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Regeneration Capacity of Cell Suspension Culture in Malaysian Rice Genotypes under Salinity Stress

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ABSTRACT

Rice, a salt-sensitive species has a substantial genetic variation for salt tolerance within the cultivated gene pool. This study describes the establishment of cell suspension culture system and analysis of proline content of the selected rice genotypes under *in vitro* salt stress. The cell suspension cultures, initiated from the friable seed-derived callus, were established in MS medium supplemented with different concentrations of 2,4-D (0-25 μM) and subjected to different concentrations of NaCl (0-250 mM). The average Settled Cell Volume (SCV) of different clumps sizes was recorded. The results showed that MR219-4 exhibited the highest cell growth rate and regeneration capacity. The 750 μm sieved clumps have higher cell mass than the 500 and 250 μm sieved cell size. The shoot-regeneration capacity was maximum in 1/2 MS medium supplemented with 10 μM BAP. However, low regeneration capacity was obtained in NaCl treatments. MR219 (control) and MR219-9 appeared to be more NaCl tolerant than MR219-4. In addition, all genotypes significantly synthesized proline under NaCl conditions as demonstrated by the proline accumulation and MR219-4 revealed the lowest content among genotypes studied. These genotypes can be good model systems for studying the physiological mechanisms associated with *in vitro* selection for salt stress. This finding also suggests that proline may play a crucial role in protecting rice cells under salinity stress.

Key words: Cell suspension, *in vitro* studies, rice (*Oryza sativa*), shoot regeneration, salinity stress, proline

INTRODUCTION

Rice is the most economically important cereal crop in many parts of the world and considered as a salt sensitive species. Soil salinity is one of the environmental hazards in agriculture worldwide because it limits crop yield and restricts the use of land previously cultivated. Rice yields can be

reduced by up to 50% when grown under moderate salinity levels (Zeng *et al.,* 2002). Saline soils are one of the primary biotic stresses that adversely affect the overall metabolic activities and cause plant demise, growth and development (Galvani, 2007; Roychoudhury *et al.,* 2008). It has been estimated that over two million acres of agricultural lands are lost from production each year due to the occurrence of high Na^+ and Cl^- levels in soils. Na^+ and Cl^- derived from NaCl salts contaminate the soils which are well known as toxic ions that can damage the plant cells in both ionic and osmotic effects. In addition, plant growth and development are directly inhibited, leading to low yields prior to plant death (Chinnusamy *et al.,* 2005).

In order to maintain homeostasis during stress condition, plants need to have special mechanisms for adjusting the internal osmotic conditions and changing of osmotic pressure inside the cells. Some systems have been proved successful in the study of salinity problem but the approach of cell culture technique has been shown to be more effective in selecting salt tolerant lines. At the same time, cell suspension cultures are particularly suitable for physiological, biochemical and molecular cellular response studies. The salt tolerance or sensitivity of a plant is the result of fundamental differences in cellular level adaptations. One approach to study cellular mechanisms of tolerance is to use undifferentiated cell cultures. The external concentration of NaCl required to inhibit plant growth to 50% of its maximum growth rate has been the criterion (Wu *et al.,* 2005).

The problem of salinity has been approached through better management practices and introduction of salt-tolerant varieties in the affected areas. The cell suspension selection is most suitable for stress tolerance because all the cells are in direct contact with the stress agent in the process. To date, the cell suspension method has been used by many scientists generally for crop improvement under salinity and other stresses. However, reports on similar research in rice are limited. Some of *in vitro* selections of salt tolerant cell lines and regenerated plants have been reported in some species, such as potato, wheat, sunflower and sugarcane (Gandonou *et al.,* 2005). Therefore, the objective of this study was to determine the plant regeneration capacity of cell suspension and also accumulation of proline in selected Malaysian rice MR219 and its mutants under salinity conditions.

MATERIALS AND METHODS

Cell suspension cultures and plant regeneration: This study was investigated that at the tissue culture laboratory of Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia in 2007. MR219-4 and MR219-9 genotypes are two potential rice mutants that were generated from MR219.

A total of 0.2 g actively friable callus were placed in a 150 mL conical flask containing 25 mL liquid medium and incubated on a horizontal incubator-shaker with agitation at 120 rpm to establish the suspension culture. Different sizes of sterile filters (250, 500 and 750 μm) were used to separate large cell aggregates and establish a suspension culture. The cultures were sub-cultured at one week interval and the homogenous cell line was obtained by sequentially screening the cultures using 750, 500 and 250 μm sterile sieves. The Settled Cell Volume (SCV) was measured by transferring the suspension cell to a graduated centrifuge tube and was allowed to sediment or centrifuge before measurement was taken.

Approximately, 2 g of cell suspension were first transferred to a single layer of dry sterilized filter paper for 10 min prior to transfer to regeneration media. The regeneration media formulation was based on full and half strengths of MS (Murashige and Skoog, 1962) media with

6-benzylaminopurine (BAP), Kinetin (KIN), Zeatin and Thidiazuron (TDZ) as a preliminary test and followed by 5-25 μM BAP combination. The frequency of regeneration percentage was calculated as follows:

$$\text{Regeneration percentage (\%)} = \frac{\text{Number of plants recovered}}{\text{Number of cell clumps plated}} \times 100$$

***In vitro* cell suspension and plant regeneration under salinity condition:** This experiment was carried out to determine the effects of increasing NaCl concentration on the growth of cell suspension culture. The cells were sub-cultured and transferred to a liquid MS medium supplemented with different concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM). The average cell growth rates were measured. The established cell suspensions of the three rice genotypes were sub-cultured before transferring them to the solid regeneration medium. The suspension cells were then transferred to the best solid regeneration medium (following the above results) supplemented with different concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM).

Determination of proline content: Proline was extracted from the NaCl-treated plantlets (Bates *et al.*, 1973). The 4 week old leaf tissues (0.1 g) were ground in a mortar and mixed with 5 mL aqueous sulfosalicylic acid (3% w/v) and filtered through the Whatman filter paper (Whatman, England). The filtrate of 1 mL reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 g of ninhydrin, 30 mL of glacial acetic acid and 20 mL of 6 M HPO_4) and incubated for 1 h in boiling water. The reaction was terminated by placing the reaction tubes in an ice bath. The reaction mixture was vigorously mixed with 4 mL toluene. After warming at 25°C, it was measured for proline at 520 nm with the spectrophotometer. The standard curve for proline was prepared by dissolving it in distilled water covering the concentration range of 1-10 μg mL^{-1}. The proline concentration was determined from a standard curve and calculated on the fresh weight basis as follows:

$$[(\mu g \text{ proline/mL} \times \text{mL toluene})/115.5 \ \mu g/\mu g \text{ mole}^{-1}]/[(\text{g sample})/5] = \mu\text{moles proline/g of fresh weight material}$$

All the experiments were carried out in three replications. A completely randomized design using SAS and MSTATC computer programmes was employed and a comparison of means was carried out to test for significance using the LSD test at 0.05 level of probability.

RESULTS

Establishment of cell suspension culture and plant regeneration: This study was undertaken to assess the influence of different concentrations of 2,4-D with MS media on the initiation of cell suspension culture. MS medium with 10 μM 2,4-D showed significantly faster growth for the initiation and growth of suspension cells as shown by the cell growth rate data. The results revealed that 2.85±0.294 mL for MR219-4 followed by 2.73±0.102 mL for MR219-9 and 2.3±0.176 mL for MR219 (control) using 750 μm sieve size (Fig. 1). In addition, the degree of response also varied among the genotypes and the different sizes of sieve. The lowest cell growth rate was found in MR219 (control) followed by MR219-9 and MR219-4 at the size of 250 μm sieve.

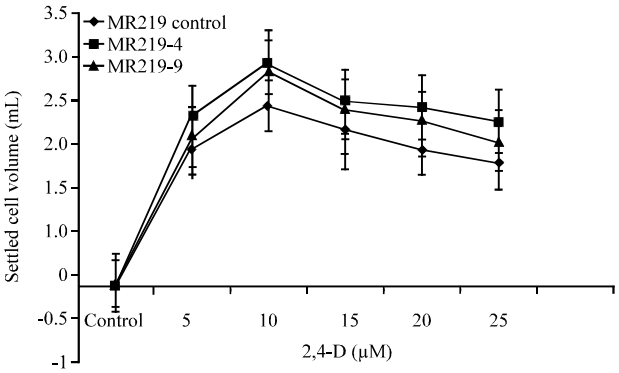

Fig. 1: The Settled Cell Volume from different rice genotypes in the MS liquid medium supplemented with different concentrations of 2,4-D

Plant regeneration: To improve the crop at cellular level, it is important that the source of plant regeneration cell come from cell suspension culture. Identifying the suitable medium for plant regeneration in cell suspension was attempted and suitable hormone combinations were investigated. The callus-derived suspension cultures from three rice genotypes were established. Reports on plant regeneration from cell suspension culture in rice are limited (Boissot *et al*., 1990) but in this study the complete plant regeneration from cell suspension of three genotypes was obtained. Rice cell aggregates with the size of 750 µm carried the distinctive character of regenerable suspension cell and these were transferred to regeneration media. As a preliminary test the friable and fast growing cell was transferred to the regeneration medium (MS and 1/2 MS medium supplemented with BAP, KIN, Zeatin and TDZ). The mean of the regeneration potential for each genotype were statistically difference. Among the eight selected mediums, regeneration percentage varied widely with respect to genotypes. The values were ranging from 3.76 to 12.3% in combination of full and 1/2 MS with 5 µM of KIN, Zeatin, TDZ and BAP in three genotypes (Fig. 2). These were resulted that combination of 1/2 MS with BAP at the concentration of 5 µM was higher (12.3%) than with KIN, Zeatin and TDZ combination. However, the regeneration percentage is considered very low hence the combination of full and 1/2 MS with specific concentration of BAP (5-25 µM) were tested. In this condition, combination with BAP was found ranging from 6.82 to 24.33% (Fig. 3). The best regeneration percentage found in 1/2 MS+BAP 10 µM (24.33%) in MR219 line 4 and followed by MR219 control (18.58%) and MR219 line 9 (17.75%). In plant regeneration, striking differences were observed between the genotypes tested. This media combination appears to be most suitable for plant regeneration. From the result it could be summarized that the 1/2 MS medium supplemented with BAP at the concentration of 10 µM were the highest regeneration capacity.

***In vitro* salt treatment**

Cell suspension under salinity condition: In this study, experiment was carried out to investigate the effects of NaCl concentration on the cell suspension culture of the three rice genotypes. The suspension culture cells established from the friable callus masses and maintained in the liquid culture over several generations. It was evident from the six salinity levels tested; 0, 50, 100, 150, 200 and 250 mM, rice callus grew well up to 150 mM NaCl. The growth of the cell suspension of all genotypes decreased with the increasing of NaCl concentration. The cell growth

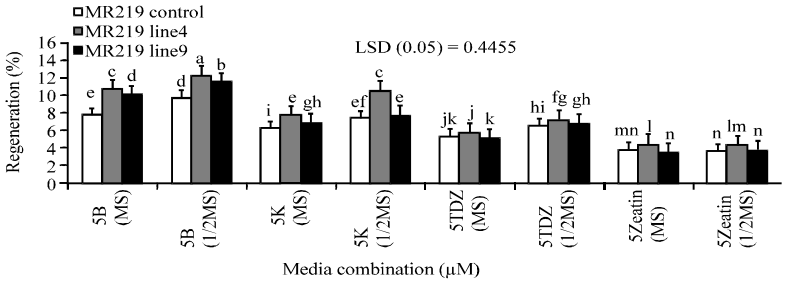

Fig. 2: Regeneration percentage of the three genotypes cell clump in full and 1/2 MS media combination with TDZ, Zeatin, KIN and BAP (5 µM). The bar shows SE in the three replications. Different letters indicate values are significantly different and the same letter are not significantly different at $p<0.05$

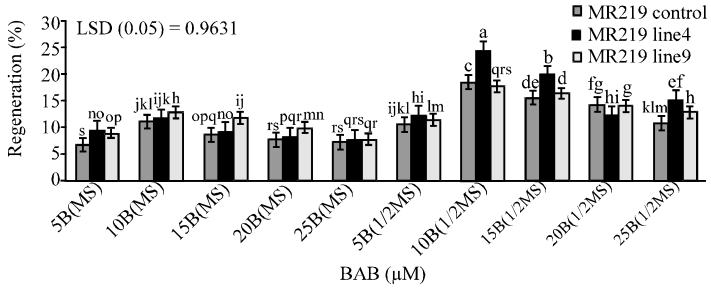

Fig. 3: Regeneration percentage of the three genotypes cell clumps in full MS and 1/2 MS media supplemented with BAP. The bar shows SE in the three replications. Different letters indicate values are significantly different and the same letter are not significantly different at $p<0.05$

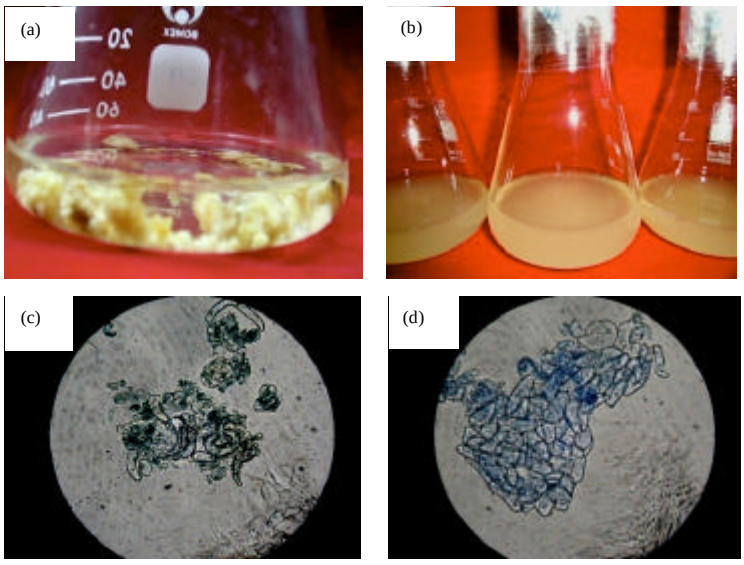

Fig. 4: (a) and (b) Steps of cell suspension culture in liquid media (MS+2,4-D 10 µM), (c) Cell suspension of MR219 line 4 without NaCl concentration and (d) Cell suspension of MR219 line 4 under NaCl (250 mM) concentration

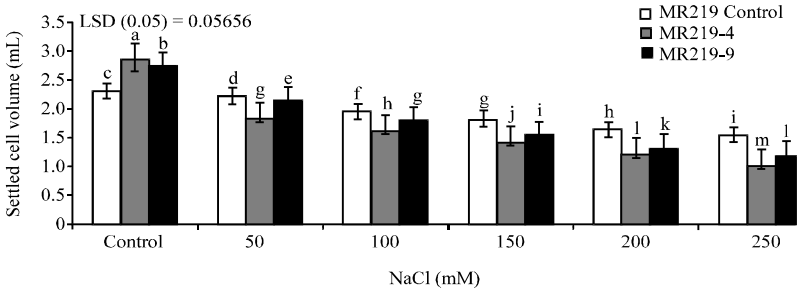

Fig. 5: The settled cell volume from different five rice genotypes cell clumps in the MS liquid media supplemented with different concentration of NaCl. The bar shows SE in the three replications. Different letters indicate values are significantly different and the same letter are not significantly different at $p<0.05$

for all genotypes was stopped and constant at 250 mM NaCl. The microscopic analysis of the cultured cells showed the cell clusters and aggregates of various sizes (Fig. 4a-d) died in high concentration of NaCl and the abnormal shape could be observed under the microscope.

The suspension cells were sieved regularly to maintain their well-being. After establishing the suspension cells in the liquid medium (MS+10 μM 2,4-D), they were transferred to a liquid medium supplemented with different concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM). Apparently, the response of Settle Cell Volume varied among the three genotypes. Comparing the three genotypes, the cell growth reached as high as 1.54±0.073 mL for MR219 (control), followed by MR219-9 with 1.18±0.023 mL and MR219-4 with 1.01±.045 mL (Fig. 5). Following these results, the cell fraction of the three genotypes under salt stress was presented in the following order: MR219 (line 4) <MR219 (line 9) <MR219 (control).

REGENERATION

From the cell suspension, all the cell aggregates of regenerable suspension cells were transferred to the regeneration medium. On the regeneration medium, the cells grew and produced yellow callus clumps from the sieve size of 750 μm and these calluses were sub-cultured on the same medium. Other sizes of cell (smaller size of sieve) responded to low regeneration capacity. The callus derived from the suspension culture were established and tested for regeneration capacity and cultured on 0, 50, 100, 150, 200 and 250 mM NaCl stress media. They exhibited the shoot regeneration that was significantly greater than that of the plants grown on NaCl free medium. The green part appeared on the callus proportion, which developed into plantlets and the callus was entirely covered with green shoot buds as shown in (Fig. 6a, b). During sub-culture, the shoot buds further elongated and multiplied vigorously. However, no significant differences in the shoot regeneration were noted when the plants were grown on NaCl stress at the concentration of 50 and 100 mM in the medium. The plantlets were regenerated from both the NaCl free (24.37%) and stress containing 50 and 100 mM (20.44 and 14.48%) of regeneration medium in MR219-4 as shown in Fig. 7. In addition, the shoot multiplication rates were comparatively low in 250 mM NaCl treatment.

Furthermore, the regenerated plantlets were comparatively not so green and healthy as compared to the plantlets regenerated from partially NaCl treated media. The regeneration frequency values in MR219-4 were 9.55, 6.89 and 4.56% on 150, 200 and 250 mM NaCl

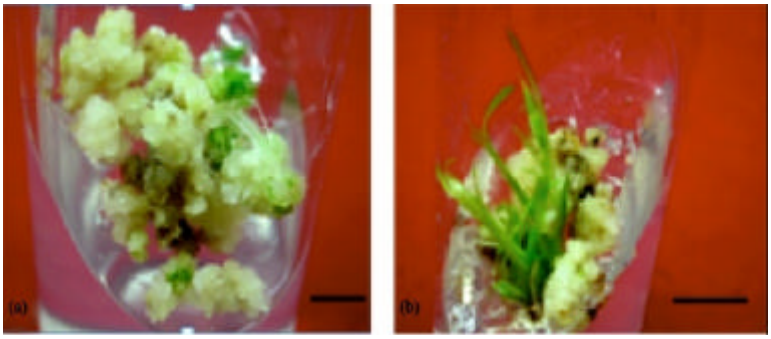

Fig. 6: (a) Green callus and (b) plant regeneration of MR219-4 in 1/2 MS+10 μM BAP with 250 mM NaCl media combination

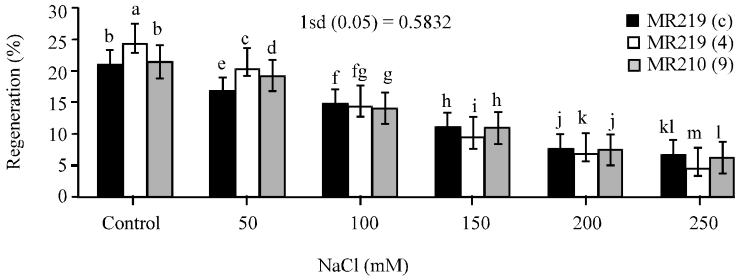

Fig. 7: Plant regeneration percentage of the three rice genotypes cell clump in NaCl treated media. The bar shows SE in the three replicates. Different letters indicate values are significantly different and the same letter are not significantly different at $p<0.05$

Table 1: Analysis of variance between the effects of regeneration media containing NaCl of three genotypes with three different cell sizes for plant regeneration capacity. The results showed that the media and cell size were significantly different in all genotypes and media at $p<0.001$. The analysis also revealed significant effect of media and cell size interaction and media, genotype and cell size interaction. But the genotype and interaction between the media and genotype interaction are not significantly different ($p>0.001$)

Source of variation	df	Mean square	F value
Regeneration media with NaCl	5	532.7664	481.07**
Genotype	2	5.508317	4.97ns
Cell size	2	897.7127	810.6**
Media×genotype	10	4.160635	3.76ns
Media×cell size	10	31.27654	28.24**
Media×genotype×cell size	24	5.739904	5.18**
Error	108	1.10747	

** = Significant at p= 0.001, ns = non significant, Coefficient of variation = 11.4

containing media, respectively. In MR219 (control), the maximum regeneration frequency values was observed as follows: 11.15, 7.69 and 6.76% for 150, 200 and 250 mM NaCl, respectively. The analysis of variance between the effects of the regeneration media and cell size were significantly ($p<0.001$) different in all genotypes and media. The analysis also revealed that the effects of media and cell size interaction, as well as media, genotype and cell size interaction were significantly different. On the contrary, interaction between the media and genotypes were not significantly different ($p>0.001$) (Table 1).

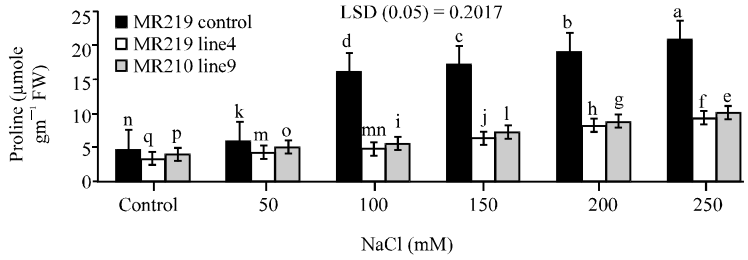

Fig. 8: Proline concentration of three rice genotypes in NaCl treated media. Different letters indicate values are significantly different and the same letter are not significantly different at $p<0.05$. The bar shows SE in the three replicates

Table 2: The analysis of variance between the effects of NaCl regeneration media on three genotypes for proline accumulation. The results showed that the media, genotypes, interaction between media and genotypes were significantly different in all genotypes and media at $p<0.001$

Source of variation	df	Mean square	F-value
NaCl Reg media	5	129.21	1446.07**
Genotype	2	349.41	3910.47**
Media×Genotype	10	24.64	275.80**
Error	36	0.089	

** = Significant at p = 0.001, Coefficient of variation = 3.39

Determination of proline accumulation: The proline contents of salt-stressed and control plants are illustrated in Fig. 8. Proline contents in the salt stressed cells were approximately 3-5 times higher than that of control plant. Proline accumulation was already observed in the stress exposure of the three genotypes but it was always higher in the salt-resistant MR219 control than the salt-sensitive genotype of MR 219-4. The highest proline content in the salt-stressed cells was about 20.78 µmole g/FW. Due to the analysis of variance, the effects of the regeneration media with NaCl and genotypes on proline content were significantly different in all genotypes and media, respectively at $p<0.001$ as shown in Table 2.

DISCUSSION

In vitro regeneration of plants via somatic embryogenesis through cell suspension culture was achieved in selected Malaysian rice. Maximum frequency of cell growth rate 2.99 g observed on MS medium with 10 µM 2,4-D. It was effective to achieve a high frequency of cell growth rate for further development. The suspension cell did not develop in the medium without 2,4-D combination and caused browning in higher concentration of 2,4-D. Hence, the presence of high concentration of 2,4-D was not suitable for initiation of cell suspension, whereas low concentration of 2,4-D resulted in an increased compactness of cell clumps. Similarly, Boissot *et al.* (1990) found that 9 µM 2,4-D in the MS medium was suitable for the initiation of cell suspension in rice. Also in legume, among different auxins tested, 2,4-D was most effective for inducing somatic embryogenesis in a liquid medium (Vasarisai Mohamed *et al.*, 2004).

This suspension culture was sieved regularly to maintain the well being of the cells. This sieving step was repeated three times for sub-culture and replacement of fresh media. Bregitzer *et al.*

(1995) reported that the regular media replacement and culture sieving were important for maintaining well grown cell lines. The cells grew from a thin density to a thick suspension of cells immediately after sub-culturing. Likewise, Iantcheva *et al.* (2005) found that, once the cells were separated in a few flasks with fresh medium, they started to divide approximately 10-15% of the single cells.

The establishment of embryogenic suspension cultures for the regeneration of plants is an ideal tool for the efficient *in vitro* selection and production of transgenic plants (Christou, 1997). To establish the high frequency of plant regeneration system, the cell line must have a high potential for regeneration that should be determined. In rice, the genotype is an important factor affecting the potential for regeneration apart from the nutrient composition in the media. The highest plant regeneration capacity was found in 1/2 MS medium combination with 10 μM BAP. Under *in vitro* salt treatment the callus grown on the medium without NaCl was green and as salinity increased, the colour became lighter until at the concentration of 250 mM, it turned light brown. From the data presented in this study, there are good indications that MR219 control can grow and develop in the presence of NaCl (at least up to 250 mM).

In addition, the colour change of callus in response to salinity was also observed at 100 and 150 mM NaCl while there was no color change in the callus without NaCl. The browning of tissue could be an indication of necrosis, tissue damage, or production of stress-response compounds such as phenolics. The colour changes of callus in response to salinity was also observed in *S. bicolor* at 100 and 150 mM NaCl while there was no color change in callus of *S. halepense*, a salt-tolerant variety (Yang *et al.,* 1990) and durum wheat (Arzani and Mirodjah, 1999). Binch *et al.* (1993) observed that the NaCl concentration of 128 mM inhibited the cell growth by inducing the compactness in the cell cluster which agreed with the findings of this study.

Proline content in response to environmental stresses has been considered by a number of investigators as an adaptive trait concerned with stress tolerance (Rhodes and Hanson, 1993). Salt stress can affect the synthesis of organic compatible solutes such as proline, betaine and soluble sugars and accumulation of proline has been reported in many plants under salt stress (Baloch *et al.,* 2003). It may act as an enzyme protectant, stabilizes membranes and cellular structures during stress conditions (Delauney and Verma, 1993; Hong *et al.*, 2000). In this study, MR219 control cells accumulated high level of proline under salt stress as compared to the control condition (Fig. 8). The increase of proline content under stress condition may be due to breakdown of proline rich protein or *de novo* synthesis of proline (Tewari and Singh, 1991). It also could be due to prevention of feedback inhibition of the biosynthetic enzyme caused by decreased activity of enzymes involved in degradation of proline such as proline dehydrogenase and proline oxidase (Girija *et al.*, 2002).

In summary, this study applied the profiling analysis to investigate the responses of the three selected Malaysian rice genotypes that were tolerant and sensitive to salinity. In the absence of NaCl, the induced cell and plant regeneration were observed to be normal. The addition of NaCl caused a decrease in the cell growth or an increase in the necrosis for all genotypes and a significant difference in the cell growth rate and plant regeneration. It was observed that MR219 (control) showed a significantly greater response than MR219-4 and MR219-9 for cell suspension and plant regeneration capacity under salt stress. Likewise, the significantly higher level of proline accumulation in MR219 (control) clearly demonstrated that in NaCI-treated plants was inversely correlated with their ability to withstand salinity stress.

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