show an improved ability for nutrient uptake and tolerance against biotic and abiotic stresses (Pozo *et al.*, 2015). Regarding its effect on drought, in most cases studied AM symbiosis alleviates the negative effects induced by the stress, making the host plant more tolerant to drought (Abbaspour *et al.*, 2012, Aroca *et al.*, 2012, Augé *et al.*, 2015, Bárzana *et al.*, 2012, Bárzana *et al.*, 2014, Porcel *et al.*, 2006), although the signalling and transduction processes involved in these effects are not well known yet (Ruiz-Lozano *et al.*, 2012). Therefore, understanding the mechanisms that enhance plant drought tolerance is crucial to develop new strategies to cope with this stress and to guaranty world food production (Chaves & Oliveira, 2004).

AM symbiosis establishment and functioning requires a high degree of coordination between the two partners, which implies a signal exchange that leads to mutual recognition (Andreo-Jiménez *et al.*, 2015, Bucher *et al.*, 2014, Gutjahr & Parniske, 2013). The molecular

1   dialogue - the so-called pre-symbiotic stage - starts with the production and exudation into the

2   rhizosphere of strigolactones (SLs) by the host plant. SLs are perceived by AM fungi by a so

3   far uncharacterized receptor and stimulate hyphal growth and branching, increasing the

4   chance of encountering the host root (Akiyama *et al*., 2005, Besserer *et al*., 2006). While the

5   importance of SLs in the initial stages of mycorrhizal colonization is well accepted, it is not

6   clear whether they also play a role in subsequent steps of the symbiosis or in the responses to

7   environmental stresses. In addition to molecular cues in the plant-AM fungi interaction, in the

8   rhizosphere SLs also act as host detection signals for root parasitic plants of the

9   Orobanchaceae, including *Striga*, *Orobanche* and *Phelipanche* species, where they stimulate

10   seed germination (Bouwmeester *et al*., 2007, López-Ráez *et al*., 2011b). Accordingly to their

11   role as signalling molecules in the rhizosphere, SLs are mainly produced in the roots and they

12   have been detected in the root extracts and root exudates of both monocot and dicot plants

13   (Xie *et al*., 2010). Since 2008, SLs are classified as a new class of hormones that control

14   several processes in plants. They play a pivotal role as modulators of the coordinated

15   development of roots and shoots in response to nutrient deprivation, especially phosphorus

16   shortage. They regulate above- and below-ground plant architecture, adventitious root

17   formation, secondary growth, reproductive development, leaf senescence and defence

18   responses (reviewed in Ruyter-Spira *et al*., 2013). SLs biosynthetically derive from

19   carotenoids (López-Ráez *et al*., 2008, Matusova *et al*., 2005) by sequential oxidative cleavage

20   by two carotenoid cleavage dioxygenases - CCD7 and CCD8 - belonging to the

21   apocarotenoids as the phytohormone ABA (Walter & Strack, 2011). In addition to their role

22   in the response of plants to abiotic stress, it was shown that ABA is necessary for a proper

23   AM symbiosis establishment and functioning (Martín-Rodríguez *et al*., 2010, Pozo *et al.*,

24   2015). Interestingly, a regulatory role of ABA in SL biosynthesis has been proposed since a

25   correlation between ABA and SL content was observed (Aroca *et al*., 2013, López-Ráez *et*

1 *al*., 2010). More recently, a relationship ABA-SLs has also been shown in Lotus plants under

2 osmotic stress (Liu *et al*., 2015), and a role of SLs in drought stress tolerance in the non-

3 mycorrhizal plant Arabidopsis has been proposed (Ha *et al*., 2014, Bu *et al.*, 2014). However,

4 how ABA-SLs regulation is involved in these water-related stress responses and how it is

5 affected by AM symbiosis is so far unknown.

6 We previously showed that AM symbiosis alleviates the negative effects of salt stress

7 in lettuce by affecting the hormonal profiles and plant physiology (Aroca *et al.*, 2013). In the

8 present study, the effects of drought on AM symbiosis establishment and on the production of

9 the phytohormones SLs and ABA were investigated in two agronomically important crops

10 such as tomato and lettuce. Three different water regimes were used and their effects

11 investigated at early, middle and well-established symbiosis stages. Physiological parameters

12 such as plant biomass, stomatal conductance and efficiency of photosystem II, associated to

13 drought, as well as hormonal levels and the expression of molecular makers associated to SLs

14 and ABA were assessed in mycorrhizal and non-mycorrhizal plants.

15

16 **MATERIALS AND METHODS**

17

18 **Experimental design**

19 The experiment consisted of a factorial design with two inoculation treatments: (1) non-

20 inoculated control plants (NM) and (2) plants inoculated with the AM fungus *Rhizophagus*

21 *irregularis* (Schussler & Walker, 2010) (formerly *Glomus intraradices*) strain EEZ 58 (Ri)

22 and three irrigation treatments: (i) plants cultivated under well-watered conditions, (ii) plants

23 cultivated under moderate drought stress, (iii) plants cultivated under severe drought stress.

24 Two plant species were used with the same experimental design, tomato (*Solanum*

25 *lycopersicum*, cv. Reimlams Rhums) and lettuce (*Lactuca sativa*, cv. Romana). For each plant

1 species, fifteen replicates of each of these treatments were used, totalling 180 pots (90 pots

2 containing tomato plants and 90 pots containing lettuce plants, one plant per pot). Thus, five

3 individual plants of each treatment (30 in total per plant species) were harvested after 4 weeks

4 of cultivation, another plant set after 6 weeks and the last plant set after 8 weeks.

5

Soil and biological materials

7 A loamy soil was collected from Dúrcal (Granada, Spain). The soil had a pH of 8.2 (measured

8 in water, 1:5 w/v); 1.8% organic matter, total nutrient concentrations (g kg-1): N, 2.5; P, 6.2

9 ($NaHCO_3$-extractable P); K, 13.2. The soils was sieved (5 mm), diluted with quartz-sand (<2

10 mm) and vermiculite (2:2:1, soil:sand:vermiculite, v:v:v) in order to avoid excessive

11 compaction, and sterilized by steaming (100 ºC for 1 h on 3 consecutive days).

12 Seeds of tomato and lettuce were sown in trays containing sterile moist sand for

13 germination during 1 week. After that, individual seedlings were transferred to pots

14 containing 1000 grams of the soil/sand/vermiculite mixture described above.

15 Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted

16 of soil, spores, mycelia and infected root fragments. The AM fungus was *R. irregularis*

17 (Schenck and Smith), strain EEZ 58. Ten grams of inoculum with about 60 infective

18 propagules per gram (according to the most probable number test), were added to appropriate

19 pots at sowing time. Non inoculated control plants received the same amount of autoclaved

20 mycorrhizal inoculum together with a 3 ml aliquot of a filtrate (<20 μm) of the AM inoculum

21 in order to provide a general microbial population free of AM propagules.

22

Growth conditions

24 The experiment was carried out under greenhouse conditions with temperatures ranging from

25 19 to 25ºC, 16/8 light/dark period, a relative humidity of 50-60% and an average

1 photosynthetic photon flux density of 800 µmol m^{-2} s^{-1}, as measured with a light meter

2 (LICOR, Lincoln, NE, USA, model LI-188B).

3   Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd.,

4 Cambridge, UK). For treatments cultivated under well-watered conditions, water was supplied

5 daily to maintain soil close to 100% of field capacity. The 100% soil water holding capacity

6 corresponded to 22% volumetric soil moisture measured with the ThetaProbe, as determined

7 experimentally in a previous experiment using a pressure plate apparatus. For treatments

8 cultivated under moderate drought stress, water was supplied daily to maintain soil close to

9 75% of field capacity, which corresponded to 14% of volumetric soil moisture measured with

10 the ThetaProbe. Finally, for treatments cultivated under severe drought stress, water was

11 supplied daily to maintain soil close to 55% of field capacity, which corresponded to 8%

12 volumetric soil moisture measured with the ThetaProbe. The soil water content was daily

13 measured with the ThetaProbe ML2 before rewatering (at the end of the afternoon). The

14 amount of water lost was added to each pot in order to keep the soil water content at the

15 desired levels of volumetric soil moisture (Porcel & Ruiz-Lozano, 2004). The drought stress

16 treatments were imposed from the beginning of the experiment, just after seedlings

17 transplantation to the pots. Plants were maintained under these conditions until harvest at 4, 6

18 and 8 weeks. At harvest, the shoot and root system of each plant was separated and weighed.

19 The shoot system was used for the dry weight measurement and the root system frozen in

20 liquid nitrogen and stored at -80 ℃ until use.

21

22 **Parameters measured**

23 *Biomass production and symbiotic development*

24 The shoot dry weight (DW) for each plant at the different time points (4, 6 or 8 weeks after

25 sowing) was measured after drying in a forced hot-air oven at 70 ℃ for 2 days. Five

independent replicates per treatment and time point were analyzed. The mycorrhizal dependency (MD) was calculated for each drought treatment by using the following formula provided by Kumar et al. (2010). MD (%) = (DW of mycorrhizal plant - DW of non-inoculated plant)/DW of mycorrhizal plant X 100, and is an estimation of the plant response to mycorrhizal colonization in terms of biomass enhancement.

At each harvest, the percentage of mycorrhizal fungal colonization in tomato and lettuce plants was estimated by visual observation according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti & Mosse, 1980) in five replicates per treatment.

Stomatal conductance

One day before harvests, stomatal conductance was measured two hours after the onset of photoperiod with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user manual instructions. Stomatal conductance measurements were taken in the second youngest leaf from five different plants from each treatment after 4, 6 and 8 weeks of plant cultivation.

Photosynthetic efficiency

The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. FluorPen quantifies the quantum yield of photosystem II as the ratio between the variable fluorescence in the light-adapted state (FV') and the maximum fluorescence in the light-adapted state (FM'), according to Oxborough and Baker (1997). An actinic light intensity of 1000 µmol (photons) $\cdot$ m^{-2} $\cdot$ s^{-1}

1 was used. Measurements were taken in the second youngest leaf of five different plants of

2 each treatment after 4, 6 and 8 weeks of plant cultivation.

3

4 *ABA content*

5 ABA extraction, purification and quantification were carried out using the method described

6 by Bacaicoa et al. (2009), but using 0.25 g of frozen root tissue (previously ground to a

7 powder in a mortar with liquid nitrogen) instead of 0.5 g. ABA content was measured after 6

8 weeks of plant cultivation. ABA was quantified by liquid chromatography coupled to a 3200

9 Q TRAP (HPLC/MS/MS) system (Applied Biosystems/MDS Sciex, Ontario, Canada),

10 equipped with an electrospray interface, using an reverse-phase column (Synergi 4 mm

11 Hydro-RP 80A, 150x2 mm, Phenomenex, Torrance, CA). A linear gradient of methanol (A)

12 and 0.5% acetic acid in water (B) was used: 35% A for 1 min, 35%-95% A in 9 min, 95% A

13 for 4 min and 95%-35% A in 1 min, followed by a stabilization time of 5 min. The flow rate

14 was 0.20 mL/min, the injection volume was 40 μL and the column and sample temperatures

15 were 30 and 20 ºC, respectively. Detection and quantification were performed by multiple

16 reaction monitoring (MRM) in the negative-ion mode, employing a multilevel calibration

17 graph with deuterated hormones as internal standards. Compound dependent parameters are

18 described in Bacaicoa *et al*. (2009). The source parameters were: curtain gas 25 psi, GS1 50

19 psi, GS2 60 psi, ion spray voltage -4000 V, CAD gas medium, and temperature 600 ºC.

20

21 **Gene expression analysis by real-time RT-PCR (qPCR)**

22 After 6 weeks of plant cultivation, total RNA was isolated from tomato and lettuce roots using

23 Tri-Reagent (Sigma-Aldrich, St Gallen, Switzerland) according to the manufacturer's

24 instructions. The RNA was treated with RQ1 DNase (Promega, Madrid, Spain), purified

25 through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel, Hoerd,

1 France) and stored at -80 ºC until use. Real-time quantitative RT-PCR (qPCR) was performed

2 using the iCycler iQ5 system (Bio-Rad, Hercules, CA, USA) with SYBR Premix Ex Taq

3 (Takara, Saint-Germain, France) and specific primers for genes *LeNCED1*, *Le4*, *SlCCD7,*

4 *SlCCD8, LsNCED2* and *LsLEA1* (Table S1). The first strand cDNA was synthesized with 1

5 µg of purified total RNA using the PrimeScript RT Master Mix kit (Takara) according to the

6 manufacturer's instructions. Three independent biological replicates were analysed per

7 treatment. Relative quantification of specific mRNA levels was performed using the

8 comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Expression values were normalized

9 using the housekeeping genes *SlEF-1*, encoding an elongation factor-1 α and *LsBtub3*,

10 encoding a beta-tubulin 3, for tomato and lettuce, respectively.

11

12 **Quantification of strigolactone content**

13 *Extraction and indirect quantification of strigolactones from roots*

14 For SL analysis in root extracts, 0.5 g of tomato and lettuce roots from each treatment

15 harvested 6 weeks after sowing, were ground in a mortar with liquid nitrogen and extracted

16 with 0.5 mL of 40% acetone in a 2 mL eppendorf tube. Tubes were vortexed for 2 min and

17 centrifuged at 4 ºC for 5 min at 8000 g in a table top centrifuge. The 40% acetone fraction was

18 discarded. Then, the roots were extracted twice with 0.5 mL of 50% acetone. This fraction,

19 containing the main SLs (López-Ráez *et al.*, 2008), was carefully transferred to 2 mL glass

20 vials and stored at -20 ºC until use. Germination bioassays with *P. ramosa* seeds were

21 performed as described in López-Ráez *et al*. (2008).

22

23 *Strigolactone analysis by multiple reaction monitoring liquid chromatography-tandem mass*

24 *spectrometry (UPLC-MS/MS)*

1 SLs were extracted from 0.5 g of tomato roots as previously described (López-Ráez *et al.*,

2 2008). The analysis and quantification of SLs were performed using a Waters Xevo tandem

3 quadruple mass spectrometer equipped with an electrospray ionization source and coupled to

4 an Acquity UPLC system (Waters, Yvelines Cedex, France) (UPLC-MS/MS) as described in

5 Kohlen *et al*. (2011). The mass spectrometer was operated in positive ESI mode. MRM was

6 used to search for the different SLs by comparing retention times and MRM mass transitions

7 with those of the SL standards. The three major tomato SLs - solanacol and the two

8 didehydro-orobanchol isomers 1 and 2 - were analysed. For simplicity, the two didehydro-

9 orobanchol isomers, hereinafter called DDH, were quantified together. MRM transitions were

10 optimized for each standard using the Waters IntelliStart MS Console. Data acquisition and

11 analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters). The summed

12 area of all the corresponding MRM transitions was used for statistical analysis.

13

14 **Statistical Analysis**

15 Statistical analysis was performed using the software SPSS Statistics v. 20 (SPSS Inc.,

16 Chicago, IL, USA). All data were subjected to analysis of variance (ANOVA) with

17 inoculation treatment and water regime as sources of variation. For the percentage of

18 mycorrhizal root length, the sources of variation were harvest time and water regime.

19 Percentage values were arcsine [squareroot(X)] transformed before statistical analysis. Post

20 Hoc comparisons with the LSD test were used to find out differences between groups.

21

22 **RESULTS**

23

24 **AM root colonization**

1   The root colonization by *R. irregularis* of tomato and lettuce plants increased steadily over

2   time and was higher in plants subjected to the drought stress treatments than under well-

3   watered conditions (Figure 1). Thus, after 4 weeks the values of mycorrhizal root length

4   colonization in lettuce plants ranged from 5% under well-watered conditions to 22% under

5   severe drought stress. In tomato, these values ranged from 8% under well-watered or

6   moderate drought stress conditions to 16% under severe drought stress conditions. After 6

7   weeks, the root colonization increased significantly in lettuce plants to 18% (well-watered

8   conditions), 28% (moderate drought) and 36% (severe drought). In contrast, in tomato plants

9   the root colonization was similar to the values obtained 4 weeks after sowing. At the last

10   harvest (8 weeks), the AM root length in lettuce plants ranged from 34% under well-watered

11   conditions to about 63% under moderate or severe drought stress conditions. In tomato plants,

12   the mycorrhizal root length was 21% (WW conditions), 41% (moderate drought) and 54%

13   (severe drought).

14

15   **Shoot dry weight and mycorrhizal dependency**

16   In this study, tomato and lettuce plants were cultivated under well-watered conditions or

17   subjected to moderate or severe drought stress treatments during the whole plant growth

18   period and harvested 4, 6 or 8 weeks after sowing. At the first harvest (4 weeks), a significant

19   plant biomass reduction due to the drought stress treatments was already observed both in

20   tomato and in lettuce plants. However, AM tomato and lettuce plants grew better than non-

21   AM ones at whatever water regime (Table 1). The growth differences were more evident for

22   tomato plants, with mycorrhizal dependency (MD) values of about 60 and 67% under

23   moderate and severe drought stress, respectively. In lettuce plants the differences were more

24   evident under well-watered conditions, with a MD of 60%.

1   At the second harvest (6 weeks), the drought stress treatments significantly ($p < 0.05$)

2   decreased plant biomass production as compared to well-watered treatments, both in tomato

3   and in lettuce plants (Table 1; Supplemental Figures S1 and S2). In any case, AM plants

4   always exhibited improved growth than non-mycorrhizal ones, regardless of the water regime

5   (Table 1; Supplemental Figures S1 and S2). Indeed, for lettuce plants the MD values were 45,

6   39 and 31% under well-watered, moderate drought or severed drought stress conditions,

7   respectively. For tomato plants, these values were 22, 33 and 30%, respectively.

8   At the last harvest (8 weeks), tomato and lettuce plants maintained a similar pattern of

9   biomass production as in the previous harvest. Thus, both drought stress treatments decreased

10   significantly plant growth as compared to well-watered conditions, but AM plants always

11   maintained a higher shoot dry weight than non-AM plants. For lettuce plants, the MD at this

12   harvest was 22, 34 and 26% under well-watered, moderate drought or severed drought stress

13   conditions, respectively. For tomato plants, these values were 20, 29 and 17%, respectively

14   (Table 1). Since drought effect showed a similar trend on plant development in all the three

15   time points investigated, only plants harvested at 6 weeks were used for the following

16   analyses.

17

18   **Stomatal conductance and efficiency of photosystem II**

19   The stomatal conductance of tomato and lettuce plants was measured before each harvest.

20   Data and trends were similar in all harvests and we are showing only data corresponding to

21   the second harvest (6 weeks after sowing). At this time, the stomatal conductance of both

22   plant species decreased due to the drought stress, being this decrease statistically significant

23   under severe drought stress as compared to well-watered conditions (Figure 2). The behaviour

24   of stomatal conductance was similar for AM and non-AM plants, and differences between

25   AM and non-AM plants were not significant.

The efficiency of photosystem II was also measured before each harvest, although we are showing only data corresponding to the second harvest (6 weeks after sowing) since the patterns of values were similar at all harvests. The efficiency of photosystem II was negatively affected by the drought stress treatments in both plant species, being significantly reduced by severe drought stress both in AM and in non-AM plants (Figure 3). However, AM tomato and lettuce plants maintained higher values of efficiency of photosystem II under well-watered and under drought stress treatments. The maximum differences in this parameter between AM and non-AM plants were observed in lettuce plants under severe drought, where AM plants enhanced this parameter by 16% over non-AM plants.

ABA accumulation and expression of ABA-biosynthesis and ABA-responsive genes

ABA is a phytohormone critical for plant growth and development, generally associated to plant responses against abiotic stresses such as drought (Christmann *et al.*, 2006). ABA has been also related to AM symbiosis (Herrera-Medina *et al*., 2007, Martín-Rodríguez *et al.*, 2010). The accumulation of ABA in mycorrhizal and non-mycorrhizal lettuce and tomato roots was quantified in plants harvested at 6 weeks (Figures 4 and 5). Non-AM lettuce plants steadily enhanced the accumulation of ABA by about 60 and 450% under moderate and severe stress, respectively. In AM plants, the increase was about 400 and 760% under these conditions (Figure 4A). The expression of the lettuce ABA-biosynthesis gene *LsNCED2*, encoding a 9-*cis*-epoxycarotenoid dioxygenase (Sawada *et al*., 2008), was significantly upregulated by the severity of the drought stress both in AM and in non-AM plants (Figure 4B), following the same pattern as ABA levels. The expression of the ABA-responsive marker gene *LsLEA1*, which encodes for a dehydrin (LEA protein) (Aroca *et al*., 2008b), was not affected in roots by the water regime and by the AM fungal inoculation (Figure 4B).

1   A similar pattern as for lettuce was observed for ABA levels in tomato roots (Figure

2   5A). Non-AM tomato plants enhanced the accumulation of ABA by about 88% as a

3   consequence of drought stress (similar levels obtained under moderate and under severe

4   drought). AM plants also increased the accumulation of ABA by 58% under moderate

5   drought, although the ABA levels achieved were lower than in non-mycorrhizal plants. Under

6   severe drought, AM tomato plants enhanced the accumulation of ABA by 200% compared to

7   control well-watered plants, reaching similar levels to non-AM plants. The expression of the

8   tomato ABA-biosynthesis gene *LeNCED1* (Thompson *et al.*, 2000) was not affected in roots

9   by the water regime and by the AM fungal inoculation (Figure 5B). In contrast, the ABA-

10   inducible gene *Le4* (Kahn *et al.*, 1993), encoding a dehydrin and used as a marker of plant

11   response to the drought stress imposed, was induced by the severity of the water regime in

12   AM and in non-AM plants (Figure 5B). Other phytohormones analysed such as jasmonic acid,

13   salicylic acid and auxin were not altered in roots neither by drought nor AM symbiosis (data

14   not shown).

15

16   **Strigolactone production and expression of strigolactone-biosynthesis genes**

17   SLs are important molecules in the rhizosphere favouring AM symbiosis establishment

18   (Akiyama *et al.*, 2005, Bouwmeester *et al.*, 2007). Therefore, SL production was analysed.

19   They are also germination stimulants of root parasitic plant seeds (Bouwmeester *et al.*, 2003,

20   Cook *et al.*, 1972). Because of this germinating activity, bioassays based on seed germination

21   using a specific fraction of the host plant extracts or exudates can be used as a reliable indirect

22   way to quantify the levels of SLs (López-Ráez *et al.* 2011a, López-Ráez *et al.*, 2008,

23   Matusova *et al.*, 2005). To quantify SL production by tomato and lettuce plants, we first

24   performed a germination bioassay with seeds of *P. ramosa* using the 50% acetone fraction of

25   root extracts from plants harvested at 6 weeks (Figure 6A, B). The synthetic germination

16

stimulant GR24 (10^{-9} and 10^{-10} M), used as a positive control, always induced germination of pre-conditioned *P. ramosa* seeds. Water, used as a negative control, only induced a basal germination. Germination induced by the lettuce or tomato root extracts were in a similar range than that induced by GR24 and always below 70%, indicating that saturation of the germination response did not occur at the root extract dilutions used in the bioassays.

The germination stimulatory activity of root extracts from non-mycorrhizal plants decreased steadily with increasing severity of the drought stress applied, especially in plants cultivated under severe drought stress, both in lettuce and tomato (Figure 6A, B). This effect was more evident in tomato plants, where the germination stimulatory activity of extracts decreased over 40% from well-watered to moderate drought conditions and about 60% to severe drought stress. These data suggest a negative effect of drought stress on SL production in non-AM plants. Conversely, the germination stimulatory activity of root extracts from mycorrhizal plants increased significantly ($p < 0.05$) with increasing severity of the drought stress applied. Again, this effect was more evident in tomato plants, where the germination stimulatory activity increased from 47% under well-watered conditions to 52% under moderate drought and to 65% under severe drought stress. Thus, these data suggest a positive effect of drought stress on SL biosynthesis in AM plants.

As a second approach, we quantified the accumulation of the main SLs in tomato - solanacol and the didehydro-orobanchol isomers (DDH) (López-Ráez *et al.*, 2008) - by liquid chromatography coupled mass spectrometry (UPLC-MS/MS). Orobanchol, other SL described in tomato, was also detected but its concentration was too low for accurate quantification. In lettuce, the production of SLs is extremely low (Yoneyama *et al*., 2012), making their quantification difficult. The quantification in tomato was carried out with root samples from plants harvested at 6 weeks (Figure 7A), and repeated with root samples from tomato plants harvested at 8 weeks, obtaining similar results. Both solanacol and DDH

1 followed a similar behaviour, which confirmed the pattern observed in the seed germination

2 bioassay. Indeed, in non-mycorrhizal tomato plants solanacol and DDH steadily decreased

3 with increasing severity of the drought stress, while both SLs increased with increasing

4 severity of the drought stress imposed in mycorrhizal plants.

5 As a third approach, the expression of two tomato genes - *SlCCD7 and SlCCD8* -

6 involved in the biosynthesis of SLs (Kohlen *et al*., 2012, Vogel *et al*., 2010) was quantified by

7 real-time quantitative RT-PCR (qPCR). The expression of *SlCCD8* was not altered by the

8 water regime and mycorrhizal status (Figure 7B). However, *SlCCD7* expression was

9 differentially affected under these two conditions. In roots of non-AM tomato plants, the

10 expression of *SlCCD7* was down-regulated by increasing severity of the drought stress

11 imposed (Figure 6B). Conversely, *SlCCD7* expression was clearly up-regulated by increasing

12 severity of the drought stress in AM roots (Figure 7B), following the same pattern as for SL

13 accumulation (Figure 7A).

14

15 **DISCUSSION**

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17 Water-related stresses, including drought, adversely impact plant physiology, growth and

18 productivity (Bray, 2004, Farooq *et al.*, 2014, Golldack *et al.*, 2014, Osakabe *et al.*, 2014,

19 Ruiz-Lozano *et al.*, 2012). Along evolution, plants have evolved mechanisms to flexibly adapt

20 to these unfavourable conditions (Pierik & Testerink, 2014, Ruiz-Lozano *et al.*, 2012). One of

21 these strategies is the establishment of AM symbiosis. It is widely accepted that this

22 mutualistic association is a key component in helping plants to cope with adverse

23 environmental conditions, including drought stress (Miransari *et al.*, 2014, Pozo & Azcón-

24 Aguilar, 2007, Ruiz-Lozano *et al.*, 2012, Ruiz-Sánchez *et al.*, 2010). Interestingly, we show

25 here for the first time and in two plant species that drought steadily enhances colonization

1   rates based on the water regime (Figure 1). The beneficial effects of different AM fungi on

2   plant growth and development under drought have been shown in a number of plant species

3   such as maize, rice, citrus, barley and pistachio (Abbaspour *et al.*, 2012, Bárzana *et al.*, 2014,

4   Ruiz-Sánchez *et al.*, 2010, Wu & Zou, 2009). We previously showed that mycorrhizal tomato

5   plants also performed better than non-mycorrhizal ones under drought stress upon a well-

6   established symbiosis (Aroca *et al*., 2008a). However, in that experiment AM symbiosis was

7   established prior to the application of drought stress. Here, we show that the beneficial effect

8   of the symbiosis on plant performance also takes place when the stress is applied from the

9   beginning of the growing period, which resembles more to natural conditions. Remarkably,

10   the same behaviour was observed in mycorrhizal lettuce plants, indicating that this beneficial

11   effect of AM symbiosis on plant performance under drought stress is conserved across plant

12   species. In both cases, the promotion of plant growth started from early stages of mycorrhizal

13   colonization (after 4 weeks), where less than 10% root colonization was achieved. Growth

14   promotion was maintained until 8 weeks of treatment, where mycorrhizal stressed plants grew

15   at a similar rate than non-stressed control plants. Thus, AM symbiosis alleviates drought

16   stress and allows mycorrhizal plants to grow better under these unfavourable conditions,

17   taking place this beneficial effect from the very beginning of the association. Interestingly, the

18   same effect of AM symbiosis was previously observed in lettuce plants exposed to salt stress

19   (Aroca *et al.*, 2013), suggesting a conserved behaviour for different osmotic-related stresses.

20   Plant growth and productivity is closely associated to the drought stress level

21   experienced by plants. This induces a decrease in the leaf water potential and in stomatal

22   opening, negatively affecting photosynthesis and CO_2 availability (Augé *et al.*, 2015, Osakabe

23   *et al.*, 2014). It has been described that AM symbiosis can alter stomatal behaviour, thus

24   affecting plant productivity (Augé *et al.*, 2015). In our experiment, in addition to a better

25   growth rate, tomato and lettuce AM plants exhibited a better performance of photosystem II

19

1 both under well-watered and stressed conditions. The increase was higher in plants under a

2 severe stress, an effect that was previously shown in tomato (Bárzana *et al.*, 2012). Likely,

3 this positive effect has also contributed to the enhanced plant growth of mycorrhizal plants,

4 probably by enhancing CO_2 fixation. In this sense, several studies have shown a correlation

5 between tolerance to drought stress and maintenance of efficiency of photosystem II, which

6 also sustained plant productivity (Loggini *et al.*, 1999, Ruiz-Sánchez *et al.*, 2010). The higher

7 values of photosynthetic efficiency in mycorrhizal plants indicate that the photosynthetic

8 apparatus of these plants is less damaged by the drought stress imposed (Bárzana *et al.*, 2012,

9 Sperdouli & Moustakas, 2012). The same pattern for plant growth and physiological

10 parameters was previously observed in mycorrhizal lettuce plants subjected to salinity (Aroca

11 *et al.*, 2013), suggesting, once again a common effect of AM symbiosis to different osmotic-

12 related stresses.

13   The enhanced tolerance of mycorrhizal plants against water-related stresses has been

14 associated to an alteration of the phytohormone homeostasis, for which ABA signalling is the

15 most intensively studied (Calvo-Polanco *et al.*, 2013, Osakabe *et al.*, 2014, Ruiz-Lozano *et al.*,

16 2012). Plants have to adjust their ABA levels continuously in response to changing

17 physiological and environmental conditions. Indeed, ABA is considered as the 'stress

18 hormone', as it accumulates rapidly in response to salinity and drought (Hong *et al.*, 2013).

19 As expected, a steady increase in ABA content was observed in roots from non-AM plants as

20 a consequence of drought in both tomato and lettuce, reaching the maximum ABA levels

21 under the most severe stress (Figures 4 and 5). An induction in ABA was also detected in AM

22 plants, showing a similar trend as for non-AM plants. As for the physiological parameters, a

23 similar pattern in ABA content was previously observed in lettuce plants under salt stress,

24 especially in mycorrhizal plants (Aroca *et al.*, 2013). In that study, we showed a correlation

25 between ABA levels and the expression of the ABA-biosynthesis gene *LsNCED2*, coding for

the ABA rate-limiting enzyme (Taylor *et al*., 2005). Here, a correlation between these two parameters has also been observed in lettuce under drought stress in AM and non-AM plants, indicating a *de novo* ABA biosynthesis in stress conditions. In the case of tomato, in agreement with previous observations, the expression of *LeNCED1* was not regulated by drought or by ABA (Aroca *et al.*, 2008a, Thompson *et al.*, 2000). However, the expression pattern of the ABA-responsive gene *Le4* perfectly matched with that of ABA levels, indicating an efficient activation of the ABA signalling pathway under drought. In addition to its role as a 'stress phytohormone', ABA is also important for symbiosis establishment and functioning (Herrera-Medina *et al.*, 2007, Martín-Rodríguez *et al.*, 2010). Therefore, the increased ABA levels in stressed plants would serve not only to promote tolerance against stresses in non-AM and AM plants, but also to enhance and maintain the symbiosis in mycorrhizal plants. Hormonal results, together with those of other physiological parameters, support that AM symbiosis improves plant fitness under water-related stress conditions.

As mentioned above, AM symbiosis establishment requires a finely regulated molecular dialogue between the two partners, in which SLs have arisen as essential cues (Bucher *et al.*, 2014, Gutjahr & Parniske, 2013, López-Ráez *et al.*, 2011b). It is well known that SL production is promoted by nutrient deficiency, mainly phosphorus starvation (López-Ráez *et al.*, 2008, Yoneyama *et al*, 2007) to promote AM fungal development and symbiosis establishment (Andreo-Jiménez *et al.*, 2015, Kapulnik & Koltai, 2014). In addition to nutritional stress, an increased SL production in the presence of *R. irregularis* under salt stress was proposed in lettuce (Aroca *et al.*, 2013). However, the SL promotion did not take place under stress conditions in the absence of the AM fungus. Actually, a reduction of the SL levels in root extracts was observed. In the present study, a promotion of SL production in mycorrhizal plants under drought stress is also shown. Root extracts from AM plants showed a steadily increased germination-stimulatory activity of *P. ramosa* seeds with increasing stress

severity, both in tomato and lettuce. Interestingly, this SL promotion correlated with an increase in the levels of root colonization (Figures 1 and 6). Conversely, as in the case of salinity, drought stress negatively affected SL production in non-AM plants. The germination bioassay data were analytically and transcriptionally confirmed by LC-MS/MS and qPCR, respectively, in tomato. The same pattern was observed for the main tomato SLs - solanacol and DDH - in non-AM and AM plants, although here the total amount of SLs was higher in AM plants. We do not have an explanation for this difference. We checked the possibility of ion suppression in the non-AM plants, but this was not the case. It might be that the germination-stimulatory capacity in these plants is increased by other non-described SL in tomato or by other active compound(s). In any case, it is clear that the production of SLs was steadily promoted by drought in mycorrhizal plants, while it was reduced in non-mycorrhizal ones. Accordingly, the expression of the SL-biosynthesis gene *SlCCD7* was up-regulated in AM plants and down-regulated in non-AM plants by drought based on the severity of the stress (Figure 7). Overall, the results suggest that the host plant is sensing the presence of the AM fungus under these unfavourable conditions and induces the production of SLs to improve mycorrhizal colonization. A positive regulatory role of SLs in plant responses to water-related stresses was recently proposed (Ha *et al.*, 2014). These authors showed that Arabidopsis SL-deficient and SL-response mutants were more susceptible to drought and salinity than the corresponding wild-type genotypes. The same effect has been observed in Lotus plants, where it was shown that the antisense line *Ljccd7*, affected in the expression of *CCD7*, was more susceptible to drought stress (Liu *et al.*, 2015). These results, together with our observation in tomato and lettuce, confirm the involvement of the SL signalling pathway in the plant tolerance against water-related stresses.

The increase on SL production under drought in the presence of AM fungus fitted with that of the ABA content (Figures 4, 5 and 6). This fact was also observed in lettuce plants

1 subjected to salt stress (Aroca *et al.*, 2013), and suggests a cross-talk between these two

2 phytohormones, which is important for AM symbiosis under abiotic stress. An interaction

3 SLs-ABA under water-related stress conditions has also been recently described in Lotus (Liu

4 *et al.*, 2015). Liu and co-workers found out that osmotic stress decreased SL content in roots

5 and root exudates, and that this reduction was associated with an increase in ABA levels. The

6 authors proposed that the stress-induced reduction in SLs is needed to allow the local increase

7 of ABA and thus, the plant response to the stress. This is the effect we observed in tomato and

8 lettuce under drought in non-AM plants. Therefore, this negative correlation SL-ABA might

9 be a general plant strategy to cope with water-related stresses in the absence of AM symbiosis.

10   In conclusion, we show here that AM symbiosis alleviates the negative effects of

11 drought in tomato and lettuce plants by altering the hormonal profiles, thus affecting plant

12 physiology and development. The results confirm the role of arbuscular mycorrhizas in

13 protecting host plants under unfavourable environmental conditions and their potential use as

14 a sustainable strategy in agriculture. The involvement of SLs in plant responses against water-

15 related stresses, as well as their interaction with ABA is also evidenced in this study, showing

16 a different behaviour depending on the presence or absence of AM symbiosis. However,

17 further research is required to elucidate the intrinsic mechanisms of this SL-ABA cross-talk

18 and how it is modulated by AM symbiosis.

19

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REFERENCES

Abbaspour H., Saeidi-Sar S., Afshari H. & Abdel-Wahhab M.A. (2012) Tolerance of Mycorrhiza infected Pistachio (*Pistacia vera* L.) seedling to drought stress under glasshouse conditions. *Journal of Plant Physiology*, **169**, 704-709.

Akiyama K., Matsuzaki K. & Hayashi H. (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature*, **435**, 824-827.

Albacete A.A., Martínez-Andújar C. & Pérez-Alfocea F. (2014) Hormonal and metabolic regulation of source-sink relations under salinity and drought: from plant survival to crop yield stability. *Biotechnology Advances*, **32**, 12-30.

Andreo-Jiménez B., Ruyter-Spira C., Bouwmeester H. & López-Ráez J.A. (2015) Ecological relevance of strigolactones in nutrient uptake and other abiotic stresses, and in plant-microbe interactions below-ground. *Plant and Soil*. *In press.* doi: 10.1007/s11104-015-2544-z.

Aroca R., Del Mar Alguacil M., Vernieri P. & Ruiz-Lozano J.M. (2008a) Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (*sitiens*). *Microbial Ecology*, **56**, 704-719.

Aroca R., Porcel R. & Ruiz-Lozano J.M. (2012) Regulation of root water uptake under abiotic stress conditions. *Journal of Experimental Botany*, **63**, 43-57.

Aroca R., Ruiz-Lozano J.M., Zamarreño A.M., Paz J.A., García-Mina J.M., Pozo M.J. & López-Ráez J.A. (2013) Arbuscular mycorrhizal symbiosis influences strigolactone production under salinity and alleviates salt stress in lettuce plants. *Journal of Plant Physiology*, **170**, 47-55.

Aroca R., Vernieri P. & Ruiz-Lozano J.M. (2008b) Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. *Journal of Experimental Botany*, **59**, 2029-2041.

Augé R.M., Toler H.D. & Saxton A.M. (2015) Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza*, **25**, 13-24.

Bacaicoa E., Zamarreño A.M., Leménager D., Baigorri R. & García-Mina J.M. (2009) Relationship between the hormonal balance and the regulation of iron deficiency stress responses in Cucumber. *Journal of the American Society for Horticultural Science*, **134**, 589-601.

Badri D.V., Weir T.L., van der Lelie D. & Vivanco J.M. (2009) Rhizosphere chemical dialogues: plant-microbe interactions. *Current Opinion in Biotechnology*, **20**, 642-650.

Barea J.M., Pozo M.J., Azcon R. & Azcon-Aguilar C. (2005) Microbial co-operation in the rhizosphere. *Journal of Experimental Botany*, **56**, 1761-1778.

Bárzana G., Aroca R., Bienert G.P., Chaumont F. & Ruiz-Lozano J.M. (2014) New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize

plants under drought stress and possible implications for plant performance. *Molecular Plant-Microbe Interactions*, **27**, 349-363.

Bárzana G., Aroca R., Paz J.A., Chaumont F., Martinez-Ballesta M.C., Carvajal M. & Ruiz-Lozano J.M. (2012) Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. *Annals of Botany*, **109**, 1009-1017.

Bárzana G., Aroca R. & Ruiz-Lozano J.M. (2015) Localized and non-localized effects of arbuscular mycorrhizal symbiosis on accumulation of osmolytes and aquaporins and on antioxidant systems in maize plants subjected to total or partial root drying. *Plant, Cell and Environment*, In press. doi: 10.1111/pce.12507.

Besserer A., Puech-Pages V., Kiefer P., Gomez-Roldan V., Jauneau A., Roy S., Portais J.C., Roux C., Becard G. & Sejalon-Delmas N. (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *Plos Biology*, **4**, 1239-1247.

Bohnert H.J., Nelson D.E. & Jensen R.G. (1995) Adaptations to environmental stresses. *Plant Cell*, **7**, 1099-1111.

Bouwmeester H.J., Matusova R., Zhongkui S. & Beale M.H. (2003) Secondary metabolite signalling in host-parasitic plant interactions. *Current Opinion in Plant Biology*, **6**, 358-364.

Bouwmeester H.J., Roux C., López-Ráez J.A. & Bécard G. (2007) Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends in Plant Science*, **12**, 224-230.

Bray E.A. (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. *Journal of Experimental Botany*, **55**, 2331-2341.

Bu Q., Lu T., Shen H., Luong P., Wang J., Wang Z., Huang Z., Xiao L., Engineer C., Kim T.H., Schroeder J.I., Huq E. (2014) Regulation of drought tolerance by the F-box protein in Arabidopsis. *Plant Physiology*, **164**, 424-439.

Bucher M., Hause B., Krajinski F. & Küster H. (2014) Through the doors of perception to function in arbuscular mycorrhizal symbioses. *New Phytologist*, **204**, 833-840.

Calvo-Polanco M., Sánchez-Romera B. & Aroca R. (2013) Arbuscular mycorrhizal fungi and the tolerance of plants to drought and salinity. In: *Symbiotic endophytes* (ed R. Aroca), pp. 271-288. Springer, Berlin Heidelberg.

Cook C.E., Whichard L.P., Wall M.E., Egley G.H., Coggon P., Luhan P.A. & McPhail A.T. (1972) Germination stimulants. 2. The structure of strigol-a potent seed germination stimulant for witchweed *(Striga lutea* Lour.). *Journal of the American Chemical Society*, **94**, 6198-6199.

Chaves M.M. & Oliveira M.M. (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of Experimental Botany*, **55**, 2365-2384.

Christmann A., Moes D., Himmelbach A., Yang Y., Tang Y. & Grill E. (2006) Integration of abscisic acid signalling into plant responses. *Plant Biology*, **8**, 314-325.

Farooq M., Hussain M. & Siddique K.H.M. (2014) Drought stress in wheat during flowering and grain-filling periods. *Critical Reviews in Plant Sciences*, **33**, 331-349.

Farooq M., Wahid A., Kobayashi N., Fujita D. & Basra S.M.A. (2009) Plant drought stress: Effects, mechanisms and management. In: *Agronomy for Sustainable Development*, pp. 185-212. Springer Netherlands.

Giovannetti M. & Mosse B. (1980) Evaluation of Techniques for Measuring Vesicular Arbuscular Mycorrhizal Infection in Roots. *New Phytologist*, **84**, 489-500.

Golldack D., Li C., Mohan H. & Probst N. (2014) Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Frontiers in Plant Science*, **5**, 151.

Gutjahr C. & Parniske M. (2013) Cell and developmental biology of arbuscular mycorrhiza symbiosis, pp. 593-617.

Ha C.V., Leyva-Gonzalez M.A., Osakabe Y., Tran U.T., Nishiyama R., Watanabe Y., Tanaka M., Seki M., Yamaguchi S., Dong N.V., Yamaguchi-Shinozaki K., Shinozaki K., Herrera-Estrella L. & Tran L.S.P. (2014) Positive regulatory role of strigolactone in plant responses to drought and salt stress. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 851-856.

Herrera-Medina M.J., Steinkellner S., Vierheilig H., Bote J.A.O. & Garrido J.M.G. (2007) Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. *New Phytologist*, **175**, 554-564.

Hong J.H., Seah S.W. & Xu J. (2013) The root of ABA action in environmental stress response. *Plant Cell Reports*, **32**, 971-983.

Kahn T.L., Fender S.E., Bray E.A. & Oconnell M.A. (1993) Characterization of expression of drought and abscisic acid-regulated tomato genes in the drought-resistant species *Lycopersicon pennellii*. *Plant Physiology*, **103**, 597-605.

Kapulnik Y. & Koltai H. (2014) Strigolactone involvement in root development, response to abiotic stress, and interactions with the biotic soil environment. *Plant Physiology*, **166**, 560-569.

Kohlen W., Charnikhova T., Lammers M., Pollina T., Toth P., Haider I., Pozo M.J., de Maagd R.A., Ruyter-Spira C., Bouwmeester H.J. & López-Ráez J.A. (2012) The tomato *CAROTENOID CLEAVAGE DIOXYGENASE8 (SlCCD8)* regulates rhizosphere signaling, plant architecture and affects reproductive development through strigolactone biosynthesis. *New Phytologist*, **196**, 535-547.

Kohlen W., Charnikhova T., Liu Q., Bours R., Domagalska M.A., Beguerie S., Verstappen F., Leyser O., Bouwmeester H. & Ruyter-Spira C. (2011) Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host Arabidopsis. *Plant Physiology*, **155**, 974-987.

Kumar A., Sharma S. & Mishra S. (2010) Influence of arbuscular mycorrhizal (AM) fungi and salinity on seedling growth, solute accumulation, and mycorrhizal dependency of *Jatropha curcas* L. *Journal of Plant Growth Regulation*, **29**, 297-306.

Liu J., He H., Vitali M., Visentin I., Charnikhova T., Haider I., Schubert A., Ruyter-Spira C., Bouwmeester H.J., Lovisolo C. & Cardinale F. (2015) Osmotic stress represses strigolactone biosynthesis in *Lotus japonicus* roots: exploring the interaction between strigolactones and ABA under abiotic stress. *Planta*, **241**, 1435-1451.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2_DDC_t method. *Methods*, **25**, 402-408.

Loggini B., Scartazza A., Brugnoli E. & Navari-Izzo F. (1999) Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. *Plant Physiology*, **119**, 1091-1099.

López-Ráez J.A., Charnikhova T., Fernández I., Bouwmeester H. & Pozo M.J. (2011a) Arbuscular mycorrhizal symbiosis decreases strigolactone production in tomato. *Journal of Plant Physiology*, **168**, 294-297.

López-Ráez J.A., Charnikhova T., Gómez-Roldán V., Matusova R., Kohlen W., De Vos R., Verstappen F., Puech-Pages V., Bécard G., Mulder P. & Bouwmeester H. (2008) Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. *New Phytologist*, **178**, 863-874.

López-Ráez J.A., Kohlen W., Charnikhova T., Mulder P., Undas A.K., Sergeant M.J., Verstappen F., Bugg T.D.H., Thompson A.J., Ruyter-Spira C. & Bouwmeester H.

(2010) Does abscisic acid affect strigolactone biosynthesis? *New Phytologist*, **187**, 343-354.

López-Ráez J.A., Pozo M.J. & García-Garrido J.M. (2011b) Strigolactones: A cry for help in the rhizosphere. *Botany*, **89**, 513-522.

Martín-Rodríguez J.A., León-Morcillo R., Vierheilig H., Ocampo J.A., Ludwig-Muller J. & García-Garrido J.M. (2010) Mycorrhization of the *notabilis* and *sitiens* tomato mutants in relation to abscisic acid and ethylene contents. *Journal of Plant Physiology*, **167**, 606-613.

Matusova R., Rani K., Verstappen F.W.A., Franssen M.C.R., Beale M.H. & Bouwmeester H.J. (2005) The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp are derived from the carotenoid pathway. *Plant Physiology*, **139**, 920-934.

Mendes R., Garbeva P. & Raaijmakers J.M. (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, **37**, 634-663.

Miransari M., Abrishamchi A., Khoshbakht K. & Niknam V. (2014) Plant hormones as signals in arbuscular mycorrhizal symbiosis. *Critical Reviews in Biotechnology*, **34**, 123-133.

Osakabe Y., Osakabe K., Shinozaki K. & Tran L.S.P. (2014) Response of plants to water stress. *Frontiers in Plant Science*, **5**, 86.

Oxborough K. & Baker N.R. (1997) Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - Calculation of qP and Fv'/Fm' without measuring Fo'. *Photosynthesis Research*, **54**, 135-142.

Peleg Z. & Blumwald E. (2011) Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology*, **14**, 290-295.

Phillips J.M. & Hayman D.S. (1970) Improved Procedures for Clearing Roots and Staining Parasitic and Vesicular-Arbuscular Mycorrhizal Fungi for Rapid Assessment of Infection. *Transactions of the British Mycological Society*, **55**, 158-161.

Pierik R. & Testerink C. (2014) The art of being flexible: How to escape from shade, salt, and drought. *Plant Physiology*, **166**, 5-22.

Porcel R. & Ruiz-Lozano J.M. (2004) Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. *Journal of Experimental Botany*, **55**, 1743-1750.

Porcel R., Aroca R., Azcón R. & Ruiz-Lozano J.M. (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. *Plant Molecular Biology*, **60**, 389-404.

Pozo M.J. & Azcón-Aguilar C. (2007) Unravelling mycorrhiza-induced resistance. *Current Opinion in Plant Biology*, **10**, 393-398.

Pozo M.J., López-Ráez J.A., Azcón C. & García-Garrido J.M. (2015) Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. *New Phytologist*, **205**, 1431-1436.

Ruiz-Lozano J.M., Porcel R., Bárzana G., Azcón R. & Aroca R. (2012) Contribution of arbuscular mycorrhizal symbiosis to plant drought tolerance: State of the art. In: *Plant Responses to Drought Stress* (ed R. Aroca), pp. 335-362. Springer-Verlag, Heidelberg.

Ruiz-Sánchez M., Aroca R., Muñoz Y., Polón R. & Ruiz-Lozano J.M. (2010) The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress. *Journal of Plant Physiology*, **167**, 862-869.

Ruyter-Spira C., Al-Babili S., van der Krol S. & Bouwmeester H. (2013) The biology of strigolactones. *Trends in Plant Science*, **18**, 72-83.

Sawada Y., Aoki M., Nakaminami K., Mitsuhashi W., Tatematsu K., Kushiro T., Koshiba T., Kamiya Y., Inoue Y., Nambara E. & Toyomasu T. (2008) Phytochrome- and gibberellin-mediated regulation of abscisic acid metabolism during germination of photoblastic lettuce seeds. *Plant Physiology*, **146**, 1386-1396.

Schussler A. & Walker C. (2010) *The Glomeromycota: a species list with new families and new genera*. Gloucester, UK.

Shinozaki K. & Yamaguchi-Shinozaki K. (2006) Global analysis of gene networks to solve complex abiotic stress responses. In: *Cold Hardiness in Plants: Molecular Genetics, Cell Biology, and Physiology* (eds T.H.H. Chen, S. Uemura, & S. Fujikawa), pp. 1-10. CABI Publishing.

Smith S.E. & Read D.J. (2008) *Mycorrhizal symbiosis*, London.

Sperdouli I. & Moustakas M. (2012) Interaction of proline, sugars, and anthocyanins during photosynthetic acclimation of *Arabidopsis thaliana* to drought stress. *Journal of Plant Physiology*, **169**, 577-585.

Taylor I.B., Sonneveld T., Bugg T.D.H. & Thompson A.J. (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. *Journal of Plant Growth Regulation*, **24**, 253-273.

Thompson A.J., Jackson A.C., Parker R.A., Morpeth D.R., Burbidge A. & Taylor I.B. (2000) Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. *Plant Molecular Biology*, **42**, 833-845.

Ton J., Flors V. & Mauch-Mani B. (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science*, **14**, 310-317.

Trenberth K.E., Dai A., Van Der Schrier G., Jones P.D., Barichivich J., Briffa K.R. & Sheffield J. (2014) Global warming and changes in drought. *Nature Climate Change*, **4**, 17-22.

Vogel J.T., Walter M.H., Giavalisco P., Lytovchenko A., Kohlen W., Charnikhova T., Simkin A.J., Goulet C., Strack D., Bouwmeester H.J., Fernie A.R. & Klee H.J. (2010) *SlCCD7* controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. *Plant Journal*, **61**, 300-311.

Walter M.H. & Strack D. (2011) Carotenoids and their cleavage products: Biosynthesis and functions. *Natural Product Reports*, **28**, 663-692.

Wu Q.S. & Zou Y.N. (2009) Mycorrhiza has a direct effect on reactive oxygen metabolism of drought-stressed citrus. *Plant, Soil and Environment*, **55**, 436-442.

Xie X.N., Yoneyama K. & Yoneyama K. (2010) The strigolactone story. *Annual Review of Phytopathology*, **48**, 93-117.

Yoneyama K., Xie X., Kusumoto D., Sekimoto H., Sugimoto Y., Takeuchi Y. & Yoneyama K. (2007) Nitrogen deficiency as well as phosphorous deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. *Planta*, **227**, 125-132.

Yoneyama K., Xie X., Kim H.I., Kisugi T., Nomura T., Sekimoto H., Yokota T. & Yoneyama K. (2012) How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation? *Planta*, **235**, 1197-1207.

1 **Table 1**. Influence of drought and AM symbiosis on growth in lettuce and tomato plants.
2

Treatment	WW		M		S	
	NM	Ri	NM	Ri	NM	Ri
Lettuce						
Week 4						
SDW (g plant^{-1})	0.23 ± 0.04b	0.58 ± 0.13a	0.14 ± 0.04d	0.20 ± 0.03bc	0.07 ± 0.02e	0.17 ± 0.04cd
MD (%)	-	60	-	30	-	59
Week 6						
SDW (g plant^{-1})	0.66 ± 0.22c	1.20 ± 0.27a	0.49 ± 0.03d	0.80 ± 0.04b	0.36 ± 0.09e	0.52 ± 0.04d
MD (%)	-	45	-	39	-	31
Week 8						
SDW (g plant^{-1})	1.41 ± 0.21b	1.80 ± 0.14a	0.83 ± 0.08d	1.25 ± 0.13c	0.66 ± 0.15e	0.89 ± 0.14d
MD (%)	-	22	-	34	-	26
Tomato						
Week 4						
SDW (g plant^{-1})	0.36 ± 0.05b	0.49 ± 0.09a	0.13 ± 0.04c	0.33 ± 0.09b	0.10 ± 0.05c	0.31 ± 0.07b
MD (%)	-	25	-	60	-	67
Week 6						
SDW (g plant^{-1})	0.76 ± 0.08bc	0.97 ± 0.12a	0.59 ± 0.08de	0.88 ± 0.13ab	0.49 ± 0.09e	0.70 ± 0.09cd
MD (%)	-	22	-	33	-	30
Week 8						
SDW (g plant^{-1})	0.99 ± 0.13b	1.23 ± 0.07a	0.67 ± 0.10c	0.96 ± 0.08b	0.58 ± 0.06d	0.70 ± 0.07c
MD (%)	-	20	-	29	-	17

3
4 Shoot dry weight (SDW, g plant^{-1}) and mycorrhizal dependency (MD, %) of *Lactuca sativa* and *Solanum lycopersicum* plants subjected to
5 drought stress. Plants were inoculated with the AM fungus *R. intraradices* (Ri) or remained as non mycorrhizal controls (NM). Plants were
6 cultivated under well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought since the beginning of the experiment and
7 harvested 4, 6 or 8 weeks after inoculation. Within each harvest time, data represent means ± SD. Data with different letters differ significantly
8 ($P < 0.05$), as determined by the Duncan's multiple range test (n = 5).
9

TABLE AND FIGURE LEGENDS

2

3 **Table 1.** Influence of drought and AM symbiosis on growth in lettuce plants.

4 Shoot dry weight (SDW, g plant^{-1}) and mycorrhizal dependency (MD, %) of lettuce and

5 tomato plants subjected to drought stress. Plants were inoculated with the AM fungus *R.*

6 *irregularis* (Ri) or remained as non-mycorrhizal controls (NM). Plants were cultivated under

7 well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought since the

8 beginning of the experiment and harvested 4, 6 or 8 weeks after inoculation. Within each

9 harvest time, data represent means ± SD. Data with different letters differ significantly ($P <$

10 0.05), as determined by the Duncan's multiple range test (n = 5).

11

12 **Figure 1.** Effect of drought and time on the percentage of AM root colonization in lettuce

13 (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) plants. Intensity of mycorrhizal

14 colonization by *R. irregularis* in the roots. Plants were cultivated under well-watered (WW)

15 conditions or subjected to moderate (M) or severe (S) drought since the beginning of the

16 experiment and harvested 4, 6 or 8 weeks after inoculation. Within each harvest time, data

17 represent means ± SD. Data with different letters differ significantly ($P < 0.05$), as determined

18 by the Duncan's multiple range test (n = 5).

19

20 **Figure 2.** Influence of arbuscular mycorrhizal (AM) symbiosis and drought stress on the

21 physiological status of lettuce (**A**) and tomato (**B**) plants. Effect on the stomatal conductance at

22 6 weeks. Plants were cultivated under well-watered (WW) conditions or subjected to moderate

23 (M) or severe (S) drought. Closed bars represent non-inoculated control plants (NM) and grey

24 bars represent plants inoculated with *R. irregularis* (Ri). Bars represent the means of five

1 replicates (± SE). Bars with different letters are significantly different ($P < 0.05$) according to

2 Duncan's multiple range test.

3

4 **Figure 3.** Influence of arbuscular mycorrhizal (AM) symbiosis and drought stress on the

5 physiological status of lettuce (**A**) and tomato (**B**). Effect on the efficiency of photosystem II at

6 6 weeks. See legend of Figure 2.

7

8 **Figure 4.** Effect of drought and AM symbiosis in abscisic acid (ABA) signalling pathway in

9 lettuce. **A**, ABA content in lettuce roots after 6 weeks. **B**, Gene expression analysis by real

10 time qPCR for the ABA biosynthesis gene *LsNCED2* (closed bars) and for the ABA-

11 responsive gene *LsLEA1* (grey bars) in lettuce roots after 6 weeks. See legend of Figure 2. Bars

12 represent the means of five (A) or three (C) replicates (± SE).

13

14 **Figure 5.** Effect of drought and AM symbiosis in abscisic acid (ABA) signalling pathway in

15 tomato. **A**, ABA content in tomato roots after 6 weeks. **B**, Gene expression analysis by real

16 time qPCR for the ABA biosynthesis gene *LeNCED1* (closed bars) and for the ABA-

17 responsive gene *Le4* (grey bars) in tomato roots after 6 weeks. See legend of Figure 2. Bars

18 represent the means of five (A) or three (C) replicates (± SE).

19

20 **Figure 6.** Influence of drought and mycorrhizal colonization on strigolactone production.

21 Germination of *Phelipanche ramosa* seeds induced by root extracts of lettuce (**A**) and tomato

22 (**B**) plants after 6 weeks. Dilution 1:200 in demineralised water of each root extract was used.

23 GR24 (10^{-9} and 10^{-10} M) and demineralised water (C) were used as positive and negative

24 controls, respectively. For treatments and statistics see legend of Figure 2.

25

Figure 7. Influence of drought and mycorrhizal colonization on strigolactone production. **A**, SL content in tomato root extracts at 6 weeks analyzed by LC-MS/MS. Data correspond to the amount (according to the peak area) of the SLs solanacol and the didehydro-orobanchol isomers 1 and 2 (DDH) from tomato plants colonized by *R. irregularis* (Ri) and non-colonized (NM). **B**, Gene expression analysis by real time qPCR for the SL biosynthesis genes *SlCCD7* (closed bars) and *SlCCD8* (grey bars) in tomato after 6 weeks. Bars represent the means of five (A) or three (B) replicates (± SE). For treatments and statistics see legend of Figure 2.

SUPPLEMENTAL MATERIAL

Table S1. Primer sequences used in the real time qPCR analysis.

Figure S1. Phenotypic comparison of non-mycorrhizal (NM) and mycorrhizal (Ri) lettuce plants growing at 6 weeks under different water regime: well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought.

Figure S2. Phenotypic comparison of non-mycorrhizal (NM) and mycorrhizal (Ri) tomato plants growing at 6 weeks under different water regime: well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought.