Assessment of some Physiological and Biochemical Parameters of Three Thai Sugarcane Cultivars under Salt- and Drought-stress

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Submission: 20 July 2018 Revised: 25 October 2019 Accepted: 3 December 2019

ABSTRACT

Lack of tolerance to drought and salt are major problems in growing sugarcane in Thailand. For testing these physiological stress conditions, we compared the Thai cultivars KPS 01–01–25, KPS 01–01–12 and LK92–11 genotypes in assays with different levels of sodium chloride (0–2% NaCl) and polyethylene glycol (0–15% PEG–6000) for a period of 4 weeks. We observed that both the salt and PEG treatments significantly increased the proline and glycine betaine contents in the leaves. The compound solutes accumulation can indicate osmotic adjustment in sugarcane, whereas the chlorophyll stability index and water relation content decreased significantly in treatments with salt treatments of 2% NaCl. In addition, membrane damage rates responded to only high concentrations of PEG. The results suggested that sugarcane responds differently regarding organic osmolytes accumulation and its physiological mechanisms in response to NaCl and PEG stress. Our studies allowed us to define the sugarcane KPS 01–01–25 as moderately tolerant to salt and drought stress compared to that of sugarcane PS 01–01–12 (tolerant) and LK92–11 (susceptible).

Keywords: Drought tolerance, osmolyte analysis, *Saccharum*, salt stress, sugarcane

Thai J. Agric. Sci. (2019) Vol. 52(4): 220−231

INTRODUCTION

Sugarcane is one of the top sugar-producing crops in the world. Its sugar and byproducts are processed for food and bioenergy (D'Hont *et al*., 2008). Sugarcane grows more prominently in warm-temperate and tropical regions with a minimum of 60 cm annual rainfall, throughout South Asia, the southern states of the USA, South America and Melanesia. In Thailand, the crop is cultivated all over the country, producing more than 100 million t of sugar per year over an area of approximately 1.4 million hectares (Office of Agricultural Economics, 2015). The farming of the crop in the central and northeast provinces covering 50% of the total sugarcane area is often problematic due to severe drought and high salt conditions that are key limiting factors of yield production (Bartels and Sunkar, 2005).

Drought stress generally occurs when the concentration of solutes in soil water is equal to or

higher than in root cells, preventing the plant osmotic system from taking water from its environment. Responses to this stress takes place through biochemical, morphological and physiological mechanisms. For example, the relative leaf water content was found to be involved in drought during water stress in sugarcane cultivars (Silva *et al*., 2011). Osmoregulator accumulation as measured by the free proline content increased by adding mannitol in the media of plantlets under *in vitro* culture (Cha-um and Kirdmanee, 2008). The photosynthetic pigments in the sugarcane leaf, the chlorophyll a/b ratio and carotenoids were reduced by drought (which added up to 200 mM mannitol under *in vitro* testing) (Jangpromma *et al*., 2010; Cha-um *et al*., 2012).

Moreover, plant salt stress responses look like the symptoms of ion stress or water deficit that have influenced the decrease in leaf water potential and reduce plant viable water (Grattan and Grieve, 1999; Romero-Aranda *et al*., 2001; Yadav *et al*., 2011). Salinity causes a decrease in the relative water content that is expressed as losing water maintenance for cell extension processes (Ghoulam *et al*., 2002). Synthesis of compound solutes occurs as glycine betaine and proline. In research related to this subject, soluble proteins or proline levels were found to increase in leaves of plants such as rice (Lutts *et al*., 1996), *Amaranthus tricolor* (Wang and Nii, 2000) and sugarcane (Vasantha and Rajlakshmi, 2009).

Modern sugarcane varieties have been selected from *Saccharum officinarum* X *S. spontaneum* hybrids, of which 80–90% of the highly polyploid crop are of *S. officinarum* origin, providing the genetic context for high sugar yield, while from the remaining 10–20%, *S. spontaneum* controls the various sources of resistance/tolerance against biotic and abiotic stress. There are also a few interspecific recombinant chromosomes (Piperidis and D'Hont, 2001; Cuadrado *et al*., 2004). Sugarcane breeding programs in Thailand are needed more than ever to increase the yield and sugar content, and to adapt to drought and other environmental stress problems. However, the crop's cycle means a long time for crossing and selection based on field trials. One alternative solution is a physiological trait screen that might increase the success of breeding sugarcane for abiotic stress (Jangpromma *et al*., 2012). For example, information on drought response among cultivars has to be acquired in a way that it can be used to target a cultivated selection (Inman-Bamber and Smith, 2005). Biochemical and physiological parameters can facilitate the crop improvement process as they can be used as fast tools to screen tolerance to drought or salt. Such parameters can be considered as indication points of stress. Plants displayed multiples biochemical (accumulation of proline, polyamine, treha-lose, increasing of nitrate reductase activity and storage of carbohydrate at cellular and organism levels) and physiological (transpiration, water-use efficiency, stomatal activity and osmotic adjustment) responses under drought and salt stress (Kumar *et al*., 2018). Some of the most effective strategies are the chlorophyll stability index (CSI) that is used to measure the total chlorophyll content. A high CSI level helps the plants to resist stresses through a better photosynthetic rate and to produce more dry matter (Lichtenthaler, 1987; Silva *et al*., 2007; Silva *et al*., 2011). The leaf relative water content (RWC) that leads to photosynthetic rate and stomatal conductance is another sensible manifestation of water-deficit stress. It is used to evaluate plant water status that can then be used to appraise drought tolerance cultivars in sugarcane (Patade *et al*., 2011). The membrane damage rate (MDR) is one of the parameters playing a major role in membrane injury, cellular damage and pigment degradation (Patade *et al*., 2008). The MDR in the plant is induced by relative oxygen species (ROS), which also damage proteins, nucleic acid and lipid macromolecules. Compatible solute accumulation is often used to indicate a water deficit and salt stress. Glycine betaine and proline are required to prevent ion toxicity and dehydration for the maintenance of cell turgor which is essential to survive and recover from stress (Munns and Tester, 2008). Such parameters help to assess how much the plant demonstrates resistance to stress.

An effective screening process is needed for additional parameters to resist sugarcane drought in Thai sugarcane breeding programs. To this end, we tested the above-mentioned assessments for levels

of tolerance to drought and salt using comparisons between known tolerant and susceptible cultivars—the Thai tester (KPS 01–01–25), tolerant (KPS 01–01–12) and susceptible (LK92–11) genotypes. Using assays with different levels of sodium chloride (0–2% NaCl) and polyethylene glycol (0–15% PEG–6000) treatments for a period of 4 weeks, we investigated the total chlorophyll content, leaf water relation content, leaf membrane damage rate, and the proline and glycine betaine accumulations.

MATERIALS AND METHODS

Plant Materials

The three Thai sugarcane cultivars that we selected were the tester KPS 01–01–25, the tolerant KPS 01–01–12 and susceptible LK92–11 and 5 plant samples were used for each treatment. All the testers have been generated through the testing and screening of the sugarcanes for salt and drought stress in tissue culture. The stalks were collected from single eye buds of 11–month-old plants. Cuttings (5–7.5 cm) were sown in peat moss and sandy soil (1:1) in pots with 30 cm diameter and 40 cm height. The pots were kept in a greenhouse covered with an agricultural plastic sheet (0.10 mm thick) under natural sunlight and water conditions. After 12 weeks, we moved the pots to individual semi-hydroponic trays for 4 weeks (weeks 13rd to 16th) before starting the treatments with NaCl and PEG–6000 (Sigma). The semi-hydroponic trays were always kept in about 2.5 cm of water. All three cultivars were tested of each treatment as explanation in five parameters below.

Plant Stress Treatments

After 4 weeks of semi-hydroponic adaptation, we started the physiological testing. Plants were exposed to saline stress treatments of control, 0.5%, 1.0%, 1.5% and 2.0% NaCl (w/v) (Patade *et al*., 2011) and drought stress treatments of control, 5%, 10%, 15% PEG–6000 (w/v) (Abbas *et al*., 2014). The transpiration and evaporation losses were compensated by replacing the solutions daily. The third leaves were randomly sampled from each cultivar after 15 days and control experiments were established without NaCl or PEG–6000.

We used the following parameters for the salt and drought stress ability: (1) total chlorophyll content in leaves, (2) plant water relations, (3) membrane damage rate, (4) glycine betaine accumulation and (5) proline accumulation.

Total Chlorophyll Content

Chlorophyll a, b and total carotenoids were determined from the colour shade of the fresh leaf sample following the method of Lichtenhaler (1987). We selected 200 mg of the middle third leaves and cut the sample into small pieces before grinding to a fine power in a chilled mortar (5–7°C) in 80% (v/v) acetone. The mixture of fine sugarcane leaves was filtered and the level of extracting solution adjusted to 10 ml using cold acetone. Using a UV-VIS spectrophotometer (LABTRONICS), the absorbance levels of chlorophyll a, b and carotenoids of the samples were measured at 644, 647 and 470 nm, respectively. The chlorophyll stability index (CSI) was measured using the formula:

$$CSI = 100 \times \frac{\text{Total chlorophyll content in stressed leaves}}{\text{Total chlorophyll content in control leaves}}$$

Plant Water Relations

The relative water content (RWC) of the leaves was determined from the third leaf sample of five independent plants per treatment. The fresh weight (FW) of 2 cm^2 pieces was recorded immediately after harvesting the leaves. The turgid weight (TW) was measured after 24 hours of saturation with de-ionized water in the dark. The dry weight (DW) was determined after drying the leaves for 48 hr in a hot-air oven at 70°C. This method was applied from Patade *et al*. (2011) and the RWC was calculated using the formula:

$$RWC = 100 \times \frac{FW - DW}{TW - DW}$$

Membrane Damage Rate

Third leaf pieces (2 cm^2) of samples were collected and then washed using distilled water before transferring to autoclaved tubes containing 10 ml distilled water. The tubes were then incubated for 24 hr with intermittent shaking. After incubation,

the initial electro conductivity (EC) reading (EC1) of the bathing solution was recorded. Tubes were capped and then autoclaved at 121°C for 20 min to completely exterminate the tissues and release all electrolytes, EC recording (EC2) was measurement after cooling the solution to room temperature. The values EC1 and EC2 were used to calculate the MDR using the formula:

$$MDR(\%) = 100 \times \frac{EC_1}{EC_2}$$

Proline Analysis

The free proline content was determined according to Bates *et al*. (1973). Leaf samples (200 mg) were prepared using a homogenizer (T25 digital ULTRA-TURRAX) at 10,000 rpm in aqueous sulfosalicylic acid (3% w/v, 12 ml) until the leaf pieces were thoroughly mixed. The ground tissue was added with 5 ml aqueous solution of 3% 5'-sulfosalicylic acid. The filtered homogenate (2 ml) was reacted with equal volumes each of acid ninhydrin and acetic acid at 100°C for 1 hr and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and vigorously mixed for 10–15 seconds. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The absorbance of the coloured solution was recorded at 520 nm using toluene as a blank. The proline concentration (mg/g FW) was determined from a standard curve using L-proline (1–100 nmoles for each assay).

Glycine Betaine Analysis

Extract was prepared from ground fresh leaf samples (200 mg) in liquid nitrogen. The fine powder was mixed with 20 ml of deionized water for 16 hours at 25°C. The samples were then filtered using Whatman No1 filter paper. The filtrate was stored in the freezer until analysis.

Thawed extracts were diluted 1:1 with 2 N sulfuric acid. An aliquot (500 µl) was measured in a test tube and cooled in ice water for 1 hr. Cold potassium iodide-iodine reagent (200 µl) was added and the mixture was gently mixed by vortexing. The samples were shaken at 0–4°C for 16 hr and centrifuged at 0°C and 10,000×*g* for 15 minutes. The supernatant was carefully removed using a 1 ml micropipette. As the solubility of the peridotite complexes in the acid reaction mixture increases markedly with temperature, it was important that the tubes be kept cold until the peridotite complex was separated from the acidic solution. The peridotite crystals were dissolved in 9 ml of 1,2-dichloroethane (reagent grade). Vigorous vortex mixing was applied until the crystals had dissolved. The solutions were then set aside for 2.0–2.5 hr before being measured at 365 nm using a UV-visible spectrophotometer (Labtronics LT–291) (modified after Grieve and Grattan,1983).

Statistical Analyses

The treatments and control were replicated five times, with three repeats for each. The completely randomized design tests and the treatment means were tested for significance at $P \leq 0.05$ using Tukey's range test. All descriptive statistics were calculated using a spreadsheet in Microsoft Excel.

RESULTS AND DISCUSSION

Plant Growth Response of Salt and Drought Stress Conditions

Drought and saline soils cause severe losses in crop growth and reproduction and are known as main abiotic stress problems in agriculture nowadays. Salt stress effects cover ion toxicity and metabolic imbalance, while drought stress affects osmotic balance (Munns and Tester, 2008). Physiological responses to drought and salt conditions have been observed that might be used to estimate the ability of the sugarcane cultivars regarding abiotic stress conditions (Cha-um and Kirdmanee, 2009; Silvar *et al*., 2011; Jangpromma *et al*., 2012; Cha-um *et al*., 2012).

The total chlorophyll and carotenoid contents were used to evaluate the sugarcane cultivars subjected to 0–2.0% NaCl in the salt stress test. The colour on the third leaf measured at 0.5%, 1.0%, 1.5% and 2.0% NaCl ranged from light green to yellowish, respectively. Colour analysis focused on the KPS 01–01–25 leaf response as the chlorophyll

content was significantly reduced in 1% (by 22.40%), 1.5% (by 37.58%) and 2.0% NaCl (by 63.42%) compared to the control condition. Moreover, the chlorophyll content of the tolerant KPS 01–01–12 was 24.01–30.47% reduced in 1–1.5% NaCl and 58.34% at 2.0%, whereas the chlorophyll content of the susceptible LK 92-11 was 13.71% reduced in 1% NaCl and 51.64% and 70.34% with 1.5% and 2.0% NaCl, respectively. At 0.5% NaCl and in the control treatment, the leaves of all cultivars displayed the same green colour. At the highest concentration of sodium chloride (2.0%), we observed that most of the total chlorophyll content was lost in LK 92–11, KPS 01–01–25 and KPS 01–01–12. The total chlorophyll and carotenoid contents under salt stress conditions for KPS 01–01–25 were an intermediate chlorophyll centent as the chlorophyll lost was in between the KPS 01–01–12 and LK 92–11 (Figure 1a).

Under drought conditions, the cultivars were grown on substrate with 0–15% PEG (w/v). The total chlorophyll contents of the cultivars decreased as the percentage of PEG increased. In particular, they were considerably reduced in LK 92–11 with 5%, 10% and 15% PEG (w/v) by 63.40%, 60.38% and 56.36%, respectively. The KPS 01–01–12 chlorophyll content slightly decreased with 5–10% PEG but was significantly reduced in 15% PEG by 74.40%. The amount of chlorophyll in KPS 01–01–25 reduced to 86.21, 77.29 and 65.48 for a PEG ratio of 5%, 10% and 15%, respectively. KPS 01–01–12 was significantly more tolerant than LK 92–11. The chlorophyll stability index of KPS 01–01–25 indicated that it was more resistant to drought than the drought-sensitive samples but less resistant than the drought-tolerant samples (Figure 1b).

The present study demonstrated how drought and salt stress affect physiological traits and osmosis in supposedly tolerant Thai sugarcane cultivars in field conditions. The total chlorophyll (A, B, C) and carotenoid contents decreased in all cultivars under different NaCl and PEG conditions. At the same level of NaCl and PEG, the percentage of those contents played a role for each cultivar, the contents were reduced in LK 92–11 more than in KPS 01–01–12 while KPS 01–01–25 was intermediate in that respect. PEG stress and salt stress resulted in approximately 40% chlorophyll loss in general plants and the two stresses exerted equivalent osmotic pressure (Patade *et al*., 2011) and significantly dropped depending on the genotype (Cha-um and Kirdmanee, 2008). It has been reported that oxidative stress generated in plant cells by PEG causes a reduction in photosynthetic rate, which later effects chlorophylls and carotenoid content (Meher *et al*., 2018).

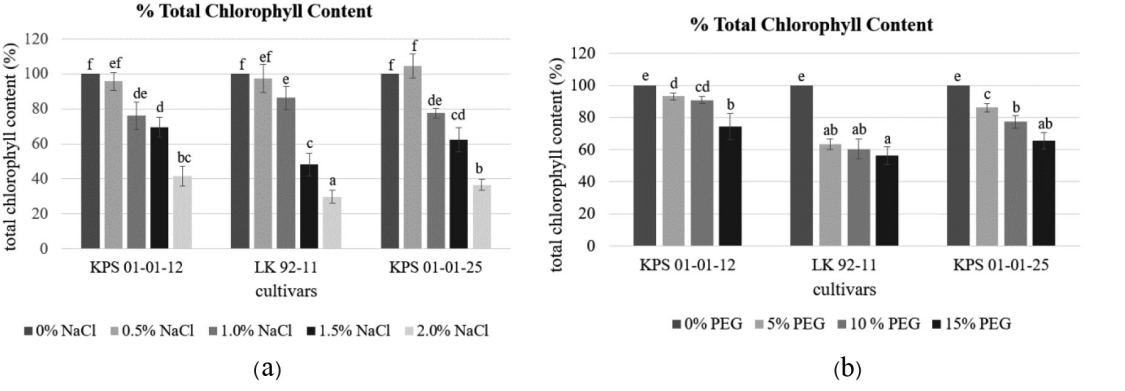

Figure 1 Comparison of total chlorophyll contents for three sugarcane cultivars under salt stress (a) and drought stress conditions (b)

Plant Water Relations

The RWC of sugarcane samples in salinity condition was investigated. The result showed that RWC in all cultivars constantly decreased with increased concentration of sodium chloride. In particular, LK92–11 (susceptible) in 2.0% sodium chloride showed 61.62% RWC which was a significant decrease, while KPS 01–01–12 (tolerant) and KPS 01–01–25 had very similar abilities at 76.18% and 76.53% RWC, respectively. The RWC results for KPS 01–01–25 indicated its salt tolerance ability as a control (Figure 2a).

The drought experiments in 0–15% PEG–6000 showed that % RWC gradually decreased in all cultivars with increased % PEG. The results at 15% PEG showed a significant reduction compared with 0% PEG being 74.10, 76.90 and 75.86 for KPS01–01–12, LK92–11 and KPS 01–01–25, respectively. However, the RWC values in the three cultivars at each PEG level were not significantly different, indicating that the RWC cannot be used as a drought screening parameter with PEG testing (Figure 2b).

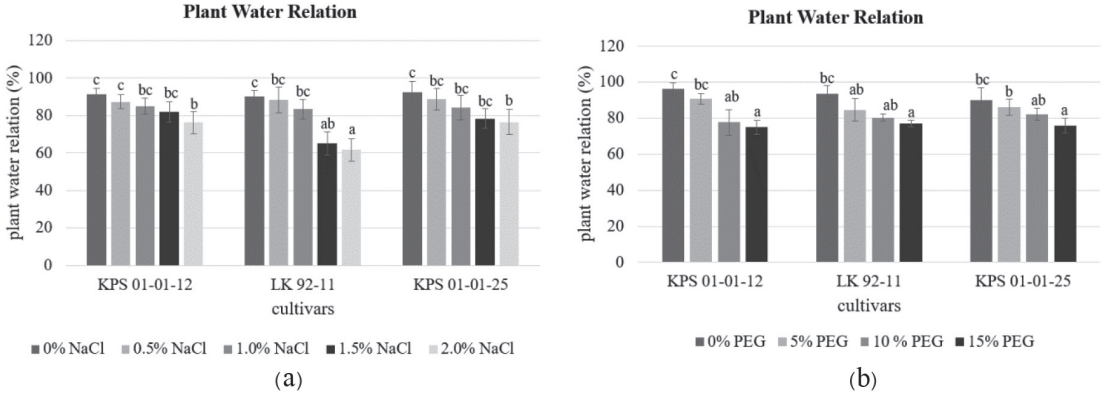

Figure 2 Comparison of water content for three sugarcane cultivars under salt stress (a) and drought stress conditions (b)

There were similar results for the leaf relative water content due to the type of cultivar, salt and drought treatments. The pattern of stress slightly decreased when the levels of NaCl or PEG increased. The relative water content is a parameter for indicating cell and tissue hydration in physiological and growth processes and has been used to estimate drought tolerance in many studies (Matin *et al*., 1989; Jamaux *et al*., 1997; Silva *et al*., 2007). At higher salt concentrations, there were significant differences in the leaf relative water content with the susceptible cultivar (LK 92–11) maintaining a lower average percentage than in the cultivar KPS 01–01–12 and KPS 01–01–25 such as reported by Silva *et al*. (2007). While there were no significant differences among the PEG conditions of this study regarding the relative water content in all cultivars, the relative water content did decrease when the PEG concentrations increased. All cultivars under the PEG conditions had relatively high leaf relative water contents that might help to maintain protoplast hydration for a longer duration under drought.

Membrane Damage Rate

Salinity and drought stress resulted in cell or tissue damage. Ions in the membrane that leak out of the cell can be measured using an electrical conductivity meter (EC meter). The membrane damage rate indicates the ability of the membrane to resist drought or/and salinity. This study tested at levels of 0, 0.5, 1.0, 1.5 and 2.0% (w/v) NaCl. The results showed that the outer ions of damaged cells

can be slightly released at all levels of added NaCl. The lowest ions released out of the membrane at 2.0% NaCl were from KPS 01–01–25 at 15.12%, which was significantly different from LK 92–11 at 20.45%, while value for KPS 01–01–12 was in between these two at 18.96%. However, KPS 01–01–25 and KPS 01–01–12 did not show any significant damage rates indicating that KPS 01–01–25 showed tolerance ability as a control. (Figure 3a).

The drought screening test using PEG (0, 5, 10 and 15%) showed that damage rates were in the same direction. This mean that all three sugarcane cultivars had the ability to maintain ions within the cell for 0–10% PEG but for 15% PEG, there was a significant release of ions between the susceptible and tolerant genotypes with 20.86% and 14.12%, respectively. KPS 01–01–25 also had the lowest ion release with membrane damage at 13.10% indicating it was tolerant (Figure 3b).

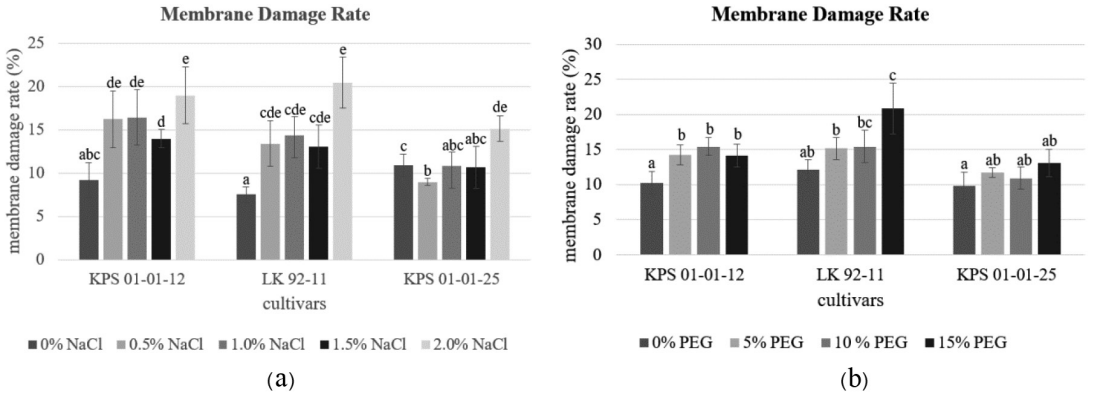

Figure 3 Comparison of membrane damage rates for three sugarcane cultivars under salt stress (a) and drought stress conditions (b)

As the NaCl or PEG concentration increased, the relative electrolytic leakage in the membranes was significant at the highest concentration. In particular, the susceptible LK 92–11 was more affected than KPS 01–01–12 and KPS 01–01–25. At the lower concentration, in terms of membrane damage rate was relatively more in exposed to NaCl than in PEG. This could be purported to the ion toxicity of salt rather than water deficiency effects. It has been reported that increasing the salt concentration might increase the membrane damage rate (Karpe *et al*., 2012) due to reactive oxygen species (ROS) and superoxide in the cell so that membrane lipids and structural proteins of the cell membrane are oxidized (Asada, 2006; Munns and Tester, 2008; Karpe *et al*., 2012).

Osmotic Adjustment of Salinity and Drought Stress Conditions

During observation, the glycine betaine accumulation in the sugarcane cultivars was gradually increased by increasing the percentage of NaCl. The samples were placed in 0, 0.5, 1.0, 1.5 and 2.0% NaCl. The glycine betaine level rapidly accumulated in KPS 01–01–12, KPS 01–01–25 and LK92–11, especially in the 2.0% NaCl treatment which had significant amounts of 25.19, 19.02 and 16.30, respectively. (Figure 4a). Drought resistance was tested using different levels of PEG–6000 concentration. The glycine betaine contents increased in KPS 01–01–12, KPS 01–01–25 and LK 92–11 at 2.0% PEG, with the results being 27.80, 18.21 and

16.42, respectively. However, there was no significant difference between KPS 01–01–25 and LK 92–11, although KPS 01–01–25 was higher than LK92–11 (Figure 4b).

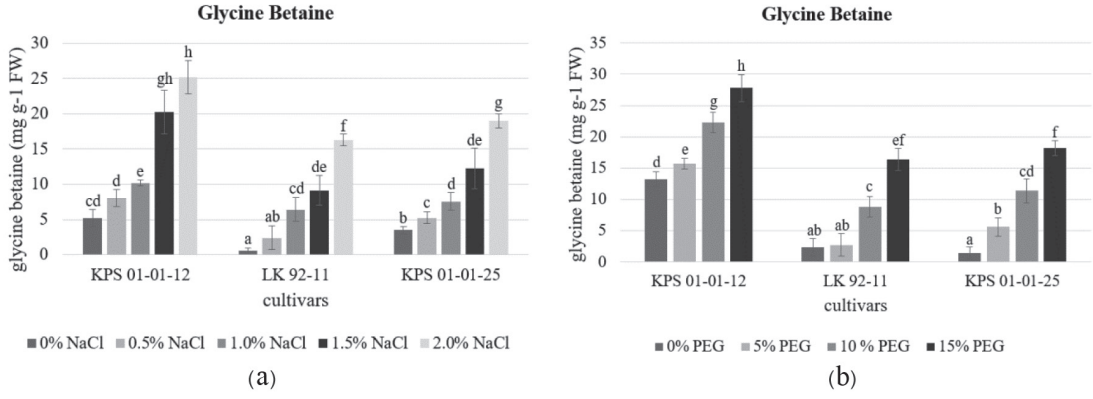

Figure 4 Comparison of glycine betaine contents for three sugarcane cultivars under salt stress (a) and drought stress conditions (b)

The proline contents in stressed plants (both drought and salt stress) showed a response for osmotic adjustment. The mechanism can help the plant to survive the stress for a period of time. In this study, the proline contents increased with higher NaCl concentrations. In particular, for 2.0% NaCl, the proline contents rapidly accumulated in the leaves of KPS 01–01–25 (12.28) and KPS 01–01–12 (9.63) while in LK92–11 (6.78) they did not play a role in determining stress ability (Figure 5a). In the drought stress test in this study, the proline contents increased with PEG addition. The results showed that KPS 01–01–12 in 10–15% PEG and KPS 01–01–25 in 15% PEG produced proline contents of 6.94, 6.58 and 5.79, respectively (Figure 5b).

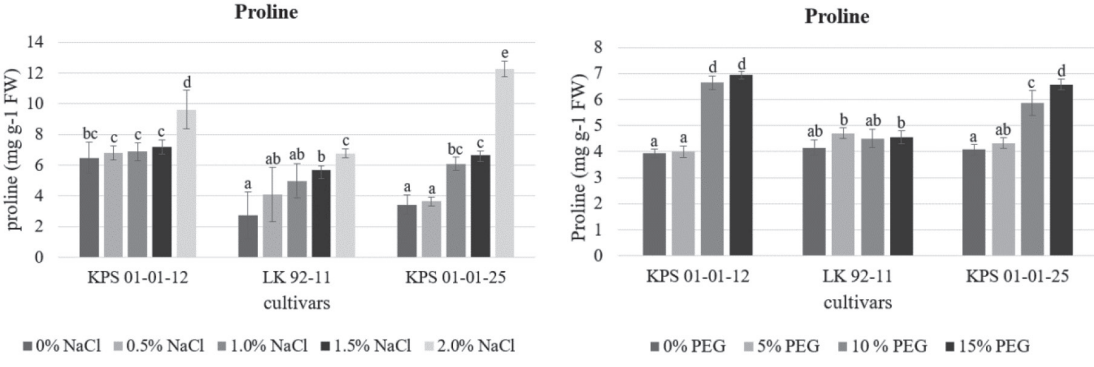

Figure 5 Comparison of proline contents for three sugarcane cultivars under salt stress (a) and drought stress conditions (b)

Higher glycine betaine and proline accumulating ability could be a reliable guide of drought

and salt tolerance in breeding programs as has been shown with cotton (Naidu *et al*., 1998), barley (Jargendorf and Takabe, 2001) and sugarcane (Abbas *et al*., 2014). The current study showed that the glycine betaine and proline contents increased when the PEG or NaCl concentration increased. Tolerance was shown at high glycine betaine and proline accumulation levels that significantly differed with the low level of susceptibility in the PEG and NaCl treatments. KPS 01–01–25 had a glycine betaine accumulation level between those of KPS 01–01–12 (tolerant) and LK 92–11 (susceptible) that could be described as an intermediate type. This may be partly due to glycine betaine and proline have effects on enzyme and membrane integrity along with adaptive roles in mediating osmotic adjustment and able to retain water within the cells in plants grown under osmotic stress conditions (Ashraf and Foolad, 2007).

It has not been clearly confirmed the relating proline accumulation and plant salt tolerance in several plants. Moreover, the studies of correlation between proline functions and salt adaptation are always carried out in growth chambers and are not successfully verified in field conditions (Mansour and Ali, 2017). They also reported that the proline play positive role in respond to improve salinity tolerance in some species/cultivars, but may not be relevant in others. In the present study, we can conclude that proline and glycine can be used as a parameter for indicating drought and salt stress of sugarcane in field conditions.

To this end, an initial drought and salt stress ability test could use physiological parameters for estimation. In addition, the genome of sugarcane is a complex polyploid, for which breeding programs and select improved cultivars could be combined with genetic information, to improve the understanding of gene regulation and expression or phosphoproteomics and redox proteomic analysis (Vital *et al*., 2017). Guo *et al*. (2017) reported that proline levels in sugarcanes were also induced by phytohormones (abscisic acid) and senescence associated genes (SAGs). The two alternative forms of transcript (ScMYB2S1 and ScMYB2S2) of a sugarcane R2R3–MYB gene (ScMYB2) play important roles in respond to drought-induced senescence in sugarcane. (Guo *et al*., 2017). Effort has also been made to generate genetically engineered stress tolerance in major-crops and model-plants, addressing these transgenic plants would help us in better understanding how they are tolerating salinity and drought (Wani *et al*., 2017).

CONCLUSIONS

Our results suggested that proline and glycine betaine accumulation can be used as parameters for screening salt stress and drought tolerance in sugarcane in field conditions, whereas the chlorophyll stability index and water relation content parameters related to high salt condition in sugarcane. In addition, membrane damage rates in sugarcane also related to high concentrations of PEG. In conclusion, sugarcane KPS 01–01–25 showed an intermediate response to salt stress and drought tolerance compared to that of sugarcane KPS 01–01–12 and LK92–11. The results of this study would enable us to gain more insights in understanding and information for further research in sugarcane salt and drought tolerance mechanisms.

ACKNOWLEDGEMENTS

This study was supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Higher Education, Science, Research and Innovation. (AG-BIO/PERDO-CHE), Thailand. I would like to thank Dr. Rewat Lersrutaiyotin from the Cane and Sugar Research and Development Center of Kasetsart University, Bangkok, Thailand collected the sugarcane samples.Dr. Cattleya Chutteang from the Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University helped with information about the PEG experiments.

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