Radial water transport in arbuscular mycorrhizal maize plants under drought stress conditions is affected by indole-acetic acid (IAA) application

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Abstract

Drought stress is one of the most devastating abiotic stresses, compromising crop growth, reproductive success and yield. The arbuscular mycorrhizal (AM) symbiosis has been demonstrated to be beneficial in helping the plant to bear with water deficit. In plants, development and stress responses are largely regulated by a complex hormonal crosstalk. Auxins play significant roles in plant growth and development, in responses to different abiotic stresses or in the establishment and functioning of the AM symbiosis. Despite these important functions, the role of indole-3acetic acid (IAA) as a regulator of root water transport and stress response is not well understood. In this study, the effect of exogenous application of IAA on the regulation of root radial water transport in AM plants was analyzed under well-watered and drought stress conditions. Exogenous IAA application affected root hydraulic parameters, mainly osmotic root hydraulic conductivity (Lo), which was decreased in both AM and non-AM plants under water deficit conditions. Under drought, the relative apoplastic water flow was differentially regulated by IAA application in non-AM and AM plants. The effect of IAA on the internal cell component of root water conductivity suggests that aquaporins are involved in the IAA-dependent inhibition of this water pathway.

Keywords: arbuscular mycorrhizal symbiosis; drought stress; IAA; radial water transport

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45 **Introduction**

46 Drought stress is one of the most devastating abiotic stresses, compromising crop
47 growth, reproductive success and ultimately yield (Hasanuzzaman et al., 2014). In
48 addition, climate change is intensifying the effects of drought worldwide (Trenberth et al.,
49 2014). Roots are the first organs to sense the stress, as they are in contact with soil
50 moisture changes. Therefore, they must adapt to it morphologically and physiologically in
51 order not to be damaged. In this sense, the arbuscular mycorrhizal symbiosis between
52 *Glomeromycotina* fungi and the roots of most terrestrial plants has been demonstrated to
53 be beneficial in helping the plant to bear with water deficit (Chitarra et al., 2016; Essahibi
54 et al., 2017; Yooyongwech et al., 2016). This association contributes to the uptake of water
55 and nutrients thanks to a vast network of extraradical mycelium in exchange of carbon
56 compounds and lipids. In addition to a better access of nutrients and water in soil, the
57 relieve of drought stress is achieved by the alteration of root hydraulic properties (Aroca et
58 al., 2007; Bárzana et al., 2012; Quiroga et al., 2019a).

59 In roots, radial water movement occurs through three main parallel pathways
60 according to the composite transport model: the apoplastic (around the cell walls),
61 symplastic (crossing cells via plasmodesmata) and transcellular (involving water passage
62 across cell membranes). The last two pathways are commonly referred as to cell-to-cell
63 pathway (Steudle and Peterson, 1998). During non-stressful conditions the apoplastic
64 pathway usually dominates, following the transpiration stream. However, during water
65 deficit conditions in the soil such pathway is hampered due to stomatal closure and
66 transpiration decline and the cell-to-cell pathway is enhanced. Aquaporins play a key
67 regulatory role of root cell water transport in higher plants both under normal and under
68 stressful conditions (Maurel et al., 2008), participating in the cell-to-cell water transport.
69 Arbuscular mycorrhizal symbiosis has been shown to modulate the switch between water

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transport pathways in roots, and a previous study showed an increase in the relative apoplastic water flow in AM plants under both well-watered and drought stress conditions (Bárzana et al., 2012). In addition, these changes in root water conductivity (Lpr) by AM symbiosis were found to be largely mediated by changes in plant aquaporins (Ruiz-Lozano and Aroca, 2010, 2017). In fact, several maize aquaporins were differently regulated by the AM fungus depending on the intensity and duration of the drought stress imposed (Bárzana et al., 2014).

In plants, development and stress responses are mainly regulated by a complex hormonal crosstalk (Munné-Bosch and Müller, 2013). Thus, auxins play significant roles in plant growth and development, as well as, in response to different abiotic stresses (Ullah et al., 2018). In concert with other hormones, the roles of auxin include meristem maintenance, leaf primordia and lateral root initiation, tropic responses, development of vascular tissues, root and shoot elongation and control of apical dominance. At cell level, auxins also affect cell division, elongation, differentiation and polarity (Naser and Shani, 2016). During AM symbiosis, a complex molecular dialog is established between both symbiotic partners and phytohormones also play an important role on this process. In fact, some evidences showed that auxin signalling is required for normal AM infection, and the exchange of diffusible signals between plant and fungus is mediated by host auxin responses (Hanlon and Coenen, 2011). Besides, auxin was found to be required for arbuscule development (Etemadi et al., 2014). In trifoliate orange, higher root indole-3acetic acid (IAA) levels were found in AM plants and this enhancement (together with other hormonal increases) was positively related with drought tolerance in different studies (Liu et al., 2016, 2018).

Many studies have shown that abscisic acid (ABA) biosynthesis and concentrations increase under drought stress (Rowe et al., 2016). ABA modulates plant water status

through regulation of important plant processes such as root hydraulic conductance (Lpr) and transpiration rate, as well as, by inducing genes encoding for proteins involved in cellular dehydration tolerance (Schraut et al., 2005; Zhang et al., 2006; Hirayama and Shinozaki, 2007; Yao et al., 2019). Jasmonic acid (JA) and methyl jasmonate (MeJA; a derivate of JA) are hormones implicated in plant development but also with a protective role against abiotic and biotic stresses. For instance, Anjum et al. (2011) observed that MeJA-treated plants were more resistant to drought conditions, enhancing their antioxidant response and their leaf relative water content. Salicylic acid (SA) is also involved in the regulation of important plant physiological processes under stress conditions, such as photosynthesis, antioxidant defence system, nitrogen metabolism and plant water relations, thereby providing protection in plants against abiotic stresses (Khan et al., 2015; Faried et al., 2017).

Regarding the effects of plant hormones on water transport, ABA generally enhances root water transport capacity by increasing Lpr (Aroca et al., 2008; Mahdieh and Mostajeran, 2009; Kudoyarova et al., 2011). On the other hand, MeJA was also shown to increase Lpr in three different plant species (*Solanum lycopersicum*, *Phaseolus vulgaris* and *Arabidopsis thaliana*) in a calcium- and ABA-dependent way (Sánchez-Romera et al., 2014; 2016), while both SA and IAA were shown to decrease Lpr (Boursiac et al., 2008; Peret et al., 2012; Quiroga et al., 2018).

Despite the important functions of IAA, its role as a regulator of root water transport and stress response is not well understood (Wani et al., 2016). Some evidences point to a role in drought stress tolerance. Aux/IAA proteins accumulate in response to auxin signalling. In the absence of auxin, these proteins dimerize with Auxin Response Factors (ARF) to prevent ARF-mediated transcriptional regulation of early auxin response genes. However, when auxin is present, Aux/IAA proteins are ubiquitinated, allowing ARF-

120 mediated transcriptional regulation of response genes. Some Aux/IAA genes in rice were
121 induced by drought stress, and in particular *OsIAA6* was confirmed to be involved in
122 drought stress responses (Jung et al., 2015). In another study, plants overexpressing
123 *YUCCA6*, a gene involved in the tryptophan-dependent IAA biosynthesis pathway, was
124 associated with drought stress tolerance in poplar (Ke et al., 2015). Additionally, auxin
125 treatment was found to regulate tissue hydraulics and reduce root hydraulic conductivity,
126 at cell and whole-organ level, and to repress aquaporin genes through the auxin response
127 factor ARF7 (Péret et al., 2012).

128 ABA is considered the most important signal transduction pathway among all the
129 plant responses to stresses (Hirayama and Shinozaki, 2007; Kim, 2014). The induction of
130 ABA synthesis is one of the fastest hormonal responses of plants to drought stress,
131 thereby triggering ABA-inducible gene expression. This generally causes stomatal closure
132 and reduces water loss via transpiration. However, ABA-independent regulatory systems
133 are also involved in stress-responsive gene expression. Moreover, a complex network with
134 extensive interactions between the different hormone signalling pathways exists in plants.
135 For instance, JA interacts with the ABA-regulated stomatal closure by increasing Ca^{2+}
136 influx and data support the concept of common signalling components for ABA and MeJA,
137 including nitric oxide (Riemann et al., 2015). Several studies have revealed that under
138 osmotic stress conditions, ABA regulates plant development through the interaction with
139 auxins, cytokinin and ethylene (Rowe et al., 2016). At the same time, auxin and cytokinin
140 can mutually inhibit their biosynthesis (Nordstrom et al., 2004; Jones and Ljung, 2011),
141 while cytokinin promotes ethylene biosynthesis (Stepanova et al., 2007) and ethylene
142 promotes auxin biosynthesis (Ruzicka et al., 2007; Stepanova et al., 2007). Therefore, the
143 signalling responses of auxin, ABA, cytokinin and ethylene all play their roles in plant
144 physiological changes produced by osmotic stress (Rowe et al., 2016).

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Based on all these previous results, we hypothesized that hormonal treatment can affect the AM modulation of water transport in roots, especially during drought stress conditions and probably through aquaporin regulation. To ascertain this hypothesis, we externally applied IAA or 6-Fluoroindole (6-FI), an inhibitor of the tryptophan-dependent IAA biosynthesis (Ludwig-Müller et al., 2010), to AM or non-AM plants subjected or not to drought stress. The aim was to analyze the effect of this hormone on root water transport pathways and aquaporins in AM plants subjected to drought stress. The results obtained from this study shed further light on the AM regulation of root water transport during drought stress conditions.

Materials and methods

Experimental design

The experiment consisted of a factorial design with three factors: (1) inoculation treatment, with plants inoculated with the AM fungus *Rhizophagus irregularis*, strain EEZ 58 (AM) and non-inoculated control plants (non-AM); (2) watering treatment, so that half of the plants were subjected to drought stress (DS) for 15 days before harvest while the other half was grown under well-watered (WW) conditions throughout the entire experiment; (3) chemical treatment, so that one group of each inoculation treatment was maintained untreated, another group of plants was treated with 20 μM of the auxin indole-3acetic acid (IAA), and the last group was treated with 75 μM of 6-fluoroindol (6-FI), as an inhibitor of IAA biosynthesis. Both chemicals were applied 6 h before measurements and harvest, according to preliminary trials to determine the doses and exposure time. The different combination of these factors gave a total of 12 treatments. Each treatment had 10 replicates, giving a total of 120 plants.

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170 **Biological material and growth conditions**

171 The growing substrate consisted of a mixture of soil and sand (1:9 v/v). The soil was
172 collected at the grounds of Instituto de Investigación y Formación Agraria y Pesquera
173 (IFAPA, Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) and sterilized
174 by steaming (100°C for 1 h) on 3 consecutive days. The undiluted soil had a pH of 8.1
175 (water); 0.85% organic matter, nutrient concentrations (mg kg^{-1}): P, 10 ($NaHCO_3$-
176 extractable P); N, 1; K, 110. The soil texture was made of 47.1% silt, 38.3% sand and
177 14.6% clay.

178 Seeds of *Zea mays* L. were provided by Pioneer Hi-Bred (Spain), cultivar PR34B39
179 that was also used in previous studies (Quiroga et al., 2017; 2018). Seeds were pre-
180 germinated in sand and then transferred to 1.5 L pots containing 1250 g of the above-
181 described substrate. At planting time, half of the plants were inoculated with ten grams of
182 AM inoculum with *Rhizophagus irregularis* (Schenck and Smith), strain EEZ 58. The
183 inoculum consisted of spores, mycelia, infected root fragments and soil. Non inoculated
184 plants received a 10 mL aliquot of an inoculum filtrate (<20 μm), in order to provide the
185 natural microbial population present in the inoculum, but free of AM propagules.

186 Plants were grown under greenhouse conditions (average photosynthetic photon
187 flux density 800 μmol m-2 s-1, 25/20ºC, 16/8 light dark period and 50-60% RH) for a total
188 of eight weeks. Plants were irrigated three times per week with 50 mL of Hoagland nutrient
189 solution (Hoagland and Arnon, 1950) modified to contain only 25% of P, in order to avoid
190 the inhibition of AM symbiosis establishment. Plants received the same amount of water
191 on alternate days. In order to avoid a combination of drought stress plus nutrient
192 deficiency, droughted treatments received 2X Hoagland nutrient solution, so that 25 mL
193 provided the same nutrient levels as 50 mL of the 1X Hoagland nutrient solution used with
194 well-watered plants. This water stress is considered as a severe stress and was similar to

195 that imposed in previous studies (Quiroga et al., 2017; 2018).

196 Indole-3-acetic acid 20 μM and 6-FI 75 μM were applied with the nutrient solution 6
197 hours before harvesting. The dose of phytohormone and its inhibitor, as well as, the
198 exposure time needed to affect root hydraulic conductivity were previously established in a
199 preliminary experiment. In such preliminary experiment maize plants were cultivated under
200 the same substrate, duration and conditions as described here. We tested 0.5, 5 and 20
201 μM IAA, as well as, 25, 50 and 100 μM of 6-FI, applied with the nutrient solution 1 h, 6 h,
202 12 h or 24 h before measurement of the osmotic root hydraulic conductivity (Lo). Results
203 showed that 1h and 24h after application of the compounds there were not significant
204 effects on Lo, regardless of the compounds or the doses used (data not shown). The most
205 pronounced effects on Lo were found 6 h and 12 h after application of 20 μM of IAA,
206 producing a decline of Lo values over 75% and 60%, respectively, as compared to
207 untreated controls (data not shown). The lower doses of IAA did not affect significantly Lo
208 values. Regarding 6-FI, analogous effects were found 6 h and 12 h after application of the
209 compound, with similar values of Lo recovery for the doses of 50 and 100 μM 6-FI. In all
210 these cases 6-FI application recovered Lo to values comparable to untreated controls.
211
212 **Parameters measured**
213 **Biomass production and symbiotic development**
214 Five replicates per treatment were collected from roots and shoots and dried in a
215 hot-air oven at 70 ºC for 2 days to measure dry weight.
216 To differentiate fungal structures, roots of maize plants were stained according to
217 Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated in five
218 replicates per treatment according to the gridline intersect method (Giovannetti and
219 Mosse, 1980).

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220

221 **Stomatal conductance**

222 Stomatal conductance (*gs*) was measured in the second youngest leaf from 10
223 plants per treatment two hours after the onset of photoperiod and one day before harvest
224 with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK), following
225 the manufacter's recommendations.

226

227 **Photosynthetic efficiency**

228 Photosystem II efficiency was measured with FluorPen FP100 (Photon Systems
229 Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant
230 photosynthetic performance by measuring chlorophyll a fluorescence. It quantifies the
231 quantum yield of photosystem II as the ratio between the current fluorescence yield in the
232 light-adapted state (FV') and the maximum fluorescence yield in the light-adapted state
233 (FM'), according to Oxborough and Baker (1997). Measurements were taken one day
234 before harvest in the second youngest leaf of 10 different plants of each treatment.

235

236 **Membrane electrolyte leakage**

237 Leaf samples from 10 plants per treatment were washed with deionized water to
238 remove surface-adhered electrolytes. Samples were placed in 15 mL falcon tubes
239 containing 10 mL of deionized water and incubated on a rotary shaker (at 100 rpm) at 25
240 ^{o}C during 3 hours. Then the electrical conductivity of the solution (E_0) was determined
241 using a conductivity meter (Mettler Toledo AG 8603, Switzerland). Samples were
242 subsequently placed at -80 ºC for 2 hours. Afterwards, tubes were incubated again at
243 room temperature under smoothly agitation for 3 hours and the final electrical conductivity
244 (E_f) was obtained. The electrolyte leakage was defined as follows: $[(E_0 - E_{water})/(E_f - E_{water})]$

X 100, where E_{water} is the electrical conductivity of the deionized water used to incubate the samples.

Osmotic (*Lo*) and hydrostatic (*Lpr*) root hydraulic conductivities

Lo was measured at noon in five plants (n=5) per treatment by the free exudation method (Benabdellah et al., 2009) on detached roots exuding under atmospheric pressure. Under such conditions, water moves through the cell-to-cell path following only an osmotic gradient (Steudle and Peterson, 1998). The exuded sap was collected and weighed after 2 h of exudation. A cryoscopic osmometer was used to measure the osmolarity of the exuded sap and the nutrient solution, needed for *Lo* calculation, according to Aroca et al. (2007). Lo was calculated as $Lo = Jv/\Delta\Psi$, where Jv is the exuded sap flow rate and $\Delta\Psi$ the osmotic potential difference between the exuded sap and the nutrient solution where the pots were immersed. Measurements were carried out 6 h after starting the chemical treatment.

The *Lpr* was determined at noon in five plants (n=5) per treatment with a Scholander pressure chamber, 6 h after starting the chemical treatment as described by Bárzana et al. (2012). The detached roots received a gradual increase of pressure (0.2, 0.3 and 0.4 MPa) at 2-minutes intervals. Sap was collected after 2 minutes at the three pressure points. Then the sap flow was plotted against pressure, with the slope being the root hydraulic conductance (*L*) value. Finally, Lpr was determined by dividing *L* by root dry weight (RDW) and expressed as mg H_2O g RDW^{-1} MPa^{-1} h^{-1}. The collected sap was also used for subsequent hormonal determination.

Relative apoplastic water flow

Relative changes in apoplastic water flux were estimated using a high molecular

weight dye (light green SF yellowish; Sigma-Aldrich Chemical, Gillingham, Dorset; colour index 42095, molecular weight 792.85 g mol^{-1}), which has the ability to move only through the apoplast (López-Pérez et al., 2007). For that, detopped roots from five plants (n=5) per treatment were immersed in 250 μmol L^{-1} dye solution inside the pressure chamber 5 min before pressure application and kept in this solution during measurement. Sap was collected after 2 min at 0.2, 0.3 and 0.4 MPa in a Scholander pressure chamber. At the end, the concentration of the dye was determined at 630 nm (Bárzana et al., 2012) in the whole collected sap. The percentage of apoplastic pathway was calculated from the ratio between dye concentration in the sap flow and in the nutrient solution, being the concentration of dye in the nutrient solution of each treatment considered to be 100%.

Sap and tissues hormonal content

In sap, IAA, ABA, salicylic acid (SA) and jasmonic acid (JA) contents were analysed according to Albacete et al. (2008) with some modifications. Thus, xylem sap samples were filtered through 13 mm diameter Millex filters with nylon membrane having 0.22 μm pore size (Millipore, Bedford, MA, USA). Ten μl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 μg L^{-1}).

In plant roots and leaves, IAA, ABA, SA, JA and jasmonate isoleucine (JA-Ile) were analysed using high-performance liquid chromatography-electrospray ionization-high-resolution accurate mass spectrometry (HPLC-ESI_HRMS) as described in Ibort et al.

295 (2017).

296

297 **PIP aquaporins abundance and phosphorylation status**

298 Isolation of microsomal fraction and ELISA were performed as described previously
299 by Calvo-Polanco et al. (2014). As primary antibodies we used two antibodies recognizing
300 several PIP1s and PIP2 isoforms, and three antibodies recognizing the phosphorylation of
301 PIP2 proteins in the C-terminal region: PIP2A (Ser-280), PIP2B (Ser-283) and PIP2C (Ser-
302 280/Ser-283) (Calvo-Polanco et al., 2014) at (dilution 1:1000).

303

304 **Statistical analysis**

305 Within each watering regime, data were analysed using SPSS Statistics (version
306 23, IBM Analytics) and subjected to analysis of variance (ANOVA) with inoculation
307 treatment and chemical treatment as sources of variation. Post-hoc comparisons were
308 performed with Duncan's test (P<0.05). Correlations between the different parameters
309 were performed by calculating the Pearson correlation coefficients.

310

311 **Results**

312 **Root mycorrhization, plant growth and ecophysiological parameters**

313 The chemical treatment for only 6 h did not affect parameters presented in Table 1,
314 thus only the inoculation treatment and the water regime are considered in these data.

315 Uninoculated plants did not show AM root colonization. Mycorrhizal root length of
316 plants inoculated with *Rhizophagus irregularis* (AM) was 64.7% under well-watered
317 conditions and 65.8% under drought stress conditions (data not shown).

318 Drought stress negatively affected plant dry weight (between 36 and 42% of
319 decrease), but under well-watered conditions AM plants maintained higher plant dry weight

than non-AM ones (Table 1). Membrane electrolyte leakage (EL) increased significantly in non-AM plants after drought stress. In contrast, AM plants maintained levels of well-watered plants (Table 1). Stomatal conductance (*gs*) was significantly reduced after two weeks of drought stress treatment in non-AM plants (Table 1), while AM plants exhibited higher gs levels than under well-watered conditions. The efficiency of photosystem II was reduced by water deficit both in AM and in non-AM plants (Table 1).

Hydrostatic and osmotic root hydraulic conductivities and percentage of apoplastic water flow

Hydrostatic root hydraulic conductivity (L_{pr}) was not significantly affected by IAA treatment or its inhibitor under well-watered conditions, regardless of AM inoculation. In contrast, under drought stress AM plants had considerably higher Lpr values (118%) than non-AM ones. Application of IAA enhanced Lpr levels in non-AM plants under drought stress, almost doubling control values, while AM ones maintained their high Lpr values but with no further increment due to IAA. The application of 6-FI, the IAA biosynthesis inhibitor, decreased significantly Lpr levels only in AM plants (Figure 1A).

Lo levels remained also unchanged in the case of well-watered plants, both in non-AM and AM treatments. However, an evident drop of Lo levels occurred under drought conditions in both non-inoculated and inoculated plants. Moreover, under these conditions an additional inhibition of Lo by IAA occurred in both AM and non-AM plants (more than 70% inhibition in both cases), and the inhibitor 6-FI had a weak effect restoring Lo levels but without reaching the control levels (Figure 1B).

As for the other parameters, well-watered plants did not featured changes in the relative percentage of water circulating by the apoplastic pathway, with the exception of the inhibition produced by 6-FI in AM plants. This effect seems to be compensated with Lo

345 contribution in these plants, thus not being reflected in changes of Lpr levels. Under
346 drought stress, IAA application differently affected the percentage of water circulating by
347 the apoplastic pathway in non-AM and AM plants. Hence, in non-AM plants IAA produced
348 a significant increase of this percentage, while in AM plants the hormone significantly
349 diminished the flux of water circulating through this pathway. The use of 6-FI restored this
350 parameter in non-AM plants and had a similar effect than IAA in AM ones (Figure 1B).
351
352 **Aquaporin protein abundance and post-translational regulation**
353 The abundance PIP1 and PIP2 aquaporin proteins was measured. Moreover, the
354 PIP2 phosphorylation state was quantified in roots, as aquaporin water channel activity is
355 affected by this post-translational modification. In this context, the contents of PIP2 protein
356 phosphorylated at Ser-280 (PIP2A), at Ser-283 (PIP2B) and double phosphorylated at
357 Ser-280 and Ser-283 (PIP2C) were quantified.
358 There was not a clear response of aquaporin accumulation to the chemical
359 treatment, probably due to the short time of application. Under well-watered conditions, 6-
360 FI increased PIP1 and PIP2 protein levels in non-AM plants. IAA also increased PIP2
361 accumulation in non-AM plants under these conditions. However, it had the opposite effect
362 in AM plants. No significant effect on protein levels was observed under drought stress
363 conditions either by the hormones or by fungal inoculation (Figure 2A and B).
364 AM inoculation generally decreased the abundance of phosphorylated proteins
365 under well-watered conditions in plants treated with IAA or 6-FI. No significant changes in
366 phosphorylation levels were observed in droughted plants (Figure 2C, D and E).
367
368 **Sap and tissues phytohormones contents**
369 In sap IAA content resulted not significantly affected by the applied chemical

370 treatments, AM inoculation or water regime (Figure 3A). Under well-watered conditions,
371 sap ABA concentration was higher in AM plants compared to non-AM ones when IAA or 6-
372 FI were applied. The application of the inhibitor did not affect sap ABA levels in non-AM
373 plants but increased its levels in AM plants under the same conditions. No significant
374 changes were observed under water deprivation conditions (Figure 3B). No clear effect
375 was observed in sap SA and JA levels under any of the studied conditions (Figure 3C and
376 D). In the case of sap iP, a precursor of cytokinins, no significant effect was observed
377 under well-watered conditions. However, under drought stress, IAA treatment significantly
378 decreased sap iP levels of AM plants, being their levels restored by 6-FI (Figure 3E).
379 In roots, IAA content increased significantly after application of this compound in AM
380 plants under fully-irrigation conditions, being this effect reversed by the application of the
381 inhibitor 6-FI. Under drought stress conditions, AM plants presented higher root IAA
382 concentration compared to non-AM plants, and the application of IAA further increased
383 IAA levels (Figure 4A). However, in leaves, the increase in IAA content after the
384 application of the hormone was only observed in non-AM plants during drought stress
385 (Figure 5A).
386 Root ABA concentration increased with AM inoculation regardless of the water
387 treatment. However, during drought stress, this increment was significant only with the
388 application of the hormone or its inhibitor, which strongly elevated ABA levels (Figure 4B).
389 In leaves, the trend was similar, but the increase was only significant in well-watered AM
390 roots treated with 6-FI and in droughted AM roots with both chemical treatments (Figure
391 5B). Root SA content presented the same pattern than ABA, being increased by the fungal
392 presence in both watering conditions, especially under drought stress (Figure 4C). In
393 contrast, SA concentration in leaves decreased in AM plants under well-watered
394 conditions when untreated or treated with IAA. Under drought stress, SA content in leaves

was not significantly affected by the different treatments (Figure 5C).

Root JA content suffered a drop in well-watered AM plants regardless of hormonal treatment, and 6-FI slightly increased JA levels in both non-AM and AM plants under these conditions. The same increment was observed in non-AM plants during drought after application of IAA or 6-FI (Figure 4D). In leaves, a slight increase in JA concentration occurred during drought in non-AM plants when IAA was applied (Figure 5D).

Interestingly, JA-Ile content in roots was also increased by 6-FI in roots of AM plants under well-watered conditions. Under drought stress, levels of this hormone were unaffected by any of the treatments (Figure 4E). In the case of leaves, JA-Ile levels were not modified under well-watered conditions, but they were increased by IAA application in AM plants under drought stress (Figure 5E).

Tissue hormonal content data was used to perform a principal component analysis (PCA) in order to compare inoculation treatments, as well as, differences related to water and hormonal treatments. PCA analysis separated treatments by inoculation (Non-AM and AM plants). Axes PC1 and PC2 explained 56.3% of data variability. PCA analysis also revealed that hormonal contents were clearly separated in groups; one group was constituted by ABA content in roots and leaves as well as SA and IAA in roots; another group was constituted by JA in leaves and roots, JA-Ile in leaves and roots and SA in leaves; IAA content in leaves was separated from both groups and negatively correlated with JA in leaves and JA-Ile in roots and leaves (Figure 6).

Correlations among root hydraulic properties and the different parameters analyzed

Under well-watered conditions, Lpr was negatively correlated with root IAA content, as reflected by Pearson correlation coefficient (Table 2). However this correlation was not found in the case of Lo (Table 2). The percentage of apoplastic water flow showed a

420 strong negative correlation with sap IAA, ABA and JA, as well as, with root JA-Ile (Table
421 2). Sap IAA content was also positively correlated with sap JA content and root JA-Ile.
422 Moreover, root IAA content showed a positive correlation with IAA content in leaves (Table
423 2).

424 Under drought stress conditions, Lpr did not correlate with any of the studied
425 parameters (Table 2). In contrast, Lo positively correlated with sap and leaf ABA content
426 (Table 2). In addition, the percentage of water flowing through the apoplastic pathway
427 presented a negative correlation with JA-Ile in leaves. The data also revealed a strong
428 negative correlation of sap IAA with sap JA, as well as with root IAA, ABA, SA and JA-Ile
429 (Table 2). Moreover, this parameter correlated negatively with PIP1 protein abundance
430 and positively with PIP2B protein content. Finally, root IAA content presented a positive
431 correlation with ABA, SA and JA-Ile in roots, and a negative correlation with PIP2B and
432 PIP2C (Table 2).

433

434 **Discussion**

435 In this study, the beneficial effect of AM colonization on plant physiology was
436 evidenced by the higher dry weight of AM plants compared to non-AM ones when plants
437 were well-watered (Table 1). This may be the consequence of a better plant hydration and
438 nutrition due to the increased uptake surface of the fungal hyphae. Under drought stress,
439 AM plants also presented lower electrolyte leakage than non-inoculated plants, which
440 suggest a higher membrane stability of these plants (Table 1). This parameter was
441 considered a good indicator of the plant tolerance to water stress (Ortiz et al., 2015).

442 The information available about the IAA effect on water relations and drought
443 tolerance is scarce. Moreover, to the best of our knowledge, its interactive effect with
444 mycorrhizal inoculation is almost elusive. In our study, exogenous application of IAA

445 negatively affected osmotic root hydraulic conductivity (Lo) in both non-AM and AM plants,
446 although only during drought stress (Figure 1B). This could be due to a different dynamic
447 of droughted roots for water uptake as compared to well-watered roots, and therefore, to
448 higher avidity for the uptake of the chemical from the nutrient solution in droughted roots.
449 The application of 6-FI partially recovered the negative effect of IAA on Lo, but the effect
450 was not enough to be statistically significant (Figure 1B). As this parameter is an
451 estimation of the water flowing by the cell-to-cell pathway, it is thought that its values are
452 largely determined by water channel activity. However, there was not a clear effect of the
453 application of IAA or 6-FI on aquaporin accumulation or in the phosphorylation status of
454 PIP2 aquaporins (Figure 2). This may be due to the short time of application of the
455 hormonal treatment (only 6 hours before harvesting). On the other hand, the antibodies
456 used in this study are not isoform-specific, but general for the whole PIP1 and PIP2
457 subfamilies, which means that changes in specific aquaporins may be diluted by other
458 isoforms and are, thus, not detected.

459 In contrast, our result is indeed in agreement with previous findings from Péret *et al.*
460 (2012), which showed that IAA inhibited Lpr and root aquaporins in Arabidopsis during
461 lateral root formation. Different studies have shown that hormonal treatment can affect
462 hydraulic conductivity and probably aquaporins. In maize roots, exogenous application of
463 ABA enhanced Lpr especially at root cortical cell level, which suggest the implication of
464 aquaporins in this process (Hose et al., 2000; Ruiz-Lozano et al., 2009). In other studies,
465 SA was found to inhibit Lpr and this was attributed to internalization of PIPs in cell vesicles
466 (Boursiac et al., 2008) or to a fine regulation in roots of the aquaporins ZmPIP2;4 and
467 ZmTIP1;1 (Quiroga et al., 2018).

468 In this study, hydrostatic root hydraulic conductivity (Lpr) was enhanced when IAA
469 was applied in both non-AM and AM plants during drought (although it was not significant

for AM plants). This effect could be explained by a mechanism compensating the drop of Lo in non-AM plants treated with IAA, which enhanced the proportion of water flowing by the apoplastic water pathway (Figure 1C). Nevertheless, in AM plants, such an increase in apoplastic water flow was not observed when treated with IAA, and the high levels of Lpr in these plants (Figure 1A) may be due to a higher water transport by the own AM fungal hyphae (Allen, 2009; Ruth et al., 2011). Interestingly, the same opposite response of apoplastic water flow to the hormonal treatment in AM plants compared to non-AM plants was also found in a previous study after the external application of SA (Quiroga et al., 2018), suggesting that when these hormones are applied exogenously, the fungus differentially modulates the water flowing by the apoplastic pathway (Bárzana et al., 2012). The possible involvement of nitric oxide in this process was discussed (Quiroga et al., 2018), although it remains to be elucidated yet.

Aquaporin activity can be regulated by phosphorylation events in different residues, and previous studies have shown that the phosphorylation of PIP2s at Ser-280 and Ser-283 was related to the regulation of plant hydraulic conductivity (Prado et al., 2013). In this case, phosphorylation generally decreased with the AM colonization under WW conditions, but the differences were not significant under drought stress (Figure 2C, D and E). This is in agreement with previous results on mycorrhizal maize plants (Quiroga et al., 2019b, 2019a), and it may suggest a decrease in the activity of these proteins when the mycorrhizal fungus is present in the roots.

Although the external application of IAA was enough for affecting root hydraulic conductivity levels, it is possible that its concentration was not enough to alter internal plant contents in all tested tissues. Indeed, despite the application of IAA, the levels of this hormone enhanced only in roots of AM plants, especially under drought stress conditions and in leaves of non-AM plants under well-watered conditions (Figure 4A, B). In fact,

495 during drought, root IAA levels in AM plants were generally much higher in all treatments,
496 compared to the other plants. Elevated IAA levels in AM roots were also observed in
497 trifoliate orange during drought stress, and they were related to greater root-hair growth,
498 enhancing drought tolerance (Liu et al., 2018). In the case of leaves, the increase of IAA
499 levels was only observed in non-AM plants during drought stress (Figure 4B). Curiously,
500 although IAA sap levels were not significantly affected by the chemical treatments,
501 isopentenyl-adenine (iP, a naturally occurring cytokinin) sap levels were highly diminished
502 with IAA treatment in AM plants under DS conditions (Figure 3E). This can be easily
503 understood, due to the well-known auxin/cytokinin antagonism (Moubayidin et al., 2009;
504 Rowe et al., 2016).

505 Root ABA concentration was increased in AM plants, regardless of the water
506 treatment (Figure 4C). This is not surprising, as ABA is necessary for a proper AM
507 symbiosis establishment and functioning, being related to arbuscule formation (Herrera-
508 Medina et al., 2007; Martín-Rodríguez et al., 2011; Pozo et al., 2015). Moreover, it was
509 shown that AM fungi could enhance endogenous ABA levels during plant colonization and
510 during drought stress (Ludwig-Müller, 2010; Ruiz-Lozano et al., 2016). Therefore, it was
511 suggested that increased ABA levels in stressed AM plants would serve not only to
512 promote tolerance against stresses in these plants but also to enhance and maintain the
513 symbiosis (Ruiz-Lozano et al., 2016). The same pattern of accumulation occurred for SA
514 levels in roots, being increased by the fungal presence (Figure 4C). SA has been
515 suggested to play a role in AM colonization, although mainly at early stages of the
516 symbiosis (Foo et al., 2013). Besides the relation of these hormones with the own AM
517 symbiosis, ABA is the so called “stress hormone” and regulates a wide number of plant
518 physiological processes in response to drought stress. ABA signalling involves
519 PYR/PYL/RCAR ABA receptors and the phosphatase/kinase enzyme pairs PP2Cs and

SnRK2s, having opposite functions. In the end, several transcription factors under the control of SnRK2s activate the ABA dependent gene expression, which lead to drought responses (Kim, 2014). Moreover, it has been shown that ABA usually increases Lpr values under drought stress (Mahdieh and Mostajeran 2009; Kudoyarova et al. 2011) and combined effects of ABA and mycorrhizal symbioses on root water transport capacity and aquaporins regulation have been reported (Aroca et al. 2008; Ruiz-Lozano et al. 2009; Calvo-Polanco et al., 2019).

It is noteworthy, that plant hormones act in synchrony with each other, and an intricate crosstalk is established in order to regulate plant physiology and development (Munné-Bosch and Müller, 2013). During this interaction, hormones can mutually influence their endogenous contents. For instance, it is known that under osmotic stress conditions, ABA regulates important plant processes such as root growth through the interaction with auxins, cytokinin and ethylene (Rowe et al., 2016). In our study, this fact is suggested through the strong correlations among root and sap IAA and root ABA, SA and JA-Ile levels during drought stress (Table 2; Figure 6). In a recent study, ABA and SA alleviated drought stress through the maintenance of membrane stability and leaf water status, and had effects on common metabolic pathways (Li et al., 2017). The fact that these hormones were only correlated during water deficit suggests a tight hormonal control of plant physiology under these conditions, and highlights their mutual relationship.

Conclusions

Here, the effect of IAA on the regulation of root radial water transport of AM plants during well-watered and drought conditions was analyzed. The alteration of SA, ABA and JA-Ile levels by the IAA application under drought confirms that water transport in roots is regulated by the combined action of different hormones.

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Interestingly, under water deficit conditions the percentage of relative apoplastic water flow was differentially regulated in AM and non-AM plants by IAA application, which is in line with previous studies. The exogenous IAA application affected root hydraulic parameters, mainly Lo, during water deficit conditions, which was decreased in both AM and non-AM plants. This effect of IAA on the internal cell component of root water conductivity (Lo) suggests that aquaporins are involved in the IAA-dependent inhibition of this internal cell pathway, although this was not reflected at protein levels for the analyzed antibodies, and further studies are needed to confirm this hypothesis.

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Table 1: Plant dry weight (DW), electrolyte leakage (EL), stomatal conductance (gs) and photosystem II efficiency in the light-adapted state (∆Fv/Fm') in maize plants inoculated or not with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS).

	Plant DW (g plant^{-1})	**EL (%)**	**gs (mmol H_2O m^{-2} s^{-1})**	**∆Fv/Fm'**
WW non-AM	**14.3 ± 0.51 b**	**10.5 ± 1.12 b**	**210.5 ± 21.4 a**	**0.61 ± 0.01 a**
WW AM	**18.6 ± 0.95 a**	**8.1 ± 0.68 b**	**112.3 ± 9.39 b**	**0.64 ± 0.01 a**
DS non-AM	**9.15 ± 0.22 c**	**17.7 ± 2.33 a**	**115.3 ± 10.8 b**	**0.55 ± 0.02 b**
DS AM	**10.7 ± 0.27 c**	**6.43 ± 0.23 b**	**199.0 ± 15.8 a**	**0.52 ± 0.01 c**

Data represents the means of thirty values ± SE for plant DW or twelve values ± SE for EL, gs and ∆Fv/Fm'. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test.

Table 2. Pearson correlation coefficients between hydrostatic root hydraulic conductivity (Lpr), osmotic root hydraulic conductivity (Lo), relative apoplastic water flow, sap and root IAA concentrations and the measured sap and root hormones, root aquaporin abundance in well-watered and drought stressed plants (n = 5). * Significant at p < 0.05; ** Significant at p < 0.01; *** Significant at p < 0.001.

		Well-watered					Drought				
		Lpr	Lo	% Apoplastic water flow	Sap IAA	Root IAA	Lpr	Lo	% Apoplastic water flow	Sap IAA	Root IAA
Sap hormones	IAA	-0.757	-0.171	**-0,854***		0.499	-0.451	-0.061	0.477		**-0.841***
	ABA	-0.525	0.047	**-0.860***	0.795	0.502	-0.293	**0.810***	-0.173	-0.232	0.290
	SA	-0.381	-0.010	-0.687	0.556	-0.106	0.014	-0.644	0.318	0.722	-0.577
	JA	-0.609	-0.332	**-0.846***	**0.915***	0.324	0.661	0.235	-0.278	**-0.906****	0.758
	iP	0.408	-0.509	-0.523	0.235	-0.529	-0.706	0.752	0.141	0.184	-0.387
Root hormones	IAA	**-0.825***	0.301	-0.194	0.499		0.377	-0.123	-0.675	**-0.841***	
	ABA	-0.313	0.117	-0.152	0.195	0.638	0.145	-0.011	-0.689	**-0.854***	**0.943***
	SA	-0.609	0.077	-0.602	0.634	0.651	0.440	-0.074	-0.598	**-0.835***	**0.993*****
	JA	0..321	0.100	0.032	-0.130	-0.458	-0.267	-0.601	-0.031	0.052	-0.229
	Ja-Ile	-0.530	0.186	**-0.929****	**0.861***	0.396	0.384	0.319	-0.394	**-0.838***	**0.869***
Leaf hormones	IAA	-0.669	-0.023	-0.165	0.550	**0.802***	0.085	-0.649	0.604	0.526	-0.506
	ABA	0.274	0.743	0.231	-0.178	-0.383	0.108	**0.922****	0.365	-0.068	-0.148
	SA	0.078	-0.110	0.142	-0.125	-0.540	0.269	-0.466	-0.469	-0.021	0.216
	JA	0.791	0.041	0.283	-0.520	-0.550	-0.267	-0.605	0.601	0.040	-0.192
	JA-Ile	0.093	-0.078	-0.591	0.447	-0.065	0.278	-0.195	**-0.824***	-0.583	0.767
Root protein abundance	PIP1	0.531	0.309	0.206	-0.384	-0.169	0.588	-0.116	-0.321	**-0.965****	0.792
	PIP2	0.766	0.453	0.465	-0.784	-0.552	0.026	0.187	0.759	0.286	-0.743
	PIP2A	0.598	0.396	0.557	-0.768	-0.600	-0.209	-0.569	0.088	0.230	-0.450
	PIP2B	0.557	0.143	0.461	-0.572	-0.616	-0.345	-0.422	0.478	**0.832***	**-0.834***
	PIP2C	0.632	0.236	0.472	-0.635	-0.589	-0.626	-0.008	0.356	0.788	**-0.916***
Root hydraulic parameters	Lpr		-0.135	0.433	-0.757	**-0.825***		-0.243	0.259	-0.451	0.377
	Lo	-0.135		-0.032	-0.171	0.301	-0.243		0.126	-0.061	-0.123
	% Apoplastic water flow	0.433	-0.032		**-0.854***	-0.194	0.259	0.126		0.477	-0.675

Figure 1.

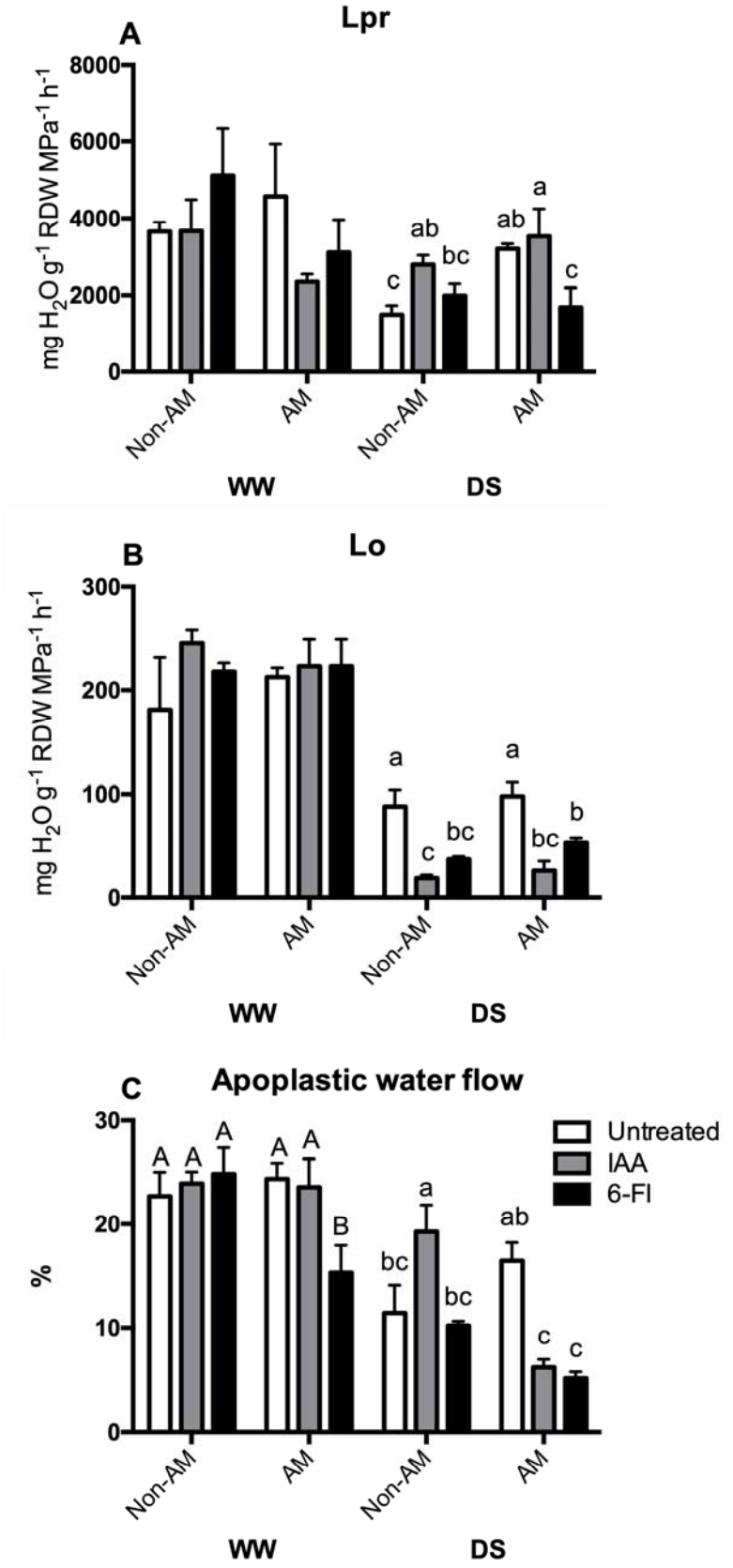
A
Lpr
mg H_2O g^{-1} RDW MPa^{-1} h^{-1}
8000
6000
4000
2000
0
c
ab
bc
ab
a
c
Non-AM
AM
Non-AM
AM
WW
DS
B
Lo
mg H_2O g^{-1} RDW MPa^{-1} h^{-1}
300
200
100
0
a
c
bc
a
bc
b
Non-AM
AM
Non-AM
AM
WW
DS
C
Apoplastic water flow
%
30
20
10
0
A
A
A
A
A
B
bc
a
bc
ab
c
c
Untreated
IAA
6-FI
Non-AM
AM
Non-AM
AM
WW
DS

Figure 1. (A) Hydrostatic root hydraulic conductivity (Lpr), (B) osmotic root hydraulic conductivity (Lo) and (C) relative apoplastic water flow in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences between treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Figure 2

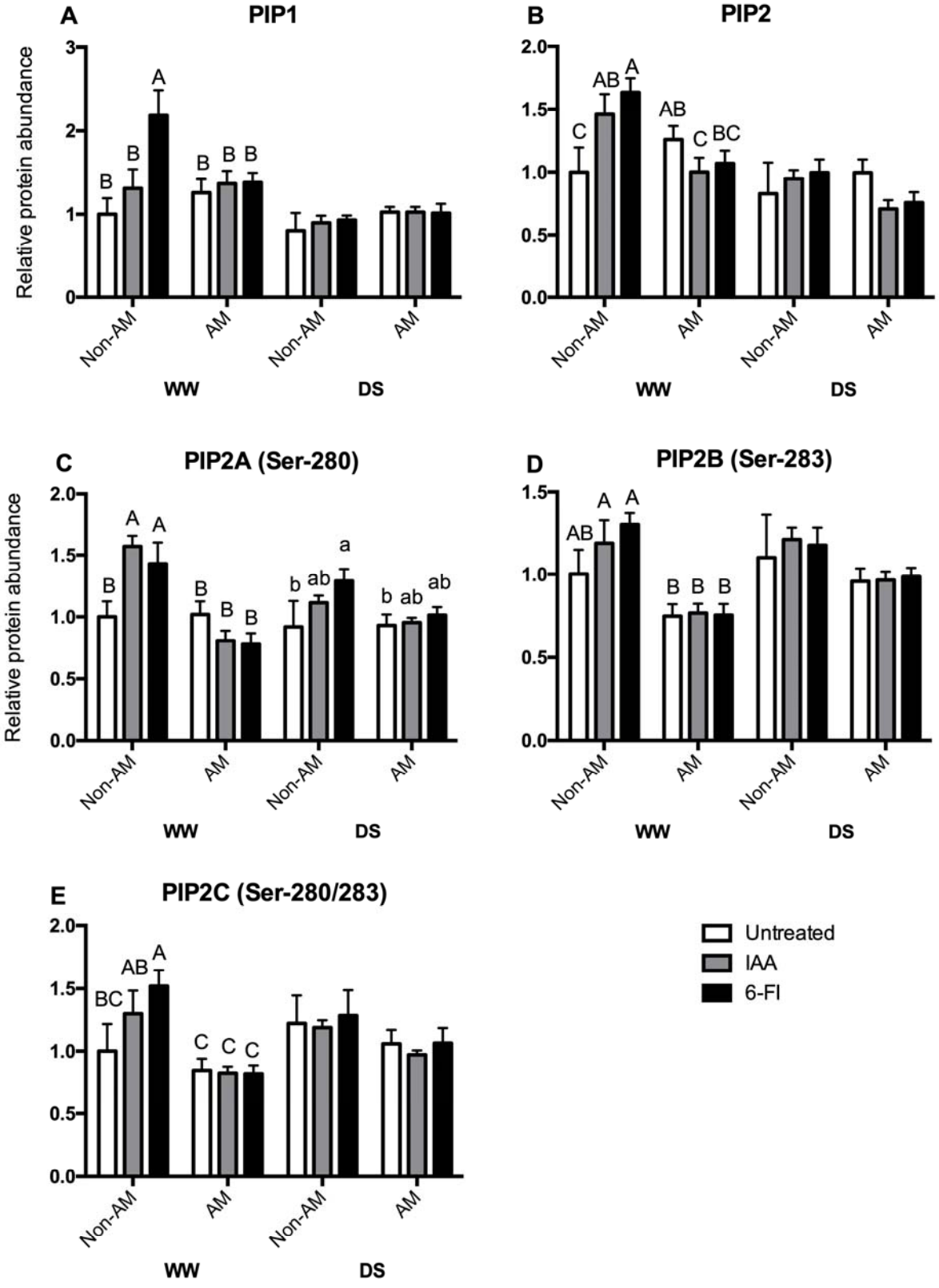
A
PIP1
Relative protein abundance
B
PIP2
C
PIP2A (Ser-280)
Relative protein abundance
D
PIP2B (Ser-283)
E
PIP2C (Ser-280/283)
Non-AM
AM
WW
DS
Untreated
IAA
6-FI

Figure 2. (A) PIP1, (B) PIP2, (C) PIP2A, (D) PIP2B, (E) PIP2C relative protein abundance in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Figure 3

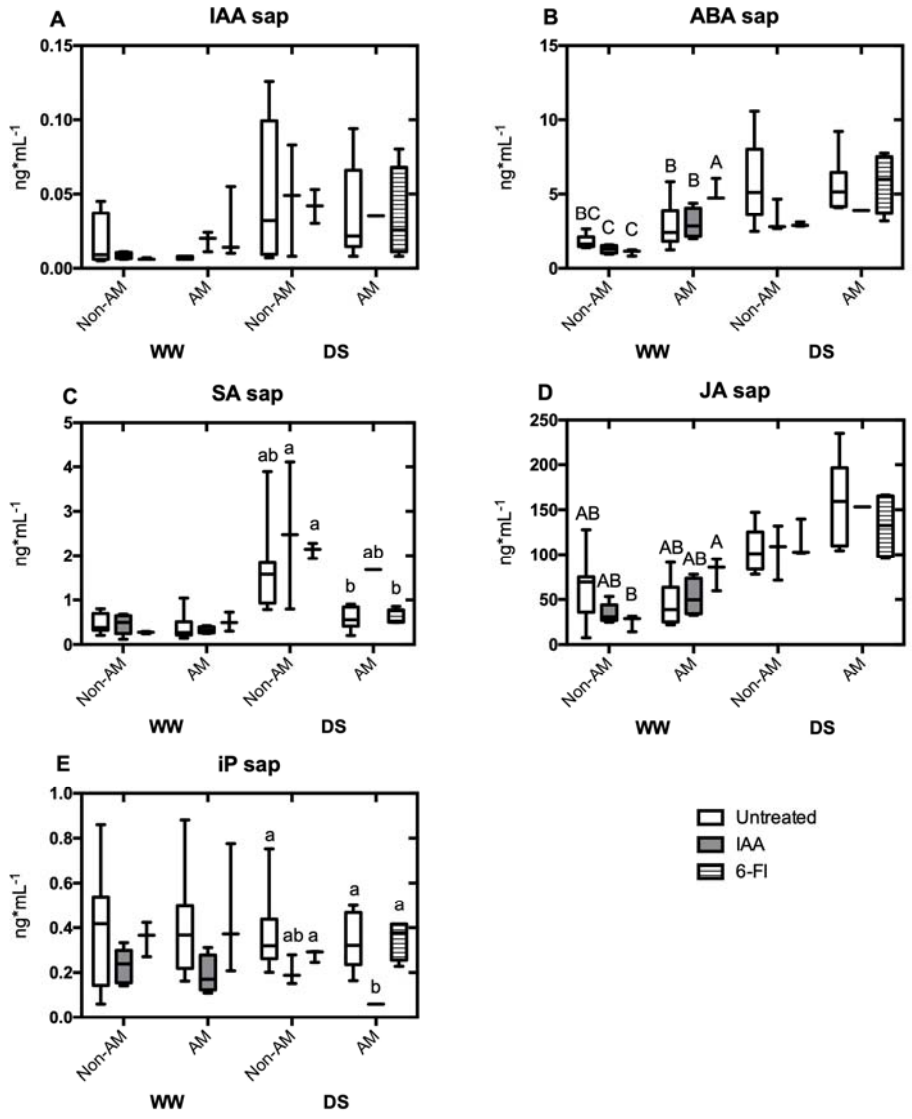

Figure 3. (A to E) Boxplots representing the sap concentrations of IAA, ABA, SA, JA and iP in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test.

Figure 4

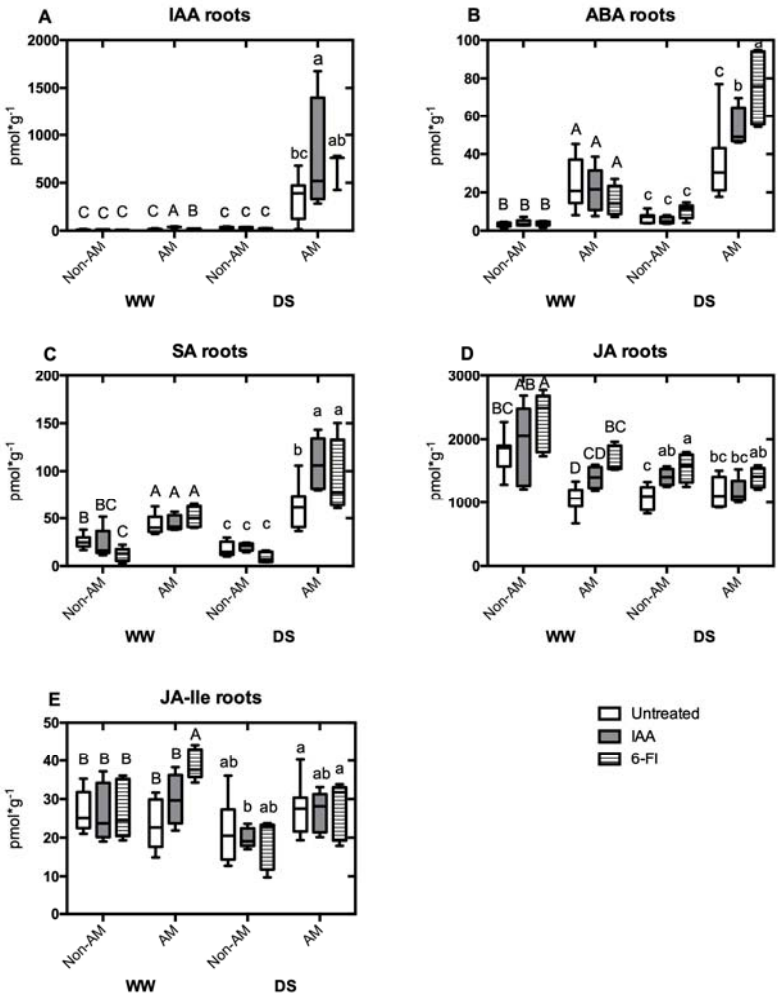

Figure 4. (A to E) Boxplots representing root concentrations of IAA, ABA, SA, JA and JA-Ile in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Figure 5

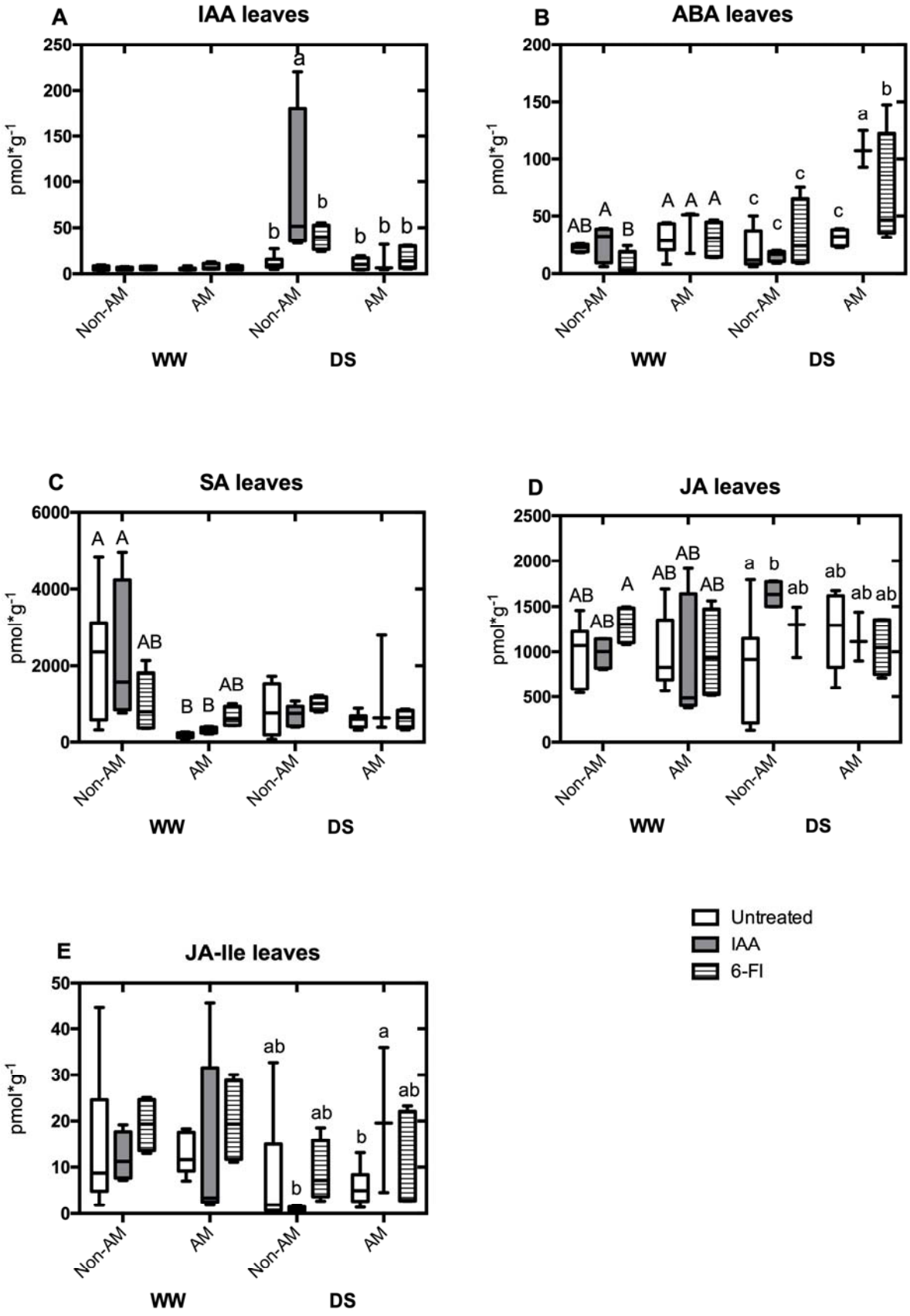
A
IAA leaves
pmol*g-1
250
200
150
100
50
0
a
b
b
b
b
b
Non-AM
AM
Non-AM
AM
WW
DS
B
ABA leaves
pmol*g-1
200
150
100
50
0
AB
A
B
A
A
A
c
c
c
c
a
b
Non-AM
AM
Non-AM
AM
WW
DS
C
SA leaves
pmol*g-1
6000
4000
2000
0
A
A
AB
B
B
AB
Non-AM
AM
Non-AM
AM
WW
DS
D
JA leaves
pmol*g-1
2500
2000
1500
1000
500
0
AB
AB
A
AB
AB
AB
a
b
ab
ab
ab
ab
Non-AM
AM
Non-AM
AM
WW
DS
Untreated
IAA
6-FI
E
JA-Ile leaves
pmol*g-1
50
40
30
20
10
0
ab
b
ab
b
a
ab
Non-AM
AM
Non-AM
AM
WW
DS

Figure 5. (A to E) Boxplots representing leaf concentrations of IAA, ABA, SA, JA and JA-Ile in maize plants inoculated (AM) or not (Non-AM) with the AM fungus *Rhizophagus irregularis* and submitted to two water regimes (well-watered, WW or drought stress, DS). Plants remained untreated or received exogenous IAA or 6-Fluoroindole (6-FI) as an inhibitor of IAA biosynthesis. Data represents the means of five values ± SE. Different letter indicates significant differences among treatments ($p < 0.05$) based on Duncan's test for well-watered (uppercase) and drought stressed (lowercase) plants. The absence of letters indicates that no significant differences among treatments were found.

Figure 6

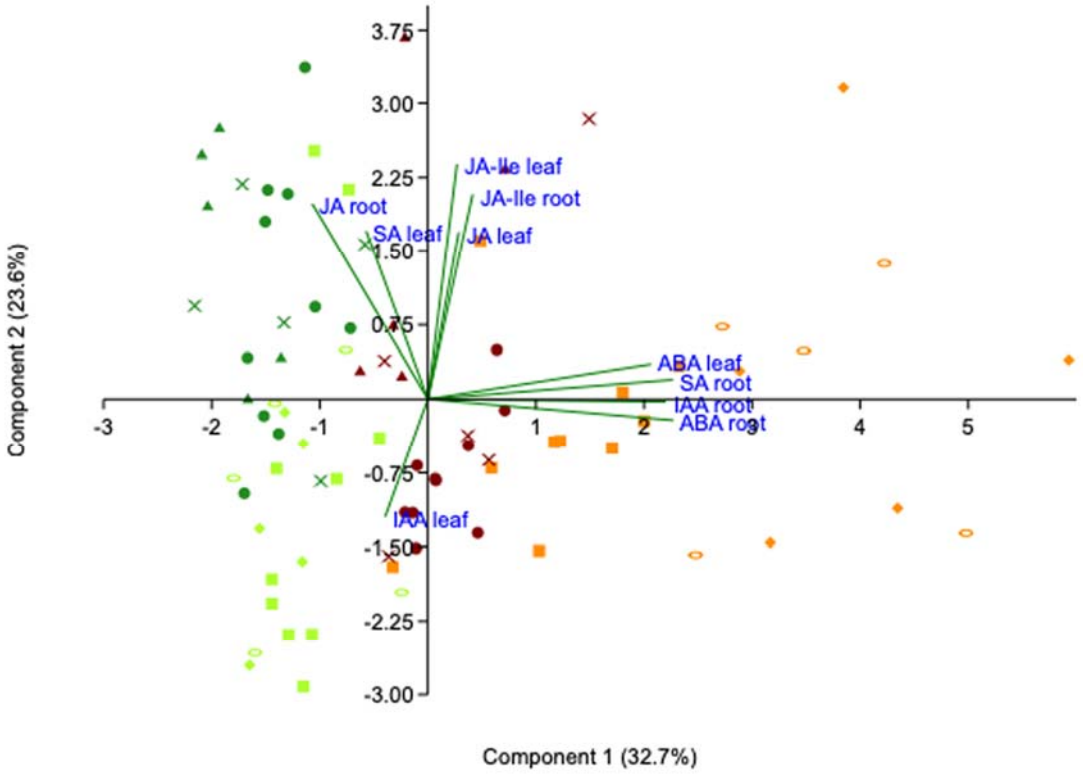

Figure 6. Principal components analysis (PCA) for tissue hormonal content data presented in Figures 4 and 5. Plot with data run in PAST software, showing the two principal components (PC1 and PC2) which separated the samples by inoculation treatment. Black dots represent non-AM plants under WW, black-filled squares represent non-AM plants under DS, circles represent AM plants under WW and unfilled squares represent AM plants under DS.