Original Article

Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato

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ABSTRACT

Arbuscular mycorrhizal (AM) symbiosis alleviates drought stress in plants. However, the intimate mechanisms involved, as well as its effect on the production of signalling molecules associated with the host plant–AM fungus interaction remains largely unknown. In the present work, the effects of drought on lettuce and tomato plant performance and hormone levels were investigated in non-AM and AM plants. Three different water regimes were applied, and their effects were analysed over time. AM plants showed an improved growth rate and efficiency of photosystem II than non-AM plants under drought from very early stages of plant colonization. The levels of the phytohormone abscisic acid, as well as the expression of the corresponding marker genes, were influenced by drought stress in non-AM and AM plants. The levels of strigolactones and the expression of corresponding marker genes were affected by both AM symbiosis and drought. The results suggest that AM symbiosis alleviates drought stress by altering the hormonal profiles and affecting plant physiology in the host plant. In addition, a correlation between AM root colonization, strigolactone levels and drought severity is shown, suggesting that under these unfavourable conditions, plants might increase strigolactone production in order to promote symbiosis establishment to cope with the stress.

Key-words: abscisic acid; AM symbiosis; drought stress; phytohormones; strigolactones.

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 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 not only 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

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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 guarantee world food production (Chaves & Oliveira, 2004).

Arbuscular mycorrhizal 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 dialogue – the so-called pre-symbiotic stage – starts with the production and exudation into the rhizosphere of strigolactones (SLs) by the host plant. SLs are perceived by AM fungi by in so far uncharacterized receptor and stimulated hyphal growth and branching, increasing the chance of encountering the host root (Akiyama *et al.*, 2005, Besserer *et al.*, 2006). While the importance of SLs in the initial stages of mycorrhizal colonization is well accepted, it is not clear whether they also play a role in subsequent steps of the symbiosis or in the responses to environmental stresses. In addition to molecular cues in the plant–AM fungi interaction, in the rhizosphere, SLs also act as host detection signals for root parasitic plants of the Orobanchaceae, including *Striga*, *Orobanche* and *Phelipanche* species, where they stimulate seed germination (Bouwmeester *et al.*, 2007, López-Ráez *et al.*, 2011b). Accordingly to their role as signalling molecules in the rhizosphere, SLs are mainly produced in the roots, and they have been detected in the root extracts and root exudates of both monocot and dicot plants (Xie *et al.*, 2010). Since 2008, SLs are classified as a new class of hormones that control several processes in plants. They play a pivotal role as modulators of the coordinated development of roots and shoots in response to nutrient deprivation, especially phosphorus shortage. They regulate above-ground and below-ground plant architecture, adventitious root formation, secondary growth, reproductive development, leaf senescence and defence responses (reviewed in Ruyter-Spira *et al.*, 2013). SLs biosynthetically derive from carotenoids (López-Ráez *et al.*, 2008, Matusova *et al.*, 2005) by sequential oxidative

cleavage by two carotenoid cleavage dioxygenases – CCD7 and CCD8 – belonging to the apocarotenoids as the phytohormone ABA (Walter & Strack, 2011). In addition to their role in the response of plants to abiotic stress, it was shown that ABA is necessary for a proper AM symbiosis establishment and functioning (Martín-Rodríguez *et al.*, 2010, Pozo *et al.*, 2015). Interestingly, a regulatory role of ABA in SL biosynthesis has been proposed because a correlation between ABA and SL content was observed (Aroca *et al.*, 2013, López-Ráez *et al.*, 2010). More recently, a relationship ABA–SLs has also been shown in lotus plants under osmotic stress (Liu *et al.*, 2015), and a role of SLs in drought stress tolerance in the non-mycorrhizal plant Arabidopsis has been proposed (Ha *et al.*, 2014, Bu *et al.*, 2014). However, how ABA-SLs regulation is involved in these water-related stress responses and how it is affected by AM symbiosis is so far unknown.

We previously showed that AM symbiosis alleviates the negative effects of salt stress in lettuce by affecting the hormonal profiles and plant physiology (Aroca *et al.*, 2013). In the present study, the effects of drought on AM symbiosis establishment and on the production of the phytohormones SLs and ABA were investigated in two agronomically important crops such as tomato and lettuce. Three different water regimes were used, and their effects were investigated at early, middle and well-established symbiosis stages. Physiological parameters such as plant biomass, stomatal conductance and efficiency of photosystem II, associated with drought, as well as hormonal levels and the expression of molecular makers associated with SLs and ABA were assessed in mycorrhizal and non-mycorrhizal plants.

MATERIALS AND METHODS

Experimental design

The experiment consisted of a factorial design with two inoculation treatments: (1) non-inoculated control plants (NM) and (2) plants inoculated with the AM fungus *Rhizophagus irregularis* (Schussler & Walker, 2010) (formerly *Glomus intraradices*) strain EEZ 58 (Ri) and three irrigation treatments: (i) plants cultivated under well-watered conditions; (ii) plants cultivated under moderate drought stress; and (iii) plants cultivated under severe drought stress. Two plant species were used with the same experimental design, tomato (*Solanum lycopersicum*, cv. Reimlams Rhums) and lettuce (*Lactuca sativa*, cv. Romana). For each plant species, 15 replicates of each of these treatments were used, totalling 180 pots (90 pots containing tomato plants and 90 pots containing lettuce plants, one plant per pot). Thus, five individual plants of each treatment (30 in total per plant species) were harvested after 4 weeks of cultivation, another plant set after 6 weeks and the last plant set after 8 weeks.

Soil and biological materials

A loamy soil was collected from Dúrcal (Granada, Spain). The soil had a pH of 8.2 (measured in water, 1:5 w/v); 1.8% organic matter, total nutrient concentrations (g kg^{-1}): N, 2.5; P, 6.2 (NaHCO3-extractable P); K, 13.2. The soils were sieved

(5 mm), diluted with quartz sand (<2 mm) and vermiculite (2:2:1, soil:sand:vermiculite, v:v:v) in order to avoid excessive compaction and sterilized by steaming (100 °C for 1 h on 3 consecutive days).

Seeds of tomato and lettuce were sown in trays containing sterile moist sand for germination during 1 week. After that, individual seedlings were transferred to pots containing 1000 g of the soil:sand:vermiculite mixture described previously.

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

Growth conditions

The experiment was carried out under greenhouse conditions with temperatures ranging from 19 to 25 °C, 16/8 light/dark period, a relative humidity of 50–60% and an average photosynthetic photon flux density of 800 μmol m^{-2} s^{-1}, as measured with a light meter (Li-Cor, Lincoln, NE, USA; model LI-188B).

Soil moisture was measured with an ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK). For treatments cultivated under well-watered conditions, water was supplied daily to maintain soil close to 100% of field capacity. The 100% soil water holding capacity corresponded to 22% volumetric soil moisture measured with the ThetaProbe, as determined experimentally in a previous experiment using a pressure plate apparatus. For treatments cultivated under moderate drought stress, water was supplied daily to maintain soil close to 75% of field capacity, which corresponded to 14% of volumetric soil moisture measured with the ThetaProbe. Finally, for treatments cultivated under severe drought stress, water was supplied daily to maintain soil close to 55% of field capacity, which corresponded to 8% volumetric soil moisture measured with the ThetaProbe. The soil water content was daily measured with the ThetaProbe ML2 before rewatering (at the end of the afternoon). The amount of water lost was added to each pot in order to keep the soil water content at the desired levels of volumetric soil moisture (Porcel & Ruiz-Lozano, 2004). The drought stress treatments were imposed from the beginning of the experiment, just after seedlings transplantation to the pots. Plants were maintained under these conditions until harvest at 4, 6 and 8 weeks. At harvest, the shoot and root system of each plant was separated and weighed. The shoot system was used for the dry weight measurement and the root system frozen in liquid nitrogen and stored at −80 °C until use.

Parameters measured

Biomass production and symbiotic development

The shoot dry weight (DW) for each plant at the different time points (4, 6 or 8 weeks after sowing) was measured after drying in a forced hot-air oven at 70 °C for 2 days. Five independent replicates per treatment and time point were analysed. 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 × 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 2 h 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) · m^{-2} · s^{-1} was used. Measurements were taken in the second youngest leaf of five different plants of each treatment after 4, 6 and 8 weeks of plant cultivation.

Abscisic acid content

Abscisic acid extraction, purification and quantification were carried out using the method described by Bacaicoa *et al.* (2009), but using 0.25 g of frozen root tissue (previously ground to a powder in a mortar with liquid nitrogen) instead of 0.5 g. ABA content was measured after 6 weeks of plant cultivation. ABA was quantified by liquid chromatography coupled to a 3200 Q TRAP (high-performance liquid chromatography–tandem mass spectrometry) system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using a reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150 × 2 mm, Phenomenex, Torrance, CA, USA). A linear gradient of methanol (A) and 0.5% acetic acid in water (B) was used: 35% A for 1 min, 35–95% A in 9 min, 95% A for 4 min and 35–95% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 mL min^{-1}, the injection volume was 40 μL, and the column and sample

temperatures were 30 and 20 °C, respectively. Detection and quantification were performed by multiple reaction monitoring (MRM) in the negative-ion mode, employing a multilevel calibration graph with deuterated hormones as internal standards. Compound-dependent parameters are described in Bacaicoa *et al.* (2009). The source parameters were as follows: curtain gas 25 psi, GS1 50 psi, GS2 60 psi, ion spray voltage −4000 V, Charged Aerosol Detector (CAD) gas medium and temperature 600 °C.

Gene expression analysis by real-time quantitative RT-PCR

After 6 weeks of plant cultivation, total RNA was isolated from tomato and lettuce roots using Tri-Reagent (Sigma-Aldrich, St Gallen, Switzerland) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega, Madrid, Spain), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel, Hoerd, France) and stored at −80 °C until use. Real-time quantitative RT-PCR (qPCR) was performed using the iCycler iQ5 system (Bio-Rad, Hercules, CA, USA) with SYBR Premix Ex Taq (Takara, Saint-Germain, France) and specific primers for genes *LeNCED1*, *Le4*, *SlCCD7*, *SlCCD8*, *LsNCED2* and *LsLEA1* (Table S1). The first strand cDNA was synthesized with 1 μg of purified total RNA using the PrimeScript RT Master Mix kit (Takara) according to the manufacturer's instructions. Three independent biological replicates were analysed per treatment. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Expression values were normalized using the housekeeping genes *SlEF-1*, encoding an elongation factor-1 α and *LsBtub3*, encoding a beta-tubulin 3, for tomato and lettuce, respectively.

Quantification of strigolactone content

Extraction and indirect quantification of strigolactones from roots

For SL analysis in root extracts, 0.5 g of tomato and lettuce roots from each treatment harvested 6 weeks after sowing, were ground in a mortar with liquid nitrogen and extracted with 0.5 mL of 40% acetone in a 2 mL eppendorf tube. Tubes were vortexed for 2 min and centrifuged at 4 °C for 5 min at 8000 g in a table top centrifuge. The 40% acetone fraction was discarded. Then, the roots were extracted twice with 0.5 mL of 50% acetone. This fraction, containing the main SLs (López-Ráez *et al.*, 2008), was carefully transferred to 2 mL glass vials and stored at −20 °C until use. Germination bioassays with *P. ramosa* seeds were performed as described in López-Ráez *et al.* (2008).

Strigolactone analysis by multiple reactions monitoring liquid chromatography–tandem mass spectrometry

Strigolactones were extracted from 0.5 g of tomato roots as previously described (López-Ráez *et al.*, 2008). The analysis and quantification of SLs were performed using a Waters Xevo tandem quadruple mass spectrometer equipped with an electrospray ionization (ESI) source and coupled to an Acquity UPLC system (Waters, Yvelines Cedex, France) (liquid chromatography–tandem mass spectrometry) as described in Kohlen *et al.* (2011). The mass spectrometer was operated in positive ESI mode. MRM was used to search for the different SLs by comparing retention times and MRM mass transitions with those of the SL standards. The three major tomato SLs – solanacol and the two didehydro-orobanchol isomers 1 and 2 – were analysed. For simplicity, the two didehydro-orobanchol isomers, hereinafter called DDH, were quantified together. MRM transitions were optimized for each standard using the Waters IntelliStart MS Console. Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters). The summed area of all the corresponding MRM transitions was used for statistical analysis.

Statistical Analysis

Statistical analysis was performed using the software SPSS Statistics v. 20 (SPSS Inc., Chicago, IL, USA). All data were subjected to analysis of variance (ANOVA) with inoculation treatment and water regime as sources of variation. For the percentage of mycorrhizal root length, the sources of variation were harvest time and water regime. Percentage values were arcsine [squareroot (X)] transformed before statistical analysis. Post hoc comparisons with the Least Significant Difference (LSD) test were used to find out differences between groups.

RESULTS

AM root colonization

The root colonization by *R. irregularis* of tomato and lettuce plants increased steadily over time and was higher in plants subjected to the drought stress treatments than under well-watered conditions (Fig. 1). Thus, after 4 weeks, the values of mycorrhizal root length colonization in lettuce plants ranged from 5% under well-watered conditions to 22% under severe drought stress. In tomato, these values ranged from 8% under well-watered or moderate drought stress conditions to 16% under severe drought stress conditions. After 6 weeks, the root colonization increased significantly in lettuce plants to 18% (well-watered conditions), 28% (moderate drought) and 36% (severe drought). In contrast, in tomato plants, the root colonization was similar to the values obtained 4 weeks after sowing. At the last harvest (8 weeks), the AM root length in lettuce plants ranged from 34% under well-watered conditions to about 63% under moderate or severe drought stress conditions. In tomato plants, the mycorrhizal root length was 21% (WW conditions), 41% (moderate drought) and 54% (severe drought).

Shoot dry weight and mycorrhizal dependency

In this study, tomato and lettuce plants were cultivated under well-watered conditions or subjected to moderate or severe
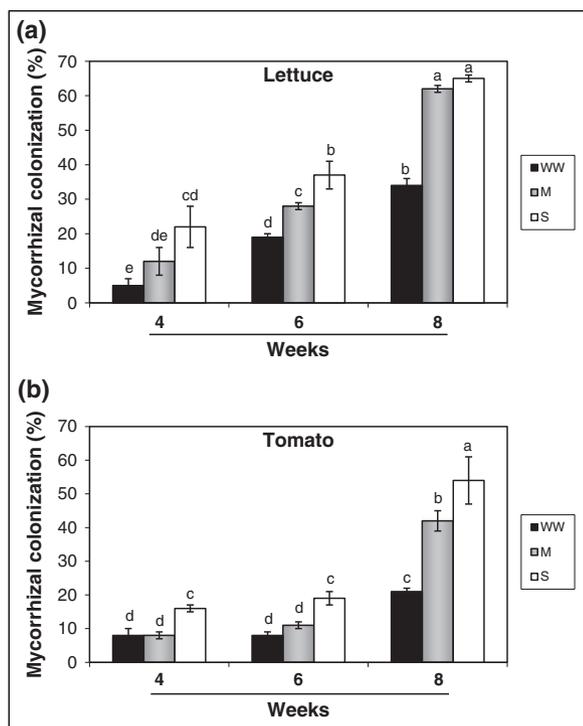
Figure 1. Effect of drought and time on the percentage of AM root colonization in (a) lettuce (*Lactuca sativa*) and (b) tomato (*Solanum lycopersicum*) plants. Intensity of mycorrhizal colonization by *R. irregularis* in the roots. Plants were cultivated under well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought since the beginning of the experiment and harvested 4, 6 or 8 weeks after inoculation. Within each harvest time, data represent means ± SD. Data with different letters differ significantly ($P < 0.05$), as determined by the Duncan's multiple range test ($n = 5$).

drought stress treatments during the whole plant growth period and harvested 4, 6 or 8 weeks after sowing. At the first harvest (4 weeks), a significant plant biomass reduction due to the drought stress treatments was already observed both in tomato and in lettuce plants. However, AM tomato and lettuce plants grew better than non-AM ones at whatever water regime (Table. 1). The growth differences were more evident for tomato plants, with mycorrhizal dependency (MD) values of about 60 and 67% under moderate and severe drought stress, respectively. In lettuce plants, the differences were more evident under well-watered conditions, with an MD of 60%.

At the second harvest (6 weeks), the drought stress treatments significantly ($P < 0.05$) decreased plant biomass production as compared with well-watered treatments, both in tomato and in lettuce plants (Table 1; Supporting Information Figs S1 and S2). In any case, AM plants always exhibited improved growth than non-mycorrhizal ones, regardless of the water regime (Table 1; Supporting Information Figs S1 and S2). Indeed, for lettuce plants, the MD values were 45, 39 and 31% under well-watered, moderate drought or severed drought stress conditions, respectively. For tomato plants, these values were 22, 33 and 30%, respectively.

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

previous harvest. Thus, both drought stress treatments decreased significantly plant growth as compared with well-watered conditions, but AM plants always maintained a higher shoot dry weight than non-AM plants. For lettuce plants, the MD at this harvest was 22, 34 and 26% under well-watered, moderate drought or severed drought stress conditions, respectively. For tomato plants, these values were 20, 29 and 17%, respectively (Table 1). Because drought effect showed a similar trend on plant development in all the three time points investigated, only plants harvested at 6 weeks were used for the following analyses.

Stomatal conductance and efficiency of photosystem II

The stomatal conductance of tomato and lettuce plants was measured before each harvest. Data and trends were similar in all harvests, and we are showing only data corresponding to the second harvest (6 weeks after sowing). At this time, the stomatal conductance of both plant species decreased because of the drought stress, being this decrease statistically significant under severe drought stress as compared with well-watered conditions (Fig. 2). The behaviour of stomatal conductance was similar for AM and non-AM plants, and differences between 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) because 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 (Fig. 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

Abscisic acid is a phytohormone critical for plant growth and development, generally associated with 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 (Figs 4 & 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 (Fig. 4a). The expression of the lettuce ABA-biosynthesis gene *LsNCED2*, encoding a 9-*cis*-epoxycarotenoid dioxygenase (Sawada

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

Treatment	Well-watered		Medium		Severe	
	Non-mycorrhizal	*R. intraradices*	Non-mycorrhizal	*R. intraradices*	Non-mycorrhizal	*R. intraradices*
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 ± 0.04 cd
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 ± 0.09 cd
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

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

et al., 2008), was significantly upregulated by the severity of the drought stress both in AM and in non-AM plants (Fig. 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 (Fig. 4b).

A similar pattern as for lettuce was observed for ABA levels in tomato roots (Fig. 5a). Non-AM tomato plants enhanced the accumulation of ABA by about 88% as a consequence of drought stress (similar levels obtained under moderate and under severe drought). AM plants also increased the accumulation of ABA by 58% under moderate drought, although the ABA levels achieved were lower than in non-mycorrhizal plants. Under severe drought, AM tomato plants enhanced the accumulation of ABA by 200% compared with control well-watered plants, reaching similar levels to non-AM plants. The expression of the tomato ABA-biosynthesis gene *LeNCED1* (Thompson *et al.*, 2000) was not affected in roots by the water regime and by the AM fungal inoculation (Fig. 5b). In contrast, the ABA-inducible gene *Le4* (Kahn *et al.*, 1993), encoding a dehydrin and used as a marker of plant response to the drought stress imposed, was induced by the severity of the water regime in AM and in non-AM plants (Fig. 5b). Other phytohormones analysed such as jasmonic acid, salicylic acid and auxin were not altered in roots neither by drought nor AM symbiosis (data not shown).

Strigolactone production and expression of strigolactone biosynthesis genes

Strigolactones are important molecules in the rhizosphere favouring AM symbiosis establishment (Akiyama *et al.*, 2005, Bouwmeester *et al.*, 2007). Therefore, SL production was analysed. They are also germination stimulants of root parasitic plant seeds (Bouwmeester *et al.*, 2003, Cook *et al.*, 1972). Because of this germinating activity, bioassays based on seed germination using a specific fraction of the host plant extracts or exudates can be used as a reliable indirect way to quantify the levels of SLs (López-Ráez *et al.* 2011a, López-Ráez *et al.*, 2008, Matusova *et al.*, 2005). To quantify SL production by tomato and lettuce plants, we first performed a germination bioassay with seeds of *P. ramosa* using the 50% acetone fraction of root extracts from plants harvested at 6 weeks (Fig. 6a,b). The synthetic germination stimulant GR24 (10^{-9} and 10^{-10} M), used as a positive control, always induced germination of preconditioned *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
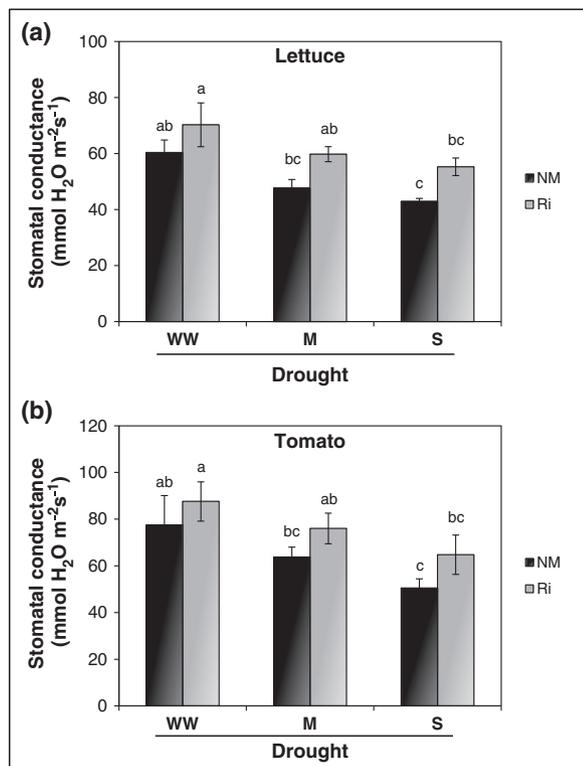
Figure 2. Influence of arbuscular mycorrhizal (AM) symbiosis and drought stress on the physiological status of lettuce (a) and tomato (b) plants. Effect on the stomatal conductance at 6 weeks. Plants were cultivated under well-watered (WW) conditions or subjected to moderate (M) or severe (S) drought. Closed bars represent non-inoculated control plants (NM), and grey bars represent plants inoculated with *R. irregularis* (Ri). Bars represent the means of five replicates (±SE). Bars with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

Figure 3. Influence of arbuscular mycorrhizal (AM) symbiosis and drought stress on the physiological status of lettuce (a) and tomato (b). Effect on the efficiency of photosystem II at 6 weeks. See legend of Figure 2.

(Fig. 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 DDH isomers (López-Ráez *et al.*, 2008) – by liquid chromatography coupled mass spectrometry. 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 (Fig. 7a), and
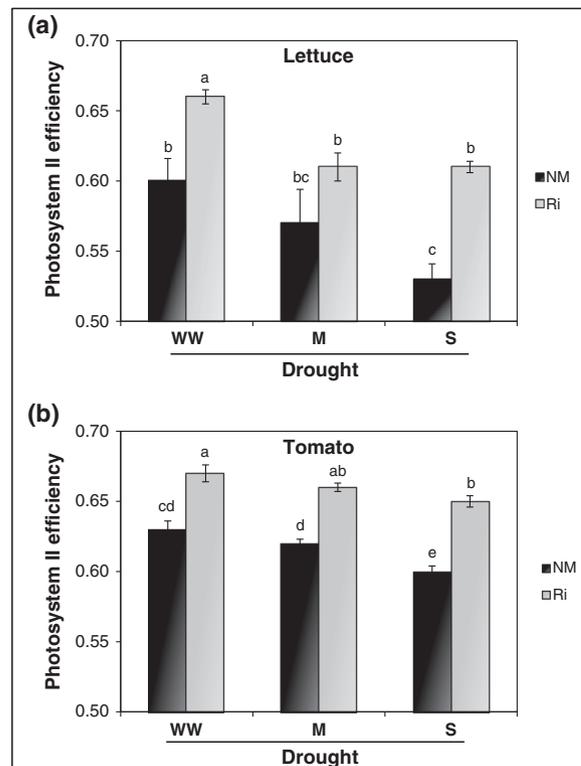
repeated with root samples from tomato plants harvested at 8 weeks, obtaining similar results. Both solanacol and DDH followed a similar behaviour, which confirmed the pattern observed in the seed germination bioassay. Indeed, in non-mycorrhizal tomato plants, solanacol and DDH steadily decreased with increasing severity of the drought stress, while both SLs increased with increasing severity of the drought stress imposed in mycorrhizal plants.

As a third approach, the expression of two tomato genes – *SlCCD7* and *SlCCD8* – involved in the biosynthesis of SLs (Kohlen *et al.*, 2012, Vogel *et al.*, 2010) was quantified by real-time quantitative RT-PCR (qPCR). The expression of *SlCCD8* was not altered by the water regime and mycorrhizal status (Fig. 7b). However, *SlCCD7* expression was differentially affected under these two conditions. In roots of non-AM tomato plants, the expression of *SlCCD7* was down-regulated by increasing severity of the drought stress imposed (Fig. 6b). Conversely, *SlCCD7* expression was clearly up-regulated by increasing severity of the drought stress in AM roots (Fig. 7b), following the same pattern as for SL accumulation (Fig. 7a).

DISCUSSION

Water-related stresses, including drought, adversely impact plant physiology, growth and productivity (Bray, 2004, Farooq *et al.*, 2014, Golldack *et al.*, 2014, Osakabe *et al.*, 2014, Ruiz-Lozano *et al.*, 2012). Along evolution, plants have evolved mechanisms to flexibly adapt to these unfavourable conditions
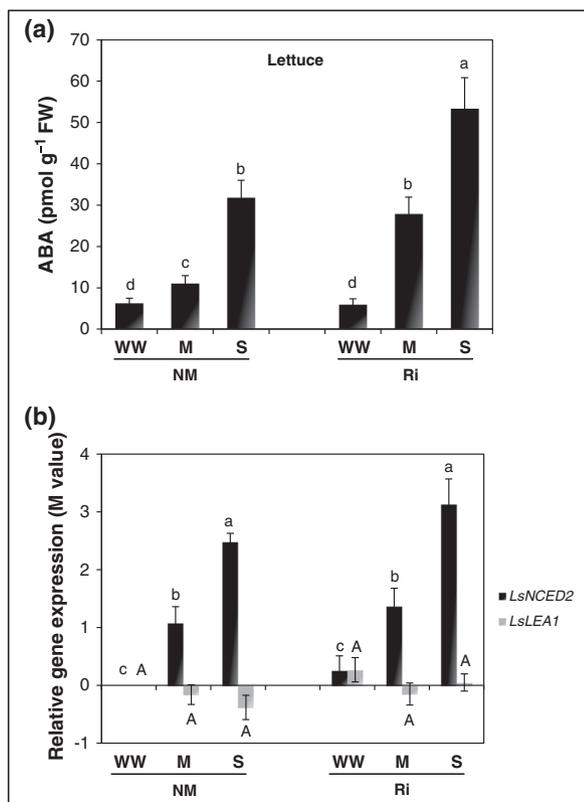
Figure 4. Effect of drought and AM symbiosis in abscisic acid (ABA) signalling pathway in lettuce. (a) ABA content in lettuce roots after 6 weeks. (b) Gene expression analysis by real-time qPCR for the ABA biosynthesis gene *LsNCED2* (closed bars) and for the ABA-responsive gene *LsLEA1* (grey bars) in lettuce roots after 6 weeks. See legend of Fig. 2. Bars represent the means of five (a) or three (b) replicates (±SE).

Figure 5. Effect of drought and AM symbiosis in abscisic acid (ABA) signalling pathway in tomato. (a) ABA content in tomato roots after 6 weeks. (b) Gene expression analysis by real-time qPCR for the ABA biosynthesis gene *LeNCED1* (closed bars) and for the ABA-responsive gene *Le4* (grey bars) in tomato roots after 6 weeks. See legend of Fig. 2. Bars represent the means of five (a) or three (b) replicates (±SE).

(Pierik & Testerink, 2014, Ruiz-Lozano *et al.*, 2012). One of these strategies is the establishment of AM symbiosis. It is widely accepted that this mutualistic association is a key component in helping plants to cope with adverse environmental conditions, including drought stress (Miransari *et al.*, 2014, Pozo & Azcón-Aguilar, 2007, Ruiz-Lozano *et al.*, 2012, Ruiz-Sánchez *et al.*, 2010). Interestingly, we show here for the first time and in two plant species that drought steadily enhances colonization rates based on the water regime (Fig. 1). The beneficial effects of different AM fungi on plant growth and development under drought have been shown in a number of plant species such as maize, rice, citrus, barley and pistachio (Abbaspour *et al.*, 2012, Bárzana *et al.*, 2014, Ruiz-Sánchez *et al.*, 2010, Wu & Zou, 2009). We previously showed that mycorrhizal tomato plants also performed better than non-mycorrhizal ones under drought stress upon a well-established symbiosis (Aroca *et al.*, 2008a). However, in that experiment, AM symbiosis was established prior to the application of drought stress. Here, we show that the beneficial effect of the symbiosis on plant performance also takes place when the stress is applied from the beginning of the growing period, which resembles more to natural conditions. Remarkably, the same behaviour was observed in mycorrhizal lettuce
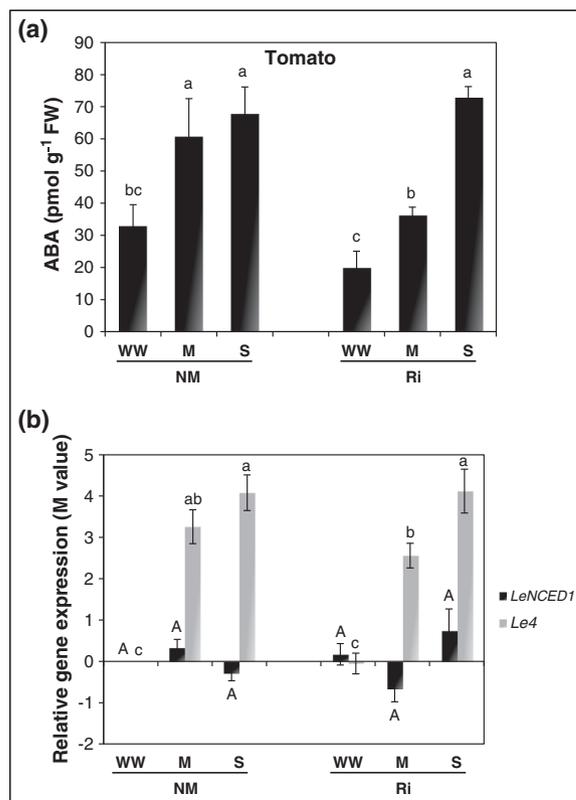
plants, indicating that this beneficial effect of AM symbiosis on plant performance under drought stress is conserved across plant species. In both cases, the promotion of plant growth started from early stages of mycorrhizal colonization (after 4 weeks), where less than 10% root colonization was achieved. Growth promotion was maintained until 8 weeks of treatment, where mycorrhizal stressed plants grew at a similar rate than non-stressed control plants. Thus, AM symbiosis alleviates drought stress and allows mycorrhizal plants to grow better under these unfavourable conditions, taking place this beneficial effect from the very beginning of the association. Interestingly, the same effect of AM symbiosis was previously observed in lettuce plants exposed to salt stress (Aroca *et al.*, 2013), suggesting a conserved behaviour for different osmotic-related stresses.

Plant growth and productivity is closely associated with the drought stress level experienced by plants. This induces a decrease in the leaf water potential and in stomatal opening, negatively affecting photosynthesis and CO_2 availability (Augé *et al.*, 2015, Osakabe *et al.*, 2014). It has been described that AM symbiosis can alter stomatal behaviour, thus affecting plant productivity (Augé *et al.*, 2015). In our experiment, in addition to a better growth rate, tomato and lettuce
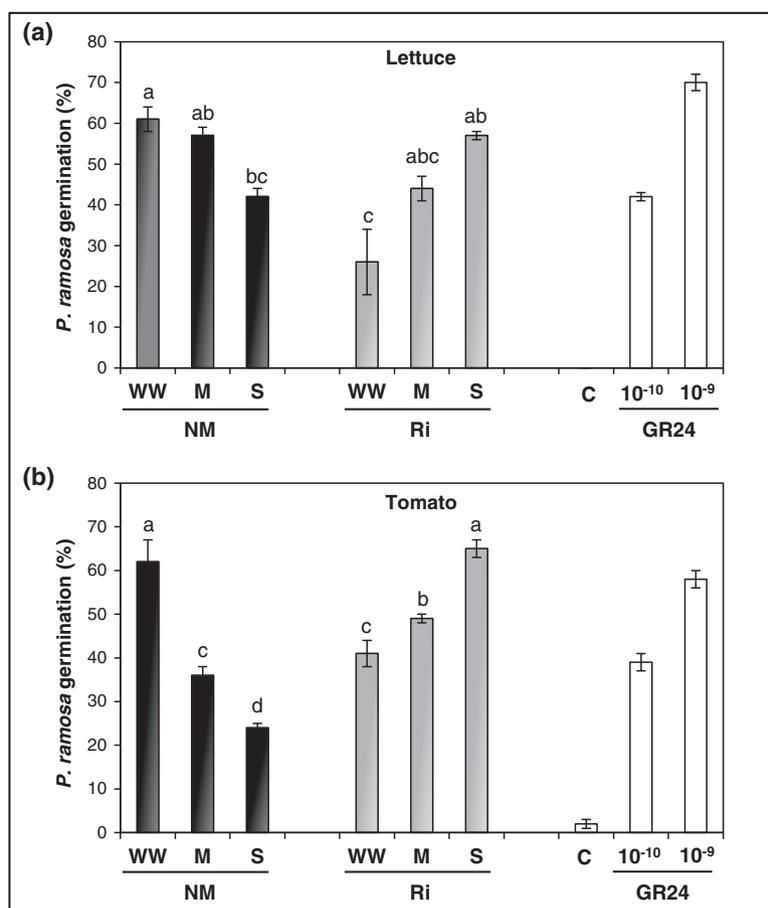
Figure 6. Influence of drought and mycorrhizal colonization on strigolactone production. Germination of *Phelipanche ramosa* seeds induced by root extracts of lettuce (a) and tomato (b) plants after 6 weeks. Dilution 1 :200 in demineralized water of each root extract was used. GR24 (10^{-9} and 10^{-10} M) and demineralized water (C) were used as positive and negative controls, respectively. For treatments and statistics, see legend of Fig. 2.

AM plants exhibited a better performance of photosystem II both under well-watered and stressed conditions. The increase was higher in plants under a severe stress, an effect that was previously shown in tomato (Bárzana *et al.*, 2012). Likely, this positive effect has also contributed to the enhanced plant growth of mycorrhizal plants, probably by enhancing CO_2 fixation. In this sense, several studies have shown a correlation between tolerance to drought stress and maintenance of efficiency of photosystem II, which also sustained plant productivity (Loggini *et al.*, 1999, Ruiz-Sánchez *et al.*, 2010). The higher values of photosynthetic efficiency in mycorrhizal plants indicate that the photosynthetic apparatus of these plants is less damaged by the drought stress imposed (Bárzana *et al.*, 2012, Sperdouli & Moustakas, 2012). The same pattern for plant growth and physiological parameters was previously observed in mycorrhizal lettuce plants subjected to salinity (Aroca *et al.*, 2013), suggesting, once again a common effect of AM symbiosis to different osmotic-related stresses.

The enhanced tolerance of mycorrhizal plants against water-related stresses has been associated with an alteration of the phytohormone homeostasis, for which ABA signalling is the most intensively studied (Calvo-Polanco *et al.*, 2013, Osakabe *et al.*, 2014, Ruiz-Lozano *et al.*, 2012). Plants have to adjust their ABA levels continuously in response to changing physiological and environmental conditions. Indeed, ABA is considered as the 'stress hormone', as it accumulates rapidly in response to salinity and drought (Hong *et al.*, 2013). As expected, a steady increase in ABA content was observed in roots from non-AM plants as a consequence of drought in both tomato and lettuce, reaching the maximum ABA levels under the most severe stress (Figs 4 & 5). An induction in ABA was also detected in AM plants, showing a similar trend as for non-AM plants. As for the physiological parameters, a similar pattern in ABA content was previously observed in lettuce plants under salt stress, especially in mycorrhizal plants (Aroca *et al.*, 2013). In that study, we showed a correlation 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
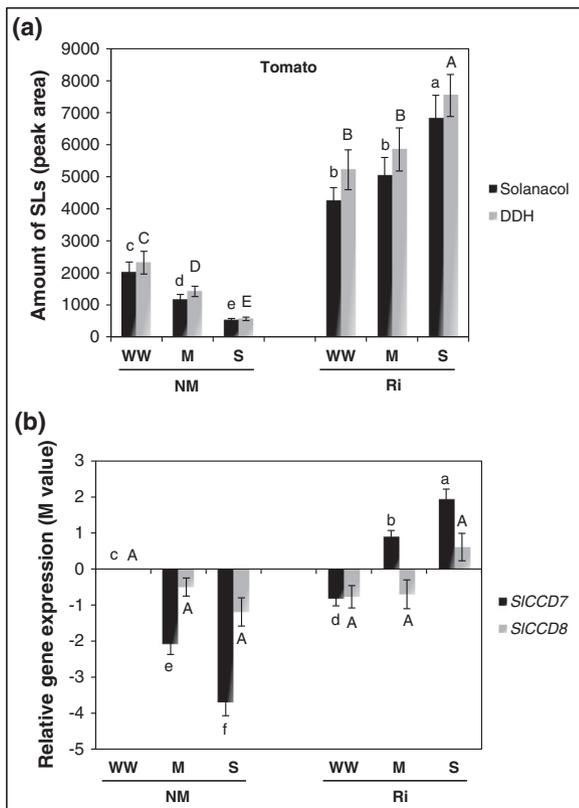
Figure 7. Influence of drought and mycorrhizal colonization on strigolactone production. (a) SL content in tomato root extracts at 6 weeks analysed by LC-MS/MS. Data correspond to the amount (according to the peak area) of the SLs solanacol and the didehydro-orobanchol isomers 1 and 2 (DDH) from tomato 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 Fig. 2.

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 previously, 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 (Figs 1–6). 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 (Fig. 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 (Figs 4–6). This fact was also observed in lettuce plants subjected to salt stress (Aroca *et al.*, 2013) and suggests a crosstalk between these two phytohormones, which is important for AM symbiosis under abiotic stress. An interaction SLs–ABA under water-related stress conditions has also been recently described in lotus (Liu *et al.*, 2015). Liu *et al.* found out that osmotic stress decreased SL content in roots and root exudates, and that this reduction was associated with an increase in ABA levels. The authors proposed that the stress-induced reduction in SLs is needed to allow the local increase of ABA and thus, the plant response to the stress. This is the effect we observed in tomato and lettuce under drought in non-AM plants. Therefore, this negative correlation SL–ABA might be a general plant strategy to cope with water-related stresses in the absence of AM symbiosis.

In conclusion, we show here that AM symbiosis alleviates the negative effects of drought in tomato and lettuce plants by altering the hormonal profiles, thus affecting plant physiology and development. The results confirm the role of arbuscular mycorrhizas in protecting host plants under unfavourable environmental conditions and their potential use as a sustainable strategy in agriculture. The involvement of SLs in plant responses against water-related stresses, as well as their interaction with ABA is also evidenced in this study, showing a different behaviour depending on the presence or absence of AM symbiosis. However, further research is required to elucidate the intrinsic mechanisms of this SL–ABA crosstalk and how it is modulated by AM symbiosis.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

We declare that we do not have any conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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. There is a phenotypic comparison of non-mycorrhizal (NM) and mycorrhizal (Ri) tomato plants.