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Response of stomatal conductance and xylem sap abscisic acid concentration of *Sorghum bicolor* (L.) Moench cv. Tegemeo under re-watered and drought-stressed conditions

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Abstract

The sorghum plant grows in a wide range of soils and environments, and its agronomic and economic importance worldwide is incrementing yearly. This crop has several traits that make it a model for research for the study of C4 species and stress tolerance. In this research, three methods for creating a controlled environment to grow the sorghum plant using diverse substrates and nutrient solution combinations were tested. After trials, the method that was composed of sand and nutrient solution by infertile sandy soils was effective and sorghum plants were significantly bigger in height, and heavier in fresh and dry weight compared to those that were cultivated by other two methodologies. Using this artificial susbtrate, sorghum plants were grown-up and then were subjected to a drought conditions. Following the drought-stressed period, the stomatal conductance was significantly reduced and xylem sap abscisic acid concentration significantly increased by about 64% in plants developed in both substrates. This experimental system can be used for future research contributing to sorghum study of mechanisms in overcoming abiotic constrains such as drought and water relations and possible adaptation to tropical and subtropical climates.

Sorghum, Drought, ABA Xylem Sap, Stomatal Conductance

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1. Introduction

Sorghum is the fifth most important cereal in the world, and its relevance is increasing because of its low demand for water, coupled with its potential adaptation to global climate change (Tsuboi, 2017). Furthermore, sorghum is categorized as a competitive coarse grain in the world markets and as an important food supply to overcome the accelerated expansion of human population (Thomason *et al*., 2017). In the same line, sorghum planted area worldwide has been increased by 66% over the past 50 years, and the yield has improved by 2.4 fold (Qi *et al*., 2016).

This cereal is cultivated in at least 73 countries throughout Africa, America and Asia in tropical and subtropical regions (Upadhyaya *et al*., 2016). In the semiarid tropics of undeveloped countries with low income human populations, sorghum remains the primary source for human nutrition.

Countries such as the United States, Argentina, and Australia have been the major sorghum producers for primarily commercial use in livestock feed, bioenergy, and other non-food and industrial uses (Qi, 2016, O'Brien, 2016). Recently, sorghum has been useful for phytoremediation in poluted lands, whereby soil contaminants (usually heavy metals) are taken up by the plant and removed from the environment (Tsoboi, 2017).

Sorghum plants possess inherent abilities to survive and exceed under extreme temperatures and drought conditions, and it has also gained interest because contains most of the traits in a model plant species such as large embryos that are easy to rescue, plenty of seeds production, moderate genome size and available whole-genome sequences (Paterson *et al*., 2009; Calviño and Messing, 2012; Rizal *et al*., 2014).

Sorghum also has a high photosynthetic efficiency and use of water due to the effective C4 carboxylation pathway (Nielsen and Vigil, 2017) and harbors genes for higher biomass and other yield-related traits.

In spite of being one of the major crops in the world, the use of sorghum in scientific research lags far behind other cereals (Calviño and Messing, 2012). Moreover, the outcomes of sorghum research have not been used widely in crop improvement compared with other cereals like maize and rice (Izawa and Shimamoto, 1996; Rensink and Buell, 2004).

As a consequene, in order to promote sorghum as a model species it is necessary to get a better knowledge about sorghum responsivness to water deficit and drought starting with simple experimental model designs, and then scaling up to bigger trials to understand its adaptation under severe environmental conditions (Calviño and Messing, 2012; Neri-Luna *et al*., 2016; Chen *et al*., 2017).

Despite the known resilience of sorghum to drought stress, little is known about the responses to water deficit and its drought tolerance, specifically, the role of non-hydraulic root-to-shoot signalling (*i.e*. changes in concentration and flux rate of abscisic acid, ABA) considered significant in regulating shoot growth and water use when soil is drying, without any demonstrable change in shoot water or nutrient status (Hansen and Dörffling, 2003).

The xylem sap ABA concentration has a distinctive role in stomatal regulation (which is the main mechanism used by plants to control gas exchange and transpiration) of hydric status in water stress conditions (Dodd, 2003; Pospíšilová, 2003).

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The aims of this research are: (i) to compare three different methods for the establishment and growth of sorghum plants under controlled conditions in order to select the most suitable; (ii) to provide the optimum approach in order to evaluate the stomata responses to changes in the concentration of ABA in the xylem sap under a drought-stressed and re-watered period.

2. Materials and Methods

2.1. Experimental set up

In this research three different artificial shorgum culture methods suggested by Brundrett *et al*. (1996), INVAM (1997), YMRG (1996) were tested. Plastic pots (2 L, 15cm x 15cm x 12cm) were thoroughly washed with water, and disinfected with 1% (w/v) Virkon solution (Antec International Limited) and rinsed with destillated water before use (Vimard *et al*., 1999).

For each method, the pots (n= 7) were filled with the adequate substrate and the containers were watered to field capacity according to the appropriate nutrient solution (Table 1).

Sorghum bicolor (L.) Moench cv. Tegemeo seeds were washed with running tap water, and surface sterilized with 10% NaOCl for 20 min (Jarstfer and Sylvia, 1993) followed by a final rinse with running distilled water. The seeds were placed in a baker with autoclaved distilled water for 16 h in darkness to imbibe (Maiti, 1996).

Method	Substrate	[1]Particle size /disinfection	Nutrient Solution
(a) Brundrett *et al*. (1996)	Silica sand	(0.50-0.78 mm) autoclaved twice for 1 h at 121°C.	**Snowball and Robson (g/L):** (26) KH_2PO_4, (43) K_2SO_4, (21) NH_4NO_3/2 weeks, (43) $CaCl_2 2H_2O$, (6.4) $MgSO_4 7H_2O$, (4.3) $MnSO_4 H_2O$, (3) $ZnSO_4$ $7H_2O$, (1.3) $CuSO_4 5H_2O$, (0.2) H_3BO_3, (0.1) $CoSO_4 7H_2O$, (0.05) $Na_2MoO_4 2H_2O$. Plants watered twice a week with nutrient solution
(b) INVAM (1997)	Silica sand: loamy soil (2:1 v/v).	Soil (1-0.5 mm) steamed twice for 1 h at 85°C. Sand (0.50-0.78 mm) autoclaved twice for 1 h at 121°C.	**Long Ashton (g/L):** (50.6) KNO_3, (80.25) $Ca(NO_3)_2 4H_2O$, (52) $NaH_2PO_4 2H_2O$, (46) $MgSO_4 7H_2O$, (6.7) FeNaEDTA Plants watered twice a week with 50% full strength of nutrient solution
(c) YMRG (2001)	Silica sand: calcined attapulgite clay soil conditioner (50:50) + bone meal	Sand:calcined attapulgite clay soil conditioner (0.50-1.00 mm) autoclaved twice for 1 h at 121°C.	**Rorison (g/500 mL):** (62.01) $MgSO_4 7H_2O$, (119.02) $Ca(NO_3)_2 4H_2O$, (57.69) $K_2HPO_4 3H_2O$, Trace elements: (6.250) FeEDTA, (0.560) $MnSO_4 H_2O$, (0.716) H_3BO_3, (0.046) $(NH_4)_6Mo_{24} 4H_2O$, (0.110) $ZnSO_4$ $7H_2O$, (0.099) $CuSO_4 5H_2O$ Plants watered twice a week with half to one fifth strencgth of nutrient solution

[1]Gaur and Adholeya (2000).

Table 1 The three tested methods for shorgum growth under greenhouse conditions controlled conditions

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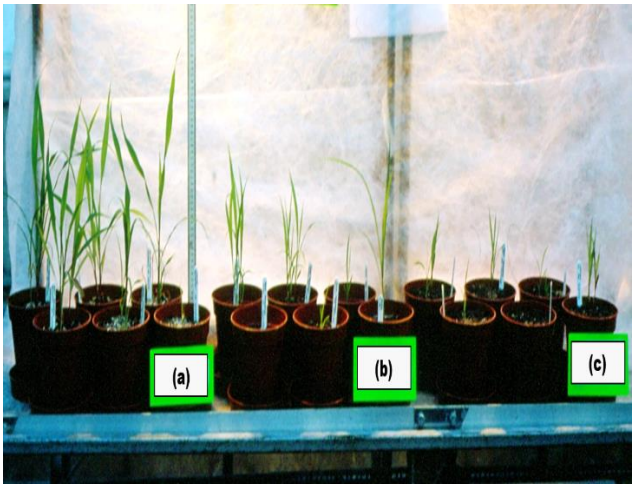

Figure 1 Experimental set up with sorghum seedlings growing with three differents methods under greenhouse controlled conditions. (a) Brundrett et al. (1996). (b) INVAM (1997). (c) YMRG (2001).

After imbibition, the sorghum seeds were distributed on a damp layer of tissue paper and placed in an envelope of aluminium foil and stored in a dark cupboard for 48 h to allow germination (Mace, 1999). After germination 5 sorghum seeds with a uniform radicle were planted in each container and covered with the substrate.

Finally, the planted pots were placed on a greenhouse bench and randomly arranged every week (Figure 1). After 2 weeks, the sorghum seedlings were thinned to 1 plant per container and received the nutrient solution and water appropriate to the corresponding treatment. Sorghum plants were cultivated in the greenhouse under controlled conditions with a photoperiod of 16h light/8h dark and a Photosynthetic Active Radiation (PAR) of 300-800 μmol m^{-2} s^{-1}. The temperature ranged from 16°C to 26°C with a relative humidity (rh) between 45-70%.

A destructive harvest was performed at 9 weeks after planting. First, the height of each plant was recorded, and the shoot components (leaves, stem, and ligules) were separated and the fresh weight (FW) was recorded.

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The plant samples were placed in paper bags, and dried at 60°C for 48 h and allowed to cool in a perspex single door desiccator chamber with silica gel and subsequently the dry weight (DW) was recorded using a Mettler AJ100 analytical balance. Statistical analyses were carried out using SPSS® v24 package.

All data was verified for normality using a Kolmogorov-Smirnov Test and for homogeneity of variances using the Levene Test. Plant parameters at harvest were compared by One-way ANOVA (Dytham, 2011). Based on these results, the following experiment was designed in order to test the sorghum response to stomatal conductance and variations in xylem sap abscisic acid concentration ([ABA_{xyl}]).

2. Stomatal conductance measurements and xylem sap collection

PVC pipe cylinders (10.5 cm diameter X 18cm height) designed to fit inside a pressure chamber were disinfected as mentioned before. Each cylinder was sealed at the base with three layers of plant fleece attached with waterproof tape. The cylinders (n=8) were packed with sand (S) substrate and watered with the nutrient solution proposed by Snowball and Robson (1984) and using the best sorghum growing system in previous experiment (Brundrett, 1996). In order to validate this artificial system, cylinders (n=8) were packed with natural field soil (NS) for comparative purposes. The natural soil is a sandy clay loam with a pH 5.9 in H_2O and 5.5 in $CaCl_2$, CEC 2.4 cmol kg^{-1}, base saturation 88.4, texture loamy sand (sand 73.9%, silt 20%, clay 6.1%), organic matter 6.4%, C_{org}3.5% and N_{org} 449.6 mgN/100g.

Sorghum seeds were washed, surface sterilized and germinated as explained before. Three germinated seeds were planted in the middle of each cylinder and the planted cylinders were placed on a greenhouse bench and randomly re-arranged every week.

After emergence, the seedlings were thinned to one per cylinder. Plants cultivated in S were watered twice per week with 120 ml of the nutrient solution (Snowball and Robson, 1984) and with deionised water at intervals (twice a week) of nutrient additions. The plants growing in NS were watered with tap water as required. They were kept in the greenhouse under the following environmental conditions: $T°C_{min}$ 23°C/$T°C_{max}$ 30°C, 16-48% rh and a mean PAR of 200 μmol m^{-2} s^{-1} at plant level for 14 h d^{-1}.

Drought period: Eight weeks after sowing, the water was withheld from half of the plants (n=4 in each substrate (S or NS). Firstly, the planted cylinders were watered to field capacity and allowed to drain overnight. In the morning, the sorghum plants were re-watered and allowed to drain until they stopped dripping, then the drought-stressed period was induced.

The initial weight of each cylinder was recorded using a digital balance (Ohaus, 5 kg capacity). This step was repeated every 4 h throughly, until the plants stopped losing weight, and at this point they were moved to the lab for xylem sap collection.

Throughout the drought-stressed period, the stomatal conductance (gs) was recorded several times using a Diffusion Porometer (AP4, ©Delta-T Devices, Cambridge, UK). Finally, the control plants (n=4) for each susbtrate, were watered as required.

Xylem sap was collected based on the method described by Seel and Jeschke (1999). The plants were sealed in the special lid of the pressure vessel (Figure 2) with the two-component silicon–based dental impression material Blend-a-gum (Coltène® PRESIDENT fast microsystem™ regular body).

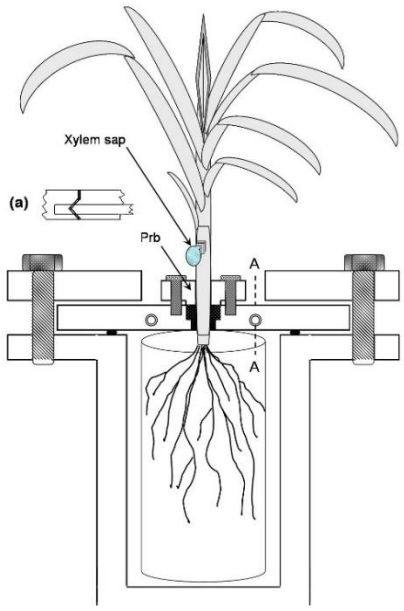

Figure 2 Schematic representation of the pressure chamber. Inset (a) shows a section through the lid along the line A-A with the V-shaped joint sealed with high vacuum grease. Sealing of the stems within the lid also is achieved by silicon-based dental impression material which is, after sealing, compressed by the pressurizing blocks (Prb)

A segment of 1 cm was cut out in the stem base with the lower cut sloping downwards at 60° to the centre to retain exuding sap.

After sealing the plants into the pressure vessel, the pneumatic pressure was raised gradually until the first drops of xylem liquid appeared; this balancing pressure *sensu* Passioura and Munns (1984) was recorded. Subsecuently, the xylem sap flow was induced by increasing the pressure to 100 kPa. Xylem sap samples (*c*. 500 μL) were collected and stored in the dark at -80°C for further analysis.

The ABA concentration was analysed in crude samples of xylem sap by the radioimmunoassay technique described by Quarrie *et al*. (1988).

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After thawing, 200 μL 50% PBS were put into 2 mL plastic centrifuge tubes. Subsequently 50 μL of ABA standard or xylem sap sample was added carefully to each tube. This solution was mixed with 100 μL of ^{3}H-ABA in γ-globulin solution (ca 9,000-10,000 cpm per vial) and 100 μL MAC252 antibody solution. The solution was mixed briefly by inverting tubes and then spun down in a microcentrifuge (Centurion 8080, Norlab) for 30s.

The assay mixture was incubated at 4°C in darkness for 45 min to allow binding to take place. Subsecuently, a saturated solution (500 μL) of $(NH_4)_2SO_4$ was added to each tube in order to precipitate the ABA-antibody complex and, after a brief shaking, the mixture was left in the dark at room temperature for 30 min. Free antibodies and those that bound to ABA in the reaction mixture were precipitated and pelleted by centrifugation for 4 min at 1400g, then the supernatant was discarded.

The pellet was washed by suspending in 1 mL of 50% saturated $(NH_4)_2SO_4$ solution in order to remove the excess unbound radioactivity (the total pellet was re-suspended by shaking the rack of vials). The tubes were centrifuged again for 4 min at 1400g and the supernatant was discarded carefully and the pellet resuspended in 100 μL of deionized water.

The tubes were again spun for 30 s and 1.25 mL of scintillation cocktail (Ecoscint-H) was added to all tubes and the content was mixed thoroughly. Finally, the tubes were put inside the glass scintillation vials and the radioactivity was quantified in a liquid-scintillation counter (Packard 4430, Berlshire, England), the tubes where count up (on protocol 8), three times for 5 min on ^{3}H setting.

Concentrations of ABA were calculated from the radioactivity (cmp) present in the pellets. A series of ABA standards was included in each batch of assays in order to construct a calibration curve. This was usually linearised by subtraction of B_{min} and plotting log-transformed counts against the natural logarithm (ln) of the unlabelled ABA present per vial, where:

$$\text{Log}\,(B/B_{max}) = \ln [B/B_{max} / 1-(B/B_{max})] \quad (1)$$

B= Corrected cpm bound in the presence of an ABA standard = (cpm-B_{min})
B_{max}= The maximal bound radioactivity when only ^{3}H-ABA reacted with MAC252 (H_2O standards).
B_{min}= The minimal bound radioactivity when a large excess of non-labelled ABA was added to the vial.

Sample ABA concentrations were calculated from this formula and the final result was expressed in pmol per mL. Statistical analyses were carried out using SPSS® v24 package. All data were verified for normality using a Kolmogorov-Smirnov Test and for homogeneity of variances using the Levene Test. An independent sample t-Test was performed in order to compare means between watered *vs.* droghted plants treatments (Dytham, 2011).

3. Results

3.1. A comparison of methods using artificial substrates

The effect of different non-natural substrates and diverse nutrient solutions on sorghum´s development was variable. For instance, sorghum plants grown using Brundrett (1996) method, were significantly bigger in height, and heavier in fresh and the dry weight compared to those that were cultivated by other methods (Table 2). It is important to pointed out that the sorghum germination process was not affected in any of the three methods used (P=0.319).

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3.2. Response of stomatal conductance and changes of [ABA_{xyl}]

The main reduction in stomatal conductance (gs) of sorghum leaves was at 53 h and 99 h after the water was withheld for plants cultivated in S and NS substrates respectively (Figure 3a). It appears that when stomatal closure (within the first 100 h after the water was suspended) the weight loss from containers stopped (Figure 3b).

The combination of stomatal conductance measurements and weight loss data clearly indicated that plants were suffering water stress. Daily weather conditions in the greenhouse during the drought-stressed period were: mean PAR of 152±8 μmol m^{-2} s^{-1} at plant height, $T°C_{min}$ 23.5°C/ $T°C_{max}$ 31°C, 16-39% rh and vapor pressure deficit between 1.7-3.4 kPa.

The mean balancing pressure (ρ_o) needed to obtain xylem sap from the stems of sorghum plants under re-watered conditions was ~150±0 kPa in both S and NS substrates. The sorghum plants subjected to a drought-stressed period needed higher balancing pressure were before xylem fluid appeared. For instance, ~525±43 kPa in those plants grown-up in sand and ~300±0 kPa in plants cultivated in NS were needed.

Following the drought-stressed period, the gs was significantly (P=0.006) reduced and [ABA_{xyl}] significantly increased (P=0.003) by about 64% in plants developed in both substrates (Figure 3c-d). During the first 8 h after withholding water, the sand substrate allowed more water loss (P<0.05) than the NS substrate, and were not observed significant differences until 76 h, when the plants developed in NS substrate lost more water.

By the end of drought-stressed period, no significant differences were found (P>0.05). It appears that sand substrate permitted more rapid gs reduction than NS, but the differences were not significant (P>0.05).

4. Discussion

Sorghum can be used as a model system for research because of its: (1) agronomic importance, (a) C4 photosynthesis, (b) low input levels of nutrients, (c) adaptation to stresses due to its reservoir of genes for tolerance to high salt, pH, drought and heat; (2) increase of its economic significance in areas as a valuable source of food, biofuel and phytoremediation (Tsuboi *et al.*, 2017); (3) representative of large genomes like sugarcane and switchgrass (Paterson *et al*., 2009); (4) ease to produce different types of breeding materials (Upadhyaya *et al*., 2016); (5) the big plant size

Plant parameters	Brundrett *et al.*	INVAM	UYRG	P
Germination (%)	77.7 ±7.02 a	49.9±16.6 a	55.5±14.0 a	NS
Height (cm^{-1})	32.19 ±3.52 b	30.2±3.55 b	18.57±2.13 a	**
Leaves				
FW (g)	1.55±0.41 b	0.50±0.08 a	0.31±0.10 a	**
DW (g)	0.44±0.09 b	0.14±0.02 a	0.07±0.01 a	**
Stem				
FW (g)	0.85±0.25 b	0.23±0.04 a	0.14±0.06 a	*
DW (g)	0.19±0.04 b	0.05±0.009 a	0.02±0.007 a	***
Total				
FW	2.41±0.67 b	0.74±0.12 a	0.46±0.16 a	**
DW	0.63±0.13 b	0.19±0.02 a	0.10±0.02 a	***

(§) Plants were harvested 9 weeks after plantation. Means ±1SE (n=7). Significance values were calculated using One-Way ANOVA at 95% CI and Student-Newman-Keuls Test. *P≤0.05; **P≤0.01; ***P≤0.001, NS= Not significant. **FW**= Fresh Weight; **DW**= Dry Weight.

Table 2 Parameters of sorghum plants (§) using three different techniques

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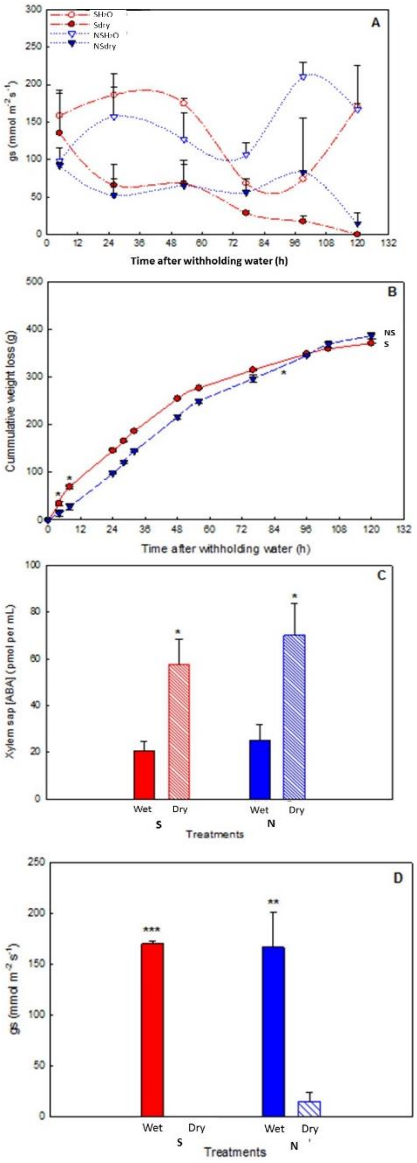

Figure 3 Response of sorghum plants under re-watered and drought-stressed conditions grown in S and NS substrates. (A) gs in leaves. (B) Cumulative weight loss. (C) Xylem sap ABA concentration. (D) gs in leaves before harvest (gs in drought-stressed plants cultivated in sand was zero). Data represent ±1SE of the mean (n=4). Significance values were calculated using a t-Test. *P≤0.05; **P≤0.01; ***P≤0.001.

With wide leaves, make it suitable for physiological measurements, leaf anatomical analysis, growth and development studies (Roozeboom and Prasad, 2016).

However, despite all this, other crop plants like maize and rice have still remained considered the primarly models for the study of cereals and C4 species (Izawa and Shimamoto, 1996; Strable and Scalon, 2009). Surprisingly, the outcomes of *Arabidopsis* research have been used widely in crop improvement (Rensink and Buell, 2004).

Therefore, it is important to consider sorghum, for biological research on stress tolerance from harsh environmental conditions due to its valuable agronomic traits (Rizal *et al*., 2014). Given the multifunctionality responses by plants to environmental factors, some researchers have pointed out the need of studies under controlled conditions in order to evaluate the results more carefully (van der Heijden and Kuyper, 2001; Lendzemo *et al*., 2005).

Therefore, in this research to get a better knowledge about sorghum responsiveness to water deficitency and drought tolerance, diverse artificial procedures (instead of the complicate use of natural soil) were tested. Our results indicate that the substrate and nutrient solution suggested by Brundrett *et al.* (1996) was effective and allowed sorghum to grow well under the greenhouse controlled environmental conditions.

Furthermore, in order to validate this experimental system, the sensitive parameters such as stomatal conductance and [ABA_{xyl}]) suggested in other drought experiments (Dodd, 2003; Hansen and Dörffling, 2003; Pospíšilová, 2003) were chosen to detect sorghum plants response when subjected to a drought-stressed period.

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The results shown that stomatal response and changes in xylem sap concentration were detectable and there were not different from those obtained using field natural soil, thus, confirming that using sand as substrate with the proper nutrient solution is suitable for further research.

Because, the germplasm of sorghum is extraordinarily diverse (Upadhyaya *et al*., 2016), could be a key species for the expanding knowledge about the functions of beneficial crops in agriculture, especially in hot and dry regions of the world.

For instance, the method used for growing sorghum under controlled conditions could be useful for trials involving sorghum germoplasm collections in order to identify new sources of variations for stress resistance, phenology, seed yield and quality, and for bioenergy uses (Upadhyaya *et al*., 2016). In addition, this system can be useful to know: (1) how sorghum proceeds from one stage to another, (2) how it accumulates dry matter along the way, and (3) how it partitions the dry matter to tissue and grain, essential for understanding how a sorghum plant is likely to respond to environmental factors (Roozeboom and Prasad, 2016).

On the other hand, a common problem in trying to scale up results from greenhouse to natural environment is the uncertainty about whether the effects that take place in these unrealistic conditions occur in field conditions (Rillig, 2004). Furthermore, in nature, plant biodiversity, fitness and adaptation rely on the development of roots in combination with soil microorganisms with complementary functions in order to promote plant growth and health. Therefore, it could be important to generate proposals involving soil microorganism that may influence water relations or lessen drought straining in sorghum plants.

5. Conclusion and future research

The method proposed by Brundrett (1996) was proven as a good alternative for sorghum development under greenhouse controlled conditions.

Data from sorghum plants grown-up in this system related to stomatal conductance and xylem sap ABA concentration subjected to a drought-stressed period were successfully measurable and were not different from those obtained using field natural soil.

Therefore, this experimental system seems to be reliable for future research in supporting the idea of sorghum as a model cereal crop. Most importantly, sorghum can be study in order to understand the plant advanced mechanisms in overcoming abiotic constrains such as water relations and drought in its development of strategies for crop stress tolerance and adaptation to harsh environmental conditions around the world.

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