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Effects of substrate water potential in root growth of *Agave salmiana* Otto ex Salm-Dyck seedlings

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ABSTRACT

The objective of this study was to test the hypothesis that root of maguey (*Agave salmiana* Otto ex Salm-Dyck) seedlings reacts during the first 24 h to low substrate water potential (Ψ_W), by anatomical modifications. Three-4 cm root length seedlings were planted in vermiculite for 24 h at Ψ_W between -0.03 and -2.35 MPa. Root dimensions, proline content and anatomy were evaluated. Substrate Ψ_W between -0.65 and -2.35 MPa did not significantly affect longitudinal root growth. However, proline content significantly increased from 1.6 to 2.1 µmoles mg^{-1}. Significant reductions of transverse root area (41 %), thickness of mucilage covering the epidermis (47 %), thickness of epidermis (between 15 and 46 %), area of the parenchyma (between 35 and 41 %) and number of vessels (up to 28 %) were observed with Ψ_W of -2.35 MPa. In contrast, thickness of xylem wall, diameter of xylem vessels and the number of cells of the cortex of the differentiation root region significantly increased (64, 17, and 97 %, respectively). The anatomical changes associated with low substrate Ψ_W indicate a net increase of root apoplatic paths; structures involved in water conduction increased their diameter under low substrate Ψ_W conditions and anatomical changes occurred during the first 24 h of water stress.

Key terms: Maguey, proline, root anatomy, root growth, water deficit, water stress.

INTRODUCTION

For the last 20 years evidence has shown that roots are more sensitive to small changes in soil water content than to any change in shoot water relations. This early warning system triggers several signals that are sent through the vascular system to regulate growth and gas exchange (Davies and Zhang, 1991). This kind of feedback reaction involves an early detection of environmental changes which may help the survival of plants (Passioura, 2002).

Physiological and biochemical changes as a result of water deficit have been widely described in annual species with C3 (*Phaseolus* spp., *Nicotiana* spp., *Vicia faba* L., *Allium cepa* L., *Lycopersicum esculentum* Miller, *Cicer arietinum* L., *Glycine max* (L.) Merr.) and C4 (*Oryza sativa* L., and *Zea mays* L., *Sorghum bicolor* (L.) Moench. *Andropogon gayanus* Kunth and *Brachiaria mutica* (Fonsk.) Stapf) photosynthetic metabolism. These changes are mainly associated with gas exchange, photosynthesis, accumulation and degradation of metabolites, alterations of cellular membranes and some enzymatic activities in leaves (Hare and Cress, 1997; Steudle, 2000; Wu and Cosgrove, 2000; Yin et al., 2004; Zlatev, 2005). However, plants with crassulacean acid metabolism (CAM) have a partially physiological description. The physiological root reactions to water availability in soil are diverse; however, metabolic factors controlling root survival in such conditions are only partially known due to the experimental difficulties in accessing intact roots (Huang and Gao, 2000).

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Several studies have revealed that in species like *Betula ermanii, B. maximowicziana, B. platyphylla, P. vulgaris* L., and *Triticcum aestivum* soil water deficit increases the root/shoot ratio due to an increase of root growth and an inhibition of shoot growth (Asseng et al., 1998; Koike et al., 2003). Experiments conducted under laboratory conditions have demonstrated that maize root continues growing at substrates with a water potential (Ψ_W) of -1.6 MPa, while shoot growth is totally inhibited at -0.8 MPa (Sharp et al., 1988 and 1990). The root response seems to have an ecological significance when the plant grows under a water deficit. Therefore, shoot growth inhibition helps to save water and to keep tissues hydrated, without significant reduction of plant metabolism. In addition, root growth maintenance improves water extraction from soil at deeper layers.

Agave salmiana as a CAM plant (Gibson and Nobel, 1986) fixes CO_2 during the night as malic acid. This acid is then stored in the vacuole and thereafter, during the day, is released from the vacuole and decarboxylated. The resulting CO_2 is then assimilated via the Calvin cycle. CAM plants are resistant to a xerothermic environment, with extremely dry soil and air, high salt content in the soil and extreme temperatures during the summer and winter. There is convincing evidence that roots of adult Agavaceae plants survive under extremely dry conditions (Ψ_W as low as -3.2 MPa for 30 days) (Nobel et al., 1990; North and Nobel, 1995; Dubrovsky, 1997). Therefore, the roots of *A. salmiana* are an interesting model to study the physiological and anatomical changes involved in drought tolerance occurring in short periods. It is well known that CAM plants are tolerant to water deficit, but root changes and physiological mechanisms under extreme soil Ψ_W are only partially known. The objective of this study was to test the hypothesis that root of maguey (*Agave salmiana* Otto ex Salm-Dyck) seedlings react during the first 24 h to low substrate water potential (Ψ_W), by anatomical modifications.

MATERIALS AND METHODS

Genetic Material

Seeds of *A. salmiana* collected in the locality of "Minera las Cuevas" (21° 56' Lat. N, 100° 35' Long. W, and 1890 masl), Zaragoza, San Luis Potosi, Mexico, were used in this study. The climate in this locality is arid, with the summer rainy season divided by a short dry season, and followed by a long dry season; daily temperatures changes can be up to 35° C and annual mean temperature fluctuates between 12 and 18° C (García, 1988). This seed lot had high percentage of germination (85%), according to Peña-Valdivia et al. (2006).

Experimental

Seeds were mechanically scarified (by making 1 mm deep incision in the marginal region using a scalpel) and soaked in water at 24 ± 2° C for 24 h. The seeds were then germinated at 25 ± 1° C in the dark between pleated paper (Ellis et al., 1985) and 35° inclination. Synchronic germination was reached after 72 h, and seedlings with short hypocotyl (3 cm) and 35 mm mean root length were obtained after 144 h. Then seedlings were transplanted into polyvinyl-chloride (PVC) tubes (40 and 100 mm inside diameter and length, respectively) filled with vermiculite at different Ψ_W in a dark room. Each PVC tube was covered at both ends with a piece of black polyethylene fixed with elastic bands to keep the vermiculite at a constant Ψ_W. The containers were maintained in the dark at 25 ± 1° C for 24 h, thereafter roots were harvested.

Different vermiculite Ψ_W (-0.03, -0.65, -1.48 and -2.35 MPa) were obtained by mixing dry vermiculite with 100, 10, 8 and 6 parts of distilled water (weight: volume) in polyethylene bags 48 h before starting the experiment. Subsequently, vermiculite Ψ_W was determined by incubating a sample of vermiculite in psychrometric chambers (Wescon C-52, Inc, Utha, USA) for 4 h. Vermiculite Ψ_W was determined connecting the chambers to a

microvoltmeter (Wescon HR-33T, Inc, Utha, USA) operated in the dew point mode. Longitudinal root growth, anatomical root characteristics, turgor pressure (Ψt), fresh and dry weight, and root proline content were evaluated.

Total root length was estimated using a calliper scaled in 1.0 mm units and 0.05 mm vernier (accuracy to ± 0.01 mm); fresh and dry weight was quantified in an analytic balance (Scientech, with 0.0001 g precision). Root dry mater and proline were determined in freeze-dried samples; samples were previously wrapped in aluminium foil, frozen in liquid nitrogen, and then freeze-dried for 72 h. Proline content was quantified using a colorimetric method and a calibration curve with a maximum concentration of 1000 nmols of proline per mL (Bates et al., 1973).

Roots were sampled and placed in cups of thermocouple-psychrometers connected to a dew point micro-voltmeter (Wescor HR-33T, Inc., Utah, USA), after 4 h of thermal and water vapour equilibrium the Ψ_W was determined. The same root samples were used for determining solute potential (Ψ_S) after they had been frozen in liquid nitrogen. Turgor potential ($\Psi\tau$) was calculated as the difference between Ψ_W and Ψ_S.

Anatomical characteristics were evaluated in cross-sectional segments belonging to the first centimetre of the root from the apex, including cell division and differentiation root regions. Tissue was fixed in an acetic acid: formaldehyde: ethanol: water solution (FAA, 5: 10: 52: 33), in a 10: 1 (w: v) fixer: sample ratio, for 4 to 6 d, and then dehydrated with butyl alcohol, followed by embedding with paraffin, sectioning into 10 µm ribbons and mounting on glass slides with chrome adhesive. Staining was done using a safranine-fast-green system, according to Johansen (cite by Sánchez, 2000). The dimensions and numbers of anatomical structures were obtained with inverted microscopy (ZEISS, Axiovert 25), equipped with a video chamber (SONY SSC-DC54), and the analysis was made with the software Image Tool 3.0 for personal computers (Wilcox et al., 2002).

The effect of 24 h of re-hydration on root growth was evaluated; 50 mL of water were added to each container that had substrate Ψ_W between -0.65 and -2.35 MPa, and 24 h after watering root growth was evaluated.

Experimental Design and Statistical Analysis

The experiment was conducted according to a completely random design with four replicates. The treatments corresponded to four substrate Ψ_W (-0.03, -0.65, -1.48 y -2.35 MPa) and each experimental unit included 10 roots. Data were analysed using the General Linear Model Procedures of SAS. Differences among treatments were considered to be significant at $P<0.05$. Means were compared using Tukey's method. Interaction effects among the anatomical characters between both root regions were analysed with the LSMEANS test. Also a Pearson correlation analysis was performed dividing the data in two treatments: Ψ_W of -0.03 MPa, and Ψ_W of -0.65, -1.48 and -2.35 MPa. Graphic representation of the data and the selection of the best mathematical adjustment were performed with Sigma Plot software of Jandel Scientific (version 7.1) for personal computer.

RESULTS AND DISCUSSION

Total Root Growth

The hypocotyl length of seedlings (144 h old) was similar to root length (33 mm); but, fresh and dry biomass was five times higher in hypocotyl than in root. Seedlings continued growing after transplantation in vermiculite, mostly at Ψ_W of -0.03 MPa. By this time (168 h after germination started) seedling growth was partially affected by vermiculite Ψ_W between -0.65 and -2.35 MPa compared to -0.03 MPa; thus, hypocotyl length diminished significantly (6.1 mm, equivalent to 17 %) even though root length was not affected. The hypocotyl reacted in part differently to low Ψ_W than did the root, this reaction became visible in

a relatively brief time, and in the following development stages root growth was also affected, as the root is partially dependent on hypocotyl reserves (Smith and Snedaker, 2000). As well, the percentage of hypocotyl and dry root biomass increased significantly (7 and 13% on average, respectively) in substrate Ψ_W lower than -0.03 MPa (Fig. 1). All these results indicated that substrate Ψ_W between -0.65 and -2.35 MPa only partially affected seedling root growth of *A. salmiana*. Substrate Ψ_W of -1.5 MPa has been reported as a growth inhibitor of maize, soybean, rice and cucumber (*Cucumis sativus* L.) roots (Spollen et al., 1993). The results of the present work also indicate that "maguey" seedlings growing in a substrate containing 6 and 10 % humidity (-2.35 and -0.65 MPa, respectively) had enough water in the roots to continue growing (Fig. 1). As well, after re-hydration the roots continued growing.

Proline

Proline in the maguey roots increased significantly (almost 100 %, from 1.6 to 2.1 mmol·mg^{-1} of dry weight) when seedlings were maintained in vermiculite at Ψ_w of -1.48 and -2.35 MPa (Fig. 2 B). Proline root accumulation, as a reaction to water deficit and low substrate Ψ_W, has been observed in several species, like maize (Raymond and Smirnoff, 2002; Sánchez-Urdaneta et al., 2005), rye and wheat (Albarrán, 2004), domesticated and wild *P. vulgaris* L. (Sánchez-Urdaneta et al., 2003; Khadri et al., 2007), and olive trees (*Olea europaea* L.) (Sofo et al., 2004). Proline accumulation in leaves and roots has been interpreted as a result of an imbalance in plant metabolism, but evidence indicate that proline can benefit a stressed plant by osmotic adjustment (Sofo et al., 2004; Xing Yuan et al., 2004). Proline has been classified as an osmoregulator in maintaining growth under water deficit and even after re-hydration (Prechamandra et al., 1992; Sánchez-Urdaneta et al., 2003 and 2004).

Proline increased in *Agave* root seedlings in the present study, but contrasted to other species that involves a several-fold increase in the net rate of proline deposition as a water-stress reaction (Sánchez-Urdaneta et al., 2005). However, the primary contribution to osmotic adjustment can be increases in factors other than proline osmoticum deposition (i.e. sugars, inorganic ions and several amino acids); besides osmotic adjustment along seedling root could be heterogeneous. In contrast to the basal root region of the growth zone of water-stressed maize roots, in the few apical millimeters this amino acid contributes up to 50% of osmotic adjustment (Sharp et al., 2004). In the present study complete 35 mm long root of seedlings were used for proline quantification.

The characteristics and mechanisms of "maguey" root that allow growth under drought conditions are unknown, but they could be related to the chemical composition of tissue. It is well known that high solute accumulation maintains tissue water absorption, tissue turgor and therefore growth in several species (Saito and Terashima, 2004; Vicré et al., 2004; Touchette, 2006; Bacelar et al., 2007). In the present study, substrate Ψ_W lower than -0.03 MPa significantly increased root $\Psi\tau$ (from 0.08 to 0.15 MPa; Fig. 2A) and an inverse relationship between them ($r^2=0.9926$ and n=40) existed. Turgor maintenance is necessary in plant cells to promote and modulate growth velocity. Loss of turgor pressure could result in total growth inhibition and eventually cell death. Osmotic adjustment is the main plant adaptation to maintain turgor when plants are under conditions of water stress or others kinds of stress (Saito and Terashima, 2004).

Growth of Anatomical Structures

Low substrate Ψ_w (-0.65 and -2.35 MPa) significantly reduced transverse root area (up to 41 %), thickness of the mucilage covering the epidermis (up to 47 %), epidermis thickness (up to 46 %), cell area of cortex (parenchyma; up to 41 %) and the number of xylem vessels (up to 28 %). In contrast, a sharp linear increase in the number of cortex cells, xylem vessel diameter (up to 17%) and xylem wall thickness (64%) was observed (Fig. 2 and 3).
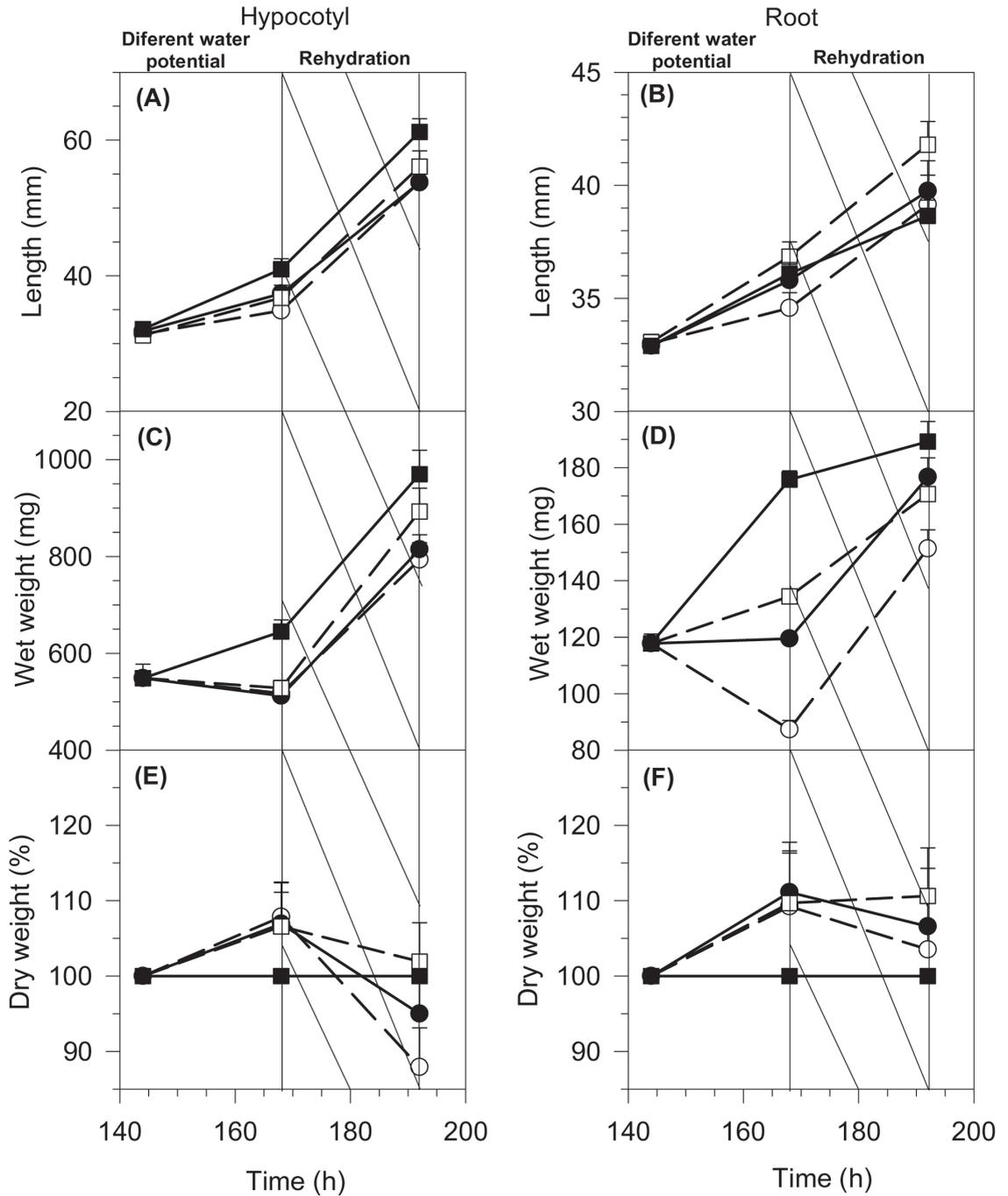
Figure 1: Hypocotyls and root growth of *Agave salmiana* Otto ex Salm-Dyck seedlings in vermiculite at different water potential: -0.03 MPa: ■, -0.65 (□), -1.48 (●) and -2.35 MPa (○) after 144 h of germination. Each point represent the average of four replicates (n=40) + standard error.
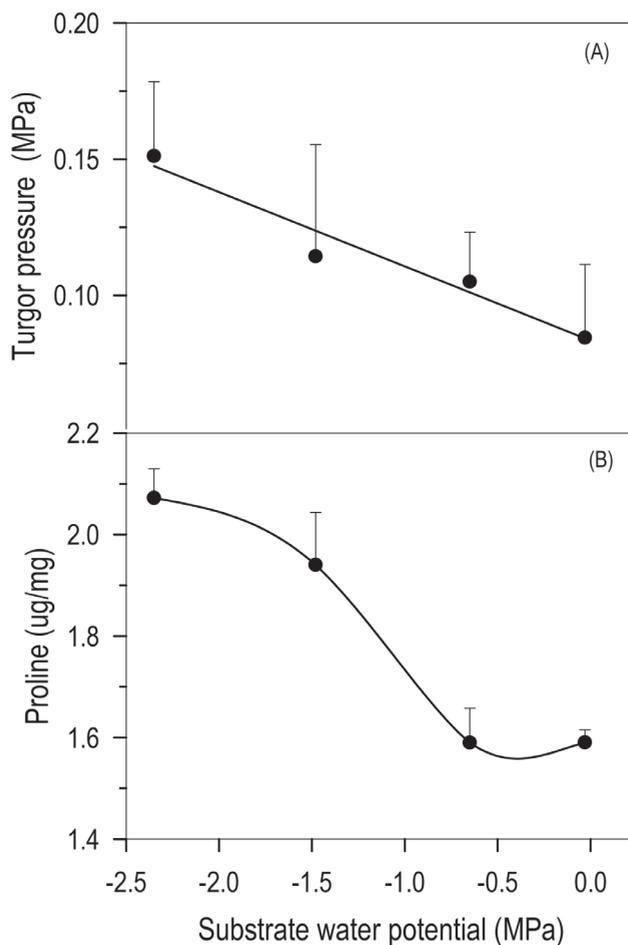
Figure 2: Effects of substrate water potential on turgor pressure (A) and proline content (B) of *Agave salmiana* Otto ex Salm-Dyck root seedlings. Each point represents the average of 10 replicates + standard error.

Longitudinal root growth of 144-168 h old seedlings, growing in vermiculite at Ψ_w of -0.03 MPa, was correlated significantly and positively with the area of parenchyma cells (r=0.459, P<0.021 and n=25) of the root cross section, but under lower vermiculite Ψ_W this relationship was not significant (r=-0.025, P<0.810 and n=91). Xylem root morphology and anatomy are relevant for water conduction along the seedling and the entire plant tissue; however, in the present study neither number and diameter of xylem vessels nor xylem wall thickness correlated significantly with any growth variable of seedlings growing at Ψ_W of -0.03 MPa.

The effect of water deficit in some anatomical root characteristics has already been documented. Seedlings of drought tolerant rice genotypes had thicker roots with broader xylem vessels than the susceptible genotype; but, water stress did not significantly affect the root and xylem thickness (Umayal et al., 2001). Root exodermis of *Agave* become suberized and the endodermis and the cortex become thicker due to drought (North and Nobel, 1995; Bobich and Gretchen, 2009). The results of the present study partially agreed with those observed in maize, where the cortical cells shrank and lost shape when relative water content (RWC) reached values of 0.5, and cells collapsed when RWC was 0.3 (Facette et al., 1999). Although there are remarkable differences in magnitude, this tendency was similar to
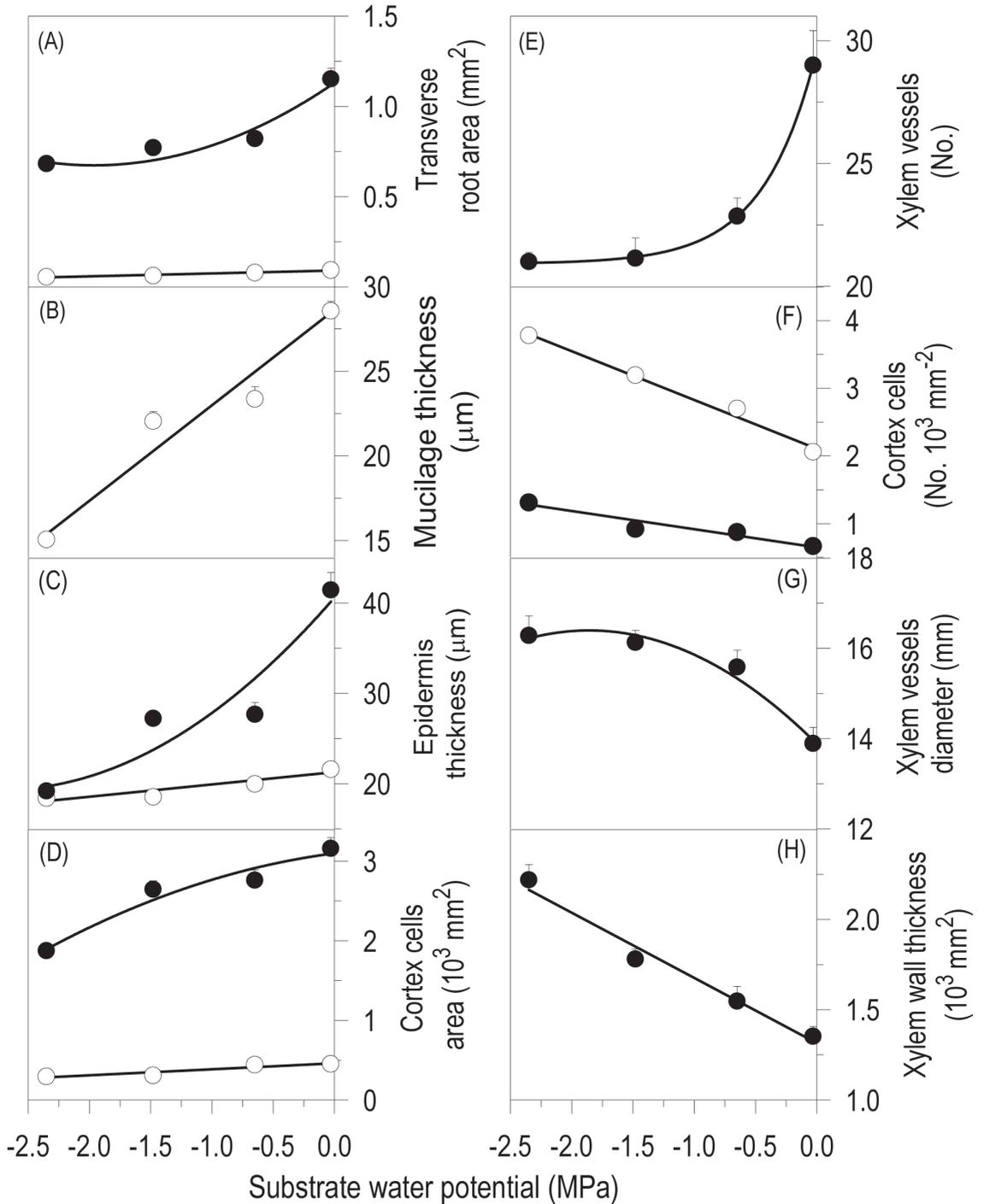
Figure 3: Effects of substrate water potential in the anatomy of root seedlings of *Agave salmiana* Otto ex Salm-Dyck. Cell division (O) and differentiation (●) root region. Each point represents the average of four root cross section replicates (n=7 and 50, respectively) + standard error.

that observed in "maguey". Epidermis thickness, cell area of cortex, and number of xylem vessels diminished, and the number of cortex cells and the xylem wall thickness increased in root cross sections of maize seedlings (Tux. Seq. C0 and Tux. Seq. C8 cultivars, drought sensitive and drought tolerant, respectively) growing in a substrate at Ψ_W lower than -0.03 MPa (Peña-Valdivia et al., 2005). These results suggest that some anatomical root changes are independent of plant species and essential for growing seedlings under low Ψ_W.

In contrast, substrate with low Ψ_W seems to affect some other characteristics depending on the cultivar, species or intrinsic plant drought tolerance. This is the case of mucilage thickness, which increased 44 and 50 % in maize root seedlings of Tux. Seq. C0 and Tux. Seq. C8, respectively, growing at -2.35 MPa (Peña-Valdivia et al., 2005), but mucilage thickness in maguey diminished 46 % in the same substrate Ψ_W (Fig. 3 B). Another case is xylem vessel diameter, which increased 15 and 16 % in Tux. Seq. C8 maize (Peña-Valdivia et al., 2005) and maguey (Fig. 3B) root seedlings growing at -2.35 MPa; but, a 10 % reduction in xylem vessels diameter was observed in maize (cv. Tux. Seq. C0) drought sensitive (Peña-Valdivia et al., 2005).

It should be stressed that under experimental conditions, in the present study adventitious roots were absent in maguey seedlings; in contrast, Peña-Valdivia et al. (2005) found in maize Tux. Seq. C0 (drought sensitive) after 72 h of germination a significantly higher number of adventitious roots than in Tux. Seq. C8 (drought tolerant) (3.3 and 2.8 adventitious roots per seedling, respectively); besides, only in Tux. Seq. C8, the number of adventitious roots diminished with the Ψ_W of vermiculite, up to 23 % at -2.35 MPa. North and Nobel (1995) showed that xylem vessel dimensions in young roots, as well in lateral roots of *A. deserti*, changed when plants were growing under water deficit (7 to 30 d), and these changes were apparently related to a reduction of root conductivity. Diameter of root structures involved in

water conduction increased significantly in *A. salmiana* seedlings growing under low substrate Ψ_W (Fig. 3 G).

Apoplastic and symplastic root barriers for water and ion flows can develop during water stress. In the present study, wall thickness of xylem roots increase significantly with the lowest substrate Ψ_W (Fig. 3 H). But, when seedlings grew with the highest Ψ_W (-0.03 MPa), the xylem vessel number, diameter of xylem vessel and its wall thickness did not correlate significantly with any other studied variable of root growth. Maize root thinning was accompanied by an increase of anticlinal cellular division, equivalent to an increase by 35 % of the number of cells in the cortex, when substrate Ψ_W is -1.61 MPa (Liang et al., 1997). Root seedlings of *Agave salmiana* at Ψ_W of -2.35 MPa were thinner (Fig. 3 A), the number of cortex cells significantly increases (Fig. 3 F) and this variable is negatively correlated with the transverse root area in both differentiation and elongation root regions (r=-0.363 and -0.661, P=0.016 and <0.001, n=44 and 32, respectively). The meaning of the increase of transverse root cells as a reaction to water deficit is unclear; it seems that root cells probably lose water at the lower substrate water potentials, thereby shrinking, and thus cell number per square millimetre increased. According to Liang et al. (1997) a large number of cells increase the total root area including more apoplatic paths and water and ion movement into the root.

The results of the present study demonstrate that *Agave salmiana* seedlings represent a good model for studying the effect of substrate Ψ_W on root growth, tissue composition and anatomical changes with minimum effects of the undeveloped shoot. The "maguey" seedlings clearly react in several ways like the common bean and maize seedlings to substrate Ψ_W and in contrast in some ways differently (Peña-Valdivia, 2005; Sánchez-Urdaneta et al., 2004 and 2005). However, it is necessary to continue studying and including more species to contrast diverse plant models to confirm the "maguey" reaction to low substrate Ψ_W more clearly

CONCLUSIONS

Root of maguey seedlings express detectable changes in growth as a reaction to low substrate Ψ_W for short periods of time (24 h) and these changes are a result of physiological, biophysical and anatomical modifications. Proline seems to be an osmoregulator for maintaining root growth at low substrate Ψ_W; the anatomical changes associated with low Ψ_W are evidence of an increase of apoplatic paths. As well, maguey root seedling structures involved in water conduction increased their diameter significantly under low substrate Ψ_W conditions.

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