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Research Article

In vitro Selection and Development of Malaysian Salt-Tolerant Rice (*Oryza sativa* L. cv. MR263) Under Salinity

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Abstract

An efficient plant regeneration protocol *via* somatic embryogenesis from *Oryza sativa* L. cv. MR263 was established in this study. MR263 seeds were cultured on MS media supplemented with ideal concentration of 2, 4-D, 1 mg/L for 30 days to produce callus. Then, callus was subjected to different concentration of NaCl (0, 100, 150, 200, and 300 mM) on the same MS media to produce salt-tolerant callus. Four months old NaCl-treated and NaCl-free callus have been screened based on the characteristics such as physiological, morphological and biochemical markers to select salt-tolerant line. As a result, callus treated with low concentration of NaCl showed yellow color and friable proliferating, however callus treated with 200 and 300 mM NaCl shows more watery, discoloration with brown patch. To induce shoot and root, selected salt-tolerant callus was sub-cultured on MS media containing auxin and cytokinin. The proline content, total soluble sugar and total protein were enhanced significantly in NaCl-treated callus. At cellular level, histological analysis of salt-tolerant MR263 callus revealed that salinity negatively affected on development somatic embryos. Therefore of cellular responses and growth performance, the plantlets that regenerated from 100 mM NaCl-treated callus were selected as salt-tolerant lines. This research suggests a novel protocol for regeneration and development salt-tolerant rice cultivar and other crops.

Keywords: Salinity; Indica Rice MR263; Regeneration; Proline Content; Salt-Tolerant Callus; Salt- Tolerant MR263

Introduction

The beginning of 21st century, agricultural sustainability has been threatened by human population and reduction in land available for cultivation. At the same time, due to the climate changes the cultivated lands are decreased. Thus, low productivity in agriculture could be related to different abiotic stresses [1]. According to Yamaguchi and Blumwald [2], soil salinization is one of the limiting factors among abiotic stresses that constraint yield of agriculture on about 20% of the cultivated and irrigated area globally. Rice (*Oryza sativa* L.) is an important and essential food item that feed more than 50% of the world population. In Asia, rice provides 40-70% of the total food calories consumed [3]. Glycophytes such as rice are very sensitive to saline soil especially at the early stage of growth. Rice has no various strategies and mechanisms to deal effectively with the excessive presence of salt and therefore does not grow well on saline soil [4].

Salinity causes ionic and osmotic stress in plants [5]. Osmotic stress leads to reduction of water uptake by root, and accumulation of two toxic ions Na^+ and Cl^- derived from NaCl can damage the plant cells through the both osmotic and ionic mechanisms. Followed by primary stresses, oxidative damage as secondary stress may occur and generate more aggressive oxygen radicals containing $^1O_2$, H_2O_2, $O^{\bullet-}_2$, and $OH^{\bullet}$, which are known as reactive oxygen species (ROS) [6]. Plants developed adaptation mechanisms to overcome the oxidative stress and ionic imbalance by osmotic adjustment (OA). Accumulation of organic solutes such as proline and total soluble sugar stabilize cellular and protect membranes structure. Meanwhile, stress condition caused production of Reactive Oxygen Species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals. Plants scavenged of reactive oxygen species by non-enzymatic and enzymatic antioxidant defenses systems (Sharma*., et al.* 2012). In general, salinity caused a negative impact on a physiological parameters, decrease the varied

number of plants per unit area, decreased seed yield, and individual grain size and even delayed heading [7].

In the recent past, in order to ensure the food security, plant cell and tissue culture techniques are used for the production and development salt-tolerant lines of crops throughout the world. Besides the use of tissue culture in selection of salt-tolerant cell lines, these lines have been used to regenerate salt-tolerant plants [8]. To date, several studies have developed salt-tolerant plants especially using *in vitro* selected NaCl tolerant rice cell lines [9-13] and various plant species such as cauliflower [14], sugarcane [15] and wheat [16]. Hence, the improvement and production of salt-tolerant crops including rice have become significant and up to date, salt-tolerant MR263, as a high yielding Malaysian rice variety, has not yet produced. Meanwhile, there is scarce report to show successful generation of *in vitro* selected salt-tolerant callus of rice with genetic stability. As a matter of fact, in *in vitro* selection salt-tolerant callus and production of stable salt-tolerant plants, the risk of genetic instability adds to the lack of regeneration potentiality as two main limitation factors. The present study aimed to assess the responses of MR263 callus toward salinity by screening and applied *in vitro* selection to produce salt-tolerant MR263 line. It was hypothesized that salt-tolerant MR263 callus withstand towards salinity with regeneration ability.

Materials and Methods

Seed material: Rice seeds MR263 were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Seberang Perai.

Seed sterilization: Mature rice seeds were soaked in 70% (v/v) EtOH for 2 minutes under aseptic conditions [17] and then surface sterilized into 10% NaOCl solution for 20 minutes, followed by rising to five times with sterile double-distilled water [18].

Induction of callus from MR263 seeds: Sterilized MR263 seeds were cultured on solid media of MS basal medium [19], 3% sucrose, 0.3% Gelrite agar and different concentrations of 2, 4-Dichlorophenoxyacetic acid (2,4-D) (0, 0.5 mg/L, 1 mg/L, 2 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, and 20 mg/L) as shown in table 1 (pH 5.8). Inoculated MR263 seeds were placed in the dark conditions at 25 ± 2°C for 28 days. Thirty replicates were used per treatment [20].

The callus induction percentage per treatment was measured and expressed according to the equation:

$$\text{Callus induction rate (\%)} = \frac{\text{Total number of explants produced callus}}{\text{Total number of explants cultured}} \times 100$$

Treatment	mg/L 2,4-D
Control	MSO
T1	MS media + 0.5 mg/L 2,4-D
T2	MS media + 1.0 mg/L2,4-D
T3	MS media + 2.0 mg/L 2,4-D
T4	MS media + 2.5mg/L 2,4-D
T5	MS media + 5.0 mg/L 2,4-D
T6	MS media + 10.0 mg/L 2,4-D
T7	MS media +15.0 mg/L 2,4-D
T8	MS media + 20.0 mg/L 2,4-D

Table 1: Treatment with different concentrations of 2, 4-D.

Production of salt-tolerant MR263 callus: The method of Queiros, Fidalgo [21] was used to directly induce salt-tolerant MR263 callus. Twenty eight days old callus was sub-cultured on MS media supplemented with 1 mg/L 2,4-D containing different concentrations of NaCl (0, 100, 150, 200 and 300 mM) to produce salt-tolerant callus of MR263 for another 28 days to establish salt-tolerant callus. Ten replicates were made per treatment. Following this, callus were sub-culture on MS media without treatment to examine the stability of callus for another 28 days. Then, callus subculture on MS media supplemented with different concentration of NaCl for 28 days.

Screening and selection of salt-tolerant MR263 callus: Salt-tolerant MR263 callus was screened according to the characteristics of callus and biochemical markers which is proline content, total protein and total soluble sugar.

Measurement of callus growth: Immediately after removal the 4 months old callus from media, the fresh weight (FW) was recorded. The callus morphology was identified by visual observation such as size, color, and form (friable, compact, watery) [20,22].

Measurement of proline content: Determination of the total proline content was done by the method of Bates, Waldren [23]. NaCl-treated and NaCl-free callus tissue (0.5g) were mixed with 3% aqueous sulfosalicylic acid (pH 7.8). Then, 2 ml of mixture was added to 2 ml acid ninhydrin (1.25 gr ninhydrin, 30 ml glacial acetic acid and 20 ml 6M phosphoric acid). The reaction incubated in 100°C for one hour. After cooling the reaction in ice bath, the mixture extracted with 4 ml toluene as solvent. The absorbance of proline-ninhydrin product was determined using an UV-visible spectrophotometer at wavelength 520 nm.

Measurement of total soluble sugar: Determination of total soluble sugar was done by the method of Watanabe, Kojima [24]. NaCl-treated and NaCl-free callus tissue (200 mg) was mixed with 10 ml of 80% (v/v) EtOH. Then, the supernatant (1 ml) was reacted with 4 ml of fresh anthrone reagent (150 mg anthrone, 100 ml 72% w/w sulphuric acid). The incubation of reaction was done in water bath at 100°C for 10 minutes. After termination the reaction in ice bath the absorbance was measured in UV-visible spectrophotometer at wavelength 620 nm.

Measurement of protein: Frozen NaCl-treated and NaCl-free callus tissue were ground in Tris-HCl (62.5 mM, pH 6.7) at 4°C and centrifuged at 4000 rpm for 15 minutes at 4°C [25]. The supernatant was proceed to protein estimation by using the method according to Bradford [26]. Total protein content was measured in UV-visible spectrophotometer at wavelength 595 nm. Bovine Serum Albumin (BSA) was used as standard for protein estimation.

Histological analysis: Callus tissues were fixed in FAA solution (Formalin: Acetic acid: Absolute ethanol) in a ratio 5:5:90 (v/v/v) for 48h before further processing. The fixation process was followed by gradual dehydration and finally embedded in paraffin according to procedure of Stobbe, Schmitt [27] with slight modification. Serial sections (4 - 10 µm) were cut using LEICA RM2, 235 microtome instruments with the staining of Haematoxylin and Eosin. Sections were then examined under a light microscope at 4X magnification.

Regeneration of salt-tolerant MR263 plantlets: Regeneration (somatic embryogenesis) of selected salt-tolerant MR263 callus has been done according to method of Rao [25]. Shoot of salt-tolerant MR263 callus was induced on MS media supplemented with 2 mg/L kinetin combined with 1 mg/L BAP. The rate of shoot induction [(the number of callus that regenerated shoot/ the number of total callus) x 100%] was counted after 30 days. Then, regenerated salt-tolerant MR263 callus was sub-cultured on MS media supplemented with 1 mg/L kinetin, and 0.5 mg/L NAA to induce root for 8 weeks.

Statistical analysis: All the experiments were repeated thrice. The collected data was analyzed using SPSS window version 22. One-way ANOVA at $p \leq 0.05$ was conducted to study the diffident among treatment followed by Duncan's multiple comparison tests at $p \leq 0.05$ for mean comparison.

Results

In this study salt-tolerant callus line was selected and developed through *in vitro* salt-tolerant callus. Optimization of plant growth regulator was determined that the best concentration for the establishment of callus is 1.0 mg/L 2, 4-D which has the highest percentage of induction rate of MR263 callus (73%). Therefore, according to table 2, 1.0 mg/L 2, 4-D has been chosen to be used in the induction of callus with NaCl to produce salt-tolerant MR263 callus (Figure 1).

Treatment	Callus induction rate (%)
Control	-
0.5 mg/L 2,4-D	33
1.0 mg/L 2,4-D	73
2.0 mg/L 2,4-D	57
2.5mg/L 2,4-D	30
5.0 mg/L 2,4-D	40
10.0 mg/L 2,4-D	40
15.0 mg/L 2,4-D	20
20.0mg/L 2,4-D	17

Table 2: Percentage of induction rate of callus.

Figure 1: A) Inoculated MR263 seed on fresh MS media with plant growth regulator (2,4-D), B) 14 days old callus, C) 21 days old callus, D) 30 days old callus

After 30 days, callus cultured on MS media supplemented with (0.5, 15.0, and 20.0 mg/L) 2, 4-D have shown watery, pale yellow callus. Callus treated by (1.0, 2.0, 2.5, 5.0, and 10.0 mg/L) 2, 4-D have shown friable proliferating and creamy as shown in table 3.

Treatment	Callus morphology
Control	-
0.5 mg/L 2,4-D	+, Py, W
1.0 mg/L 2,4-D	++, C, F
2.0 mg/L 2,4-D	+++,C, F
2.5mg/L 2,4-D	+++, C, F
5.0 mg/L 2,4-D	++, C, F
10.0 mg/L 2,4-D	++, CrW, F
15.0 mg/L 2,4-D	+, Py, W
20.0 mg/L 2,4-D	+, Py, W

Table 3: Morphology of callus per treatment.

+: Slight callus; ++: Moderate Callus; +++: Massive Callus; Py: Pale Yellow; C: Creamy; CrW: Creamy White; F: Friable; W: Watery

After 4 months, callus has different response to different concentrations of NaCl. Callus cultured on MS media without treatment (control) has normal growth with large size. Distinct different size observed in MR263 callus treated with NaCl. MR263 callus treated with 100 and 150 mM NaCl were yellow color, friable and nodular proliferating. Meanwhile, callus treated with 200 and 300 mM NaCl have shown more watery, discoloration with brown patch (Figure 2).

Figure 2: The browning effect of MR263 callus after 4 weeks of culture on MS media supplemented with different NaCl concentrations (Yellow arrow indicates the browning effects of NaCl treatment on callus).

Screening and selection of salt-tolerant MR263 callus was done by measuring the callus fresh weight and biochemical markers such as total proline content, total soluble sugar and total protein. The fresh weight of MR263 callus significantly reduced ($p \leq 0.05$) along the increased NaCl concentrations. Accumulation of total proline content of MR263 callus increased under salinity treatments ($p \leq 0.05$). The total soluble sugar gave fluctuating measurements which is not significant between NaCl-free control and NaCl-treated callus ($p \geq 0.05$). There is a significant different of protein content between the NaCl-free control and NaCl treated callus ($p \leq 0.05$) (Table 4).

Treatment	Fresh Weight	Total proline content	Total soluble sugar	Protein content
Control	$0.32^{a} \pm 0.005$	$0.21^{a} \pm 0.008$	$7.32^{b,c} \pm 0.008$	$0.33^{c} \pm 0.004$
100 mM	$0.20^{b} \pm 0.003$	$0.44^{c} \pm 0.008$	$7.31^{c} \pm 0.002$	$0.13^{d} \pm 0.005$
150 mM	$0.15^{c} \pm 0.003$	$0.63^{b} \pm 0.01$	$7.3^{c} \pm 0.003$	$0.11^{d} \pm 0.003$
200 mM	$0.12^{d} \pm 0.003$	$0.87^{a} \pm 0.01$	$7.62^{a} \pm 0.01$	$0.61^{a} \pm 0.003$
300 mM	$0.11^{e} \pm 0.005$	$0.38^{d} \pm 0.008$	$7.34^{b} \pm 0.003$	$0.51^{b} \pm 0.005$

Table 4: Fresh weight, total proline content, total soluble sugar and protein content of MR263 callus on MS media supplemented with different concentrations of NaCl after 4 months.

Morphological evaluation of salt-tolerant MR263 callus after 4 months was performed under the light microscope showed the development of somatic embryos in typical globular and heart shape. Figure 3 presents the histological section of control callus (A) which shows the cells is organized and compactly arranged with fewer vacuoles whereas in histological sections of salt-treated callus (B, C, D, and E), the cells appeared to be different in form, size and vacuolization. The majority of salt-treated cells appeared to be damaged or completely disorganized with very large vacuoles. This observation was evidently prominent in histological section of callus treated with 300 mM NaCl (E) in which the meristematic zone was not recognized due to the completely disorganized cells. The typical globular and heart shape development can be seen clearly in callus without NaCl treatment (A). The result also revealed that callus started to show scarcity in meristematic zone from 100 mM NaCl and worsen in 300 mM NaCl as mention earlier.

Salt-tolerant callus from 100 and 150 mM NaCl and the control callus (0 mM NaCl) were sub-cultured on MS media for regeneration of selection salt-tolerant callus. This study found that the MS media supplemented with combination of plant growth regulators 1 mg/L BAP, 2 mg/L KIN, and 1 mg/L NAA, and 1 mg/L KIN able

to induce shoots and roots from salt-tolerant callus starting in the fifth week of culture (Figure 4 and 5).

Figure 3: Histological section of MR263 callus after 4 months of culture on MS medium supplemented with NaCl, A) Control, globular-shaped somatic embryo and formation of meristematic zone (black arrow) B) 100 mM, meristematic zone (black arrow) C) 150 mM, meristematic zone (black arrow) D) 200 mM, meristematic zone (black arrow) E) 300 mM.

Figure 4: Regeneration after 6 weeks of culture on MS media supplemented with plant growth regulator, 1 mg/L BAP and 2 mg/L KIN. A) Control callus, B) 100 mM, C) 150 mM. The control callus (A) and 100 mM callus (B) showed no sign of shoots formation but only roots formation, 10 and 15 roots respectively. The callus was treated with 150 mM NaCl succeeded in producing both shoots (9) and roots (21).

Figure 5: Regeneration after 6 weeks of culture on MS media supplemented with plant growth regulator, 1 mg/L NAA and 1 mg/L KIN. A) Control callus, B) 100 mM, C) 150 mM. The control callus (A) and 150 mM callus (C) showed no sign of shoots formation.

Figure 6 illustrate the development of shoots and roots from 100 mM callus cultured on MS media with 1 mg/L NAA and 1 mg/L KIN in 8 weeks duration while figure 7 illustrate the development of shoots and roots of 150 mM callus cultured on MS media with 1 mg/L BAP and 2 mg/L KIN in the same period of time. It can be seen that the roots regenerate before the shoots and the shoots only start to grow in after the fifth week of culture for both combinations of plant growth regulators. It was evident that the 100 mM callus shows better development of shoots and roots as compared to 150 mM callus.

Figure 6: Regeneration of salt-tolerant callus (100 mM NaCl) in 1 mg/L NAA and 1 mg/L KIN. A) Four weeks of culture, B) Six weeks of culture, C) Seven weeks of culture, D) Eight weeks of culture.

Figure 7: Regeneration of salt-tolerant callus (150 mM NaCl) in 1 mg/L BAP and 2 mg/L KIN. A) Four weeks of culture, B) Six weeks of culture, C) Seven weeks of culture, D) Eight weeks of culture.

Despite of 1 mg/L BAP and 2 mg/L KIN, the 1mg/L NAA and 1 mg/L KIN combination succeeded in producing both shoots (10) and roots (13) from 100 mM callus. The control and 150 mM callus only succeed in producing roots which is 14 respectively. The number of replicates regenerated is shown in table 5.

Combination of plant growth regulators	NaCl concentrations	Number of shoots	Number of roots
1 mg/L BAP + 2 mg/L KIN	Control	-	10
	100 mM	-	15
	150 mM	9	21
1 mg/L NAA + 1 mg/L KIN	Control	-	14
	100 mM	10	13
	150 mM	-	14

Table 5: The number of MR263 salt-tolerant callus that successfully regenerated on MS media with combination of plant growth regulators after 8 weeks of culture.

Discussion

Production and development of salt-tolerant crops through *in vitro* selection with tissue culture technique has well known as low cost and efficient method in two past decades. In present study, selection and regeneration of salt-tolerant MR263 plantlets were successfully done through *in vitro* selection on MS media supplemented with 100 mM NaCl. MR263 callus was successfully established on MS media supplemented with different concentrations of 2,4-D to find the ideal concentration for callus induction. According to the result 1 mg/L 2,4-D shown the highest percentage of MR263 callus induction rate. Karumamkandathil, Uthup [28] suggested 2,4-D promote DNA hypermethylation in a pre-embryonic phase which was responsible to preserve the cell in highly mitotic mode. However, high concentrations of 2,4-D caused the callus become brown and low in quality which is not suitable for *in vitro* regeneration. This might be due to that high dose concentration of 2,4-D could induce a suppressive effect on callusing and *in vitro* regeneration through the effect of the remaining 2,4-D residues on re-differentiation in the mitotic stage [29].

Morphology observation depicts that NaCl has negative effect on MR263 callus growth and development. There was reduction in callus growth along the enhancement of NaCl concentrations. Meanwhile, salt-sensitive callus turns to browning, watery and acutely-necrotic after NaCl treatments. The degree of browning salt-tolerant callus was not that distinctive as in salt-sensitive callus. Salt-tolerant callus appeared with good cell proliferation and regeneration potentiality. Reddy and Vaidyanath [30] reported that prolonged exposure of rice callus to the salinity led to discoloration and restricted growth and only a small number of callus cells maintained healthy and stable growth. Other plants also have the same degree of callus morphology correspondingly changed from off-white to blackish-brown to acutely-necrotic [22].

According to our results, NaCl treatments caused change in the physiological and biochemical markers at cellular level. The detrimental effect of high intracellular NaCl concentrations on plant growth has known in two ways. First, at cellular level, accumulation of sodium chloride leads to osmotic stress that inhibited water uptake. Through osmotic challenge, water deficit and imbalance nutrition are dominated by decrease in water content and eventually reduction of fresh weight [31]. Low water availability in plant cells leads to shrink and wilt. Thus, fresh weight is often measured to reveal the growth of plants and cells in response to environmental stresses. Generally, plants under salt stress slow down their growth rate, which has been observed in a number of *in vitro* systems of halophytes and non-halophytes. Secondly, excessive level of sodium and chloride in plant cells results ionic imbalance and toxicity symptoms. Maintain intracellular ionic imbalance reduced the photosynthesis efficiency and plant growth [32]. Our finding showed that callus grew faster in non-stressed condition as compared to the callus under stressed. Understanding plants mechanisms and strategies toward salinity stress play crucial role to develop stable and tolerant crops under salinity conditions. One of the main adaptation plants mechanisms to salinity stress is osmotic adjustment (OA). This mechanism allows plant cells to cope with salinity by accumulation of compatible solutes that are defined as low molecular mass compounds that do not disturb the normal biochemical reactions [33]. Two types of solutes are involved in

osmotic adjustment; inorganic (Na^+, K^+, Ca^{2+}) and organic solutes such soluble sugar and proline accumulated in the cells along with high concentrations of NaCl to protect cellular proteins, enzymes, and cellular membranes against dehydration [34]. Liu, Zhu [35] suggested that proline plays more roles in survival rather than the plant growth maintenance. Even so, its physiological significance is yet to be fully understood [36]. However, over-accumulation of proline content in high concentrations of NaCl leads to necrosis and eventually plant cells death. Therefore, the level of proline content in MR263 callus decreased in high concentration of NaCl.

Total soluble sugar (sucrose, glucose and fructose) has been known highly sensitive compatible solutes to salinity that has several advantages when plants experienced saline condition [37]. Compatible solutes such as total soluble sugar function as osmoprotectants during severe stress time. At cellular level, total soluble sugar increased markedly in the cytoplasm as water potential dropped under salinity that promoting water retention to maintain cell turgor [38]. Meanwhile, in long term exposure to salinity, soluble sugars supplied carbohydrate to stabilize cell membrane and ROS scavenging [39]. Total soluble sugar in MR263 callus significantly increased under NaCl treatments. Sharma and Ramawat [40] reported the same trend whereby soluble sugar contents induced significantly by NaCl treatment in the callus of *Salvadora persica* (miswak) in saline conditions. The attack of ROS rapidly inactive enzymes and degrade proteins. Thus, low level of soluble proteins in MR263 is due to modification of amino acids chain as a consequence of the free radicals attack. The decreasing trend of total soluble protein also reported by Kalhori, Nulit [41] in Malaysian rice cv. MR 219 in response to salinity. Agastian, Kingsley and Vivekanandan, (2000) reported that during reactive oxygen species (ROS) attack which happened when plant undergo stresses, there is occurrence of protein degradation due to the reaction among amino acids of protein with active radical such as hydrogen peroxide and hydroxide which therefore result in low level of total soluble protein.

Hisological analysis of MR263 callus has done to understand the cellular level of adaptation mechanism in rice. As a result of current finding, callus histological analysis revealed that salt induced vacuolation in high concentration of NaCl compared to control and lower concentration of NaCl. According to Bennici and Tani [42], the major indication of the toxic salt was observed at the cytoplasm structure levels. The injured cells resulted in an extensive alteration of compatible solutes until to a total disorganization of the protoplast. Strong electron-density as a result of osmotic stress leads to reduction of the cytoplasm and increase of the vacuolar system that caused the lower meristematic zones in the cells. Different electrical potential between the plasma membrane and cytosol caused the influx of Na^+ into the cells through channels and carriers as a passive process. Meanwhile, the excess of Na^+ has blocked the transport of K^+ [43]. To decrease cytosolic Na^+ toxicity, plant cell increased the concentration ratio of K^+/Na^+ with the uptake of K^+. Meanwhile, through an active process Na^+ with Na^+/H^+ antiporter extruded to the vacuols [44]. Increase number of vacuoles, especially in the presence of the highest salt dose, is part of osmotic adjusment which may indicate the necessity of an energy supply for the selective transport of ions [45]. Same cells mechanisms were observed in variuos plants species [46-48]. Despite the increase in research about the changes which are taking place in plants under salt stress, still the mechanism is not completely understood especially on the cellular level [49].

Our results showed that, the regeneration percentage of salt-tolerant callus was low in salt treated callus which is consistent with other studies [16,50,51]. In the present finding, combination of auxin and cytokinins produce shoot and root from callus. Many auxins and cytokinins in the regeneration media are found to promote rice regeneration frequency. Earlier research reported that MS media supplemented with NAA (2.0 mg/L) and Kin at a range of 1.0 - 4.0 mg/L has the highest shoot and root formation [52]. Auxins and cytokinins may interact through synergistic, antagonistic and additive mechanisms relying on inoculated tissue culture conditions that promote development toward callogenesis and shoot formation. Moreover, Lee and Huang [53] suggested the balance between auxin and cytokinin played a major role in the initiation of regeneration of induced callus. Both cytokinins and auxins are thought to influence the cell cycle and morphogenic competence in plant growth [54]. Unbalanced ratio of auxin and cytokinin caused a decrease in regeneration frequency as the development of multiple shoot proliferation appeared to be suppressed [29]. Therefore, besides the decreasing capability to regenerate due to salt effects, control callus that had failed to regenerate might because of the genotype behavior of MR263.

Conclusion

During salt adaption, increasing proline content and total soluble sugar protected MR263 callus. At cellular level, histological analysis confirmed that salt-tolerant callus appeared to progress through typical globular and heart shape. According to the cellular responses and growth performance of the plantlets from MR263 callus that treated by 100 mM NaCl selected as salt-tolerant line with better adaptability. Therefore, it is suggested that test the yield of salt-tolerant MR263 lines under greenhouse/field conditions which have the potential to be commercialized.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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