DOI: 10.32615/bp.2019.046 BIOLOGIA PLANTARUM 63: 511-518, 2019

Exogenous spermidine enhances expression of Calvin cycle genes and photosynthetic efficiency in sweet sorghum seedlings under salt stress

A.I. EL SAYED[1]*, M.A.M. EL-HAMAHMY[2], M.S. RAFUDEEN[3], and M.K.H. EBRAHIM[4,5]

Biochemistry Department, Faculty of Agriculture, Zagazig University, 44519 Zagazig, Egypt[1]
Department of Agricultural Botany, Faculty of Agriculture, Suez Canal University, 41522 Ismailia, Egypt[2]
Department of Molecular and Cell Biology, University of Cape Town, Private Bag, 7701 Rondebosch, South Africa[3]
Biology Department, Faculty of applied sciences, Umm Al- Qura University, ***postal code?*** *Makkah Al-Mukarramah, KSA[4]*
Botany Department, Faculty of Science, Tanta University, 31257 Tanta, Egypt [5]

Abstract

Salinity adversely affects plants resulting in disruption to plant growth and physiology. Previously, it has been shown that these negative effects can be alleviated by various exogenous polyamines. However, the role of spermidine (Spd) in conferring salinity tolerance in sorghum is not well documented. The effect of exogenous Spd on the responses of sweet sorghum (*Sorghum bicolor* L.) seedlings to salt stress (150 mM NaCl) was investigated by measuring photosynthetic carbon assimilation, Calvin cycle enzyme activities, and the the expression of respective genes. Application of 0.25 mM Spd alleviated the negative effects of salt stress on efficiency of photosystem II and CO_2 assimilation and increased the activities of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and aldolase. Salt stress significantly lowered the transcriptions of genes encoding Rubisco large subunit, Rubisco small subunit, 3-phosphoglyceric acid kinase, glyceraldehyde-3-phosphate dehydrogenase, triose-3-phosphate isomerase, fructose-1,6-bisphosphate aldolase, fructose-1,6-bisphosphate phosphatase, and sedoheptulose-1,7-bisphosphatase. However, transcriptions of genes encoding phosphoribokinase and Rubisco were up-regulated. The Spd application enhanced expressions of most of these genes. It appears Spd conferred salinity tolerance to sweet sorghum seedlings by enhancing photosynthetic efficiency through regulation of gene expressions and activities of key CO_2 assimilation enzymes.

Additional key words: aldolase, CO_2 assimilation rate, NaCl, photosystem II, phylogenetic analysis, Rubisco.

Introduction

Salinity is a major environmental factor inhibiting crop growth and productivity (Parida and Das 2005). It has been observed that in various plants, salinity induces an increase in endogenous polyamines (Das *et al.* 1995, Chattopadhayay *et al.* 2002). Polyamines, such as putrescine (Put), spermidine (Spd), and spermine (Spm) are involved in various physiological and biochemical processes related to the regulation of plant responses to different environmental stresses (Takahashi *et al.* 2010, Roychoudhury *et al.* 2011). This allows the plant to be protected from these stresses by scavenging free radicals, stabilizing membranes, maintaining a cation-anion balance, and stimulation of ATP synthesis (Bouchereau *et al.* 1999, Ioannidis *et al.* 2006). Furthermore, polyamines have pivotal roles in many other cellular processes including gene expression, DNA and protein synthesis, regulation of ion channels, and providing protection from oxidative damage (Duan *et al.* 2008). Several studies reported that application of exogenous polyamines is an effective approach for enhancing salinity tolerance of plants and for protecting plant cell structure; they could be used to improve the productivity of many crops under salinity

Submitted 8 November 2018, *last revision* 21 January 2019, *accepted* 29 January 2019.
Abbreviations: $\Phi_{PS\ II}$ – photosynthetic quantumyield; Chl - chlorophyll; FBA - fructose-1,6-bisphosphate aldolase also known as aldolase (ALD); FBPase - fructose-l,6-bisphosphate phosphatase; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; MDA - malondialdehyde; PGK - 3-phosphoglyceric acid kinase; PRK - ribulose-5-phosphate kinase; PS - photosystem; Put - putrescine; RbcL - ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RbcS - ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; RCA - ribulose bisphosphate carboxylase/oxygenase activase; Rubisco - ribulose bisphosphate carboxylase/oxygenase; SBPase - sedoheptulose-1,7-bisphosphatase; Spd - spermidine; Spm - spermine; TPI - triose-3-phosphate isomerase.
Acknowledgement: The author thanks the Zagazig University for supporting the research.
* Corresponding author: e-mail: aelsayed@zu.edu.eg

stress (Chattopadhayay *et al.* 2002, Verma and Mishra 2005, Chai *et al.* 2010, Roychoudhury *et al.* 2011, Shu *et al.* 2014). Spermidine is the most important PA and is closely associated with stress tolerance in several plants (Kasukabe *et al.* 2004). It was shown that the application of Spd results in enhancing salinity tolerance of maize (Jiang *et al.* 2000) and cucumber (Duan *et al.* 2008, Shu *et al.* 2014). Our previous study reported that application of Spd successfully alleviates salt stress by enhanced antioxidant defense systems in wheat seedlings (El Sayed *et al.* 2018). Furthermore, exogenous Spd plays major roles in preventing plasma membrane damage in rice plants in response to salinity stress by preserving membrane integrity (Roy *et al.* 2005 Roychoudhury *et al*. 2011). This is supported by studies which show Spd-mediated protection of antioxidant capacity and decreased content of superoxide anion and malondialdehyde (Liu *et al.* 2006, Farooq *et al.* 2009). It is also speculated that Spd could act in stress signaling pathways (Kasukabe *et al.* 2004).

Salinity stress induces a decrease in photosynthetic efficiency that is often associated with inhibition of photosystem II (PSII) activity (Lu and Vonshak 2002, Xia *et al.* 2004, Kalaji *et al.* 2011), and it is considered to be the main site of salt stress-mediated damage to electron transport processes (Baker 1991, Mehta *et al.* 2010). Plants treated with Spd alleviate the salinity-induced inhibition of photosynthetic capacity, but this effect depends on Spd concentration and the severity of stress (Shu *et al.* 2014). It has been reported that exogenous Spd increases photosynthetic rates and photochemical efficiency of PS II of cucumber seedlings under salt stress (Duan *et al.* 2008). Despite studies showing the involvement of polyamines in plant responses to abiotic stresses, the mechanism of exogenous Spd alleviation of salt induced inhibition of photochemical efficiency in higher plants remains largely unknown.

In the present study, we tried to understand the physiological function of exogenous Spd in the tolerance of sweet sorghum to salt stress. The importance of this crop not only for feeding animals but also as an alternative source of energy has been highlighted (Smith and Buxton 1993, Steduto *et al.* 1997). In addition, various sweet sorghum cultivars are able to grow under soil salinity and water deficiency. We therefore examined how exogenous Spd affects sweet sorghum response to salinity with respect to photosynthetic capacity, carbon assimilation, Rubisco and aldolase activities, and the transcription of genes encoding enzymes involved in the Calvin cycle.

Materials and methods

Plants and treatments: Seeds of sweet sorghum (*Sorghum bicolor* L. cv. Topper-76-6) were surface sterilized with 3 % (m/v) sodium hypochlorite solution for about 15 min and rinsed several times with sterile distilled water. Aseptic seeds were germinated in Petri dishes between two sheets of filter paper moistened with sterile water, and the seeds were germinated in a growth chamber at a temperature of 29 ± 1 °C. The germinated seeds were then transferred and grown in pots containing washed quartz sand with a half strength Hoagland nutrient solution (three plants per pot) in a growth chamber with a 12-h photoperiod, an irradiance of 450 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a 60 % relative humidity. Ten-day-old seedlings were subjected to salt stress by adding NaCl to the Hoagland nutrient solution. To avoid osmotic shock, the seedlings were exposed to an initial concentration of 50 mM NaCl, which was gradually increased by 50 mM a day until the desired concentration was reached (100 or 150 mM). We used five different treatments: *1*) control - the half-strength Hoagland solution; *2*) the half-strength Hoagland solution with 100 mM NaCl); *3*) the half-strength Hoagland solution with 150 mM NaCl; *4*) the half-strength Hoagland solution with 100 mM NaCl and 0.25 mM Spd; and *5*) the half-strength Hoagland solution with 150 mM NaCl and 0.25 mM Spd. The experiments were done in triplicates. Samples of fresh seedlings were harvested, directly placed into liquid nitrogen, and stored at -80 °C before analyses. To determine fresh mass (f.m.), seedlings was weighed after washing with sterile double distilled water. Then they were dried in an oven at 70 °C for 72 h to determine dry mass (d.m.).

Chlorophyll content, PS II quantum yield, and CO_2 fixation rate: Leaves of sweet sorghum seedlings were harvested, acetone (80 %, v/v) was used for pigment extraction, and chlorophyll (Chl) *a* and Chl *b* were analyzed spectrophotometrically at the 663 and 645 nm, respectively. The relative content of Chl *a* and Chl *b* was calculated according to Lichtenthaler (1987). The quantum yield of PS II was measured as a variable to maximum Chl fluorescence ratio (F_V/F_M) using a *Mini-PAM* fluorometer (*Walz*, Effeltrich, Germany) as described by Oelze *et al*. (2012). Carbon dioxide gas exchange rates of sweet sorghum leaves under different treatments were measured with a portable gas exchange system (*GFS-3000*, *Walz*, Effeltrich, Germany).

Rubisco and aldolase activities: Rubisco activity in leaf samples (200 mg) was estimated following the method of Wang *et al*. (2009) with some modification. A homogenization buffer consisted of 33 mM Tris-HCl (pH 7.5), 0.67 mM Na_2-EDTA, 33 mM $MgCl_2$, and 10 mM $NaHCO_3$. The initial activity was determined in 150 mm^3 of assay mixture containing 100 mM Tris-HCl (pH 8.0), 2 mM Na_2-EDTA, 20 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM ATP, 0.4 mM NADH, 140 mM $NaHCO_3$, 2.5 U of GAPDH, 1 U of 3-phosphoglyceric phosphokinase, 0.06 mM ribulose-1,5-bisphosphate (RuBP) and 25 mm^3 of the enzyme homogenate. Enzyme activity was assayed by following decrease in absorbance at 340 nm for 3 min using a spectrophotometer. Aldolase activity was assessed following the method of Mustroph and Albrecht (2003). Aldolase activity was assessed by following a decrease in absorbance at 340 nm for 1 min. Total soluble protein was estimated by using the Bradford (1976) method.

Determination of mRNA content: Total RNA was isolated using a *RNeasy* mini kit (*Qiagen*, Hilden, Germany), and

cDNA synthesis were carried out using a first strand cDNA synthesis kit (*Fermentas*, Sankt Leon-Rot, Germany). The PCR program including the design of primer sets was optimized using semi-quantitative PCR with different numbers of PCR-cycles. Primer pair combinations used are listed in Table 1 Suppl. The results of reverse transcription semi-quantitative PCR of genes related to Calvin cycle enzymes are presented in Fig. 1 Suppl.

A real-time quantitative PCR analysis was performed using an *iCycler* thermal cycler (*Bio-Rad*, Hercules, USA) with *iQ SYBR Green Supermix* (*Bio-Rad*) in a final volume of 20 mm^3 following the manufacturer's instructions. The *iCycler* was programmed to 95 °C for 1 min, 45 cycles at (95 °C for 30 s, 58 °C for 40 s, and 72 °C for 45 s), and then a final extension at 72 °C for 10 min followed by melting curve program (55 - 95 °C in increasing steps of 0.5 °C). The *Actin* gene was used as a control for normalization. Efficiencies of each reaction were determined using the *LinRegPCR* software (Ruijter *et al.* 2009). Signal values were subsequently derived from the threshold cycles, with the average background subtracted, using the equation of Pfaffl (2001).

Alignment of sequences and construction of phylogenetic tree: Databank searches for homologies to Rubisco related genes [ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*RbcL*) and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*)] and key genes of saccharide metabolism [3-phosphoglyceric acid kinase (*PGK*), triose-3-phosphate isomerase (*TPI*), sedoheptulose-1,7-bisphosphatase (*SBPase*), fructose-l,6-bisphosphate phosphatase (*FBPase*), fructose-1,6-bisphosphate aldolase (*FBA*), ribulose-5-phosphate kinase (*PRK*), and ribulose bisphosphate carboxylase/oxygenase activase (*RCA*) were done (Fig. 5*G*,*H*). The transcriptional levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)] were performed using *FASTA* (https://blast.ncbi.nlm.nih.gov) and *WU-BLAST2* (https://blast.wustl.edu based on basic local alignment search tool algorithm programs. Deduced amino acids sequences were aligned using *CLUSTALW 2.1* applying the *Dayhoff PAM 250* matrix (Larkin *et al.* 2007). Phylogenetic relationships were determined using the *Geneious* program, *v. 10.2.4* (www.geneious.com). Trees were performed using the UPGMA method. Bootstrap analyses with 500 replicates were performed to assess the robustness of the branches of the phylogenetic trees. The accession numbers of sequences are listed in Fig. 2 Suppl.

Statistical analyses: All data were analyzed by one-way *ANOVA* (Steel *et al.* 1997) using the *MSTAT-C* statistical package (M-STAT 1990). Means were separated using the Fisher least significant difference test ($\alpha = 0.05$).

Results

The salt stress caused no obvious effect on the growth of sweet sorghum seedlings at 3 h after treatment (Fig. 1*A*,*B*). The treatment of Spd + 100 mM NaCl and Spd +150 mM NaCl caused an increase of fresh mass and dry mass at 15 d after treatment compared with salt stress alone (Fig. 1*A*,*B*). Carbon dioxide assimilation rate, photosynthetic quantum yield (Φ_{PSII}), and total Chl content were also investigated in sweet sorghum leaves under the same salt stress and exogenous Spd conditions (Fig. 2*A*-*D*). Differences in Chl content were observed only at 15 d and not at 3 h after treatment with 100 mM NaCl (Fig. 2*A*), however, total Chl content of the sorghum seedlings declined under 150 mM NaCl at both time points when compared to the non-stressed control (Fig. 2*A*). The treatment of Spd + NaCl caused a significant increase of total Chl content when compared with the sorghum seedlings exposed to NaCl alone. The Φ_{PSII} of the sweet sorghum seedlings was distinctly lower under salt stress treatments when compared with the control plants at both time points (Fig. 2*B*). The lowest Φ_{PSII} rate was observed under 150 mM NaCl treatment for 15 d. Exogenous Spd significantly increased Φ_{PSII} of the sorghum seedlings under salinity stress compared with the plants exposed to salt stress alone (Fig. 2*B*). Salt stress caused a significant decrease in both CO_2 assimilation rate and intercellular CO_2 concentration of the sorghum seedlings (Fig. 2*C*,*D*). The lowest CO_2 assimilation rate was observed after 3 h of 150 mM NaCl treatment. Exogenous Spd alleviated the salt stress-induced decrease in the assimilation rate and intercellular CO_2 concentration compared to NaCl stress alone (Fig. 2*C*,*D*).

Rubisco and aldolase activities were estimated in the sweet sorghum seedlings under NaCl stress and exogenous Spd treatments and compared with the untreated control plants (Fig. 3). Rubisco activity was significantly inhibited

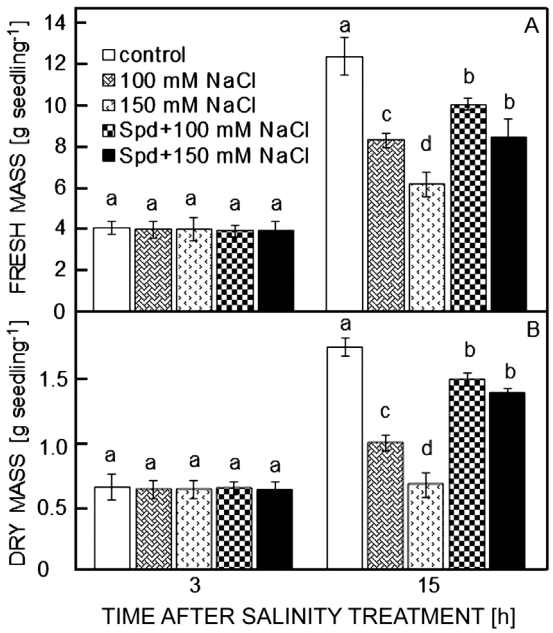

Fig. 1. Effects of 0.25 mM spermidine (Spd) on growth of sweet sorghum seedlings under 0, 100, and 150 mM NaCl measured after 3 h or 15 d (*A* - fresh mass and *B* - dry mass). Means ± SDs, $n = 6$ from three independent experiments. Different letters indicate significant differences among treatments ($P < 0.05$).

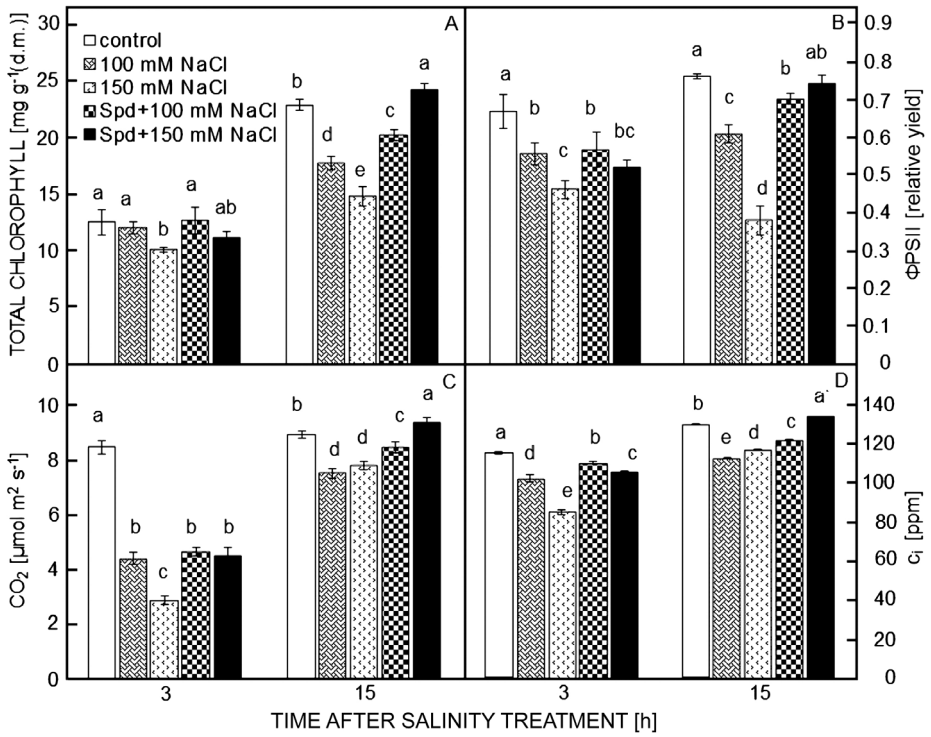

Fig. 2. Effects of 0.25 mM spermidine (Spd) on total chlorophyll content (*A*), quantum yield of PS II (ϕ_{PSII}) (*B*), CO_2 assimilation rate (*C*), and intercellular CO_2 concentration (c_i) (*D*) in sweet sorghum seedlings under 0, 100, or 150 mM NaCl. Means ± SDs, $n = 6$ from three independent experiments. Different letters indicate significant differences among treatments ($P < 0.05$).

under salt stress already at 3 h after treatment (Fig. 3*A*). The application of Spd caused an increase of Rubisco activity compared with salt stress alone and even the control plants (Fig. 3*A*). Salt stress reduced aldolase activity in the sorghum seedlings (Fig. 3*B*) and the effect of salt stress was more pronounced with 150 mM NaCl treatment at 3 h after treatment (Fig. 3*A*). The application of exogenous Spd enhanced aldose activity in comparison with salt stress alone but more at 100 mM NaCl (Fig. 3*B*).

Relative transcriptions of two genes involved in the Calvin cycle, namely ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*RbcL*) and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*) decreased in sorghum seedlings exposed only to salt stress when compared with the untreated control (Fig. 4*A*,*B*). The lowest *RbcL* expression was observed at 150 mM NaCl treatment at 3 h (Fig. 4*A*). The exogenous Spd increased the transcription of both genes under salt treatments (Fig. 4*A*,*B*).

Transcriptions of eight key genes involved in saccharides metabolism related to the Calvin cycle significantly varied after NaCl and Spd treatments (Fig. 5*A- H*). Under salt stress, the sorghum plants had significantly decreased transcriptions of *PGK*, *TPI*, *SBPase*, *FBPase*, and *FBA* at both time points (Fig. 5*A*,*C*,*D*,*E*,*F*). In contrast, there were increased transcriptions of *PRK* and *RCA* (Fig. 5*G*,*H*). Transcription of *GAPDH* decreased in response to 150 mM NaCl at 3 h but increased at 15 d compared to the unstressed control (Fig. 5*B*). Application of Spd enhanced transcriptions of *RCA* and *PRK* at 15 d compared to the unstressed plants (Fig. 5*G*,*H*). Transcriptions of *PGK, TPI, SBPase, FBPase*, and *FBA* increased when Spd was applied together with either 100 mM or 150 mM NaCl for 15 d compared with the respective salt-stressed plants. Transcriptions of *RCA, FBPase*, and *FBA* showed no significant differences between application of 100 mM NaCl and Spd + 100 mM NaCl at 3 h after treatments.

Ten genes involved in the Calvin cycle were used for phylogenetic analysis. Thirty sequences of *RbcL* gene were classified into two major groups (Fig. 2 Suppl.). Twenty divergent *RbcL* sequences were assembled in group I, which contained *Saccharum officinarum, Sorghum bicolor, Sorghastrum nutans*, and *Zea mays* with 100 % identity. Group I contained 10 sequences of *RbcL* from different plant species, with a sequence identity between 90 to 100 %. Regarding *RbcS* accessions, they were phylogenetically distant, belonging to two distinct clusters (Fig. 2 Suppl.). Sequence divergences of group I contained *RbcS* accessions (average percentage around 80 %), but *RbcS* from *Sorghum bicolor* was separated in a different group with *Saccharum officinarum, Zea mays, Miscanthus giganteus, Echinochloa crus-galli, Setaria italica*, and *Panicum hallii*. Phylogenetic analyses of amino acid sequences of *RCA*, *SBPase*, *FBA*, *FBPase*, *PGK*, *PRK* and *GAPDH* from different plant species were classified into two major groups with varied homology (Fig. 2 Suppl.) and *Sorghum bicolor* was separated into unique group associated with other *Poaceae* family species such as *Saccharum officinarum*, *Zea mays*, *Hordeum vulgare*, *Triticum aestivum*, and *Oryza sativa*. In contrast, the *TPI*

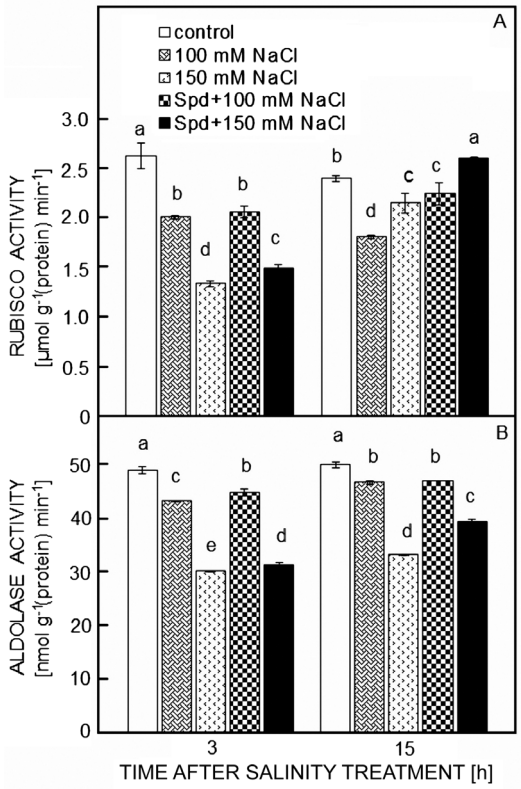

Fig. 3. Effects of 0.25 mM spermidine (Spd) on the activities of Rubisco (*A*) and aldolase (*B*) in sweet sorghum seedlings under 0, 100, or 150 mM NaCl. Means ± SDs, $n = 6$ from three independent experiments. Different letters indicate significant differences among treatments ($P < 0.05$).

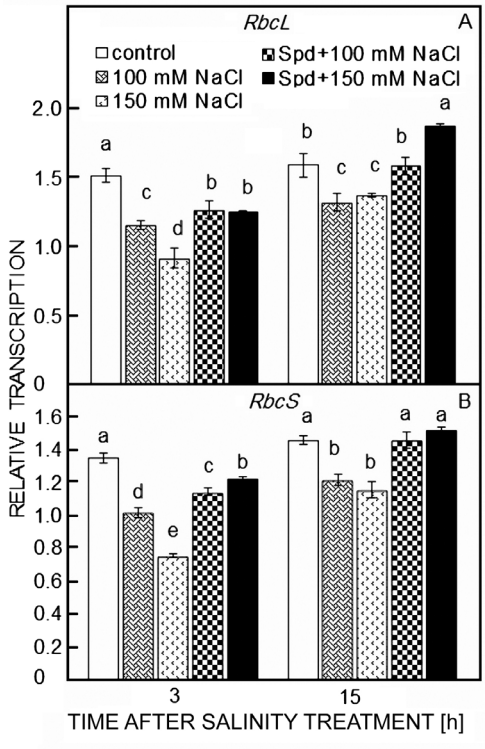

Fig. 4. Relative transcriptions of genes encoding *RbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) (*A*) and *RbcS* (ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit) (*B*) in sweet sorghum seedlings treated with 0, 100, or 150 mM NaCl alone or with of 0.25 mM spermidine (Spd) for 3 h or 15 d. Transcript amounts were quantified by quantitative PCR and made relative to *actin* transcription. Means ± SDs from three independent experiments with two technical replicates each. Different letters indicate significant differences among treatments ($P < 0.05$).

was not detected in the *Sorghum bicolor* genome according to available data presented at *GenBank*.

Discussion

Salinity is an environmental stress which results in limitation of CO_2 fixation and in further decrease of photosynthetic capacity (Barhoumi *et al*. 2007). Paschalidis and Roubelakis-Angelakis (2005) suggested that exogenous Spd can regulate various physiological processes including plant growth and development. In the present study, salt stress significantly decreased Chl content and Φ_{PSII} at both 3 h and 15 d of 150 mM NaCl treatment (Fig. 2*A*,*B*). Reduction of Chl content and damage to PS II are important factors leading to a lower photosynthetic rate. Application of Spd significantly enhanced Chl content and Φ_{PSII}, thus improving plant growth under salt stress compared with the plants grown only under NaCl stress. The increase in photosynthesis may be attributed to the ability of Spd to enhance stomatal conductance and thus increase CO_2 concentration in the cells (Shu *et al.* 2014). It was also shown that exogenous Spd prevents the degradation of Chl in salt-stressed plants and protects the integrity of PS II (Roychoudhury *et al.* 2011, Shu *et al.* 2012). These results suggest that exogenous Spd optimizes energy distribution and protects the structure and function of the photosynthetic apparatus, thereby promoting initial electron transfer in PS II (Tang *et al.* 2018). We also observed that salt stress inhibited CO_2 assimilation and intercellular CO_2 concentration of the sweet sorghum seedlings (Fig.2*C*,*D*). Exogenous Spd significantly increased CO_2 assimilation and intercellular CO_2 concentration in the sorghum seedlings which were treated with 150 mM NaCl at 15 d compared with the salt-stressed plants (Fig. 2*C*,*D*). The inhibition of photosynthesis in response to salt stress is also related to a restriction of the activity of Rubisco (Lu *et al.* 2009) . In this study, salt stress significantly inhibited Rubisco activity in the sorghum seedlings already at 3 h compared with the control plants (Fig. 3*A*). However, addition of Spd together with NaCl increased the activity of Rubisco at 15 d after treatment suggesting that exogenous Spd alleviated the reduction of Rubisco activity in the sorghum seedlings under salt stress. This suggests that application of Spd strongly regulates photosynthetic carbon assimilation (El Sayed *et al.* 2018). Photosynthetic CO_2 fixation rate is known to be limited by Rubisco activity at a relatively low CO_2 concentrations (Raines 2006). Therefore, the increased Rubisco activity, as a result of exogenous Spd, results in the maintenance of high photosynthetic CO_2 fixation rates

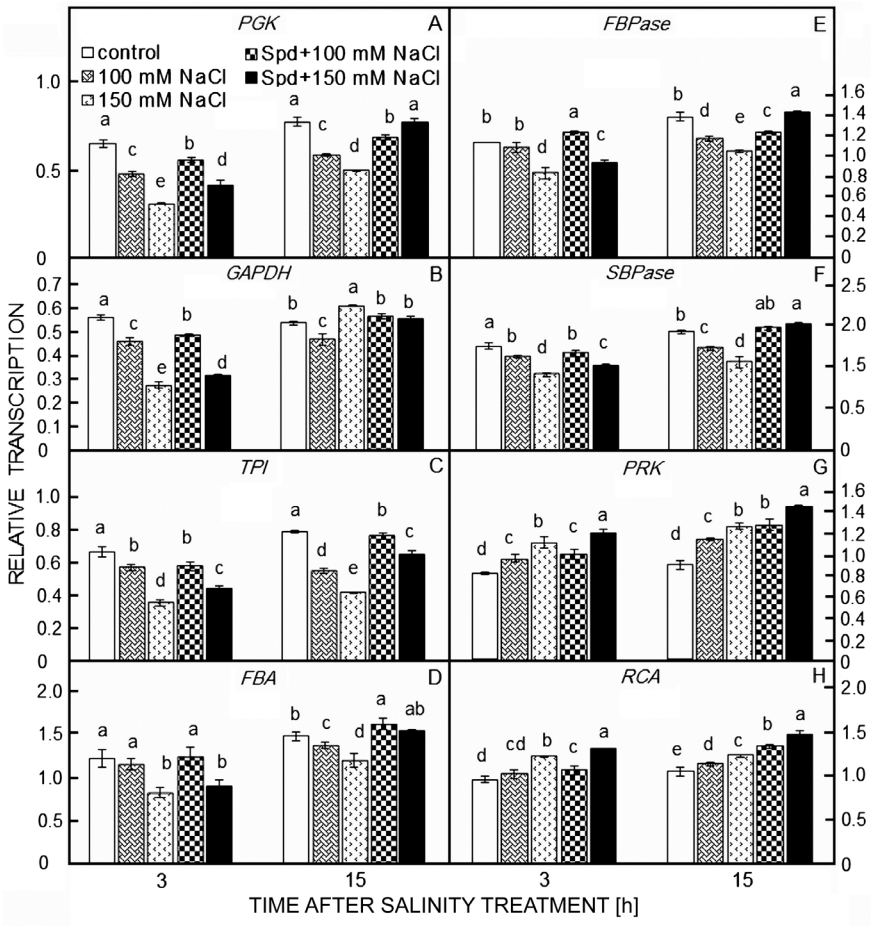

Fig. 5. Transcriptions of eight genes involved in sacharide metabolism related to the Calvin cycle in sweet sorghum seedlings treated with 0, 100, or 150 mM NaCl alone or together with 0.25 mM spermidine (Spd). *A* - *PGK* (3-phosphoglyceric acid kinase); *B* - *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase); *C* - *TPI* (triose-3-phosphate isomerase); *D* - *FBA* (fructose-1,6-bisphosphate aldolase); *E* - *FBPase* (fructose-l,6-bisphosphate phosphatase); *F* - *SBPase* (sedoheptulose-1,7-bisphosphatase); *G* - *PRK* (ribulose-5-phosphate kinase); and *H* - *RCA* (ribulose-bisphosphate carboxylase/oxygenase activase). Transcript amounts were quantified by quantitative PCR and made relative to *actin* transcription. Means ± SDs from three independent experiments with two technical replicates each. Different letters indicate significant differences among treatments ($P < 0.05$).

that lead to enhanced growth and productivity under salt stress. Aldolase activity was reduced in sorghum leaves under salt stress especially at 150 mM NaCl (Fig. 3*B*). Aldolase activity is also inhibited in tobacco plants under salt stress (Yang *et al.* 2008). In the plant, aldolase is a key enzyme involved in glycolysis, gluconeogenesis in the cytoplasm, and in regulation of CO_2 fixation (Raines 2003). Enhancement of aldolase activity could improve the flow of carbon through the Calvin cycle, which leads to an increase in amino acid and sucrose production (Raines 2003). This would also lead to production of osmolytes and so to stress tolerance (Negrao *et al* 2017). In our study, exogenous Spd increased the activity of aldolase in the salt-stressed sorghum seedlings suggesting that application of Spd accelerated the carbon assimilation pathway through the Calvin cycle, leading to enhancing photosynthetic capacity that may contribute to salt tolerance (Shu *et al.* 2014).

Calvin cycle enzymes are localized in chloroplasts and play an essential role in improvement of photosynthetic efficiency (Furbank and Taylor 1995, Raines 2003) by producing intermediates for glycolysis and/or building blocks for cellular components (Uematsu *et al.* 2012). In this study, ten genes related to enzymes involved in the Calvin cycle were investigated and their transcriptions after treatments with NaCl and Spd. Salt stress markedly decreased gene expressions of *RbcL* and *RbcS* at 3 h, but the decrease was alleviated by exogenous Spd (Fig. 4*A*,*B*). We speculate that the maintenance of photosynthesis in sorghum under salt stress by exogenous Spd could be due to the increased expressions of *RbcL* and *RbcS* genes as these two subunits regulate the structure and/or function of Rubisco (Spreitzer 2003, El Sayed *et al*. 2018). Expression of *PGK* was clearly lower under salt stress, but the application of Spd increased gene transcription. Salt stress also decreased the transcription of *GAPDH* at 3 h after treatment. Burzyński and Żurek (2007) suggested that *PGK* and *GAPDH* are enzymes necessary in the reduction

phase of the Calvin cycle. Moreover, the decreased expressions of *FBA* and *PGK* indicate that salinity stress affected carbon assimilation (Shu *et al.* 2014). However, exogenous Spd alleviated down-regulation of these genes to some extent, especially *PGK*, *TPI*, *FBA*, *FBPase*, and *SBPase*. Lv *et al*. (2017) suggested that enhancement of the activity of *FBA* could increase CO_2 assimilation in plant leaf tissues. In addition, the *FBA* gene family plays significant roles in plants in responses to abiotic stresses such as drought, salt, heat, and low temperature (Lv *et al.* 2017).

The function of *FBPase* has been observed at the branch point between the regenerative stage of the Calvin cycle and starch biosynthesis with *FBPase* and *SBPase* catalyzing irreversible reactions (Kossmann *et al.* 1994). Miyagawa *et al*. (2001) reported that the FBP/SBPase-overexpressing plants accumulate more sucrose, hexose, and starch compared to wild type plants. Tamoi *et al*. (2006) reported that increasing SBPase activity in transgenic tobacco overexpressing this gene causes an improvement in photosynthesis and increases growth rate. In this study, the up-regulation of *GAPDH*, *PRK*, and *RCA* in the sorghum seedlings may be explained by an adaptation to salt stress. The application of exogenous Spd during salt treatment could regulate the salt stress-mediated accumulation of transcripts of genes related to the Calvin cycle.

The phylogenetic analysis of enzymes involved in the Calvin cycle was performed for elucidation of the evolutionary history of these enzymes. As expected, based on the phylogenetic relatedness and the deduced amino acid sequences (Fig. 2 Suppl.) *RbcL*, *RbcS, GAPDH, RCA, FBA, FBPase, SBPase, PGK,* and *PRK* genes were identified in the *Sorghum bicolor* genome and shared a high relatedness to those in *Saccharum officinarum*, *Zea mays*, *Hordeum vulgare*, *Triticum aestivum*, and *Oryza sativa*. In contrast, the *TPI* gene was not found in the *Sorghum bicolor* genome according to available data presented at *GenBank*. Even though, *TPI* gene was expressed in the sorghum seedlings, and it was influenced by salt stress, primers were designed from the *TPI* gene of *Cucumis sativus* published on *GenBank* with accession number XM_004146969.2.

In summary, our results show that application of Spd was able to effectively up-regulate the transcription of Calvin cycle genes in order to improve and regulate the defense response of plants to salinity stress.

References

Baker, NR.: A possible role for photosystem II in environmental perturbations of photosynthesis. - Physiol. Plant. **81**: 563-570, 1991.

Barhoumi, Z., Djebali, W., Chaibi, W., Abdelly, C., Smaoui, A.: Salt impact on photosynthesis and leaf ultrastructure of *Aeluropus littoralis*. - J. Plant Res. **120**: 529-537, 2007.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J.: Polyamines and environmental challenges: recent development. - Plant Sci. **140**: 103-125, 1999.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. - Ann. Biochem. **72**: 248-254, 1976.

Burzyński, M., Żurek, A.: Effects of copper and cadmium on photosynthesis in cucumber cotyledons. - Photosynthetica **45**: 239-244, 2007.

Chai, Y.Y., Jiang, C.D., Shi, L., Shi, T.S., Gu, W.B.: Effects of exogenous spermine on sweet sorghum during germination under salinity. - Biol. Plant. **54**: 145-148, 2010.

Chattopadhayay, M.K., Tiwari, B.S., Chattopadhyay, G., Bose, A., Sengupta, D.N., Ghosh, B.: Protective role of exogenous polyamines on salinity-stressed rice (*Oryza sativa*) plants. - Physiol. Plant. **116**: 192-199, 2002.

Das, S., Bose, A., Ghosh, B.: Effect of salt stress on polyamine metabolism in *Brassica campestris*. - Phytochemistry **39**: 283-285, 1995.

Duan, J., Li, J., Guo, S., Kang, Y.: Exogenous spermidine affects polyamine metabolism in salinity-stressed *Cucumis sativus* roots and enhances short-term salinity tolerance. - J. Plant Physiol. **165**: 1620-1635, 2008.

ElSayed, A.I., Rafudeen, M.S., El-Hamahmy, M.A.M., Odero, D.C., Hossain, M.S.: Enhancing antioxidant systems by exogenous spermine and spermidine in wheat (*Triticum aestivum*) seedlings exposed to salt stress. - Funct. Plant Biol. **45**: 745-759, 2018.

Farooq, M., Wahid, A., Lee, D.J.: Exogenously applied polyamines increase drought tolerance of rice by improving leaf water status, photosynthesis and membrane properties. - Acta Physiol. Plant. **31**: 937-945, 2009.

Furbank, R.T., Taylor, W.C.: Regulation of photosynthesis in C3 and C4 plants: a molecular approach. - Plant Cell **7**: 797-807, 1995.

Ioannidis, N.E., Sfichi, L., Kotzabasis, K.: Putrescine stimulates chemiosmotic ATP synthesis. - BBA Bioenergetics **1757**: 821-828, 2006.

Jiang, X.Y., Song, J., Fan, H., Zhao, K.F.: Regulation of exogenous calcium and spermidine on ion balance and polyamine levels in maize seedlings under NaCl stress. - J. Plant Physiol. mol. Biol. **26**: 539-544, 2000.

Kalaji, H.M., Bosa, G.K., Kościelniak, J., Żuk-Gołaszewska, K.: Effects of salt stress on photosystem II efficiency and CO_2 assimilation of two Syrian barley landraces. - Environ. exp. Bot. **73**: 64-72, 2011.

Kasukabe, Y., He, L.X., Nada, K., Misawa, S.H, Ihara, I., Tachibana, S.: Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress regulated genes in transgenic *Arabidopsis thaliana*. - Plant Cell Physiol. **45**: 712-722, 2004.

Kossmann, J., Sonnewald, U., Willmitzer, L.: Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth. - Plant J. **6**: 637-650, 1994.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680, 1970.

Larkin, M.A., Blackshileds, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G.: Clustal W and Clustal X ver. 2.0. - Bioinformatics **23**: 2947-2948, 2007.

Lichtenthaler, H.K.: Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. - Methods Enzymol. **148**: 350-382, 1987.

Liu, J., Yu, B., Liu, Y.: Effects of spermidine and spermine levels on salt tolerance associated with tonoplast H^+-ATPase and H^+-PPase activities in barley roots. - Plant Growth Regul. **49**:

119-126, 2006.
Lu, C., Vonshak, A.: Effects of salinity stress on photosystem II function in cyanobacterial *Spirulina platensis* cells. - Physiol. Plant **114**: 405-413, 2002.
Lu, K.X., Cao, B.H., Feng, X.P., He, Y., Jiang, D.A.: Photosynthetic response of salt-tolerant and sensitive soybean varieties. - Photosynthetica **47**: 381-387, 2009.
Lv, G.-Y., Guo, X.-G., Xie, L.-P., Xie, C.-G., Zhang, X.-H., Yang, Y., Xiao, L., Tang Y.-Y., Pan, X.-L., Guo A.-G., Xu H.: Molecular characterization, gene evolution, and expression analysis of the fructose-1, 6-bisphosphate aldolase (FBA) gene family in wheat (*Triticum aestivum* L.). - Front. Plant Sci. **8**: 1030, 2017.
Mehta, P., Jajoo, A., Mathur, S., Bharti, S.: Chlorophyll α fluorescence study revealing effects of high salt stress on photosystem II in wheat leaves. - Plant Physiol. Biochem. **48**: 16-20, 2010.
Miyagawa, Y., Tamoi, M., Shigeoka, S.: Overexpression of a cyanobacterial fructose-1,6/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. - Nat. Biotechnol. **19**: 965-969, 2001.
Mustroph, A., Albrecht, G.: Tolerance of crop plants to oxygen deficiency stress: fermentative activity and photosynthetic capacity of entire seedlings under hypoxia and anoxia. Physiol. Plant. **117**: 508-520, 2003.
Negrão, S., Schmöckel, S.M., Tester, M.: Evaluating physiological responses of plants to salinity stress. - Ann. Bot. **119**: 1-11, 2017.
Oelze, M.L., Vogel, M.O., Alsharafa, K., Kahmann, U., Viehhauser, A., Maurino, V.G., Dietz, K.J.: Efficient acclimation of the chloroplast antioxidant defence of *Arabidopsis thaliana* leaves in response to a 10- or 100-fold light increment and the possible involvement of retrograde signals. - J. exp. Bot. **63**: 1297-1313, 2012.
Parida, A.K., Das, A.B.: Salt tolerance and salinity effects on plants: a review. - Ecotoxicol. Environ. Saf. **60**: 324-349, 2005.
Paschalidis, K.A., Roubelakis-Angelakis, K.A.: Spatial and temporal distribution of polyamine levels and polyamine anabolism in different organs/tissues of the tobacco plant. - Plant Physiol. **138**: 142-152, 2005.
Pfaffl, M.W.: A new mathematical model for relative quantification in real-time RT-PCR. - Nucl. Acids Res. **29**: e45, 2001.
Raines, C.A.: The Calvin cycle revisited. - Photosynth. Res. **75**: 1-10, 2003.
Raines, C.A.: Transgenic approaches to manipulate the environmental responses of the C3 carbon fixation cycle. - Plant Cell Environ. **29**: 331-339, 2006.
Roy, P., Niyogi, K., SenGupta, D.N., Ghosh, B.: Spermidine treatment to rice seedlings recovers salinity stress induced damage of plasma membrane and PM-bound H^+ ATPase in salt-tolerant and salt-sensitive rice cultivars. - Plant Sci. **168**: 583-591, 2005.
Roychoudhury, A., Basu, S., Sengupta, D.N.: Amelioration of salinity stress by exogenously applied spermidine or spermine in three varieties of indica rice differing in their level of salt tolerance. - J Plant Physiol. **168**: 317-328, 2011.
Ruijter, J.M., Ramakers, C., Hoogaars, W., Bakker, O., Van den Hoff, M.J.B., Karlen, Y., Moorman, A.F.M.: Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. - Nucl. Acids Res. **37**: e45, 2009.
Spreitzer, R.J.: Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch. Biochem. Biophys. **414**: 141-149, 2003.
Shu, S., Yuan, L.-Y., Guo, S.-R., Sun, J., Liu, C.-J.: Effects of exogenous spermidine on photosynthesis, xanthophyll cycle and endogenous polyamines in cucumber seedlings exposed to salinity. - Afr. J. Biotechnol. **11**: 6064-6074. 2012.
Shu, S., Chen, L., Lu, W., Sun, J., Guo, S., Yuan, Y., Li, J., Effects of exogenous spermidine on photosynthetic capacity and expression of Calvin cycle genes in salt-stressed cucumber seedlings. - J. Plant Res. **127**: 763-773, 2014.
Smith, G.A., Buxton, D.R.: Temperate zone sweet sorghum ethanol production potential. - Bioresour. Technol. **43**: 71-75, 1993.
Steduto, P., Katerji, N., Puertos-Molina, H., Unlu, M., Mastrorilli, M., Rana, G.: Water-use efficiency of sweet sorghum under water stress conditions. Gas exchange investigations at leaf and canopy scales. - Field Crops Res. **54**: 22-–234, 1997.
Steel, R.G.D., Torrie, J.H., Diskey, D.A.: Principles and Procedures of Statistics: a Biometrical Approach. 3rd Ed. - McGraw-Hill, New York 1997.
Takahashi, Y., Cong, R., Sagor, G.H.M., Niitsu, M., Berberich, T., Kusano, T.: Characterization of five polyamine oxidase isoforms in *Arabidopsis thaliana*. - Plant Cell Rep. **29**: 955-965., 2010.
Tamoi, M., Nagaoka, M., Miyagawa, Y., Shigeoka, S.: Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. - Plant Cell Physiol. **47**: 380-390, 2006.
Tang, S., Zhang, H., Li, L., Liu, X., Chen, L., Chen, W., Ding, Y.: Exogenous spermidine enhances the photosynthetic and antioxidant capacity of rice under heat stress during early grain-filling period. - Funct. Plant Biol. **45**: 911-921, 2018.
Uematsu, K., Suzuki, N., Iwamae, T., Inui, M., Yukawa, H.: Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants. - J. exp. Bot. **63**: 3001-3009, 2012.
Verma, S., Mishra, S.N.: Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. - J. Plant Physiol. **162**: 669-677, 2005.
Wang, H., Gu, M., Cui, J., Shi, K., Zhou, Y., Yu, J.: Effects of light quality on CO_2 assimilation, chlorophyll-fluorescence quenching, expression of Calvin cycle genes and carbohydrate accumulation in *Cucumis sativus*. - J. Photochem. Photobiol. B: Biol. **96**: 30-37, 2009.
Xia, J.R., Li, Y.J., Zou, D.H.: Effects of salinity stress on PSII in *Ulva lactuca* as probed by chlorophyll fluorescence measurements. - Aquatic Bot. **80**: 129-137, 2004.
Yang, X., Liang, Z., Wen, X., Lu, C.: Genetic engineering of the biosynthesis of glycinebetaine leads to increased tolerance of photosynthesis to salt stress in transgenic tobacco plants. - Plant mol. Biol. **66**: 73-86, 2008.