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RESEARCH ARTICLE

In vitro development of salt tolerant Malaysian *indica* rice 'MARDI Siraj 297' and enhancement of salinity tolerance using salicylic acid

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ABSTRACT

Among cereal crops, rice (*Oryza sativa* L.) is the most susceptible to salinity. Due to emerging salinity impacts on food security, different strategies have been implemented including developing salt tolerant varieties. Therefore, this study was conducted to produce salt tolerant lines of 'MARDI Siraj 297' through in vitro callus selection, enhancement of salinity tolerance through supplementation of salicylic acid (SA) (0, 0.5 and 1.0 mM) and regeneration of the selected salt tolerant callus. Embryogenic calli were induced and then treated in selection medium containing 0, 25, 50, 75, 100, 125 and 150 mM NaCl for 5 mo, followed by screening and selection of salt tolerant variants using morphology and biochemical parameters associated with salt tolerance. The salt tolerant calli showed reduced tissue necrosis and maintain their viability. Biochemical profile of these salt tolerant calli showed significantly ($p < 0.05$) higher content of proline (0.28 to 0.38 mg g^{-1}), total soluble sugar (6.4 to 12.9 mg g^{-1}), catalase activity (0.9 to 8.0 μmol min^{-1} mg^{-1} protein), ascorbate peroxidase activity (5.4 to 20.3 μmol min^{-1} mg^{-1} protein), malondialdehyde (6 to 17 μg g^{-1} protein) and K^+/Na^+ ratio (1.0 to 3.0) while reduced in protein content as compared to the non-tolerant control. These characteristics are typically associated with tolerance against salinity, therefore lines potentially tolerant to this stress have been regenerated. The selected salt tolerant calli were transferred into medium with SA to enhance their tolerance. Supplementation of 1.0 mM SA results in reduced morphological injury, higher regeneration frequency and shoot number (56% and 6 shoots, respectively) as compared to the non-SA-treated. Hence, this study demonstrates the protective effect of SA against salinity and provides a reliable protocol for establishment of salt tolerant rice lines through in vitro selection.

Key words: NaCl, salicylic acid, salinity, salt tolerant, tissue culture.

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important food crop that feeds about 4 billion people with an estimation of 25% increase in demand between 2010 to 2030 (IRRI, 2019; Childs and LeBeau, 2023). However, rice production in most regions has progressively been affected by various abiotic stress such as drought, flood and salinity, which are expected to worsen due to climate change as reported by Dar et al. (2021). In Malaysia, the intrusion of sea water into irrigation system and cultivation area near coastal line in Kedah, Perak and Perlis during high tide season has destroyed around 35 ha rice fields (Rahman et al., 2021). According to a report by Azimi (2021), high tide phenomenon during the monsoon season in November 2021 has affected 5849 ha of paddy field in Kedah with estimated loss of MYR 600 per hectare

or approximately MYR 3 million in total. Therefore, due to severity and potential threat of salinity problem towards the national food security, the National Agro Food Policy 2.0 in 2021 has addressed the importance of developing new crop varieties that can tolerate salinity in order to safeguard food supply in the challenging climate variability. At present, majority of farmers in Malaysia are using 'MARDI Siraj 297' for cultivation due to its high yield and resistance to rice blast. However despite its advantages, this variety is susceptible to salinity (Sazali et al., 2021).

Salinity induced various major responses in rice plants in terms of morphology, physiology, biochemical and agronomic attributes (Dramalis et al., 2021; Afzal et al., 2023). Dissolved salt in soil water causes stress that hamper cellular metabolisms, accelerate senescence, interrupt source-sink relationship and finally impair plant growth and development. Salt accumulation also negatively affects the yield-related traits such as number of productive tillers, number of fertile florets per panicle, grain weight and grain yield.

In order to survive, plants are endowed with various mechanisms to surge their tolerance during this stressful condition which includes the activation of antioxidant enzymes and enhanced accumulation of compatible solutes such as proline and soluble sugars for osmotic adjustment (Boughalleb et al., 2020). Over the past years, studies have been conducted to pinpoint the mechanisms involved in salinity tolerance and to develop salt tolerant varieties in rice. However, because of the complexity of plant responses towards salinity, the effort of developing salinity tolerant rice remains challenging. Previous studies by Reddy et al. (2017b) have demonstrated that certain physiological and morphological parameters serve as reliable indicators for salinity tolerance evaluation in rice. Thus, assessing the cumulative effect of these morpho-physiological traits and their interactions can help to build a comprehensive protocol for salinity tolerant selection and elicit the underlying mechanisms involved (Farid et al., 2021).

Apart from screening and developing salt tolerant lines, the usage of salicylic acid (SA) as a phytoprotectant for the elevation of stress has gained more attention. Exogenous SA application was proposed to give protection against abiotic stress including salinity through the regulation of physiological or biochemical mechanisms as reported in many plant species such as tomatoes, maize and wheat (Alsahli et al., 2019; Naeem et al., 2020; Ferdosi et al., 2021). Naeem et al. (2020) reported that salicylic involves in alleviation of salt stress impacts by improving fruit size, yield, fruit DM, fruit firmness and total soluble solid in tomatoes. It acts as an endogenous signal molecule during environmental stresses in plant tolerance mechanisms which confers protection against numerous abiotic stresses, as proved by other studies including Alsahli et al. (2019) and Ferdosi et al. (2021). Its application was also found to significantly increase morphological growth, osmotic adjustment and antioxidant enzyme activities in rice (Chen et al., 2022).

Research on salt tolerant rice cultivars have been conducted through various approaches including conventional breeding, transgenic manipulation and in vitro induced mutation. However, each of them imposed its own limitations (Chen et al., 2021). The in vitro technique on the other hand offers many advantages such as faster development compared to conventional breeding, high frequency of trait changes, possibility of obtaining novel variants as well as allowing the use of large population of cells for selection purpose (Deepthi, 2018). Previous findings by Hannachi et al. (2021) showed that in vitro selection is likely to have a significant role in the recovery of stable somaclonal variants with improved stress tolerance, whereby cell and tissue cultures serve as model system to investigate plants' stress response. This study was therefore looking at the feasibility of developing salt tolerant rice lines of 'MARDI Siraj 297' through in vitro selection and evaluates the role of SA in enhancing the salt tolerant ability of these selected lines.

MATERIALS AND METHODS

Plant material

Mature rice (*Oryza sativa* L.) 'MARDI Siraj 297' seeds were obtained from the Gene Bank of the Malaysian Agricultural Research and Development Institute (MARDI) (accession number: MRGB13019). The experiment was conducted at the Cell Biology Laboratory, Universiti Putra Malaysia.

Seed sterilization and callus induction

After husk removal, rice seeds were washed with running tap water for 3 min to remove debris. The seeds were then serially washed with 70% (v/v) ethanol (2 min), 5% (v/v) sodium hypochlorite (20 min) and sterilized distilled water (Kalhori et al., 2017) before blotted dry on sterilized filter paper. Following sterilization, the seeds were cultured on Murashige & Skoog (MS) basal medium (Sigma-Aldrich, St. Louis, Missouri, USA) containing 3% (w/v) sucrose, 0.3% (w/v) Gelrite, 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg L^{-1} kinetin at pH 5.8 to induce callogenesis (Sidek et al., 2022). The culture vessels were maintained in temperature-controlled growth room without light at 25 ± 2 °C. After 35 d culture, calli with embryonic structures were identified using stereo microscope (KSZ-L; Kyowa Optical, Sagamihara, Japan) and isolated for production of salt tolerant lines.

Salinity treatment

The embryogenic calli clumps (0.2 ± 0.01 g) were excised and transferred onto selection medium (MS basal medium with 3% w/v sucrose, 0.3% w/v Gelrite, 2.0 mg L^{-1} 2,4-D and 0.2 mg L^{-1} kinetin) added with different concentration of NaCl (0, 25, 50, 75, 100, 125 and 150 mM) (Kalhori et al., 2017). The culture was arranged in completely randomized design (CRD) and maintained in dark growth room at 25 ± 2 °C for 5 mo, with subculture every 2 wk to induce salinity stress and trigger cell adaptation mechanisms.

Screening and selection of 'MARDI Siraj 297' salt tolerant calli

After 5 mo, the salt treated calli were screened for salt tolerant traits using morphological and biochemical parameters. The morphology of calli was observed using stereo microscope. Selection of calli with normal morphology was done as described by Mohd et al. (2016) for further biochemical analysis.

Free-proline content was determined using acid-ninhydrin colorimetric assay described by Suekawa et al. (2019). Callus (0.2 g) was homogenized in 5 mL 3% aqueous sulfosalicylic acid (pH 7.8) and centrifuged at 6000 g at room temperature. The supernatant (2 mL) was reacted with acid-ninhydrin reagent (2 mL) and glacial acetic acid (2 mL). The reaction mixture was incubated for 1 h at 100 °C and immediately terminated in an ice bath. Toluene (4 mL) was added and the reaction mixture was left at room temperature for 5 min. The chromophore was collected for absorbance reading at 520 nm using UV-vis spectrophotometer (U-1900, Hitachi High Technologies Corporation, Tokyo, Japan), with toluene was used as blank. The proline content was determined from a standard curve of L-proline and calculated based on sample fresh weight.

Total soluble sugar (TSS) content was determined using anthrone method. Calli (0.1 g) was homogenized in 2 mL ice-chilled 80% (v/v) ethanol and centrifuged for 10 min at 11000 g (4 °C). The supernatant was added with 80% (v/v) ethanol to make up the volume to 10 mL. The mixture (1 mL) was added to 3 mL freshly prepared anthrone reagent and incubated in 100 °C water bath for 10 min then transferred to ice bath to terminate the reaction. The optical density was measured at 620 nm using UV-vis spectrophotometer with 80% (v/v) with ethanol used as blank. The TSS content was determined from a standard curve prepared using a series of known concentration of D-glucose.

Total protein content was determined using Bradford's assay. Fresh callus (0.5 g) was homogenized in 2 mL 62.5 mM Tris-HCl (pH 6.7) and centrifuged at 10 000 g (4 °C) for 30 min. The supernatant was collected and used as protein sample. The protein samples (20 µL) were added with phosphate buffered saline to make up the volume to 1 mL. Bradford's reagent (1 mL) was then added to the sample and mixed by vortexing. The optical density of sample was measured at 595 nm using UV-vis spectrophotometer after 5 min incubation at room temperature. The blank was prepared by mixing 1 mL PBS with 1 mL Bradford's reagent. Protein content was determined from a standard curve plotted using a series of known concentration of bovine serum albumin (BSA).

The catalase (CAT) activity was determined using method applied by Poli et al. (2018). Protein extract from 0.5 g fresh callus was used as sample. The sample was diluted with 50 mM potassium phosphate buffer, pH 7.0 at 1:100 (v/v). The reaction mixture consists of 2 mL sample and 1 mL 30 mM H_2O_2, making up the total volume of 3 mL. Both solutions were mixed and the reaction begins once H_2O_2 was added. The decrease in absorbance at 240 nm was recorded for about 30 s using UV-vis spectrophotometer.

The H_2O_2 oxidation rate by ascorbate peroxidase (APX) and lipid peroxidation (LPX) activity was determined using method described by Hossain et al. (2013); 1 mL of total reaction mixture contains protein sample, 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ascorbic acid. The decrease in absorbance at 290 nm was monitored between 2 to 7 s after addition of H_2O_2 to estimate the ascorbic acid oxidation rate using UV-vis spectrophotometer. Lipid peroxidation in samples was carried out by measuring its decomposition product malondialdehyde (MDA). Fresh callus (0.3 g) was homogenized in 3 mL 50 mM phosphate buffer (pH 7.0), then centrifuged at 15 000 g for 15 min. The supernatant was mixed with 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) and incubated in 95 °C water bath for 30 min (Ratio 1:2 for substrate:TBA/TCA mixture and 1:1 for TBA:TCA). The reaction mixture was immediately cooled in ice bath and centrifuged again at 10 000 g (4 °C) for 10 min. The supernatant was collected and the optical density was determined using UV-visible spectrophotometer at 532 and 600 nm.

Ratio of Na^+/K^+ was measured using flame photometer (M410; Sherwood Scientific, Cambridge, UK). Calli were dried to a constant weight in the oven at 60 °C and ground to powder. Sample (0.25 g) was transferred into 25 mL 1 N HCl. The mixture was incubated at room temperature for 24 h and then sieved through a Whatman N° 1 filter paper. The filtrate was collected and concentration of Na^+ and K^+ ions was measured. A 25 mL 1 N HCl was prepared as blank.

Enhancement of salt tolerance using SA

The viable salt tolerant calli from previous selection were transferred into salt tolerant enhancement medium (MS basal medium + 3% (w/v) sucrose + 0.3% (w/v) Gelrite + 2.0 mg L^{-1} 2,4-D + 0.2 mg L^{-1} kinetin) added with different concentrations of NaCl (0, 25, 50, 75, 100 and 125 mM) and SA (0, 0.5 and 1.0 mM) (Jini and Joseph, 2017). The calli were maintained in dark growth room at 25 ± 2 °C for 30 d, with 10 replicates for each treatment.

Regeneration of salt tolerant calli of 'MARDI Siraj 297'

Calli from SA-enhancement treatments were transferred into shoot regeneration medium which consists of MS basal salts supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (BAP), 1.0 mg L^{-1} naphthaleneacetic acid (NAA), 100 mg L^{-1} myo-inositol and 3% (w/v) sucrose at pH 5.8. The cultures were maintained in growth room at 25 ± 2 °C with 16:8 h photoperiod. Shoots and roots proliferation was observed within 50 d. Regeneration frequency and number of shoots per explant were recorded.

Statistical analysis

The results were analyzed by one way (biochemical parameters) and two-way ANOVA (regeneration parameters) using SAS software (SAS Institute, Cary, North Carolina, USA) for Windows version 9.4. The mean comparison was carried out using Duncan's multiple range test (DMRT) at $p < 0.05$.

RESULTS AND DISCUSSION

Morphology of callus in response to salinity

Calli in control medium without NaCl showed normal, globular stage somatic embryos with friable texture and cream color as shown Figure 1. Meanwhile, gradual morphological deterioration and viable callus decreased markedly with the increment of NaCl concentration in the selection medium. The similar response was also reported on salt exposed calli of some rice cultivars as reported by Rattana and Bunnag (2015). The effect of salinity on in vitro cultured rice callus includes reduction of callus size, tissue browning and unorganized meristematic zone due to enlarged vacuoles (Atabaki et al., 2018). In this study, enlargement of tissue could be observed at concentration of 50 mM NaCl, and became obvious in 100 mM NaCl. The concentration of 150 mM NaCl resulted in complete loss of cell viability due to necrosis and therefore regarded as the threshold concentration for callus survival of this cultivar. Plants at callus stage were proved to be more sensitive towards salinity compared to the plantlet stage and therefore, screening at callus stage would be more reliable for salinity tolerance evaluation (Gou et al., 2016).

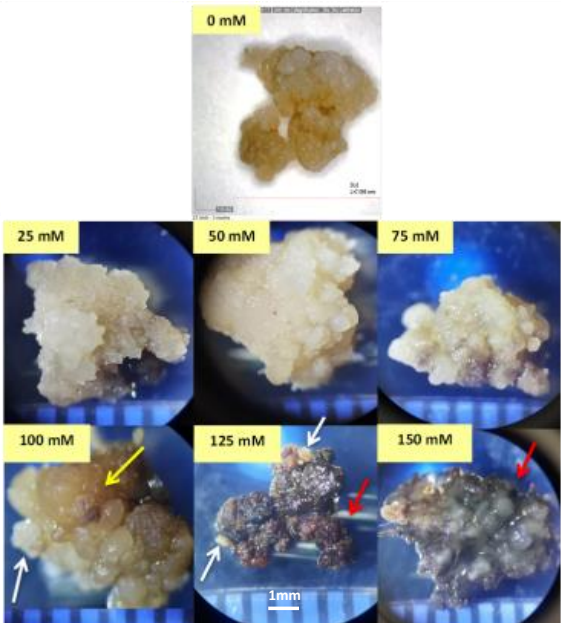

Figure 1. Morphology of MARDI Siraj 297 rice calli after 5 mo in MS medium containing NaCl under 20X magnification. The white, yellow and red arrows show the viable tissue, browning region and necrotic callus region, respectively (scale = 1 mm).

Screening and selection of 'MARDI Siraj 297' salt tolerant calli based on compatible solutes and antioxidant activities

Despite tissue injury on callus in medium with NaCl, some of them showed tolerance hence able to thrive and proliferate through the 5 mo of selection period. These viable tissues which consist of somatic embryos at globular and coleoptilar stage appears as cream-colored patches on the surviving calli in selection medium containing 25 to 125 mM NaCl. The biochemical parameters such as compatible solutes and antioxidant enzyme activities discriminate between these salt tolerant calli and the non-tolerant control as shown in Figure 2.

The proline level in salt tolerant calli was significantly higher than control in 50 to 125 mM NaCl medium. Proline accumulation was reported to have positive correlation with salinity tolerance capacity (Kalhori et al., 2017; Kibria et al., 2017). Increased proline production was proved to diminish ROS levels, thus prevents programmed cell death as reported by Hasanuzzaman et al. (2020) and Mattioli et al. (2020). Apparently, the calli in 150 mM that underwent severe necrosis showed the highest proline content compared to the calli in other treatments which were viable. These observations lead to the assumption that the proline accumulation levels in calli produced in 25 to 125 mM were sufficient to enhance antioxidant defense system so that the cellular ROS accumulation is maintained at a steady-state (Hasanuzzaman et al., 2020).

Likewise, the TSS of salt tolerant calli in 50 to 150 mM NaCl was significantly elevated as compared to control. Low molecular mass solutes such as soluble sugars such as sucrose, hexose, trehalose and sugar alcohols were reported to be produced during salinity to maintain osmotic pressure by acting as osmoticum or as respiratory substrates (Chen et al., 2021).

Significant reduction in total protein content was observed in salt tolerant calli treated in NaCl even at the lowest concentration of 25 mM. However, further salinity increases from 50 to 125 mM NaCl. Likewise, the similar reduction in protein content was also reported on the germinating maize seeds treated with 200 mM NaCl (Sangeetha, 2013). Highly saline environment increases water surface tension that causes protein and salt ions to compete for hydration; which eventually removes the essential water layer from protein surface that leads to denaturation (Mittler, 2017).

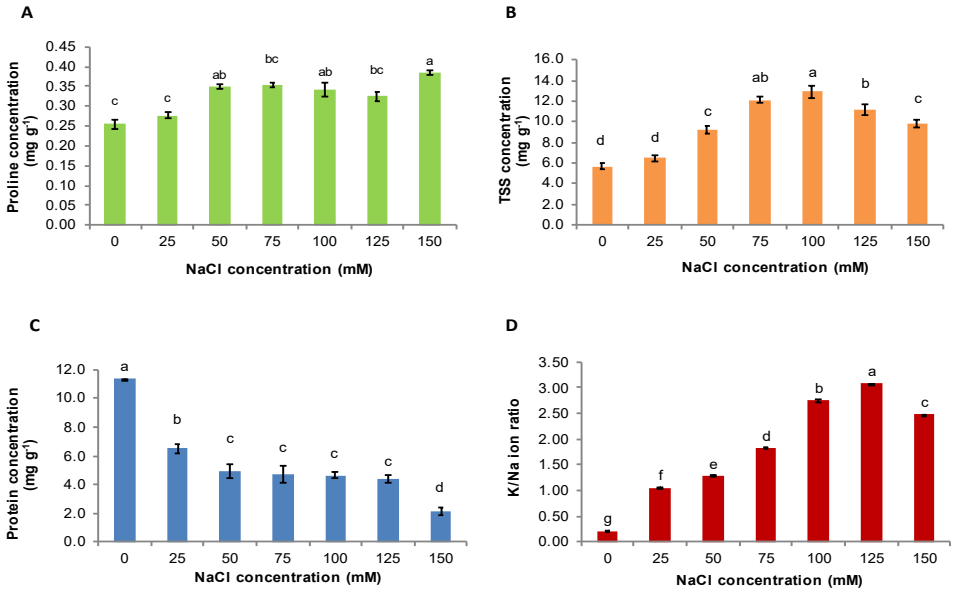

Figure 2. The total proline content (A), total soluble sugar (TSS) (B), protein content (C) and K^+/Na^+ ratio (D) of salt tolerant calli treated in different salinity level. Means with different letter(s) are significantly different at $p < 0.05$ by DMRT.

Higher K^+/Na^+ ratio was found in salt tolerant calli, with significant increase as the salinity level progressed to 125 mM NaCl, suggesting that the produced salt tolerant calli were able to maintain low cellular level of Na^+ despite the elevated Na^+ concentration in the medium hence keeping high K^+/Na^+ ratio. Maintenance of high cellular K^+/Na^+ ratio is important for survival of rice species during NaCl stress because increased Na^+ generally reduces the K^+ content due to antagonistic effect of the ions that inhibit the K^+ uptake, which is essential for growth and development as reported by Kibria et al. (2017) and Reddy et al. (2017a). Studies in different plant species proved that the elevated K^+/Na^+ ratio was responsible for the enhanced plants salinity tolerance including rice (Alhasnawi et al., 2016; Kibria et al., 2017). Therefore, K^+/Na^+ ratio could be used as an important salt tolerance indicator during callus stage.

Results found that the activity of antioxidant enzymes such as CAT, APX and LPX in calli was significantly increased in salinity as compared to control (Figure 3). Similar to this finding, a study on safflower demonstrated that salt stress induces further increases in the enzyme activity of CAT and APX, along with other antioxidant enzymes such as superoxide dismutase and guaiacol peroxidase in tolerant cultivar as compared to the sensitive ones (Vijayalakshmi et al., 2016). In addition, Kibria et al. (2017) demonstrated that increasing salinity leads to significant increase in CAT and APX activities in salt tolerant genotypes but decrease in the salt-sensitive ones. During salinity stress, the upregulation of both CAT and APX together with increased activities of dehydroascorbate reductase (DHAR) and glutathione reductase (GR) possibly provide protection to the cell membrane (Sarkar et al., 2013). Catalase and APX are regarded as the most remarkable enzymes because the former mainly involved in peroxisomes and reductant is not required for dismutation reaction catalysis. Between these two, APX has a higher affinity towards H_2O_2 that reduces it to water in chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space (Sofo et al., 2015).

Various studies reported the increase in LPX during salinity stress. For instance, Cheng et al. (2020) reported two-fold increase in LPX and total ROS content in rice root tissues in comparison with the control under salinity stress while in mung bean, double increase in MDA level was reported upon exposure to 100 mM NaCl (Ahmad et al., 2019). This study showed that the toxic effect of salinity was not limited to whole-plant level as reported in other finding (Dramalis et al., 2021), but rather appeared to be a cellular phenomenon that changed the morphology and biochemical traits at callus or tissue level. Therefore, these biochemical traits are useful for identification and selection of salt tolerant callus.

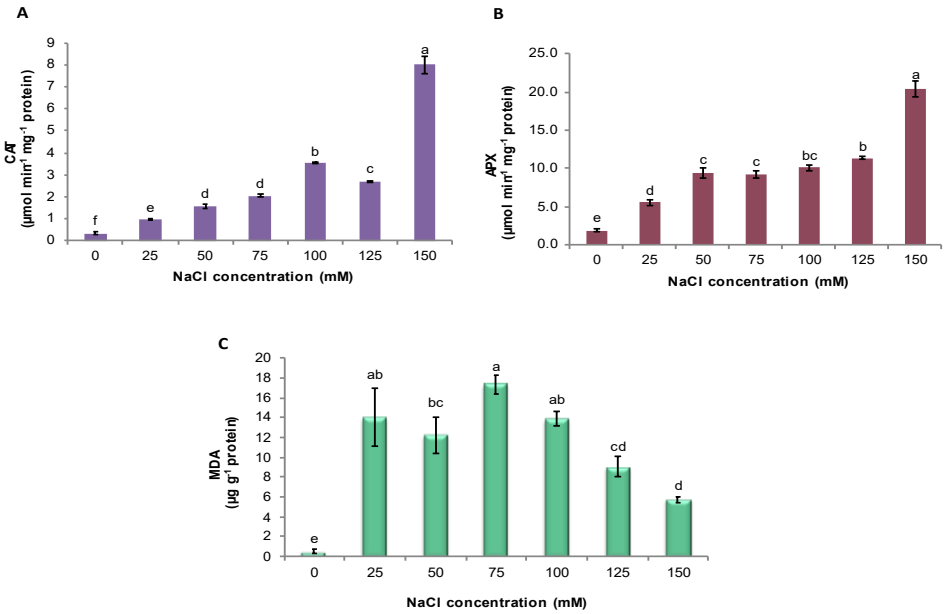

Figure 3. Catalase (CAT) activity (A), ascorbate peroxidase (APX) activity (B), and lipid peroxidation (C) in callus treated under different salinity level. Means with different letter(s) are significantly different at $p < 0.05$ by DMRT. MDA: Malondialdehyde.

Heritable somaclonal variation from a stable tissue culture derived rice line (TC-reg-2008) was previously reported by Zhang et al. (2014), which was confirmed by whole genome sequencing, Hence the possibility of obtaining stable somaclonal variant for salt tolerant line through induction of salinity stress by sodium chloride during *in vitro* culture is very promising. Since sodium and chloride are the most predominant ions in salinized soils, the use of this salt in this study mimics the real situation faced by plants in the field (Ghosh et al., 2016). In Malaysian rice fields, salinity level can fluctuates depending on the rainfall and season, which can range from very low to moderate salinity (4-8 dS m^{-1}), whereas salinity threshold level for 'MARDI Siraj 297' is 4 dS m^{-1} (Sazali et al., 2021). Therefore, the maximum salinity level imposed in this study is above this threshold to ensure the selected calli survived the threshold level.

Phytoprotective effect of SA on morphology and regeneration capacity of salt tolerant lines

The morphological features showed that the calli viability decreased as the NaCl concentration increases, which was demonstrated by the larger area of browning on the callus clumps as shown in Figure 4. However, the browning and necrosis were less severe in SA treated calli (0.5 and 1.0 mM) as compared to the non-SA treated control. It was observed that in medium without SA, the cream colored calli at 25 mM NaCl started to become browning at 50 mM NaCl. At 75 and 100 mM, the tissue became swollen and watery with only small patches of viable tissues. At the highest 125 mM NaCl, the calli became severely necrotic, watery and easily ruptured during subculture. Meanwhile, in medium with SA, the area of viable tissues was much increased as compared to calli without SA. At higher concentration of 100 and 125 mM NaCl, the calli in 1.0 mM SA were able to maintain the viability of most of their tissue, with only some browning and necrosis at the base of calli that were in contact with the medium. Oxidative browning is frequently a severe problem during in vitro plant culture as a result of phenolic compounds accumulation and oxidation, which consequently increase the prevalence of tissue browning especially in discrete cells, making them more prone to plasmolysis and rupture (Jones and Saxena, 2013). In other plants, in vitro salinity stress also induced a noticeable increase in callus browning rate, which was accompanied by salt induced damage in anatomical structures of such as reduced palisade tissue and chloroplast, poor xylem development and severe phloem tissue damage (Gou et al., 2016).

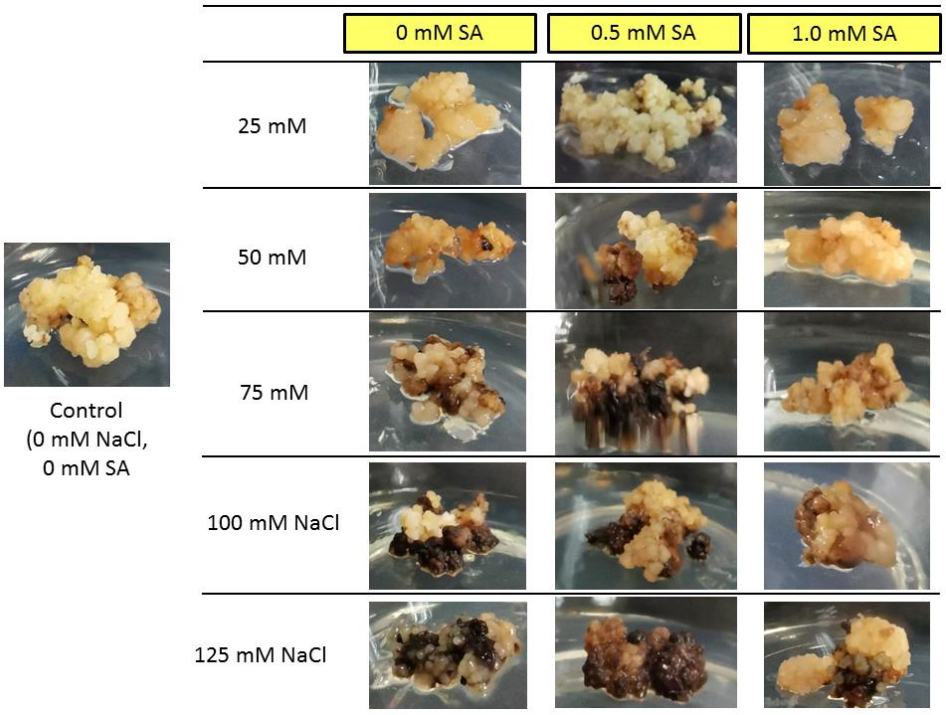

Figure 4. Morphology of rice calli after 1 mo on salinity tolerance enhancement medium with salicylic acid (SA) at different NaCl concentration.

The inherent potential of salt tolerant calli to regenerate after salt treatment and SA enhancement was evaluated by transferring the calli into MS regeneration medium. As shown in Table 1, regeneration frequency and number of shoots significantly decreased as the salinity increases. Meanwhile, significant improvement in regeneration frequency and number of shoots were observed in the presence of SA. However, the two-way ANOVA showed that there was nonsignificant interaction effect between salinity level and SA concentration in this study, meaning that the effect of salinity level on regeneration frequency and number of shoots does not depend on the SA concentration and vice versa. Calli recovered from medium enhanced with 1.0 mM SA showed the highest regeneration frequency and number of shoots, followed by 0.5 mM SA and without SA. The application of SA has been proven to ameliorate the impact of NaCl. Phytoprotective effect of SA foliar application (0.5 mM) was observed in tomatoes grown at concentration up to 90 mM NaCl, whereby salt-stressed plants treated with SA showed higher fruit length, diameter and yield as compared to the control (Naeem et al., 2020). The alleviation of salinity stress by SA was governed by different mechanisms including modulation of osmolytes, antioxidant enzymes activity and nutrient uptake (Alsahli et al., 2019). Exogenous SA application has been reported to improve the survival and growth under salt stress in many plant species such as rice (Jini and Joseph, 2017), wheat (Alsahli et al., 2019) and tomato (Naeem et al., 2020). The ability to reduce browning and improve regeneration frequency and number of shoots in this study indicates the potential of SA to be used during in vitro stress-induced selection.

The ability of salt tolerant calli to regenerate into plantlets in their respective salinized regeneration medium proves that these plantlets inherently retain its tolerance during callus stage (Figure 5). These salt tolerant plantlets were then acclimatized and transferred to the field until seeds were produced.

Table 1. Regeneration frequency and number of shoots per explants of salt tolerant plantlets produced from salicylic acid enhancement medium that contain different NaCl and SA concentrations (6 NaCl x 3 SA combinations). The plantlets generated were previously treated with NaCl during callus stage. Each value represents the mean ± standard error. Within the same column, means with different letter(s) are significantly different at $p < 0.05$ by DMRT. *, **Significant at the 0.05 and 0.01 levels, respectively; ^{ns}: nonsignificant.

Factor	Concentration	Regeneration frequency	Number of shoots per callus
	mM	%	
NaCl (N)	0	74.4 ± 3.1^{a}	7.8 ± 0.2^{a}
	25	52.2 ± 3.5^{b}	7.5 ± 0.2^{a}
	50	38.9 ± 3.6^{c}	7.0 ± 0.2^{b}
	75	34.4 ± 3.7^{cd}	6.8 ± 0.2^{b}
	100	30.0 ± 3.7^{d}	2.1 ± 0.2^{c}
	125	16.7 ± 3.5^{e}	1.1 ± 0.2^{d}
Salicylic acid (S)	0	28.9 ± 3.2^{c}	4.7 ± 0.4^{c}
	0.5	38.3 ± 3.1^{b}	5.2 ± 0.3^{b}
	1.0	56.1 ± 3.0^{a}	6.2 ± 0.4^{a}
Significance level	N	**	**
	S	**	**
	N × S	ns	ns

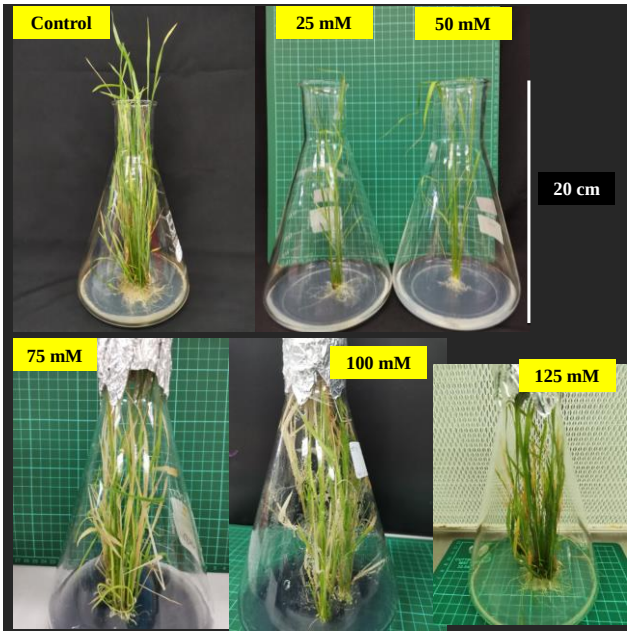

Figure 5. The selected, healthy growing salt tolerant line plantlets regenerated from the selected salt tolerant calli. The ability of salt tolerant calli to survive in salinized selection medium and subsequently regenerate into plantlets proves that these plantlets retain its tolerance during callus stage.

CONCLUSIONS

The salt tolerant lines of 'MARDI Siraj 297' rice were successfully produced in this study through in vitro selection by salinity stress induction on embryogenic calli. The biochemical profile of these surviving salt tolerant calli showed significant increase in proline, total soluble sugar, catalase, ascorbate peroxidase, malondialdehyde and K^+/Na^+ ratio as well as reduction in protein content as compared to non-tolerant control, thereby confers their tolerance against salinity. In addition, supplementation of salicylic acid (SA) in the medium enhanced the salinity tolerance of the calli which was marked by reduction in the appearance of browning and necrotic tissue, higher regeneration frequency and higher number of shoots as compared to the non-treated calli, hence suggests that SA provide protection against salinity that helped the calli recover from a prolonged salinity stress. In summary, in vitro somaclonal variation resulted through selective salinity pressure in this study was found to be useful in generating tolerant variants. The established salt tolerant lines have huge potential to be acclimatized and utilized in salinity affected areas. This study also provides a reliable protocol for the establishment of salt tolerant rice lines through tissue culture selection.

Author contribution

Conceptualization: N.S., R.N. Methodology: N.S., R.N., C.Y.S.Y., R.S. Formal analysis: N.S., R.N. Investigation: N.S. Resources: R.N. Data curation: N.S., R.N. Writing-original draft: N.S Writing-review & editing: N.S., R.N., Y.C.K., C.Y.S.Y., R.S. Supervision: N.S., R.N., Y.C.K., C.Y.S.Y., R.S. Project administration: R.N. Funding acquisition: R.N. All co-authors reviewed the final version and approved the manuscript before submission.

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