



Full Length Article

Selection, Characterizations and Somatic Embryogenesis of Malaysian Salt-tolerant Rice (*Oryza sativa* L. cv. MR219) through Callogenesis

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Abstract

Salt-tolerant lines of MR219 were produced through somatic embryogenesis from salt-tolerant callus of *Oryza sativa* L. cv. MR219 using *in vitro* selection procedure. Callus was developed aseptically from seeds on MS media supplemented with 2 mg L⁻¹ 2, 4-D. Then, callus directly was sub-cultured on MS media with different concentrations of NaCl (0, 50, 100, 200, and 300 mM) to produce salt-tolerant callus. Based on the callus characteristics which are morphological, physiological and biochemical cascades such as proline content, total protein, total soluble sugar, lipid peroxidation, activity of ascorbate peroxidase and catalase, salt-tolerant callus was screened and selected. After 4 months, callus cultured in 50 and 100 mM NaCl showed yellow color, soft, friable and nodular proliferating. However, callus cultured in 200 and 300 mM NaCl turn blackish-brown and stiff and acutely-necrotic. The selected salt-tolerant callus was sub-cultured on MS media for somatic embryogenesis. The salt-tolerant plantlets were transferred into pots individually for acclimatization purpose. Salt stress caused significant reduction in water content, fresh and dry weight of callus. The level of total soluble sugar, proline, lipid peroxidation and ascorbate peroxidase significantly increased under salt stress. Salt-tolerant callus indicated high activity of catalase that determined more protection against production of reactive oxygen species. According to growth performance and antioxidant capacity, the plantlets from 50 and 100 mM NaCl, selected as salt-tolerant line. This study suggests the methodology to produce salt-tolerant cultivar of rice which could be a step forward to commercialization. © 2017 Friends Science Publishers

Keywords: Salinity; Antioxidant; Lipid peroxidation; *Indica* rice MR219; Regeneration

Introduction

Soil salinity is a global eco-threat, which causes a reduction in productivity of agriculture all around the world (Mahajan and Tuteja, 2005). According to Wang *et al.* (2003) study, the global agricultural land will loss 30%- 50% at the year of 2050. Excess salt negatively affected on growth and biochemical cascades of rice in cellular level with osmotic stress and ionic imbalance (Yunita *et al.*, 2014). Excessive amount of Na⁺ and Cl⁻ imposed ionic imbalance and reduction capacity of water uptake by cells, which lead to oxidative stress and production of reactive oxygen species (ROS) (Wani *et al.*, 2010). The production of reactive oxygen species (ROS) causes degradation of proteins and lipids, thereby cell damage (Megdiche *et al.*, 2007). Plants cells have non-enzymatic and enzymatic defense system to control over production of ROS. Activity of antioxidant enzymes correlated with concentration of salt and plants species (Sharma and Ramawat, 2013). Generally, during

oxidative stress, plants with high antioxidant enzyme activity indicated better resistance to stresses (Parida and Das, 2005).

In recent years, the development of salt-tolerant crops has been done in different strategies to overcome salinity effects on crop productivity. Breeding program, genetic engineering, and *in vitro* selection procedure (tissue culture) are three efficient procedures for developing of salt-tolerant crops. However, *in vitro* selection is known as most feasible and low-cost procedure to select and develop salt-tolerant crops (Rai *et al.*, 2011). A plenty of research had been done to produce salt-tolerant rice using breeding program approach, genetic engineering, and tissue culture approach (Ahmad *et al.*, 2007; Khaleda *et al.*, 2007; Tariq *et al.*, 2008; Evangelista *et al.*, 2009; Abiri *et al.*, 2015; Rattana and Bunnag, 2015). However, no research has done to produce salt-tolerant MR219 lines until to date. Moreover, there are a few researches showed that *in vitro* selected salt-tolerant calli of rice were able to regenerate with genetic

stability (Basu *et al.*, 1997; Lutts *et al.*, 1999; Priya *et al.*, 2011; Mori-Gastelo *et al.*, 2015; Sankepally *et al.*, 2016). As a matter of fact, loss of regeneration potentiality or genetic instability is the major constrain in development stable salt-tolerant plants from salt-tolerant callus. Therefore, this present study attempts to produce and develop stable salt-tolerant MR219 plants by screening and selecting salt-tolerant callus line. It was hypothesized that salt stress effects on subsequent differentiation and proliferation pattern of MR219, for physiological, morphological, and biochemical cascades.

Material and Methods

Seed Materials

The seeds of MR219 rice variety provided by Malaysian Agriculture Research and Development Institute (MARDI) Serdang, Selangor, Malaysia.

Seed Sterilization and Callus Induction

The seeds of MR 219 were dehulled, and sterilized by immersion in 70% EtOH for 3 min and for 20 min, immersion in 40% NaOCl solution. Followed by rinsed three times in deionized water (Zuraida *et al.*, 2012). Sterilized seeds were cultured on MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.3% gelrite and 2 mg L⁻¹ 2, 4-D (pH 5.8) to induce the formation of MR219 callus. The cultures were maintained in the dark at 25 ± 2°C for 30 days. MR219 callus was sub-cultured in the same media for proliferation purpose.

Production, Screening and Selection of salt-tolerant MR219 Callus

Salt-tolerant MR219 callus was produced, screened and directly selected according to the modified method reported by Queiros *et al.* (2007). 30-day-old of callus was sub-cultured on same MS media as mentioned before with different concentrations of NaCl (0, 50, 100, 200 and 300) mM. Each treatment has 10 replicates. Callus incubated in the dark conditions at 25 ± 2°C for 4 weeks. Following this, callus was sub-cultured on MS media for another 4 weeks to examine the stability of the salt-tolerant callus. After this, some pockets of healthy callus were sub-cultured on MS media supplemented with different concentration of NaCl for another 4 weeks. Then, based on the callus characteristics, which are growth parameter, and biochemical cascades such as proline content, total protein and total soluble sugar to salt-tolerant MR219 callus was screened and selected.

Measurement of Tissue Water Content

Immediately after removal the callus from media, the fresh weight (FW) was recorded. The callus dried at 50°C in the

oven until constant dry weight (DW) (Lokhande *et al.*, 2010). The water content of callus cells was measured according to this formula: (Fresh weight (FW) - Dry weight (DW))/Fresh weight (FW) x 100 (Lai and Liu, 1988).

Measurement of Total Proline

Total proline was determined according to method described by Bates *et al.* (1973). Fresh stressed and non-stressed callus tissue (500 mg) were homogenized with 3% (w/v) aqueous sulfosalicylic acid (pH 7.8) and centrifuged at 800 g for 15 min. The supernatant (2 mL) was mixed with glacial acetic acid (2 mL) and ninhydrin reagent (2 mL) (1.25 g of ninhydrin, 30 mL of glacial acetic acid and 20 mL of 6 M H₃PO₄). The reaction incubated for 1 h at 100°C in water bath. After cooling the reaction, 4 mL Toluene was added. The absorbance of proline-ninhydrine product was determined at 520 nm.

Measurement of Lipid Peroxidation

The level of lipid peroxidation was assayed in terms of Malonylaldehyde (MDA) content method described by Heath and Packer (1968). Callus tissue (0.2 g) was homogenized in 5 mL of ice-cold TCA (0.1% w/v) and centrifuged for 15,000 g for 15 min at 4°C. The supernatant (1 mL) was mixed with 0.5% 2 mL TBA reagent in 2 mL 20% TCA. The reaction mixture was heated at 95°C for 30 min and immediately cooled. The mixture centrifuged (10,000 g for 10 min, at 4°C). The absorbance was measured at 532 and 600 nm. The MDA content was calculated according to formula:

$$\text{MDA } (\mu\text{g g}^{-1}\text{protein}) = [(A_{532}-A_{600}) \times V \times 1000 / \epsilon] \times \text{protein}$$

* ϵ is the specific extinction coefficient (=155 mM cm⁻¹).

*V (mL) is the volume of crushing medium.

Protein Extraction and Quantification and Enzyme Assay

Frozen callus (1 g) tissues were ground in 2 mL (62.5 mM Tris-HCl, pH 6.7), then centrifuged (20,000 × g for 30 min at 4°C). The supernatant used to measure of total soluble protein and enzyme activity assay. Total soluble protein estimate according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Measurement of Catalase Activity (Hydrogen Peroxide)

Catalase activity was measured using the method of Aebi (1974). The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL 15 mM H₂O₂ and 20 μL protein extract. The decomposition of H₂O₂ was followed by the decline in absorbance at 240 nm (ϵ = 36 mM⁻¹ cm⁻¹). Catalase activity was expressed in terms of unit mg⁻¹ protein.

Measurement of Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) activity was assayed using the method of Jebara *et al.* (2005) by measuring the decrease in absorbance at 290 nm for 1 min ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture (1.0 mL) consisted of 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 15 μL protein extract. Ascorbate peroxidase activity was expressed in unit mg^{-1} protein.

Regeneration of Salt-tolerant MR219 Plantlets from Salt-tolerant MR219 Callus

Shoot induction of salt-tolerant MR219 callus: Selected salt-tolerant callus were cultured in MS media supplemented with 2 mg L^{-1} kinetin combined with 1 mg L^{-1} BAP to induce shoot formation (Rao and Jabeen, 2013). The rate of shoot induction [(the number of callus that regenerated shoot/the number of total callus) x 100%] was counted after 4 weeks.

Root Induction of salt-tolerant MR219 callus: Selected salt-tolerant callus were sub-cultured in MS media supplemented with 0.5 mg L^{-1} BAP, 1 mg L^{-1} kinetin, 1 mg L^{-1} IBA and 0.5 mg L^{-1} NAA for another 4 weeks to induce the formation of root (Gopitha *et al.*, 2010). Following this, the regenerated plants were transferred into pots individually for acclimatization purpose.

Statistical Analysis

All the experiments were repeated thrice. The collected data was analyzed using SPSS window version 22. Oneway ANOVA at $P \leq 0.05$ was conducted to study the diffident among treatment followed by Duncan's multiple comparison test at $P \leq 0.05$ for mean comparison.

Results

In Vitro Production and Screening of Salt-tolerant MR219 Callus

NaCl-tolerant callus lines was directly selected and developed from *in vitro* callus culture under NaCl treatments. After 4 months, MR219 callus cultured in 50 and 100 mM NaCl showed yellow color, soft, friable and nodular proliferating (Fig. 1). However, callus cultured in 200 and 300 mM NaCl turn blackish-brown and stiff and acutely-nerotic. These characteristics of callus were showing the response to salinity. Results showed that increasing NaCl concentration causing fresh and dry weight of callus significantly declined ($P \leq 0.05$) (Fig. 2). There was a significant reduction ($P \leq 0.05$) in the percentage of water content among callus cultured in different concentration of NaCl (Fig. 3). Percentage of water content of callus in control (74.6%) is significantly higher than callus cultured in NaCl.

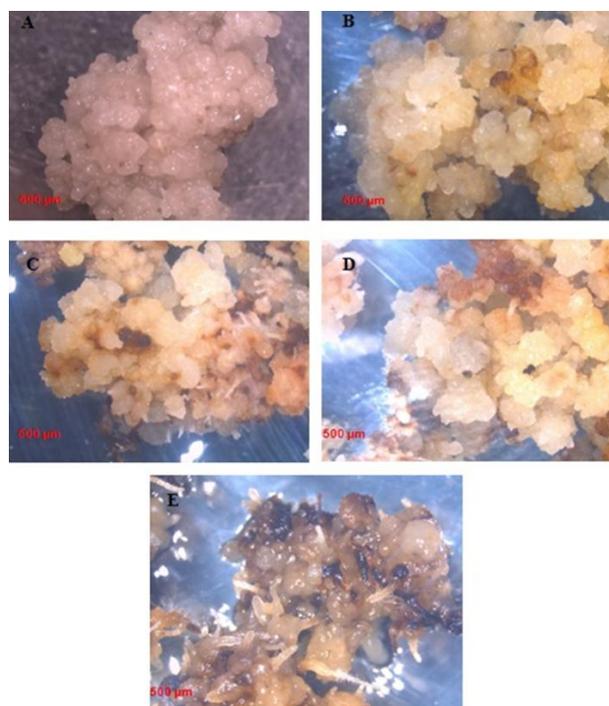


Fig. 1: Morphology of MR219 callus after 4 months on MS media supplemented with: A) Control (0 NaCl), B) 50 mM, C) 100 mM, D) 200 mM and E) 300 mM NaCl

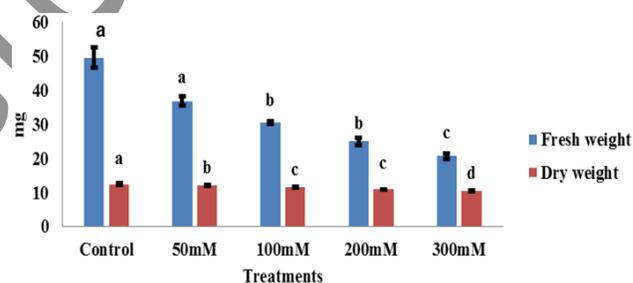


Fig. 2: Fresh and dry weight of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months

Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

As a result, at higher concentration of NaCl, the water content becomes lower. For screening and selection of salt-tolerant MR219 callus, the biochemical cascades such as total proline content, total soluble sugar, total soluble protein, malondialdehyde (MDA), activity of APX and CAT of salt-tolerant MR219 callus was measured.

Accumulation of total proline content of callus under NaCl treatments is significantly enhanced ($P \leq 0.05$) compared to control (Fig. 4). Total soluble sugar of callus correlated with increased NaCl concentration (Fig. 5). In addition, results show that the total soluble protein of callus is decreased with increasing NaCl concentration.

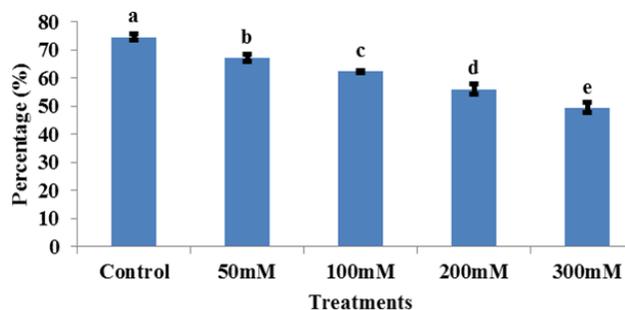


Fig. 3: Percentage of tissue water content of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

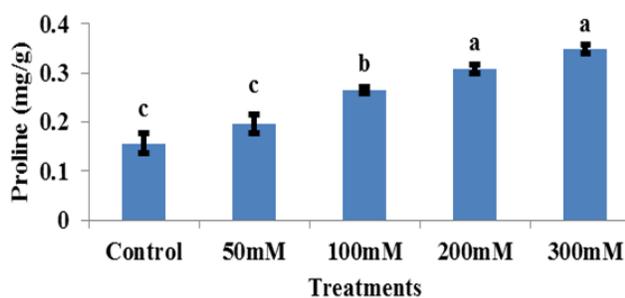


Fig. 4: Total proline content of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

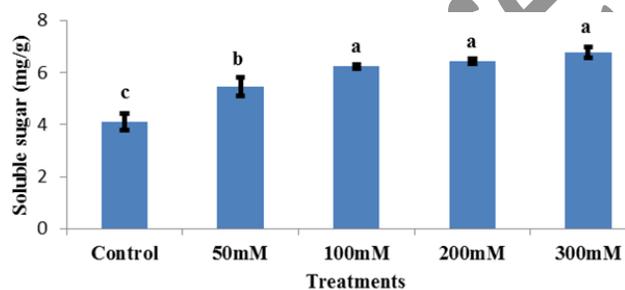


Fig. 5: Total soluble sugar (TSS) of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

The MDA contents of callus significantly ($P \leq 0.05$) enhanced under NaCl treatments (Fig. 7). As a result, there was a considerable increase in membrane damage rate in high concentration of NaCl. The MDA contents in 300 mM (107.08 $\mu\text{g g}^{-1}$ protein) and 200 mM (103.4 $\mu\text{g g}^{-1}$ protein) NaCl are significantly higher than 100 mM (78.8 $\mu\text{g g}^{-1}$ protein) and 50 mM (63.3 $\mu\text{g g}^{-1}$ protein)

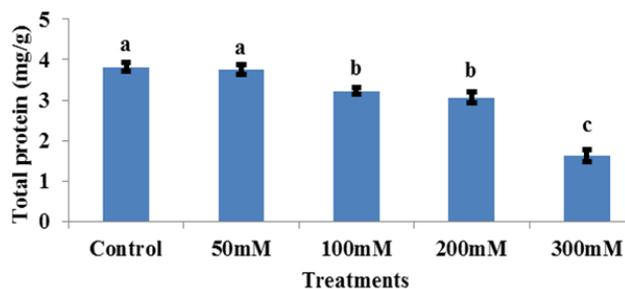


Fig. 6: Total Soluble Protein of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

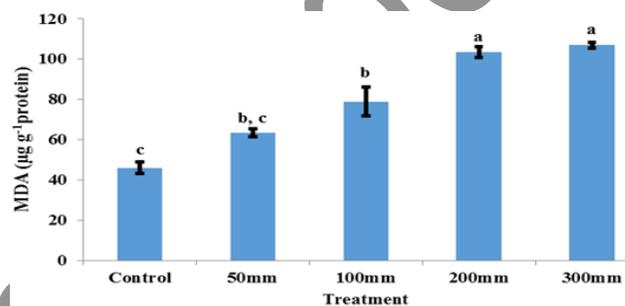


Fig. 7: Lipid peroxidation (MDA content) of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

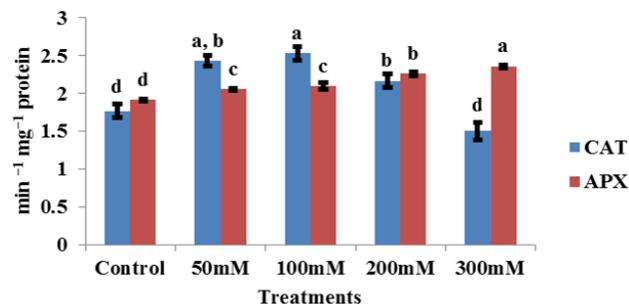


Fig. 8: Antioxidants activity of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 months
 Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

NaCl, followed by control (45.9 $\mu\text{g g}^{-1}$ protein). The activity of APX of callus significantly ($P \leq 0.05$) was increased in NaCl treatments compare with control (Fig. 8). CAT activity increased in mild level of NaCl and then significantly ($P \leq 0.05$) decreased in high level of NaCl (Fig. 8).

Selection of Salt-tolerant MR219 Callus and Regeneration of Salt-tolerant MR219 Plantlets

The viability of callus which is greenish-yellow has shown in 50 and 100 mM NaCl (Fig. 9). The callus was considered and selected as salt-tolerant line and sub-cultured in MS media for regeneration of salt-tolerant plantlets. The growth of the callus is significantly lower than control on different concentrations of NaCl ($P \leq 0.05$) (Fig. 10). Somatic embryogenesis (shoot induction) was higher in control followed by 50 mM (Fig. 11). The result indicates that the average of percentage of shoot induction in control (82%) is significantly ($P \leq 0.05$) higher than 50 mM (44%), followed by 100 mM (15%).

Discussion

In vitro selection is an efficient and cost-effective procedure to develop salt-tolerant plants. We successfully selected and developed MR219 cell lines by *in vitro* selection on MS media supplemented with 50 mM and 100 mM NaCl. It was observed that NaCl obstructed callus growth and development. Thus, sensitive callus to NaCl turn to blackish-brown and become stiff and acutely-necrotic. However, adapted callus to NaCl appeared greenish and good cell proliferation with ability of regeneration. Our results indicated that NaCl effects on different physiological and biochemical cascades at cellular level of rice (Hazman *et al.*, 2015). Increasing of NaCl concentrations caused reduction in water content, fresh and dry weight of callus cells. Reportedly salinity has two major effects on plants cells. Firstly, high concentration of NaCl produced hyperosmotic stress in cells that inhibited water uptake, which lead to decrease nutrition absorption and low water availability. Consequently in cellular level, these process lead to decrease water content, which caused reduction of fresh and dry weight (Wani *et al.*, 2010). Secondly, uptake high level of toxic ions (Na^+ and Cl^-) produced ionic stress, which poisoned plants cells and negatively affected on photosynthesis efficiency (Shahid *et al.*, 2013). Photosynthesis production for 12- 16 h provided the energy for osmotic adjustment in plants. Hence, it's one of the causes of decrease in plant growth (Warren *et al.*, 2011).

In cellular level, plants have developed complex mechanisms to adjust hyperosmotic stress and ionic imbalance by osmotic adjustment (OA). These mechanisms accumulate osmotic regulators such as sugars and proline to protect membrane integrity and stabilize enzymes against oxidative stress (Chen and Jiang, 2010). In our findings, callus treated by NaCl showed level of proline content significantly increased compare to control. Accumulation of a nitrogenous compounds such as proline leading to necrosis and tissue damage (Dibax *et al.*, 2005). Thus, accumulation of proline due to high concentrations of NaCl caused MR219 callus necrosis. Increasing production of reactive

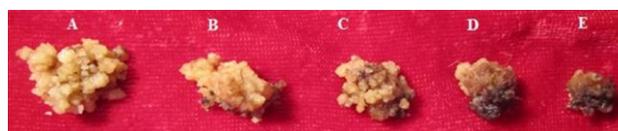


Fig. 9: Growth performance of MR219 callus under after 4 months on MS media supplemented with: A) Control (0 NaCl), B) 50 mM, C) 100 mM, D) 200 mM and E) 300 mM NaCl

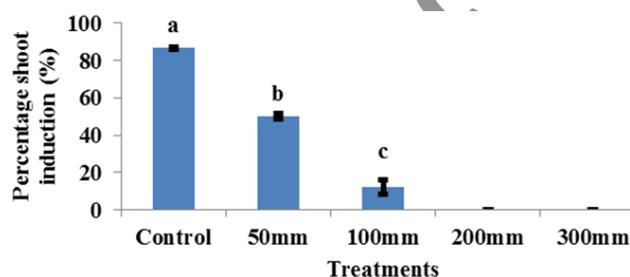


Fig. 10: Regeneration percentage of shoot induction of MR219 callus on MS media supplemented with different concentrations of NaCl after 4 month

Bars represent means \pm S.E. of three replicates and letters indicate significant ($p \leq 0.05$) differences among treatments

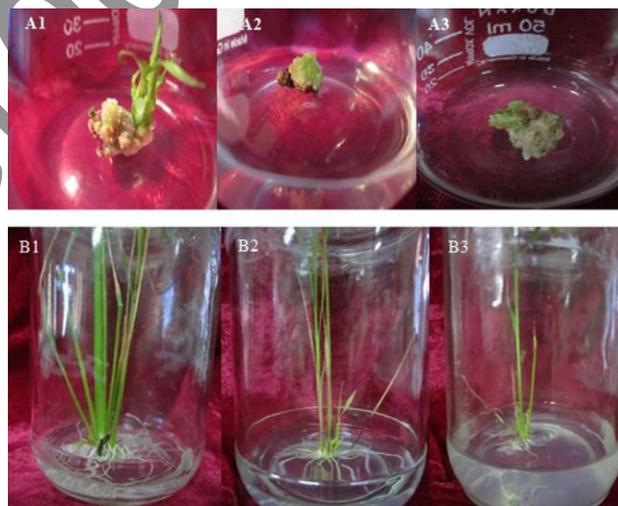


Fig. 11: Somatic embryogenesis of greenish salt-tolerant callus of MR219 on MS media supplemented with 0.5 mg L^{-1} BAP, 1 mg L^{-1} kinetin, 1 mg L^{-1} IBA and 0.5 mg L^{-1} NAA; A1) Control, A2) 50 mM, A3) 100 mM. Regeneration of MR219 salt-tolerant callus; B1) Control, B2) 50 mM, B3) 100 mM

oxygen species (ROS) causes the cell membrane damage through oxidation of lipids, protein and nucleic acids in cells (Sharma *et al.*, 2012). Lipid peroxidation (MDA) content as a salt injury index in plants cells membrane, has widely used to determining salt-tolerance and the degree of damage (Ozgun *et al.*, 2013). In this study,

salinity caused lipid peroxidation (MDA) content significantly increased. Total soluble sugar has been known as source of carbon, and ROS scavenging during salinity stress. Hence, during severe stress time, level of total soluble sugar increased to keep the plants osmotic potential balanced and provide energy for plants (Elmaghrabi *et al.*, 2013). Increasing level of total soluble sugar in MR219 callus was observed under salt stress. Low level of total soluble protein during ROS attack is due to reaction among amino acids of protein with active radical such as $O_2^{\cdot-}$, H_2O_2 , and OH^{\cdot} . Therefore, decreasing of total protein content in callus caused by ROS attack, which leading to degradation proteins (Agastian *et al.*, 2000).

Plants developed strategies to control and decreasing the harmful effects of ROS by non-enzymatic and enzymatic antioxidant defenses systems. A non-enzymatic defense system involves phenolic compounds and lipid peroxidation. An enzymatic defense system include catalase and ascorbate peroxidase (Desikan *et al.*, 2004). APX and CAT as the ROS scavenging system, were associated with the oxidative damage of the enzymatic defense system caused by environmental stresses (Tang and Newton, 2005). The role of APX is crucial in scavenging H_2O_2 within the organelles in response to environmental stresses. In our findings, APX activity is closely correlated with increasing concentration of NaCl. APX uses two molecules of electron donors (ascorbate) to convert H_2O_2 to H_2O . At the same time, two molecules of electron acceptors (Monodehydroascorbate) generates in the chloroplast (Sharma *et al.*, 2012). CAT protects plants cells and tissues against the toxicity effects of H_2O_2 . CAT is an important scavenger enzyme to convert 6 million molecules of H_2O_2 to water and oxygen per minute. Meanwhile, in long term activity of a CAT enzyme to convert H_2O_2 , the catalase-bound NADPH became oxidized to $NADP^+$. Hence, the activity of CAT fell to about one-third of the initial activity (Gill and Tuteja, 2010). According to the result, CAT activity increased in NaCl-tolerant callus and decreased in NaCl sensitive callus. The decline in activity of CAT is due to inactivation of CAT by the accumulation of H_2O_2 in high level of NaCl.

Conclusion

During salt adaption, increasing proline content didn't give the callus protection under salinity. However, increasing CAT activity indicate that callus can tolerant NaCl and regenerate. Elevated of CAT activity in NaCl-tolerant callus shows more efficient detoxification of free radicals. As a result of growth performance and antioxidant activity, the plantlets from 50 and 100 mM NaCl, could be selected as salt-tolerant line which showed better adaptability. Further studies are needed on agronomic performance and genetic stability of salt-tolerance regenerated under greenhouse/field conditions.

Acknowledgements

The authors would like to acknowledge to Universiti Putra Malaysia for the research grants, PUTRA GRANT- IPS-9446700 for financial support.

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(Received 27 June 2016; Accepted 28 October 2016)