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Original article

In vitro and *in vivo* screening for the identification of salt-tolerant sugarcane (*Saccharum officinarum* L.) clones: molecular, biochemical, and physiological responses to salt stress

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ABSTRACT

Sugarcane is a glycophyte whose growth and yield can be negatively affected by salt stress. As the arable lands with potential saline soils expand annually, the increase of salt-tolerance in sugarcane cultivars is highly desired. We, herein, employed *in vitro* and *in vivo* conditions in order to screen sugarcane plants for salt tolerance at the cellular and at the whole plant levels. Calli of sugarcane cv. Khon Kaen 3 (KK3) were selected after culturing in selective media containing various NaCl concentrations, and regenerated plants were then reselected after culturing in selective media containing higher NaCl concentrations. The surviving plants were finally selected after an exposure to 254 mM NaCl under greenhouse conditions. A total of 11 sugarcane plants survived the selection process. Four plants that exhibited tolerance to the four different salt concentrations applied during the aforementioned screening process were then selected for the undertaking of further molecular, biochemical, and physiological studies. The construction of a dendrogram has revealed that the most salt-tolerant plant was characterized by the lowest genetic similarity to the original cultivar. The relative expression levels of six genes (i.e., *SoDREB*, *SoNHX1*, *SoSOS1*, *SoHKT*, *SoBADH*, and *SoMIPS*) were found to be significantly higher in the salt-tolerance clones than those measured in the original plant. The measured proline levels, the glycine betaine content, the relative water content, the SPAD unit, the contents of chlorophyll *a* and b, as well as the K^+/Na^+ ratios of the salt-tolerant clones were also found to be significantly higher than those of the original plant.When the salt-tolerant clones were grown in a low saline soil, they exhibited a higher Brix percentage than that of the original cultivar.

1. Introduction

Salinity is a prominent abiotic factor in arid and semi to arid areas, and it is responsible for inhibiting the growth, the development, and the production of commercially important crops of glycophytic and halophytic plants (Singh et al., 2022). In fact, a number of physiological, biochemical, and metabolic mechanisms have been shown to be affected by salinity. This salinity to induced effects is known to exert a negative impact on both agricultural production and food security (Sanwal, et al., 2022). The consequences of excessive salinity on plants are quite complicated; they involve alterations in plant shape, in growth, as well as in physiological and biochemical processes due to difficulties in water absorption and the toxicity of certain ions. The suppression of the main root growth, the reduction in the quantity of lateral roots, and the withering and yellowing of the leaves are also part of the observed consequences of salinity on plants. Moreover, salinity can prompt the decomposition of chlorophyll and, thereby, impair photosynthesis. As a result, salinity can exert detrimental effects on plant production,that can even lead to a total loss of the latter (Zhu, 2016; Simões et al., 2019; van Zelm et al., 2020).

Sugarcane is a glycophytic plant with moderate salt tolerance (Wahid et al., 1997). In soils having an electrical conductivity (EC) of 10.4 dS m^{-1}, sugarcane yield can be diminished by 50% (Santana et al., 2007). Punpee (1990) has demonstrated that at

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salinities with an EC greater than 12 dS m $^{-1}$, cane yield and glucose content can be decreased by about 50%, while the N-P-K uptake can be decreased by about 50% in ECs between 16 and 20 dS m^{-1}. Most of the sugarcane to planted areas has insufficient irrigation, thereby resulting in a lower sugarcane yield than the expected standard amount (Simões et al., 2019). Both the total cultivated area as well as the production of sugarcane in Thailand have been declining due to the fact that sugarcane is mainly grown under rain-fed conditions, the cumulative annual rainfall decreases, and the plant is subjected to drought stress. The consequence of the drought is that the saline soils become more intense.

Sugarcane cultivar (cv.) Khon Kaen 3 (KK3) accounts for 85% of the sugarcane production in Thailand (Office of the Cane and Sugar Board, 2021), which is the world's fourth largest sugarcane producer (Khumla et al., 2022). As a result, breeding for sugarcane salinity tolerance is critical for the increasing of the yield and the lowering of costs, and will enhance both the productivity expansion and the food production (Pérez-Jiménez and Pérez-Tornero, 2020; Singh and Sengar, 2020a). However, conventional methods of plant breeding for salt tolerance are time-consuming and limited by low germplasm collection, and inter-specific or inter-generic hybridization has had very occasional effectiveness in increasing crop plants' stress tolerance (Fita et al., 2015). Genetically modified (GM) sugarcane is one of the options for developing salt tolerance in sugarcane. Nevertheless, Thailand's policy on GM plants prohibits all open field trials of transgenic plants. Genetic variability created by tissue culturing provides a new source of variation (within a species) for use in crop development (Rai, 2022). Tissue culture-mediated selection via the use of somatic embryos (SE) or embryogenic callus (EC) can be a powerful tool for the selection of excellent salt tolerance at the cellular level, because it allows the control of the homogeneity of the stress. SE or EC have also been used in order to create clones with enhanced salt tolerance for crops. Al Khateeb et al. (2020) selected salt-tolerant date palms from embryo genic callus exposed to varied salt levels ranging from 0 to 300 mM in MS-medium for six passages with 4 weeks between passages; the surviving calli were regenerated and showed an excellent response to the high salinity levels. In addition, various research have examined salt-tolerant plants derived from calluses, such as wheat (Barakat and Abdel-Latif, 1996), rice (Revathi and Pillai, 2015), pomegranate (El Mahdy et al., 2022) and sugarcane (Gandonou et al., 2005; Shomeili et al., 2011). This technique is simple, easy to use, and highly reproducible (Lal et al., 2015; Lu et al., 2020; El–Mahdy, et al., 2022). However, the molecular, biochemical, and physiological characteristics of the selected clones have not been extensively assessed. The aim of this study was: (i) to select salinity tolerance sugarcane clones from the calli of the KK3 cv. (by using both *in vitro* and *in vivo* screening methods) and (ii) to examine certain biochemical and physiological characteristics as well as the expression of salt stress responsive genes in the selected clones.

2. Materials and methods

2.1. Plant material and culture conditions

Sugarcane (*Saccharum officinarum* L.) cv. KK3 was obtained from the Eastern Sugar and Cane Public Company Limited, Thailand, and was used for both the *in vitro* and the *in vivo* salinity tolerance selection screenings. Shoots with young leaves from 7 – 8 – month-old sugarcanes were cut and surface-sterilized for 10 min each with 20% and 10% v/v bleach (6% NaOCl) supplemented with two drops of 0.1% Tween 20, followed by three washes in sterile distilled water (for 1 min each). The most inner young leaves were sectioned into pieces of about 0.5-cm-length, and were cultured on solid MS (Murashige and Skoog, 1962) medium supplemented with 3 mg L^{-1} of 2,4-dichlorophenoxy acetic acid, 2% sucrose, and 10% (v/v) coconut juice (callus induction medium; CIM). The explants induced embryogenic calli in darkness at 25 °C (±1°C), for 8 weeks. After 4 weeks, the embryogenic calli were separated from the explants and were subcultured in the same fresh CIM for another 4 weeks in order to allow further proliferation.

2.2. Salt tolerance selection in vitro and in vivo

There were three rounds of selection. The first round of selection started with the transfer of embryogenic calli to CIM supplemented with various concentrations of NaCl, namely 86, 128, 170, and 212 mM (10 pieces per replication and 10 replicates in each NaCl concentration). The *in vitro* selection lasted 4 weeks. The surviving calli were transferred to NaCl-free CIM for 2 weeks in order to allow for the small surviving calli to further proliferate, and were then transferred to basal MS medium in order to allow for plant regeneration.

In order to make sure that there was no escape in the cell selection, the regenerated plantlets were aseptically transferred to hormone-free MS that was supplemented with NaCl for the second round of the selection. The NaCl concentrations were increased by 42 mM (i.e., from 86 to 128 mM, from 128 to 170 mM, from 170 to 212 mM, and from 212 to 254 mM). The plantlets that survived in 86 mM NaCl were again selected after an exposure to 128 mM NaCl, the ones that survived in 128 mM NaCl were reselected at 170 mM NaCl, and so on. The survival plants were recovered after 8 weeks of selection by transferring them to MS medium supplemented with 2 mg L^{-1} BA for multiple shoots induction to obtain at least three shoots per clone. These multiple shoots were separated into a single shoot and transferred to hormone-free ½ MS medium for 4 weeks for root induction. Then they were transferred to 20-cm-diameter pots without holes, containing mixed soil, being watered regularly, and being kept in a greenhouse for 2 months. Each pot received 10 mL of 1/5 Hoagland solution once a week.

The third round of selection began two months later. All plants derived from the second round of selection and the original plants; i.e., the regenerated plants that have not gone a selection through a NaCl-selective medium were watered once with 10 mL of 1/5 Hoagland solution supplemented with 254 mM NaCl. Subsequently, each pot was watered with 500 mL of tap water at 2-day intervals. The selection was carried out for 1 month or until the original plant died. All of the surviving plants were recovered by re-potting them in normal soil mixer until the plants have fully recovered. The plants that had previously been used in biochemical and physiological analyses were transferred so as to grow hydroponically for the undertaking of quantitative real time polymerase chain reaction (qRT-PCR) analysis before being returned to their pots. The plants, including the unselected ones, were then planted in a field with low salinity soil and an EC of 4.1 dS m^{-1}.

2.3. Random amplified polymorphic DNA (RAPD)–PCR analysis of genomic DNA

The genomic DNA was extracted from leaves of four 8-month-old sugarcane plants, each deriving from each concentration of NaCl of the third round of selection and one control plant, based on the Laksana and Chanprame (2015) method. The RAPD amplification was carried out in a 50 µL total reaction volume including template DNA (50 ng), 0.2 µM of 10-mer oligo-deoxynucleotide (RAPD primer; 1st BASE, Singapore), 2.5 mM dNTPs (4 µL each), 0.25 µL of TaKaRa Ex Taq™ (5 units/µL), and 5 µL of 10 × Ex Taq Buffer. Thirty RAPD primers were used. PCR was performed in a thermal cycler (Bio-Rad) with an initial denaturation at 94 °C for

1 min, 40 cycles of 94 °C for 30 sec, 37 °C for 30 sec, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were electrophoresed in a 1.2% agarose gel with 0.5 × TBE buffer. The sizes of amplicons were compared to a 1-kb DNA ladder (Fermentas), and were photographed under UV *trans*-illuminator. The similarity analysis was undertaken by using the MEGA 11 program with the UPGMA method.

2.4. qRT-PCR analysis

Analysis of the dehydration responsive element binding (*SoDREB*), the Na^+/H^+ antiporter (*SoNHX1*), the salt overly sensitive (*SoSOS1*), the high-affinity potassium transporter (*SoHKT*), the betaine aldehyde dehydrogenase (*SoBADH*), and the *myo*-inositol-1-phosphate synthase (*SoMIPS*) gene expression was undertaken in leaves and roots through qRT-PCR. One clone from each concentration of NaCl which survived from the third round of selection and the non-selected clone were chosen. Each clone contained three plants. They were hydroponically grown in a 1/5 Hoagland solution supplemented with 170 mM NaCl. The total RNA of each plant was extracted from leaves and roots after 0, 1, 3, and 5 days of NaCl stress, following the Laksana and Chanprame (2015) method. The entire RNA was reverse-transcribed by using the SuperScript III First-Strand Synthesis System (Invitrogen) so as to generate the first-strand cDNA. The glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*; ACNO. 254672) and the eukaryotic elongation factor 1-alpha (*Eef-1a*; ACNO. EF581011.1) were used as reference genes for the normalization of the expression of the six genes assessed. Both reference genes were determined to be appropriate for the normalization of gene expression under salinity/drought conditions in sugarcane (Guo et al., 2014).

Subsequently, 500 ng of the first strand cDNA template, 1 × SensiFAST SYBR No-ROX mix buffer (Bioline Reagent Ltd), 0.4 M each of the forward and the reverse primers (Table 1) were added to a total volume of 20 μL for the undertaking of PCR reactions. The initial denaturation at 95 °C for 30 sec was followed by 40 cycles of denaturation at 94 °C for 5 sec, annealing at 58 °C for 15 sec, and an extension at 72 °C for 10 sec in a CFX96 Touch Real-Time PCR (Bio-Rad®). The gene expressions were compared to the control condition (day 0). For each sample, three biological and three technical duplicates of the reaction were conducted. The relative expression of each gene was determined by using the $2^{-\Delta\Delta Cq}$ values (Livak and Schmittgen, 2001).

2.5. Physiological and biochemical analyses

Certain physiological and biochemical characteristics concerning the salt stress response were analyzed from the leaves and the roots of the three plants of each clone as the same clone as 2.4. All measurements took place 3 weeks after the third round of selection was started, with the exception of the Brix percentage that was assessed in 8-month-old plants.

Table 1
The primers used for qRT-PCR.

Primer name	Primer sequence	Gene
SoDREB F	GCTCCTTCCCTACTGCTGTG	*SoDREB*
SoDREB R	CACTAGATGCCAGCAACGAA	
SoMIPS F	CCCCAAGTCCGTCAAGTACA	*SoMIPS*
SoMIPS R	CTTGGTCGCCCATGAGATCC	
SoSOS F	GGAACAATGTTTGTGTTCTT	*SoSOS1*
SoSOS R	TCTTCAAGCATTCCCCAGTA	
SoNHX F	TGCTCCATTTCAGCGAGGAC	*SoNHX1*
SoNHX R	AGCGCCAAGGGATATCACAG	
SoHKT F	GCTGCTGTCACCGCTGGC	*SoHKT*
SoHKT R	CAGCCTCGAGCAGCTGTA	
SoBADH F	TCCGAATTGGCTTCTGTGACT	*SoBADH*
SoBADH R	CTTGTCAACATCTGGGTGCG	
SoGAPDH F	CACGGCCACTGGAAGCA	*SoGAPDH*
SoGAPDH R	TCCTCAGGGTTCCTGATGCC	
SoEef-1a F	TTTCACACTTGGAGTGAAGCAGAT	*SoEef-1a*
SoEef-1a R	GACTTCCTTCACAATCTCATCATAA	

2.5.1. Proline content measurement

Fresh leaves (500 mg) were homogenized in 3% (w/v) sulphosalicylic acid, and were centrifuged at 4,000 g for 10 min at 4 °C. In a test tube, the supernatants were combined with acid ninhydrin and glacial acetic acid. In sealed test tubes, the mixture was heated for 30 min at 98 °C, and was then allowed to cool down before 4 mL of toluene were added and the absorbance was measured at 520 nm by using toluene as blank. The proline content in the extracts was calculated according to Bates et al. (1973) and Stoleru et al. (2019).

2.5.2. Glycine betaine (GB) levels' measurement

For GB, the fully developed uppermost leaves of plants cultivated under control and salt-stressed conditions were collected and analyzed by using the Grieve and Grattan (1983) method. In 20-mL test tubes, the leaf extracts were produced by cutting 0.5 g of leaves into 5 mL of a toluene-water mixture (0.05% toluene). The tubes were then shaken for 24 h at 25 °C and filtration, 1 mL of a 2-N HCl solution and 0.1 mL of a potassium triiodide solution were added to 0.5 mL of the extracts, and were then shaken in an ice-cold water bath for 90 min. Two layers were separated by passing a continuous air stream over the sample for half a minute; the upper aqueous layer was removed, and the optical density of the organic layer was measured at 365 nm. Quantities of GB were determined using a standard curve derived from samples of GB with varied concentrations of GB.

2.5.3. Relative water content (RWC)

Fresh leaves (1.5 g) were excised and weighed, and were then hydrated by soaking in water for 24 h, at room temperature (so as to restore their full turgor) before their turgid weight was measured. In order to determine their dry weight (DW), these leaves were rapidly dried in a hot air oven for 72 h, at 80 °C. The *RWC* was determined according to Hasanuzzaman et al. (2018).

2.5.4. Relative amount of chlorophyll content (RCC)

The RCC was determined by a handheld chlorophyll meter (Chlorophyll Meter SPAD-502; Konica Minolta, Tokyo, Japan) (Minolta, 1989). Five readings per plant were taken from the widest portion of the leaf lamina of the third fully expanded leaf and/or from still green leaves, so as to avoid major veins. The values were then averaged as SPAD units. The RCC measurement was performed on sunny days, between 9:00 and 11:00 am.

2.5.5. Pigment content

The chlorophyll *a* and chlorophyll *b* contents of leaves were determined. Approximately 200 mg of a fresh leaf were pounded into a powder in a mortar half-filled with liquid nitrogen. The pigments were extracted from the leaf powder by adding 2.0 mL of an extraction solvent chilled with ice. The extraction solvent consisted of 85 percent acetone and 15 percent Tris stock buffer (1% Tris final concentration; pH 8, adjusted with HCl). The pigments were then centrifuged for three minutes at 12,000g. One milliliter of the supernatant was diluted to three milliliters, and its absorbance at 537, 663, 647, and 470 nm was measured in a cell with a path length of one centimeter (Sims and Gamon, 2002).

2.5.6. Brix value measurement

Total soluble solids (TSSs) that include sucrose were observed in 8-month-old sugarcane plants. The sugarcane juice extracted from the bottom, the middle, and the top of the stem was mixed

together. The Brix value were measured by a refractometer probe (RX-5000α; ATAGO Co. Ltd., Tokyo, Japan).

2.5.7. Analysis of the K^+/Na^+ content

The sugarcane leaves (1 g) from before and after 3 weeks of irrigation with 1/5 Hoagland solution supplemented with 254 mM NaCl were cleaned twice with Milli Q water, were crushed in 5 mL of Milli Q water, and were centrifuged at 4,000 rpm for 10 min. The supernatants of the extracts were measured by using a Na^+ and K^+ meter (LAQUAtwin).

2.6. Statistical analysis

R program was applied to perform statistical analysis (R Core Team, 2012). To compare data differences, an ANOVA was utilized, followed by Duncan's multiple range tests. At $p < 0.05$, the differences were statistically significant.

3. Results

3.1. In vitro and in vivo salt tolerance selection

Sugarcane is a glycophyte and is very sensitive to saline soils. Biotechnology has been used in order to accelerate sugarcane breeding with the aim of improving its salt tolerance. Tissue culturing is a highly effective tool for the selection of salt-tolerant sugarcane. Plant tissue culturing was employed by this study in order to select salt tolerance sugarcane calli deriving from the KK3 cv. and to then regenerate them into complete plants.

First round of selection: Embryogenic calli were transferred directly to CIM supplemented with 86, 128, 170, and 212 mM NaCl, and were cultured for 5 weeks. Subsequently, the surviving calli were transferred to a NaCl-free CIM medium suitable for callus recovery and proliferation, and were then allowed to regenerate in hormone-free MS medium. Our results have shown that when the calli were grown in media with increasing salt concentrations, the relative growth rates of the NaCl tolerant calli decreased. However, the calli that survived from each of the selection media were able to regenerate with a significantly different number of plantlets. The calli grown in the medium containing 86 mM of NaCl produced the most shoots (38 plants), while the calli grown in the medium containing 212 mM of NaCl produced the fewest shoots (4 plants; Table 2; Fig. 1). In the second round of selection, all the surviving plantlets from each NaCl concentration were submitted to a reselection process.

Second round of selection: Each plantlet was transferred into a new medium containing an increased (by 42 mM) NaCl concentration more the respective original one in the first round of selection; for example, 128 mM instead of 86 mM, 170 mM instead of 128 mM, and so on. The selection was carried out for 8 weeks. We found that some plantlets died in this round of selection. In the media containing 128, 170, 212, and 254 mM of NaCl, the 38, 22, 15, and 4 plants deriving from the first round media containing 86, 128, 170, and 212 mM of NaCl, were reduced to 20, 14, 7, and 2 plants, respectively (Table 2). The surviving plantlets deriving from the first three NaCl concentrations (i.e., 128, 170, and 212 mM) produced multiple shoots, while the plantlets that survived after an exposure to the highest NaCl concentration (254 mM) produced no multiple shoots (Fig. 2). In order to prepare for the third round of selection, all surviving plantlets were transferred to a NaCl-free MS medium plus 2 mg L^{-1} BA, allowing them to recover and produced multiple shoots. The new roots were induced in ½ MS hormone-free medium.

Third round of selection: All recovered plants were subjected to salt stress conditions (254 mM NaCl) under greenhouse conditions. The original plants died within one month. The plants that survived after growing at NaCl concentrations between 128 and 212 mM NaCl during the second round of selection exhibited some degree of wilting and dry leaves, while the 254-mM NaCl tolerant plants looked healthy with only a few dry leaves (Fig. 3). At the end of this round of selection, we were able to recover 2, 3, 4, and 2 plants originating from media containing 128, 170, 212, and 254 mM NaCl of the second round of selection, respectively (Table 2).

Table 2
Number of plants derived from each round of selection.

NaCl (mM)	Regenerant plant (First selection)	Survival plants	
		Second selection	Third selection (254 mM)
86	38	–	–
128	22	20	2
170	15	14	3
212	4	7	4
254	–	2	2

3.2. RAPD–PCR analysis of genomic DNA

A RAPD-PCR analysis was performed on five genomic DNA samples extracted from the leaves of four 8-month-old plants deriving from the third round of selection and of one original cv. The four salt tolerance plants tolerated four different levels of salt stress. A total of 30 RAPD primers were used. Of these, 24 RAPD primers exhibited polymorphisms and scoreable bands, and a dendrogram was constructed. These sugarcane plants were divided into two groups. The first group included the control (the original cv.) and the plants surviving the first three levels of the salt stress tolerance selection process, whereas the second group only included one plant, the plant exhibiting the highest salt stress tolerance. We also discovered that the lines exhibiting lower salt tolerance resembled the original cv. more than those exhibiting higher tolerance (Fig. 4).

3.3. qRT-PCR analysis of salt stress responsive genes

The relative expressions of six salt stress responsive genes under salinity conditions were analyzed through qRT-PCR. These genes were *SoDREB*, *SoNHX1*, *SoSOS1*, *SoHKT*, *SoBADH* and *SoMIPS*. cDNA was synthesized from total RNA extracted from leaves and roots of the plants deriving from the third round of selection and subjected to 170-mM NaCl stress conditions for 0, 1, 3, and 5 days. Our findings have revealed that the transcription levels of the aforementioned six genes in the salt-tolerant plants were upregulated and exhibited a significantly different expression when compared with the control plant after being submitted to the salt stress. Most of these genes have displayed a similar pattern of expression, with the relative expression increasing as the stress duration increased. In terms of the salt stress tolerance, the more salt-tolerant plants exhibited higher expression levels of these genes than the less salt-tolerant plants did.

The expression patterns of the transcription factor-expressing *SoDREB* had a similar pattern in roots and leaves. When the salt stress duration was increased, the relative expression levels were also increased, and the relative expressions were the highest at day 5 of the stress treatment. However, the expression of this gene in the leaves was higher than that in the roots. It was also found that at each stress duration timepoint assessed, the expression levels of the gene in the more salt-tolerant plants were higher than those in the less salt-tolerant plants (Fig. 5A1 and 5A2).

SoNHX1 and *SoSOS1* are genes expressing proteins of the Na^+/H^+ antiporter group, while *SoHKT* is a high-affinity K^+ transporter gene. The expression patterns of these three genes were similar,

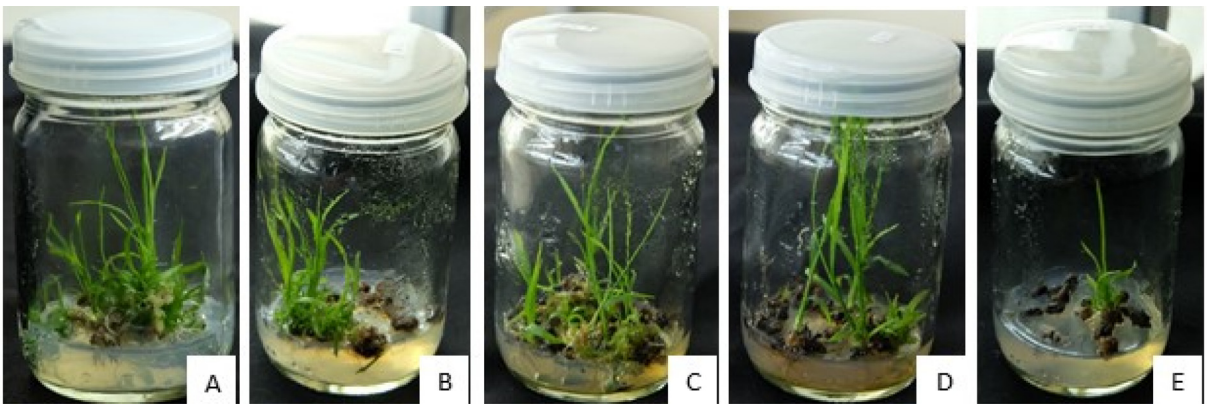

Fig. 1. The plantlets regenerated from the first round of selection at different NaCl concentrations. Note: A, control; B, 85 mM; C, 128 mM; D, 170 mM; E, 210 mM.

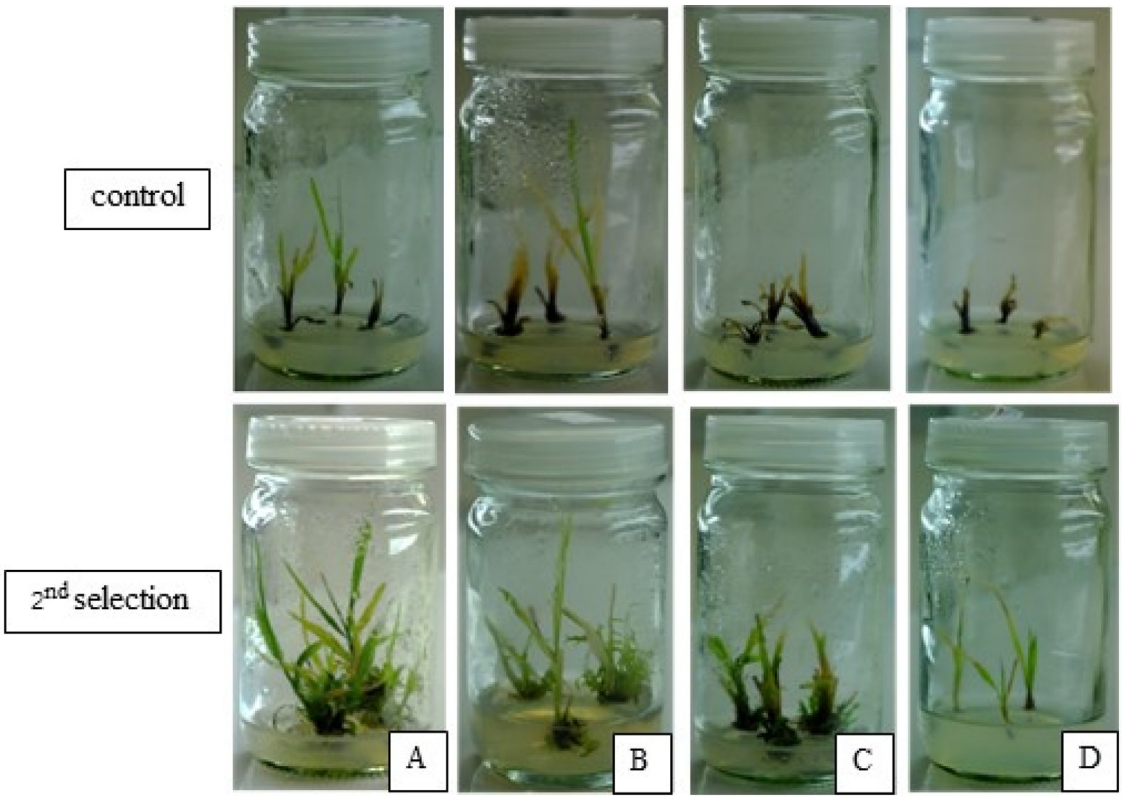

Fig. 2. The surviving plantlets after the second round of selection. Plantlets were placed in media containing higher NaCl concentrations (by 42 mM) than those they have survived from during the first round of selection: (A) 128 mM, (B) 170 mM, (C) 212 mM, and (D) 254 mM.

especially in leaves. The expression levels of the three genes in the leaves increased as the stress duration increased, and reached the highest levels at day 5 of the salt stress exposure process. The expression of these genes in the roots was more fluctuating, especially in the case of *SoNHX1*. The relative expression levels of *SoNHX1* in the root were found increased, reaching the highest levels at days 1 or 3, and then decreased at day 5. After an exposure to the same stress duration, the expression levels of the gene in the leaves were found to be higher than those in the roots. Moreover, the expression levels of *SoNHX1* in the more salt-tolerant plants were found to be higher than those in the less salt-tolerant plants (Fig. 5B1 and 5B2).

As far as the *SoSOS1* is concerned, the relative expression of the gene in leaves and in roots exhibited a similar pattern, in which the expression levels were found to be increased as the stress duration increased, and they reached their highest levels at day 5. When compared among the assessed salt-tolerant plants, the expression levels of the *SoSOS1* in the roots and the leaves of the more salt-tolerant plants were higher than those of the less tolerant ones. However, the magnitude of the gene expression was similar between the roots and the leaves (Fig. 5C1 and 5C2).

In the case of *SoHKT*, the relative expression pattern of the gene in leaves and in roots was similar, with expression levels increasing as the duration of the stress increased, and reaching their highest levels on the fifth day of trial. The relative expression levels of *SoHKT* in the roots were higher than those in the leaves, especially on the fifth day of the stress application. It was also found that the more salt-tolerant plants exhibited a higher expression of *SoHKT* than that the less tolerant ones (Fig. 5D1 and 5D2).

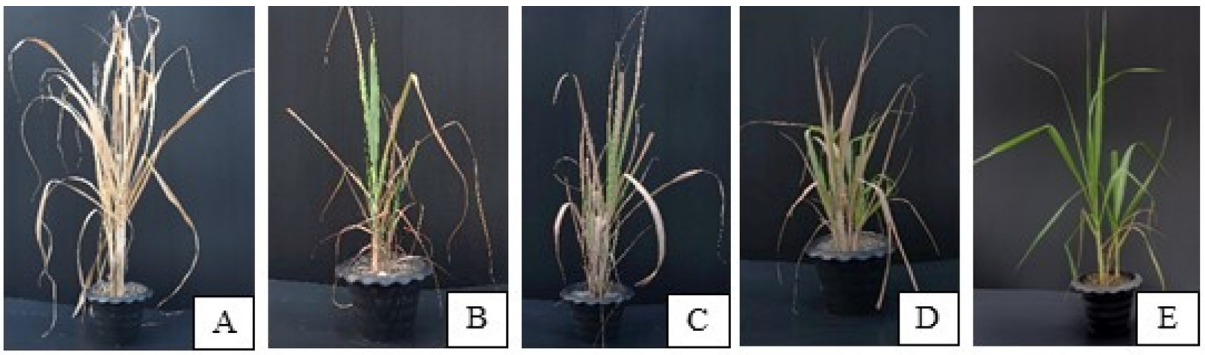

Fig. 3. The plants from the third round of selection were exposed to salt stress conditions (254 mM NaCl) in a greenhouse, for one month. (A) control; (B) second round survivor after an exposure to 128 mM of NaCl (plant no.1); (C) second round survivor after an exposure to 170 mM of NaCl (plant no.2); (D) second round survivor after an exposure to 212 mM of NaCl (plant no.3); and (E) second round survivor after an exposure to 254 mM of NaCl (plant no.4).

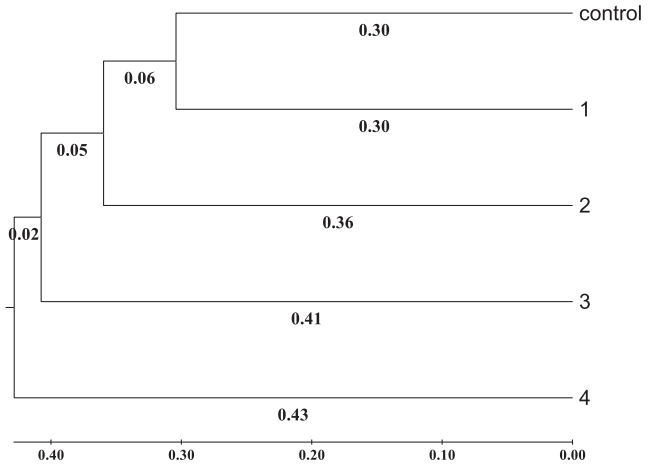

Fig. 4. Dendrogram of genetic distances among the four surviving plants from the third round of selection and the control plant (original cv.) constructed from 24 RAPD primers by using the MEGA 11 software based on the UPGMA method. The numbers 1, 2, 3, and 4 represent sugarcane plants tolerant to 128, 170, 212, and 254 mM of NaCl, respectively.

SoBADH and *SoMIPS* are genes involved in osmolyte and osmotic stabilizer production. The expression levels of *SoBADH* in the roots were more fluctuating than those in the leaves. Except for the case of the plant with a tolerance to 128 mM NaCl, whose *SoBADH* expression levels reached their highest on the fifth day of the stress trial, the expression levels of the gene in the roots reached their highest levels on days 1 or 3, and decreased on day 5. The expression levels of the gene in the leaves of highly salt-tolerant plants reached their highest levels at day 3, and decreased at day 5; on the other hand, in the less salt-tolerant plants, the expression levels of the gene increased as the stress duration increased, and reached the highest levels on day 5 (Fig. 5E1 and 5E2). As far as the *SoMIPS* is concerned, the expression levels in roots reached their highest levels on days 1 or 3, and decreased on day 5 of the stress trial, with the exception of the most salt-tolerant plant, where the highest expression was recorded on day 5. The expression pattern of the gene in the leaves differed from that in the roots. When the stress duration increased, the expression levels of the gene in the leaves also increased and reached their highest levels on day 5, while the expression of the gene in roots fluctuated (Fig. 5F1 and 5F2). At each stress duration timepoint assessed, the expression levels of both genes in the more salt-tolerant plants were higher than those in the less tolerant ones. Moreover, the relative expression levels of both genes in the leaves were higher than those in the roots.

3.4. Physiological and biochemical analyses

The plants were grown under salt stress at 254 mM NaCl. All measurements were completed 3 weeks after the third round of selection began, with the exception of the Brix value measurement that was performed on 8-month-old plants grown in low saline soil of an EC of 4.1 dS m^{-1}.

3.4.1. Proline content

The proline contents of five sugarcane plants differed significantly. The highest proline content in the leaves (82.45 μg g^{-1} DW) was found in the most salt-tolerant plant, while the lowest (12.51 μg g^{-1}DW) was found in the control plant (Fig. 6A). Under high salt stress conditions, the plants with higher salt tolerance exhibited higher leaf proline contents than those with lower salt tolerance. However, the root proline contents among the five plants assessed were very low and did not differ significantly.

3.4.2. Glycine betaine (GB) levels

Salt stress contributed significantly to the accumulation of GB in the four salt-tolerant sugarcane plants. The leaf of the most salt-tolerant plant bared the highest GB content (0.47 μg g^{-1} DW), which was significantly different from that of the rest and was followed by the 0.19, 0.12, and 0.082 μg g^{-1} DW of the second, third, and fourth most salt-tolerant plant, respectively. The control plant showed the lowest GB content (0.07 μg g^{-1} DW). GB contents in roots exhibited the same tendency as those of the leaves, but at a smaller magnitude. The GB content in the root of the most salt-tolerant plant was also significantly different from those of the less salt-tolerant plants and of the control plant (Fig. 6B).

3.4.3. Relative water content (RWC)

It was found that the RWC values were significantly different among the different salt-tolerant plants submitted to the 254-mM NaCl stress. The highest RWC (of 98.18%) was observed in the most salt-tolerant plant, while the control plant had an RWC of 84.26% (Fig. 6C).

3.4.4. Relative chlorophyll content (RCC)

RCC was expressed in SPAD units. Prior to the salt stress application, the SPAD unit of the most salt-tolerant plant was the highest (39.38), and significantly differed from that of others; however, the SPAD units among the rest of the plants were not significantly different. After 3 weeks of salt stress, the plant with the highest SPAD unit (31.89) remained the most salt-tolerant plant, but its values were not significantly different from those of the second and the third most salt-tolerant plants. It was also found that the

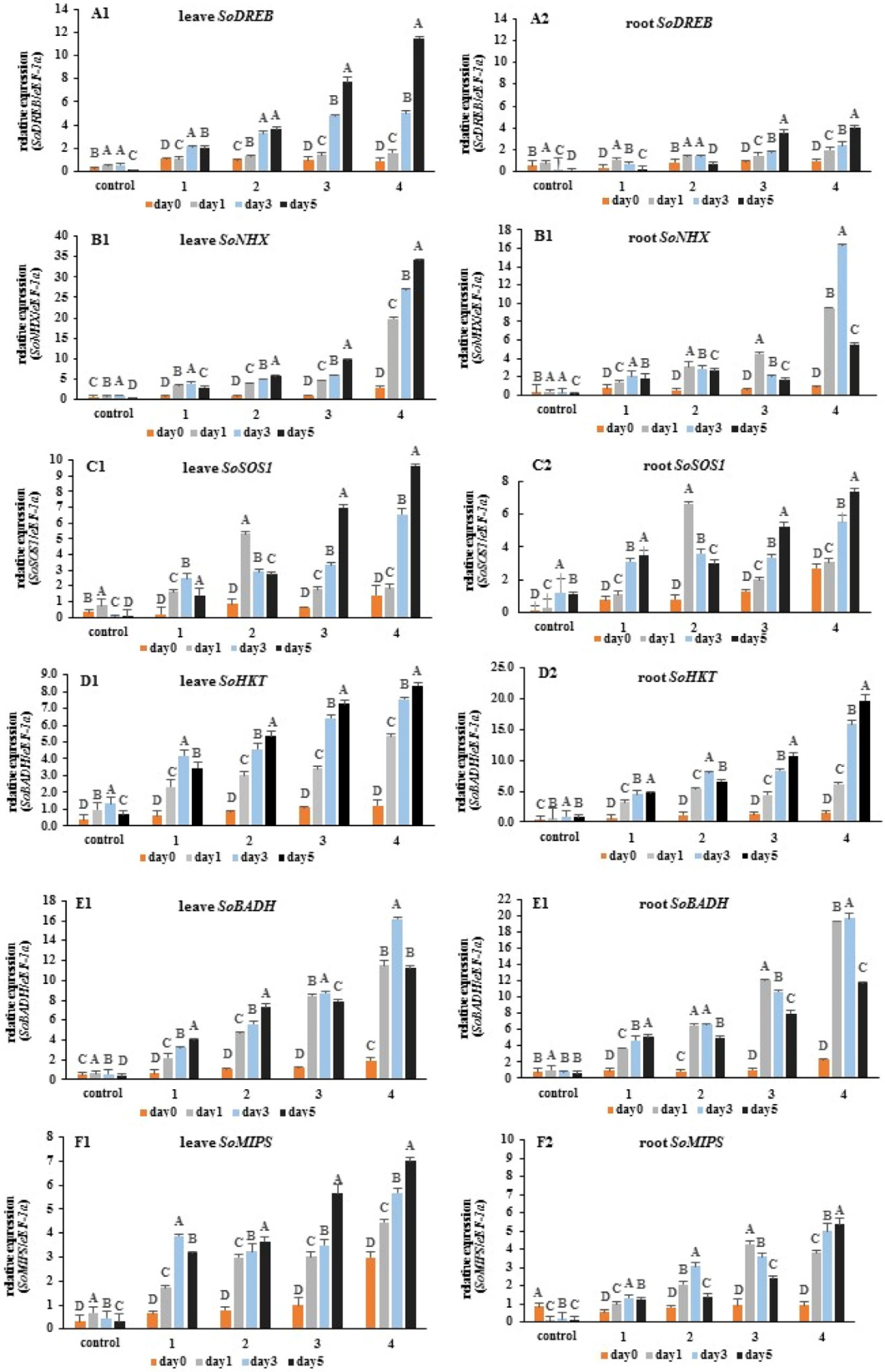

Fig 5. Relative expression levels of *SoDREB* (A1, A2), *SoNHX1* (B1, B2), *SoSOS1* (C1, C2), *SoHKT* (D1, D2), *SoBADH* (E1, E2), and *SoMIPS* (F1, F2) in the leaves and roots of the plant that survived from the third round of selection and was subjected to 170-mM NaCl stress conditions for 0, 1, 3, and 5 days. Different letters over the bars indicate significant differences at $p < 0.05$ (Duncan's test) when compared with bars referring to different days within the same plant group indicated on the x-axis. Note: Numbers 1, 2, 3, and 4 on the x-axis represent the sugarcane plants tolerating 128, 170, 212, and 254 mM of NaCl, respectively.

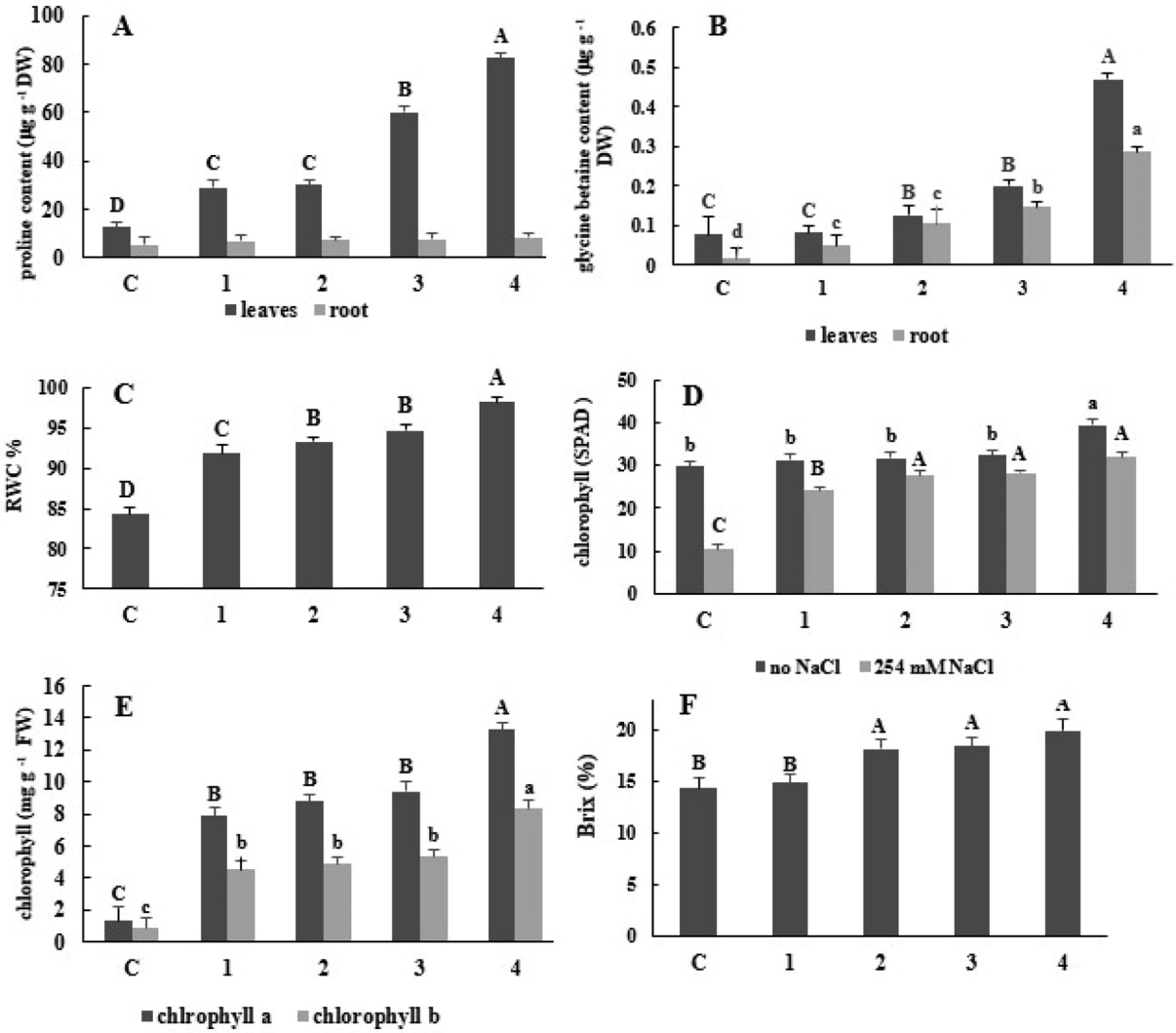

Fig. 6. The proline contents (A), the GB contents (B), the RWCs (C), the SPAD units (D), the chlorophyll contents (E), and % Brix values (F) of the control and the four salt-tolerant sugarcanes deriving from an *in vitro* selection and being submitted to an *in vivo* salt stress process. The C, 1, 2, 3 and 4 on the × axis represent control, 128, 170, 212, and 254 mM NaCl-tolerant sugarcane plants, which survived in 254 mM NaCl of the third round of selection, respectively.

SPAD units of all plants after exposure to the salt stress were lower than those before the stress application (Fig. 6D).

3.4.5. Chlorophyll a and b content

The contents of chlorophyll *a* and b in the leaves of four salt-tolerant plants and of a control plant were measured (Fig. 6E). When submitted to a salt stress condition, the most salt-tolerant plant exhibited the highest content of both pigments (13.29 mg g^{-1} FW for chlorophyll *a*; 8.35 mg g^{-1} FW for chlorophyll *b*), and these contents were significantly different from those of the other plants assessed. There were no significant differences with regard to the pigment contents among the second, the third, and the fourth most salt-tolerant plants, but these were significantly different from those of the control plant.

3.4.6. Brix value measurement

Brix values were measured in 8-month-old plants grown in low saline soil conditions. The Brix values of their juice ranged between about 14% and 20% (Fig. 6F). The Brix value of the most salt-tolerant plant were the highest (19.9%), but they were not significantly different from those of the second and the third most salt-tolerant plants. The control plant had the lowest Brix value (14.29%), but it was not significantly different from that of the fourth most salt-tolerant plant (14.78%).

3.4.7. K^+/Na^+ Ratios

The amounts of K^+ and Na^+ in the leaves were measured before and after 3 weeks of the exposure to salt stress. At normal conditions, the amount of K^+ in the most salt-tolerant sugarcane plant was the highest (15.67 μg g^{-1}), but it was not significantly different from those of the rest of the assessed plants, except for the control plant that had the minimum amount of K^+. Under salt stress conditions, the most salt-tolerant plant exhibited the highest amount of K^+ (289.33 μg g^{-1}), which was significantly different from those of the rest. In the absence of a salt stress, the amounts of Na^+ in all plants were very low and did not differ significantly. Under the herein examined salt stress condition, the highest amount of Na^+ was recorded in the control plant (115.67 μg g^{-1}) and was significantly different from those of the rest. Moreover, the most salt-tolerant plant exhibited the lowest amount of the ion (39.50 μg g^{-1}). It was found that the K^+/Na^+ ratios of all salt-tolerant plants before the salt stress exposure were lower than those recorded after the salt stress trial. In both cases, the most salt-tolerant plant exhibited the highest K^+/Na^+ ratio (7.17), which

was significantly different from those of all other plants assessed. The K^+/Na^+ ratio of the control plant was the lowest measured in both conditions (Table 3).

4. Discussion

Sugarcane (*Saccharum* sp.) is a glycophytic plant. Its yield is reduced by about 50% when the crop is grown on salt-overloaded soils (Wiedenfeld, 2008). Therefore, raising the tolerance of sugarcane to salt stress can enhance the sugarcane yield. The use of the tissue culturing for cell selection allows for the screening for salt tolerance in sugarcane to be undertaken quickly and effectively (El–Mahdy et al., 2022). Based on the findings of this study, sugarcane with enhanced salt tolerance was selected by using the tissue culture technique. The results have demonstrated once more how effectively and rapidly this technique is, and how it could be used for the screening of sugarcane for salt tolerance.

We, herein, directly selected sugarcane calli in a salt-selective medium without inducing mutations during the first round of selection. The chosen sugarcane plants could withstand *in vitro* salt stress of up to 212 mM of NaCl. A genetic variant or somaclonal variation has reportedly been produced *in vitro*. Somaclonal variation has provided a natural source for several desirable traits, and can be selected at the cellular level by culturing calli or cells on a selective medium containing appropriate selective agents. This technique has succeeded in selecting novel cvs. in many propagated crops, including sugarcane (Shomeili et al., 2011; Abdullah et al., 2013; Raza et al., 2014; Manchanda et al., 2018). Several effective *in vitro* methods for the assessment of sugarcane's salt stress tolerance have already been reported (Gandonou et al., 2005; Badawy et al., 2008; Manchanda et al., 2018). Somaclonal variation exerts several limitations: it is random, unstable, epigenetically determined, and nonheritable (Manchanda et al., 2018). In order to solve these problems, this study has employed repetitive selection aimed at eliminating such problems, including that of escape plants. The incident of escape always comes with a single selection over a short time, with the use of low concentrations of selective agents (Ayub and Reis, 2016).

The second round of selection was carried out by increasing the salt concentrations in the plants produced from the calli identified from the previous selection. This step has reduced the number of plantlets, thereby indicating that some calli escaped the cell selection during the first round of the process. We, herein, directly selected sugarcane calli in a salt-selective medium without any mutation induction. This implies that somaclonal variation in the calli is an important factor for the *in vitro* selection success.

There are two methods of *in vitro* selection. First, in the long-term and stepwise treatment, cells or calli are exposed to different levels of stress in which the stress levels are gradually increased in a stepwise manner. Second, in the shock treatment, where the cultures are grown on a selective medium containing a high concentration of a selective agent (Rai et al., 2011). In the present experiment, we combined these two methods by selecting the sugarcane calli in a rather high concentration of NaCl, and the concentrations were gradually increased within a short time. The *in vitro* selection for salt tolerance was followed by *in vivo* selection, thereby taking advantage of the somaclonal variation induced by the applied tissue culture conditions; an efficient protocol for the production of salt-tolerant sugarcane clones (Khan et al., 2004; Granja et al., 2018). Shomeili et al. (2011) have produced somaclone sugarcanes that tolerated up to 132 mM of NaCl. In the current study, the third step of selection involved an experimental procedure that selected sugarcane clones with tolerance to up to 254 mM of NaCl. In the third step of the selection, greenhouse conditions were used in order to ensure the stability of the sugarcane's salt tolerance. Repeated selection *in vitro* and *in vivo*, with the use of increasing NaCl concentrations in each round, has shown that this method works well for the selection of sugarcane clones with better salt tolerance.

Molecular, biochemical, and physiological analyses were performed so as to ensure that the selected salt-tolerant sugarcane plants did not escape or become epigenetically altered. In an attempt to study somaclonal variation at the molecular level as well as to prove that the selected salt-tolerant sugarcane plants resulted from the changing of nucleotide sequences, the RAPD-PCR and the consequent dendrogram construction were employed. This marker has proven particularly useful for the identification of genetically varied somaclones in sugarcane (Thumjamras et al., 2011; Seema et al., 2014; Mahmud et al., 2015), disease resistance, red rod resistance (Shahid et al., 2011), salt stress tolerance (Tawar et al., 2008; Younis et al., 2020; Abdelsalam et al., 2021) as well as salt-tolerance in date palm (Al-Khateeb et al., 2020). RAPD is straightforward, quick, necessitates tiny amounts of DNA, and has the capacity to discover numerous polymorphisms (Kumar et al., 2009). In the current investigation, RAPD markers revealed the absence of a DNA band on agarose gels, weak or high band intensity, and the presence of new bands in comparison to the original plant DNA. The constructed dendrogram could be divided into two groups: the first group contained the second to fourth most salt-tolerant plants as well as the original plant, while the second group only contained the most salt-tolerant plant. This finding indicates that the most salt-tolerant clone differed genetically more from the original plant than the less salt-tolerant clones did. It had been found that the regenerated plants undergo somaclonal variations as a result of the stress induced *in vitro* (Krishna et al., 2016). The results imply that the selection pressure that was applied *in vitro* to the calli tended to eliminate the wild type cells, and have accelerated the growth of variant cells. The greater the selection pressure (in this case the pressure of 254 mM of NaCl), the more genetically variable were the somaclones that were recovered, and the less genetically similar they were to the original plant.

Salinity greatly affects glycophytic plants such as sugarcane (Kaewjiw et al., 2018). Numerous genes have been shown to be affected by salt stress conditions, including those encoding transcription factors (i.e., *WRKY*, *NAC*, *ERF*, and *DREB*) (Zhang et al., 2016), Na^+/H^+ antiporters (i.e., *NHX*, *HKT*, and *SOS*) (Katiyar-

Table 3
The amount of K^+ and Na^+ in the control (original plant) and the salt-tolerant sugarcane plants before (no NaCl) and after (254 mM NaCl) the application of salt stress conditions.

Tolerant to NaCl (mM)	K^+ (μg g^{-1})		Na^+(μg g^{-1})			K^+/Na^+ ratio
	no NaCl	254 mM NaCl	no NaCl	254 mM NaCl	no NaCl	254 mM NaCl
control	14.50 ± 0.5b	110.00 ± 11.14e	7.00 ± 1.00a	115.67 ± 4.04a	1.94 ± 0.28a	0.95 ± 0.07d
128	14.67 ± 0.58ab	211.33 ± 5.51c	6.83 ± 1.44a	73.00 ± 5.20b	2.22 ± 0.49a	2.90 ± 0.17c
170	14.33 ± 0.58b	193.33 ± 5.77d	6.00 ± 1.73a	62.33 ± 4.93c	2.49 ± 0.53a	3.12 ± 0.28c
212	15.33 ± 0.58ab	271.00 ± 11.53b	5.67 ± 0.58a	42.33 ± 3.21d	2.72 ± 0.25a	6.44 ± 0.72b
254	15.67 ± 0.58a	289.33 ± 1.53a	6.00 ± 1.00a	39.50 ± 1.53d	2.67 ± 0.53a	7.17 ± 0.23a

Each value is mean ± SD and values followed by the same letter within the a column are not significantly different according to DMRT ($P < 0.05$).

Agarwal et al., 2006; Jabeen et al., 2022), and osmolytes or osmoprotectant production (i.e., *MIPS* and *BADH*) (Kido, et al., 2013; Saurabh et al., 2016). In this study, the expression levels of genes implicated in the salt stress response in sugarcane (namely, *SoDREB, SoMIPS, SoNHX1, SoHKT, SoSOS1*, and *SoBADH*) were examined by using qRT-PCR. The selected salt-tolerance plants and the non-selected plants were subjected to 170-mM NaCl stress conditions for 0, 1, 3, and 5 days. The relative expression levels of the aforementioned six genes were determined in the leaves and the roots. Our findings have revealed that the transcription levels of these six genes in the four salt-tolerant sugarcane clones were significantly upregulated when compared with those of the control plant. This finding was consistent with the research results of many researchers, such as Shohan et al. (2019) who have studied *HKT1;5* in rice, Guo et al. (2019) who have studied *HKT*, *NHX1*, *SOS1*, and *BADH* in *Atriplex canescens*, Li et al. (2021b) who have studied the *SsDREB* of *Saccharum spontaneum*, Jabeen et al. (2022) who have studied the *HvNHX* of barley, Shi et al. (2000) who have investigated the *Araabidopsis AtSOS1*, and Sharma et al. (2020) who have investigated the *MIPS* of wheat. All these studies have reported that the upregulation of these genes enhanced the salt-tolerance in the respective plant species. According to our experimental results, the most salt-tolerant plant exhibited the highest expression levels of all genes. The ion homeostasis in plants, that maintains the appropriate concentration of ions in the cytosol and prevents the transport of Na^+ to photosynthetic organs, is controlled by the transporters encoded by *HKT*, *SOS1*, and *NHX1* (Yamaguchi et al., 2013). *HKT* mediates K^+ uptake under salt stress, and this transporter is found only in monocots (Craig Plett and Møller, 2010). When plants are exposed to high NaCl environments, this gene plays an important role in maintaining a high K^+/Na^+ ratio. On the other hand, *SOS1* encodes a plasma membrane Na^+/H^+ antiporter (Shang et al., 2012) that couples the passive movement of H^+ inside the cells and can transport the excess Na^+ out of the cells, thereby regulating the electrochemical potential through the active extrusion of Na^+ and by maintaining a low Na^+ content in the cytoplasm (Yamaguchi and Blumwald, 2005; Chakraborty et al., 2012; Yue et al., 2012; Deinlein et al., 2014; Tang et al., 2015).

NHX1 (Na^+/H^+ exchanger 1) encodes the Na^+/H^+ exchanger 1, which is localized at the tonoplast of the vacuole. This exchanger is very important for maintaining a low cytoplasmic Na^+ concentration through the sequestration of Na^+ within the vacuole (Blumwald and Poole, 1985; Deinlein et al., 2014; Tang et al., 2015). Upadhyay et al. (2012) have reported that the upregulation of *VvNHX1* in grapevine receiving salinity stress has resulted into a lowering of the Na^+ content and the achievement of a higher K^+/Na^+ ratio. *BADH* encodes for betaine aldehyde dehydrogenase, which is responsible for the biosynthesis of GB; an endogenous osmoprotectant. This osmoprotectant performs an important function in cells by stabilizing the protein structure and by adjusting the cytosolic osmotic potentials in order to maintain an appropriate water content in the cell (Ashraf and Foolad, 2007), as well as by decreasing the Na^+/K^+ ratio through the restriction of the accumulation of Na^+ and the increase of the K^+ accumulation in the plant (Sofy et al., 2020; Zhu et al., 2022). This is a critical role of *BADH* in increasing tolerance to drought and salt stresses (Hashemi et al., 2018; Xu et al., 2018). The higher expression of *SoBADH* in the four salt-tolerant sugarcane clones (as compared to that of the control) has resulted in higher GB contents in the four clones. Similar results were previously observed in salt-tolerant plants such as *Sesuvium portulacastrum* L. (Lokhande et al., 2010), sugarcane (*Saccharum officinarum* L.) (Nikam et al., 2014), and *Guizotia abyssinica* Cass. (Ghane et al., 2014). On the other hand, *MIPS* encodes the rate-limiting enzyme in the *myo*-inositol biosynthesis pathway, *myo*-inositol-1-phosphate synthase (MIPS). *Myo*-inositol and its derivatives are crucial to plant stress tolerance (Rahnama et al., 2010). Recently, Hu et al. (2020) discovered that the overexpression of *MdMIPS1* from the apple can raise the *myo*-inositol concentration and improve tolerance to salt and osmotic stressors in transgenic Arabidopsis lines. As a result, the increased *SoMIPS* expression in the four elite sugarcane plants of the current study appeared to alleviate the salinity-induced osmotic stress by increasing the accumulation of additional osmoprotectants such as *myo*-inositol, though we did not examine the plants' *myo*-inositol content. A high content of both osmoprotectants, glycine betaine and *myo*-inositol, in the cells of the leaves would reduce the osmotic potential of the cells. This would have helped maintaining the appropriate cell water status, which would then result in high RWC in salt-tolerant sugarcane plants. This physiological characteristic indicates a good osmotic adjustment of the salt-tolerant sugarcane clones so as for the latter to avoid the adverse effects of osmotic shock due to the high concentration of NaCl in the soil.

DREB encodes a transcription factor that plants use for responding to stress conditions including cold, drought, and salinity (Chanprame et al., 2019). *DREB* plays an important role in the induction of the expression of various stress responsive genes in plants (Lata and Prasad, 2011). Our study has shown that due to the high expression levels of this gene in the salt-tolerant sugarcane clones, several salinity-related genes (such as *SoMIPS, SoNHX, SoHKT, SoSOS1*, and *SoBADH*) were also highly expressed, thereby allowing for the selected sugarcane to survive in saline soil conditions. Furthermore, under salt stress (254-mM NaCl) conditions, the K^+/Na^+ ratios of the four salt-tolerant sugarcane clones were much higher than those of the original plant. The K^+/Na^+ ratio is a crucial indicator for the salt-tolerance of glycophytes exposed to salt stress (Shabala and Pottosin, 2014, Assaha et al., 2017). K^+ and Na^+ transporters were highly expressed in the selected salt-tolerant sugarcane clones, thereby indicating that these ion transporters were involved in maintaining high K^+/Na^+ ratios during the adaptation of the plant. Therefore, the maintenance of a high cytosolic K^+/Na^+ ratios is relevant to the growth and tolerance to salt stress of the selected sugarcane clones.

The accumulation of an osmolyte such as proline (a water-soluble amino acid) in the leaves of the four salt-tolerant clones was much higher than that in the leaves of the control plant. The most salt-tolerant clone exhibited the highest proline content. Proline is one of the most effective compounds that a wide range of plants accumulate in response to conditions that reduce their water absorbing potential, including salinity, drought, and freezing (Polash et al., 2018; Siddique et al., 2018). Sofy et al. (2020) have recently reported that the endogenous proline levels in the common bean (*Phaseolus vulgaris* L) increase in response to NaCl stress, and allow for a better adaptation to the salt stress. In this study, the proline content in the leaves of the most salt-tolerant clone was found increased by several-fold when compared to the control. Similar results have been observed in an oil seed crop by Ghane et al. (2014). Moreover, sugarcane varieties that are more salt-tolerant than unselected varieties are frequently associated with elevated concentrations of proline (Gandonou et al., 2005; Singh and Senger, 2020b; Rao et al., 2021). However, in the present study, the effect of salt stress on the root proline accumulation remained non-significant across the examined sugarcane plants. Rhodes et al. (1986) have reported that the proline transport from the stems and the roots can also contribute to a high proline accumulation in the leaves through a combination of increased biosynthesis and slower oxidation in mitochondria. An et al. (2013) have concluded that this is the reason that results in higher proline content in the leaves than in the roots.

Brix percentage is associated with sucrose content and can be expressed as commercial cane sugar (CCS). The greater the percentage of Brix, the greater the CCS value and the sucrose content.

In the present experiment, all sugarcane plants were grown in low saline soil. Due to the fact that sugarcane is very sensitive to salt stress, the variation in the Brix percentage of the examined plants indicates the different effects of the salt stress on each sugarcane plant's growth and Brix yield. The more salt-tolerant clones have exhibited higher Brix percentages than the less salt-tolerant ones or the control plant.

Salt stress causes a decrease in chlorophyll content, reduces stomatal conductance, and impairs photosynthesis (Manchanda et al. 2018). The SPAD unit is positively correlated to the chlorophyll content (Jangpromma et al., 2010; Ling et al., 2011), and is considered as a good tool for the assessment of the integrity of the photosynthetic system (Netto et al., 2005). From the physiological study undertaken herein, we have found that the more salt-tolerant clones had a higher SPAD unit and could maintain higher chlorophyll contents than the less salt-tolerant plants. In cotton, the chlorophyll SPAD can be used in order to discriminate between salt-tolerant varieties and sensitive ones (Saleh, 2012). In low saline soil, the salt-tolerant sugarcane clones had the ability to protect their photosynthetic structures (in this case, their chlorophyll levels) and to maintain a high level of photosynthesis, which has resulted in a high Brix value or a high sugar content. This is in agreement with the findings of Borrell et al. (2000) who have found that the stay-green trait in sorghum had a significantly higher yield under drought conditions, when compared with a plant not possessing this trait. Cuin et al. (2010) and Li et al. (2021a) have also observed a strong positive correlation between the chlorophyll content and the yield in salt-tolerant wheat and broomcorn (*Panicum milliaceum*), respectively. Yang et al. (2020) have recently found that under salt stress condition, the salt-tolerant sweet sorghum was able to maintain a high shoot sugar content by protecting its photosynthesis structure and activity, as well as by inhibiting its sucrose degradation.

Base on the results of RAPD and dendrogram in conjunction with the relative expression levels of salt responsive genes, it indicated that the selected clones exhibited salt tolerance underwent changing at DNA level and gene expression and was not related to epigenetic. Together with the physiological and biochemical data, this suggests that the selected clones could adapt to salt stress conditions exceptionally well. In this study, we planted the selected clones under saline soil for eight months and they grew normally and had a higher Brix value than to the control plant. These phenomena demonstrate that the selected clones are genuinely salt-tolerant sugarcane.

5. Conclusions

The herein undertaken sugarcane cell selection for salt-tolerance through the use of selective media containing NaCl has exhibited high efficiency, was simple but precise, and was not very time-consuming. The protocol involved both an *in vitro* and an *in vivo* selection, as well as a selection at the cellular and the whole plant level in order to eliminate the escape. This method of selection depends on the degree of somatic cell variation. In sugarcane, it has been known that somaclonal variation generated by plant tissue culture is very common, and can occur at a high frequency. When combined with intense selection pressure, the new genetic stability can be assumed on salt-tolerant lines. Sugarcane is an asexually-propagated crop; therefore, the plant can take advantage of this kind of propagation. The salt-tolerance trait can pass from generation to generation without (or with less) genetic change when compared to sexually-propagated crops. We, herein, obtained 11 salt-tolerant plants with varying degrees of tolerance as a result of our experiments. In the future, we will examine them in more detail in order to find out how much they differ from the original cv. in terms of genetics, anatomy, and morphology. If they are different enough, we will release them as new cvs. and use them as a breeding stock for their salt tolerance trait as well as for the purpose of biotechnological breeding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical Disclosures

The authors announce that no experiment performed on animals and no data were collected from patient in this research.

Authors' contributions

Chanakan Laksana conceptualization and designed research, conducted experiment, wrote the original draft. Onsulang sophipan conducted experiment. Sontichai chanprame conceptualization and designed research, reviewing and editing. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103655.

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