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Effect of osmopriming on germination and initial growth of *Physalis angulata* L. under salt stress and on expression of associated genes

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ABSTRACT

This study aimed to evaluate the effects of priming on seed germination under salt stress and gene expression in seeds and seedlings of *P. angulata* L. After priming for 10 days, seed germination was tested in plastic trays containing 15 ml of water (0 dS m⁻¹ – control) or 15 ml of NaCl solution (2, 4, 6, 8, 10, 12, 14 and 16 dS m⁻¹). Fresh and dry weight of shoots and roots of seedlings were evaluated at 0, 2, 4, 6, 8 dS m⁻¹. Total RNA was extracted from whole seeds and seedlings followed by RT-qPCR. The target genes selected for this study were: ascorbate peroxidase (*APX*), glutathione-S-transferase (*GST*), thioredoxin (*TXN*), high affinity potassium transporter protein 1 (*HAK1*) and salt overly sensitive 1 (*SOS1*). At an electroconductivity of 14 dS m⁻¹ the primed seeds still germinated to 72%, in contrast with the non-primed seeds which did not germinate. The relative expression of *APX* was higher in primed seeds and this may have contributed to the maintenance of high germination in primed seeds at high salt concentrations. *GST* and *TXN* displayed increased transcript levels in shoots and roots of seedlings from primed seeds. Priming improved seed germination as well as salt tolerance and this is correlated with increased expression of *APX* in seeds and *SOS1*, *GST* and *TXN* in seedlings.

Key words: RT-qPCR, germinability, seedling growth, salinity.

INTRODUCTION

In most plant species salinity affects germination and development of the seedling, which is considered the developmental stage that is most sensitive

and vulnerable to abiotic stresses (Sosa et al. 2005, Belaqqiz et al. 2009). Delay of germination (Foolad 2004) and growth inhibition due to salinity are caused by low external water potential, ion imbalance and specific ion toxicity (Munns 2002, Khajeh-Hosseini et al. 2003, Miranda et al. 2010). Under these conditions there is a decrease in water

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absorption and an excessive absorption of ions (Akram et al. 2010).

Salt stress leads to oxidative stress and severe impairment of germination and seedling growth. Although there is extensive knowledge on physiological and molecular mechanisms that regulate salt tolerance in *Arabidopsis thaliana* (L.) Heynh (Peng et al. 2009, Tian et al. 2011), much less is known for wild species, such as *Physalis angulata* (Solanaceae). *Physalis angulata* is widely used in ethnomedicine due to the presence of secosteroid (physalins) that are produced in stems and leaves (Bastos et al. 2008). The anti-inflammatory and immunomodulatory effects of the physalins B, D, F and G, have been well documented (Vieira et al. 2005, Magalhães et al. 2006, Soares et al. 2006, Damu et al. 2007, Guimarães et al. 2009, Yu et al. 2010). Despite its important chemical and food properties, research on growth and improvement of stress tolerance of *P. angulata* is lagging behind. In this study we describe the sensitivity of germination and seedling growth *P. angulata*, to salt stress, in addition to the effect of a method involving controlled hydration of seeds followed by drying (collectively called 'seed priming') on these phenotypes. Priming is generally used to improve seed performance with respect to germination rate, uniformity and seedling emergence of vegetable and ornamental seeds (Heydecker et al. 1973, Iqbal and Ashraf 2007, Varier et al. 2010). However, seed priming can also improve resistance or tolerance of seeds to high temperatures (Yoon et al. 1997, Ligterink et al. 2007), drought (Wang et al. 2003) and salt (Sivritepe et al. 2003).

The maintenance of metabolic processes required for germination under stress may be attributed to the expression of genes specific to certain types of abiotic stresses. An increase of salinity in the medium is often associated with the expression of genes involved in water homeostasis, inorganic ion transport and metabolism, cell wall biogenesis, and signal transduction mechanisms (Bertorello and Zhu 2009, Peng et al. 2009).

The expression and activity of antiporters are highly regulated by salt stress (Bertorello and Zhu 2009), including SOS1 (salt overly sensitive) which is a member of the SOS family of antiporters. These antiporters regulate cell homeostasis by exporting Na^+ and their expression is activated when plants are subjected to saline environments (Munns and Tester 2008). Transporters with high affinity to K^+ (HKT1, HAK1), transporters with low affinity for cations (LCT1) and non-selective ion channels are considered the most likely transport systems specific to regulating the Na^+ cell influx (Davenport and Tester 2000, Amtmann et al. 2001). HAKs (High Affinity K^+ transport) are known to capture both Na^+ and K^+ in saline environments (Mian et al. 2011).

The ability of plants to counteract the production of reactive oxygen species (ROS) is an important component in the ability to withstand stress (Flors et al. 2007, Peng et al. 2009). In general, the ability to induce a high level of antioxidant systems results in higher tolerance towards stress.

The enzymatic antioxidant systems existing in seeds are superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) (Apel and Hirt 2004, Besse and Buchanan 1997, Dos Santos and Rey 2006). Thioredoxin (TXN) can also provide protection against antioxidants by allowing photochemical detoxification of H_2O_2 produced in the chloroplasts during stress (Dietz et al. 2006).

This study aimed at evaluating the effects of priming on seed germination and seedling growth under salt stress as well as changes in expression of genes related to stress signaling mechanisms in seeds and seedlings of *P. angulata*.

MATERIALS AND METHODS

PLANT MATERIAL

Physalis angulata plants were cultivated in a green house at the State University of Feira de Santana (State of Bahia, Brazil) in December 2010. The

seeds were isolated from fruits collected at a uniform stage of maturation and placed to dry over saturated KCl solution (85% RH). Water content was determined on a fresh weight basis by placing samples of 200 seeds for 17 h in an oven set at 103 °C (ISTA 2007). Remaining seeds were placed in 5 ml tubes and stored at 4 °C until further use.

SEED PRIMING AND GERMINATION

P. angulata seeds were used in two conditions: non-primed (control) and primed. To prime the seeds, samples of 1000 seeds were placed in 50 ml tubes containing 25 ml aerated -1.2 MPa polyethylene glycol solution (PEG 8000, Sigma), i.e. osmopriming, and kept in an incubator at 35° C in the dark (Villela and Beckert 2001, Souza et al. 2011). After priming for 10 days, seeds were washed with distilled water and kept at room temperature for 2 days to achieve the initial fresh weight.

Germination of non-primed and primed seeds was tested by sowing seeds on top of filter paper soaked with 15 ml of water (0 dS m⁻¹ - control) or NaCl solution (2, 4, 6, 8, 10, 12, 14 and 16 dS m⁻¹, corresponding to 17, 34, 55, 75, 92, 113, 134 and 154 mM, respectively) within plastic trays (15x21cm). The germination test was carried out in 4 replicates of 50 seeds per treatment incubated at 35° C in the dark. Germination was monitored during 10 days and seeds were considered germinated when the radicle had protruded the seed coat by at least 2 mm. Germination was assessed by measuring maximum germination percentage (G_{max}), germination rate expressed as time to reach 50% germination (t_{50}), and germination uniformity by measuring the time interval between 16% and 84% germination of viable seeds (u_{8416}) using the curve-fitting module of the 'Germinator software package' which enables the analysis of cumulative germination data (Joosen et al. 2010).

SEEDLING GROWTH

Seedlings were grown from non-primed and primed seeds sown on top of filter paper soaked with water

(0 dS m⁻¹ - control) or 15 ml of saline solutions (2, 4, 6, and 8 dS m⁻¹, i.e. normal seedlings) incubated at 35 °C and 12/12 h photoperiod. To measure root length, pictures were taken of ten day old seedlings using a digital camera (Nikon D80 with Nikkor AF-S Micro 60 mm f/2.8 G ED, Nikon), after which the seedlings were separated in shoots, roots and fresh and dry weight were determined. Dry weight was measured after placing shoots and roots at 104 °C for 24 h. The seedlings were grown randomized with 4 replicates of 25 seedlings per treatment and the data was statistically analyzed using the SISVAR software (Ferreira 2011).

RNA ISOLATION FROM SEEDS AND SEEDLINGS

RNA from seeds

Total RNA was obtained from non-primed (control) and primed seeds, both dry and 24 h imbibed in 14 dS m⁻¹ NaCl solution. Total RNA was extracted from samples of 50 seeds per treatment previously frozen in liquid nitrogen and stored at -80 °C. Frozen seeds were ground in a dismembrator at 1.200 rpm for 2 min and added to a tube containing 1.5 ml of phenol (pH 8.0):chloroform (5:1) plus 5 ml TLE grinding buffer (0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA, 1% SDS, adjusted to pH 8.2) with 5µl of β-mercaptoethanol. After subsequent centrifugation at maximum speed for 10 min, the upper phase was transferred to a new tube with 1000µl of phenol-chloroform (1:1), centrifuged at 12,000 g for 5 min and the supernatant was extracted with 1000µl of chloroform. RNA was precipitated overnight at -20 °C by adding 100µl of 10M LiCl. The samples were then centrifuged for 30 min at 12,000 g at 4 °C, the supernatant was removed, and the pellet was washed with 70% ice cold ethanol. The samples were centrifuged again for 5 min at 10,000 g at 4 °C, the supernatant was removed and the pellet was resuspended in 20µl DEPC water. The samples were DNase treated (RQ1 DNase, Promega) and further purified with RNEasy spin

columns (Qiagen) following the manufacturer's instructions.

RNA from seedlings

Total RNA was obtained from \pm 150 shoots and roots of 10 days old seedlings grown from non-primed and primed seeds imbibed in water or in 2, 4, and 6 dS m⁻¹ NaCl. Shoots and roots were initially placed in Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C. Subsequently they were homogenized in a dismembrator at 200 rpm for 1 min and RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen) following the manufacturers recommendations including a DNase treatment.

The RNA samples from seeds and seedlings (roots and shoots) were stored at -80 °C until further use. RNA quality was analyzed on a 1.2% agarose gel, stained with gel red. Concentration and purity of total RNA was assessed with a NanoDrop-ND 1000 UV-Vis Spectrophotometer (NanoDrop Technologies, New Zealand), using 1 µl of total RNA. RNA purity was estimated from the A260/A280 absorbance ratio.

Synthesis of cDNA

A cDNA iScript kit (Bio Rad, Hercules, CA, USA) was used to synthesize cDNA following the manufacturer's protocol by using 1 µg of total

seeds or seedlings RNA. The cDNA synthesis was carried out in a thermocycler (iCycler, Bio Rad, Hercules, CA, USA) using the following steps: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. A negative control, where no RNA was added to the reaction mix, was also included to check for contamination of the reagents and of the water used in the reactions.

PRIMER DESIGN AND QUANTITATIVE REAL-TIME PCR (RT-qPCR)

The target genes selected for this study were: ascorbate peroxidase (*APX*), glutathione S-transferase (*GST*), thioredoxin (*TXN*), high affinity potassium transporter protein 1 (*HAK1*) and salt overly sensitive 1 (*SOS1*). The reference genes used for normalization were SGN U-584254 and a catalytic subunit of protein phosphatase 2A (SGN U-567355) (Dekkers et al. 2012). Specific primers used for RT-qPCR reactions with seeds and seedlings (Table I) were designed based on gene sequences available in GenBank/NCBI (<http://www.ncbi.nlm.nih.gov/>) and the Sol genomics database (<http://solgenomics.net/>), from several Solanaceae species, e.g. *Lycopersicon esculentum*, *Solanum habrochaites*, *Capsicum annuum*, *Nicotiana benthamiana* using the *GeneFisher* software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/old.html>) (Giegerich et al. 1996).

TABLE I
Specific primers used in the real-time RT-qPCR reactions.

Gene	Forward (sequences written 5' to 3')	Reverse (sequences written (5' to 3'))	Amplicon length
<i>APX</i>	AGGACCTGATGTTCCCTTTCAC	AAGGTATGGGCACCAGAGAG	168
<i>GST</i>	AGYCCTCTGCTTTTGCAGATG	AAGGATCAGAAGGGAGCAAAGG	148
<i>TXN</i>	GGGYGTYGAWGAAATCCTCTG	TTTCCAGCTCCATCAGCAAG	114
<i>HAK1</i>	CGTGAGACCTGAAGAAAGGTTC	CAAACCTCTACGTCGTCCATGTG	116
<i>SOS1</i>	CCTTGTGTGCTGTGAAGT	TCGGCTTTGGTATTGCTTT	165
SGN-U 584254	GAGAGTCATGCCTAGTGGTTGG	CGAAGACAAGGCCTGAAATGTG	172
SGN-U 567355	CGATGTGTGATCTCCTATGGT	AAGCTGATGGGCTCTAGAAAT	148

Reactions were performed using a CFX96 (Bio Rad, Hercules, CA, USA) with gene specific primers, cDNA and iQ SYBR green supermix (Bio Rad, Hercules, CA, USA). The amplification protocol consisted of 3 min at 95 °C; then 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. A negative control was used in every PCR plate. Efficiency of the primers in RT-qPCR reactions was evaluated based on a standard curve generated by two-fold serial dilutions of a pooled cDNA sample, and all primer pairs had efficiencies between 90 and 110%. Gene expression was measured twice in 3 biological replicates (6x in total) for each treatment. Values of fold change in gene expression in relation to the control (dry non-primed seeds) were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with help of the qBase software (Biogazelle, Ghent, Belgium) and plotted on graphs for comparison. Stable expression of the reference genes for the studied samples was confirmed by geNORM as incorporated in the qBase software (Vandesompele et al. 2002, Hellemans et al. 2007)

RESULTS AND DISCUSSION

SEED PRIMING AND GERMINATION UNDER SALT STRESS

The effect of priming was tested on germination and seedling growth of *P. angulata* under different salt concentrations. All physiological tests were done at 35 °C since this is the optimum temperature for *P. angulata* germination and seedling growth (C.L.M. Souza, personal communication, 2010). We tested the effect of salt stress due to the severe problems with saline soils in places where *P. angulata* is grown, like for example in the northeast of Brazil (De Nys et al. 2005). Germination of both non-primed and primed seeds decreased when seeds were submitted to imbibition under increasing salt concentrations up to 16 dS m⁻¹. Differences in germination parameters between non-primed and primed seeds became significant at salt solutions with electroconductivity (EC) of 6 dS m⁻¹ or

higher (Fig. 1a). At 6 dS m⁻¹ EC the germination percentage was 90% in non-primed seeds, against 100% in primed, and the difference in germination percentage increased gradually, becoming the largest at 12 dS m⁻¹ EC, i.e 10% in non-primed, against 87% in primed seeds. Non-primed seeds failed to germinate at 14 dS m⁻¹ EC or higher in contrast with primed seeds which still germinated up to 72% at this salt concentration (Fig. 1a).

Besides germination, the non-primed *P. angulata* seeds that germinated in different salt concentrations from 8 dS m⁻¹ EC or higher, showed significantly slower germination (higher t_{50}), as well as worse uniformity (higher u_{8416}) compared to primed seeds (Fig. 1b, c). The largest difference in t_{50} between non-primed and primed seeds was at EC 8 dS m⁻¹ EC, at which t_{50} of non-primed seeds became significantly slower (higher t_{50}), keeping similar levels up to 10 dS m⁻¹ EC, above which non-primed seeds failed to germinate. Primed seeds initially kept a higher germination rate (lower t_{50}), but then gradually became slower (higher t_{50}) with increasing salt concentrations (Fig. 1b). Uniformity (u_{8416}) was better in primed seeds imbibed in water as well as in all salt solutions, but difference became larger in seeds subjected to higher salt concentrations from 8 dS m⁻¹ EC upwards (Fig. 1c).

The germination data demonstrate the positive effect of priming on germination of *P. angulata* seeds subjected to salt stress, which is consistent with the work performed by Souza et al. (2011), who demonstrated that the benefits caused by priming seeds of *P. angulata* included higher germination percentage and rate, and better uniformity in saline conditions. Priming has been confirmed as a technique to improve seed germination performance in various crops using PEG or salt solutions as osmotic agents, which control the entry of water into the cell, thereby preventing germination during the priming treatment and allowing the seed germination process to be resumed faster in suitable conditions (McDonald 2000, Sivritrepe et al. 2003,

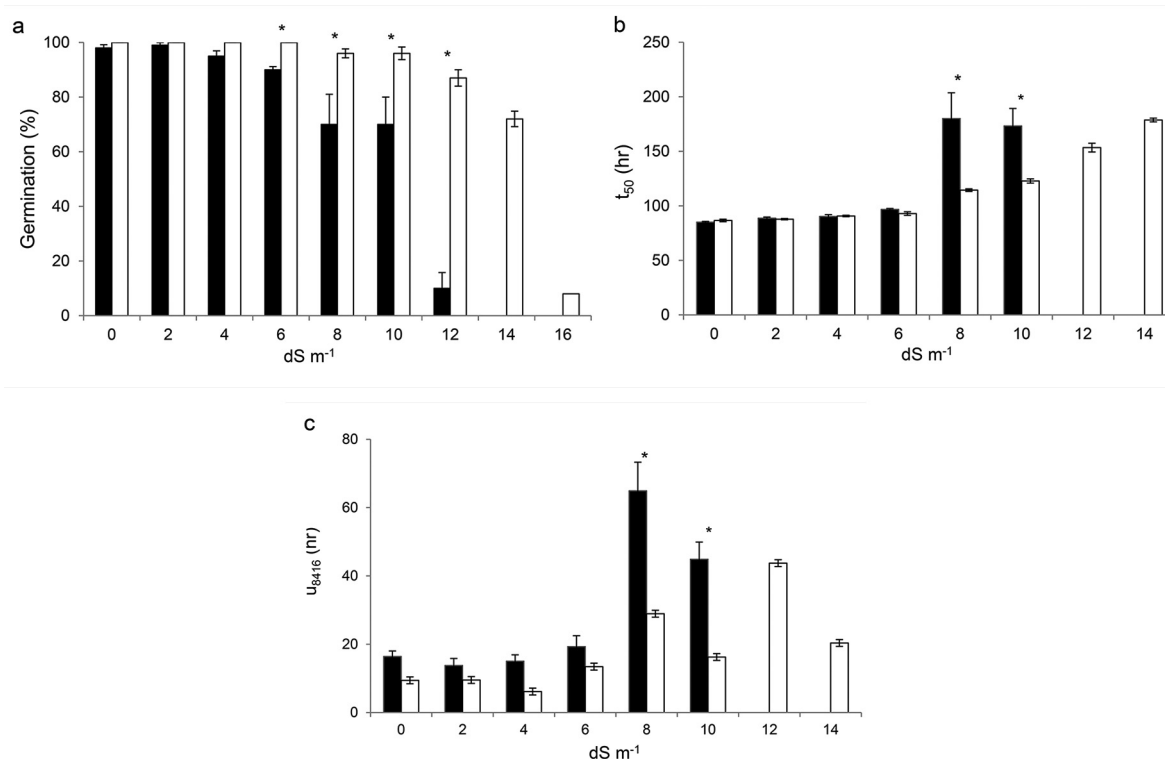


Figure 1 - Germination of non-primed (black bars) and primed seeds (white bars) of *P. angulata* under saline stress. **(a)** Maximum germination percentage (%); **(b)** Germination rate expressed as time (h) to reach 50% germination (t_{50}); **(c)** Germination uniformity expressed as the time (h) interval between 16% and 84% germination of viable seeds (u_{8416}). Salt concentrations expressed as salt electric conductivity from zero (water) up to 16 dS m⁻¹. All values are means \pm SE of 4 replicates of 50 seeds each. Asterisks represent significant differences between non-primed and primed seeds (Student t-test at $P < 0.05$).

Soeda et al. 2005, Varier et al. 2010, Souza et al. 2011, Nakaune et al. 2012).

Many studies have shown that priming is a useful technique, especially for seed lots with low vigor (Varier et al. 2010, Flors et al. 2007, Soeda et al. 2005). Agriculture has benefited from the effects of priming applied to seeds of many domesticated crop species, whereas the present results show that it can be extended to seeds of *P. angulata*, which are yet undomesticated but has increasing relevance as a plant species with significant therapeutic properties. This study shows that priming of *P. angulata* seeds results in higher percentages and rates of germination, as well as better germination uniformity, especially under adverse environmental conditions. Thus, PEG osmopriming of *P. angulata* seeds may be used to standardize and decrease the

time for germination, especially when these seeds are planted under salt stress conditions.

It has been shown that germination of *Physalis peruviana* and *Physalis ixocarpa* decreased with increasing NaCl concentration. *P. peruviana* had higher germination rates than *P. ixocarpa* with increasing salt concentrations, indicating a higher level of tolerance of *P. peruviana* to salt stress during germination. In contrast, *P. peruviana* became more sensitive to salt during emergence and early seedling stages (Yildirim et al. 2011). In fact, *P. peruviana* plants have been considered as moderately tolerant to saline conditions as measured by relative growth rate and net assimilation rate at moderate salt stress (30 mM NaCl), which corresponds to a salt solution of around 3.5 dS m⁻¹ EC (Miranda et al. 2010). In the present study

primed *P. angulata* seeds became vigorous enough to withstand salt stress and germinate well at salt concentrations between 6 and 12 dS m⁻¹ EC.

EFFECT OF PRIMING ON SEEDLING GROWTH UNDER SALINE STRESS

SHOOT GROWTH

Salinity had a significant impact on initial seedlings growth of *P. angulata*. For both fresh and dry weight, seedlings of primed seeds produced more biomass than seedlings of non-primed seeds (Fig. 2). However, the magnitude of the response to treatment with salt varied with the salt concentration. There was a tendency to stimulate the production of biomass in milder salt concentrations between 2, 4 and 6 dS m⁻¹. At the highest concentration (8 dS m⁻¹), the biomass of shoots was affected regardless of the initial treatment of the seeds (Fig. 2a, b).

These results show that, even under saline conditions, *P. angulata* seedlings show relatively normal growth, which can be attributed to mechanisms that prevent ions from acting as toxic substances. Biomass production was even stimulated by moderate salinity. This suggests that physiological mechanisms are active in *P. angulata*, especially osmotic adjustment and synthesis of important proteins involved in protection against free radicals.

The differences between shoots of seedlings grown from non-primed and primed seeds were more pronounced from 2 dS m⁻¹ upwards, in which fresh weight was increased as salt concentration increased. The values of fresh shoots obtained from plants grown from primed seeds were 4.8, 5.41 and 5.97 mg in saline solutions of 2, 4 and 6 dS m⁻¹ EC, respectively.

In saline solution of 6 dS m⁻¹ EC, the values of dry mass of shoots of seedlings from non-primed and primed seeds were 0.23 mg and 0.28 mg, respectively (Fig. 2b).

The accumulation of Na⁺ and Cl⁻ in tissues has been related to reduced plant growth (García-Legaz et al. 2005). In *Phlomis purpurea* it was

observed that the reduction in plant growth in a saline solution of 4 dS m⁻¹ EC, which would reflect the tolerance of this species to salinity (Álvarez et al. 2012).

Salt stress significantly reduced the fresh and dry weight of two species of *Physalis*. Fresh weights of *P. peruviana* and *P. ixocarpa* were reduced by 60%-75% at 30 mM NaCl (3.5 dS m⁻¹) and 72%-100% at 60 mM (7 dS m⁻¹), respectively (Yildirim et al. 2011). In that respect we can conclude that *P. angulata* seedlings are much more tolerant than its family members and this tolerance can even be increased by osmopriming of the seeds that are used to grow these seedlings.

ROOT GROWTH

Different salt concentrations had a positive effect on fresh roots weight (Fig. 2c), but no significant differences were observed when comparing root weight of non-primed with primed seeds. The length of roots of *P. angulata* showed a trend towards longer roots for seedlings grown from primed as compared to non-primed seeds and subjected to different salt concentrations, however, this difference was only statistically significant in EC 6 dS m⁻¹ where the roots from primed seedlings were much longer (Table II). Yet in EC 8 dS m⁻¹ roots, there was no significant difference between the length of roots from non-primed seedlings as compared to primed seedlings, probably reflecting a mechanism in which the effect of priming on root length is only favorable at lower salt concentration.

Sodium in the soil inhibits the uptake of potassium by the root which is an essential mineral for all plants. Furthermore, high concentrations of sodium salts hinder water absorption by the roots. Finally, the accumulation of sodium in the cytoplasm of plant cells can inhibit metabolic enzymes and may cause oxidative stress (Katiyar-Agarwal et al. 2006).

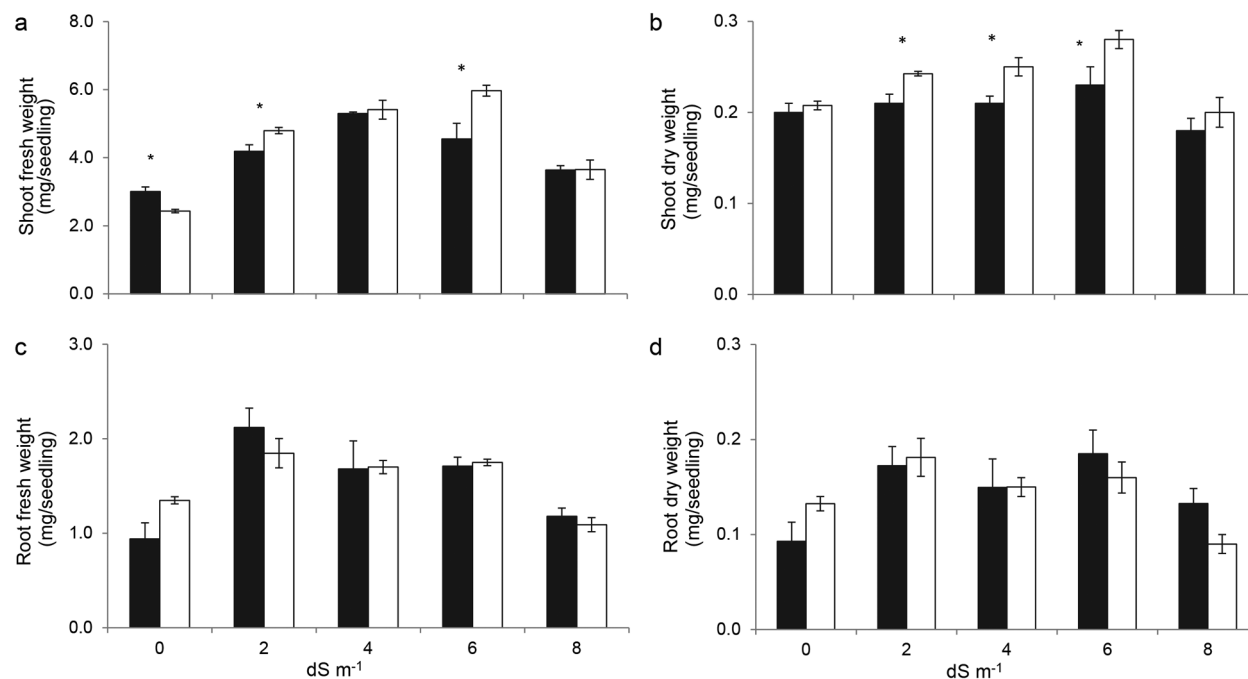


Figure 2 - Fresh and dry weights (mg/seedling) of shoots and roots of seedlings derived from non-primed (black bars) and primed seeds (white bars) of *P. angulata* grown under saline stress. (a) Shoot fresh weight; (b) Shoot dry weight; (c) Fresh root weight; (d) Root dry weight. Salt concentrations expressed as salt electric conductivity from zero (water) up to 8 dS m⁻¹. All values (mg/seedling) are means \pm SE of 4 replicates of 25 seedlings each. Asterisks represent significant differences between non-primed and primed seedlings (Student t-test at $P < 0.05$).

TABLE II
Seedling root lengths (cm) derived from non-primed and primed seed of *Physalis angulata* grown under saline stress. Salt concentration expressed as the salt electric conductivity from zero (water) up to 8 dS m⁻¹.

Salt concentrations (dS m ⁻¹)	Seeds	
	Non-Primed	Primed
0	2.95 a	3.12 ab
2	2.09 b	2.67 a
4	2.73 ab	3.04 abc
6	2.50 ab	3.38 b
8	2.33 b	1.90 c

Means followed by same lower-case letter in a column do not differ by Student t-test at $P < 0.05$.

Our results suggest that the roots of *P. angulata* may have been able to osmotically adjust themselves keeping intracellular Na⁺ and Cl⁻ concentrations at affordable levels, reducing the water potential and maintaining cellular metabolism without toxic effects.

GENE EXPRESSION IN *P. angulata* SEEDS UNDER SALINE STRESS

To find a link between the observed physiological effects of osmopriming of *P. angulata* seeds with respect to germination and seedling growth with changes in gene, we studied the expression of

genes with a described role in tolerance towards stress, in other species. The *SOS1* gene encodes a plasma membrane Na^+/H^+ antiporter responsible for the exclusion of sodium from the apoplast (Liu et al. 2000), whereas *HAK1* is a high affinity carrier for K^+ , which also regulates the influx of Na^+ in the cell (Amtmann et al. 2001).

The relative expression of *SOS1* and *HAK1* in seeds (Figs. 3a1 and 3b1, respectively) did hardly differed between treatments. Only *HAK1* expression was significantly lower in dry primed seeds as compared to non-primed dry seeds and seeds that were exposed to salt for 24 h. This incubation time was probably not sufficient to increase expression of both genes, and higher expression values of especially *HAK1* might be expected after longer exposure times, but additional experiments would be needed to prove this.

To further explore the correlation between the effect of priming on salt tolerance in *P. angulata* seeds and gene expression, we measured the expression of some antioxidant enzymes. The increase in reactive oxygen species (ROS) as a response to different types of stress, including salinity, triggers mechanisms to minimize the damaging effects caused by the presence of ROS in plant tissues. Among these mechanisms are the actions of antioxidant enzymes (Munns and Tester 2008). These enzymes include, among others, glutathione peroxidase (*GPX*), glutathione-S-transferase (*GST*), superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*) and thioredoxin (*TXN*) (McFarland et al. 1999, Wedderburn et al. 2000, Apel and Hirt 2004, Ashraf et al. 2008). We tested the expression of genes for *APX*, *GST* and *TXN*. All these genes code for important detoxifying enzymes with a proven role in general stress responses in other plant species, because of their antioxidant activity (Besse and Buchanan 1997, Su et al. 2002, Dos Santos and Rey 2006).

Expression of *APX* was induced upon salt stress with a higher up-regulation in primed seeds (Fig. 3c1). Expression of *GST* did change significantly as a result of priming in dry seeds, but its expression was lower in seeds exposed to salt for 24 h with no differences between non-primed and primed seeds (Fig. 3d1).

We observed a negative effect of priming on the expression of *TXN*, since the expression in the dry non-primed seeds was higher than in the dry primed seeds (Figs. 3e1). The expression of *TXN* in imbibed seeds is lower, which indicates that *TXN* expression might be generally repressed upon imbibition under salt stress (Figs. 3e1).

According to Varier et al. (2010) the metabolic energy of dried primed seeds is greater than that of unprimed seeds during imbibition. This might be one of the aspects of the positive effect of priming on the vigor of seeds. According to Soeda et al. (2005) expression of genes that encode for components of the protein synthesis machinery, such as protein initiation factors and translation and elongation factors, increase during priming. This fact may also partly explain why primed seeds had the highest germination percentages (Fig. 1a). It is probable that *Physalis angulata* primed seeds possess mechanisms to protect against salt stress through gene expression that adjust the enzymatic machinery, providing continuous tissue growth and radicle protrusion.

GENE EXPRESSION IN SEEDLINGS OF *P. angulata* UNDER SALINE STRESS

The changes caused by salt/oxidative stress result in molecular changes in the cell and in architectural and phenotypical patterns. Changes in molecular phenotype include changes in patterns of gene expression and protein synthesis. Changes may occur in the cytoskeleton due to a disproportionate increase in cell size, which leads to disorganized cells, resulting in impairment of physiological functions (Bertorello and Zhu 2009).

Expression of *SOS1* and *HAK1* hardly changed in the shoots and roots at the different salt concentrations to which the seedlings were subjected to the non-primed samples (Figs. 3a-2, a-3, b-2 and b-3). However, expression was higher in the shoots

of seedlings that originated from primed seeds, especially at 2 dS m⁻¹ in the shoots for *SOS1* (Fig. 3a-2) with a tendency for higher expression at 4 and 6 dS m⁻¹ and higher expression in the roots for both *SOS1* and *HAK1* at 6 dS m⁻¹ (Figs. 3a-3 and b-3).

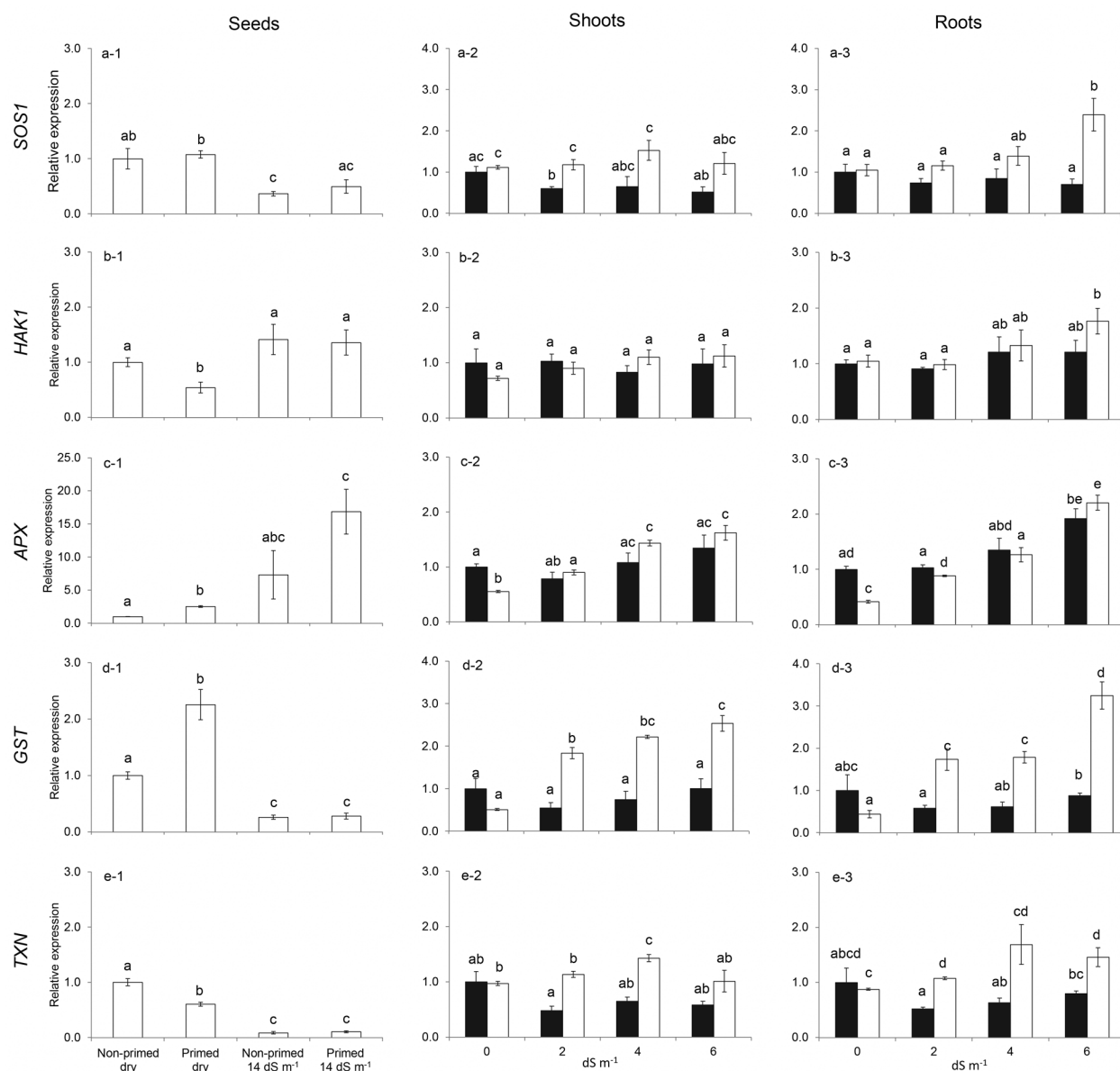


Figure 3 - Relative expression of *SOS1* (a), *HAK1* (b), *APX* (c), *GST* (d) and *TXN* (e) in non-primed and primed seeds, and in shoots and roots of seedlings derived from non-primed and primed seeds submitted to saline stress. (1) relative expression of the respective genes in non-primed and primed dry seeds and in non-primed and primed seeds subjected to saline stress for 10 days in 14 dS m⁻¹ salt solution; (2), (3) relative expression of the respective genes in shoots (2) and roots (3) of seedling derived from non-primed (black bars) and primed seeds (white bars) that were grown under saline stress from zero (water) up to 6 dS m⁻¹ salt solution. Salt concentrations expressed as salt electric conductivity (dS m⁻¹). Error bars indicate \pm SE of 3 biological replicates. Lower-case letters indicate significant differences (Student t-test at $P < 0.05$).

These results might partly be explained by the fact that priming has an important role in expression of proteins that retain the organization of the cell membranes. Many genes expressed during priming, encode for membrane proteins, which probably provided beneficial effects to the maintenance of cell functions and regulation of the ions present in greater amounts during salt stress (Varier et al. 2010). Studies with *Arabidopsis thaliana* showed that *SOS1* is not essential to plant growth and normal development, but is critical to the development of tolerance to salt (Wu et al. 1996, Shi et al. 2002). In studies with tomato Olías et al. (2009) showed that *SOS1* gene silencing resulted in negative effects on plant growth under salt stress. It was shown that, besides its main action in the extrusion of Na^+ out of the root tissue, *SOS1* is critical for the partitioning of Na^+ in plant organs, besides participating in the retention of Na^+ in stems of tomato, to prevent it from reaching photosynthetic tissues.

According to Su et al. (2002) salt stress increased the expression of family members of the *AKT* and *KAT* genes, including *HAK1* in common ice plant. Potassium channels are also important in the regulation of homeostasis. They have high affinity for K^+ , but also have affinity for Na^+ . Therefore, they can also transport Na^+ , especially when the K^+/Na^+ ratio is low (Pardo and Quintero 2002). The K^+ transporting function of *HAK1* is competitively inhibited by the presence of high concentrations of Na^+ , thereby sharing the transport route of the two monovalent cations (Santa Maria et al. 1997, Mian et al. 2011). This may partially explain the higher level of *HAK1* expression in the roots of *P. angulata* when the concentration of salt was increased.

The expression of *APX*, *GST* and *TXN* was mainly up regulated in plants that originated from primed seeds in shoots (Figs. 3c-2, d-2 and e-2) and roots (Figs. 3c-3, d-3 and e-3), although, this was partly the result of lower expression in shoots and roots of seedlings grown under control conditions.

In the shoots, up-regulation was especially observed for *GST* and *TXN* at all salt concentrations. Very similar patterns were observed for *GST* and *TXN* in the roots, whereas *APX* expression increased with increasing salt concentration in seedlings originated from both non-primed and primed seeds.

The production of reactive oxygen species in cellular compartments such as mitochondria and chloroplasts can change the nuclear transcriptome, indicating that there is a signal transmitted from these organelles to the nucleus. Although, the identity of this signal remains unknown, ROS sensors can be activated, inducing signaling cascades, and ultimately change the level of gene expression. Finally, ROS might change gene expression by altering the activities of transcription factors (Apel and Hirt 2004). Increased expression of *APX*, *GST* and *TXN* probably resulted in an increase of the synthesis of the antioxidant enzymes, which protected the shoots and roots from the negative effects of salt and oxidative damage. Our result suggest, therefore, that up-regulation of these genes is important for the observed increased salt tolerance of seedling from primed *P. angulata* seeds. It might seem conflicting that up-regulation of these genes is measured in shoots and roots, while increased biomass as a result of the priming treatment is only observed for shoots. We believe that the reason for this lies in the fact that also the roots need to be able to deal with the oxidative stress that is caused by the salt solutions in order to keep functioning optimally, and in that way be able to support better growth of the shoot.

Priming is an important technique that provides an increase in germination percentage and rate of *P. angulata* seeds, especially under salt stress. Furthermore, seedlings grown from primed seeds have a slightly higher biomass when grown under salt stress. We postulate that this may partly be the result of up-regulation of genes related to ion transport and genes coding for anti-oxidant enzymes. Although, further research is needed,

these results will help elucidate the molecular processes related to the link between priming and salt tolerance in *P. angulata*.

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RESUMO

Este estudo teve como objetivo avaliar os efeitos do osmocondicionamento na germinação de sementes submetidas ao estresse salino e expressão gênica em sementes e plântulas de *P. angulata*. Após serem osmocondicionadas por 10 dias, a germinação foi testada em bandejas plásticas contendo 15 ml de água (0 dS m⁻¹ – controle) ou 15 ml de solução de NaCl (2, 4, 6, 8, 10, 12, 14 e 16 dS m⁻¹). Avaliou-se massa fresca e seca da parte aérea e raízes de plântulas a 0, 2, 4, 6, 8 dS m⁻¹. O RNA total foi extraído de sementes e plântulas seguido de RT-qPCR. Os genes-alvo selecionados para este estudo foram: ascorbato peroxidase (*APX*), glutationa-S-transferase (*GST*), tioredoxina (*TXN*), proteína transportadora para o potássio 1 (*HAK1*) proteína altamente sensível ao sal 1 (*SOS1*). A uma eletrocondutividade de 14 dS m⁻¹ as sementes osmocondicionadas apresentaram germinação de 72%, em contraste com as sementes não osmocondicionadas que não germinaram. A expressão relativa de *APX* foi maior em sementes osmocondicionadas, o que pode ter contribuído para a manutenção de uma alta taxa de germinação em concentrações salinas elevadas. *GST* e *TXN* apresentaram aumento nos níveis de transcritos na parte aérea e raízes de plântulas provenientes de sementes osmocondicionadas. O osmocondicionamento incrementou a germinação de sementes, bem como a tolerância à salinidade, o que está correlacionado com

o aumento da expressão de *APX* em sementes e *SOS1*, *GST* e *TXN* em plântulas.

Palavras-chave: RT-qPCR, germinabilidade, crescimento de plântulas, salinidade.

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