Plant Omics Journal POJ

POJ 8(3):244-249 (2015) ISSN:1836-3644

Comparative analyses of osmotically and ionically adapted cell lines of rice and their response to regeneration

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Abstract

Production of callus and its subsequent regeneration are imperative in the development of pure cell lines and to increase the stress tolerance responses in crops. In the present study, cell lines of *Oryza sativa* variety indica, cv. Swat-1 were adapted to osmotic (20% PEG) and ion toxicity (20mM LiCl) stresses by incremental increase of PEG and LiCl concentrations in the medium. Analyses of these cell lines revealed a substantial increase in Ca^{2+} level and significant reduction in Mg^{2+} contents on adaptation to PEG stress, while no prominent differences in accumulating organic and inorganic cytosolutes were observed between unadapted and LiCl adapted cell lines. SDS-PAGE results revealed that adaptation to PEG and LiCl was evident by appearance of six different polypeptides, among these four polypeptide bands 15, 17, 26 and 68 kDa were common, while other two polypeptide bands of 60 kDa and 32 kDa were present only in PEG and LiCl adapted cell lines respectively. The adapted cell lines were growing on respective stresses over a period of 55 passages (each passage contain15 days) and their tolerance was stable at least up to 6^{th} generation on stress free medium. However, regeneration frequency of unadapted and LiCl adapted cells line decreased by 30 and 10 % respectively after 55 passages. On the other hand, about 80% increase in regeneration frequency of PEG adapted might have increased intracellular attachment with balanced shear forces that may allow enhanced cell totipotency. These results indicate that adaptation to ionic stress (LiCl) inhibit the regeneration frequency while adaptation to osmotic (PEG) stress enhances totipotency/regeneration capabilities of the cell lines.

Key Words; regeneration, LiCl, PEG, stress.

Introduction

Plant development and productivity are negatively regulated by various environmental stresses that may be broadly divided into two classes (a) biotic stress such as diseases, insects/ pests and weeds etc. (b) abiotic (physical environment) such as salinity, drought, heat, cold etc. (Dhariwal, *et al.*, 1997). Abiotic stresses represent key elements limiting agricultural productivity worldwide. According to Serrano *et al.*, (1999) loss by diseases and insects typically decrease crops yields less than 10%, but severe abiotic stresses may account for up to 65% yield reduction. Thus, developing crop plants with the ability to tolerate abiotic stresses are of paramount significance and require the involvement of modern novel strategies. Employing contemporary tools and techniques from all branches of science, attempts are being made worldwide to understand how plants respond to abiotic stresses with an aim to enhance plant performance under these stresses (Alexieva et al., 2003). Unlike animals plants cannot escape environmental stresses that affect their activities. Therefore, plants have to adapt various strategies to cope with these abiotic stresses. Plants must sense environmental stimuli before being able to respond appropriately. Due to the complex nature of the stress response, multiple sensors rather than a single sensor are more likely to be responsible for perception of a stress. After the initial stress recognition, a single transduction cascade is raised. Secondary messengers relaying on the signal, ultimately activates stress responsive genes generating the initial stress response. Further, stress induced gene products can be divided into two major groups: those involved in stress tolerance and those involved in signal transduction. These include the synthesis of chaperones and enzymes for osmolytes biosynthesis and detoxification, to change in composition of membrane lipids as found with cold stress. Gene product can also act as transcription regulators controlling sets of stress specific genes or those involved in the production of regulatory molecules, such as plant hormone ABA. It has been demonstrated that multiple signalling pathways can be activated during exposure to stress, leading to a similar response to different triggers (Rohila et al., 2002). During adaptation plants are able to restore their normal functions under stressed conditions (Alexieva et al., 2003) and there are a lot of evidences that suggests that software adaptation of cell/plant is superior to changing of hardware by inserting new genes (Bohnert and Cushman, 2003). While incremental adaptation/tolerance is the most direct approach of defining the mechanisms of stress perception, signal transduction and regulation of gene expression involved in environmental stresses. Incremental adaptation involves gradual increase requirements to the plant or tissues or cells to adjust themselves to an environment and this is initiated by disturbance of normal metabolic processes (Alexieva et al., 2003). Moreover, adaptation of cells, plants or tissues against one stress that confers the tolerance to a range of different stresses are referred as cross tolerance

(Streb et al., 2008). For instance, salt stress adapted cells/plant shows tolerance to cold stress (Ryu et al., 1995; Shah et al., 2002) and heat stress increase tolerance against heavy metal toxicity (Bonham-Smith et al., 1987), Polyethylene Glycol (PEG) and Lithium Chloride (LiCl) adapted cell lines of rice tolerate heat, cold and salt stresses (Shah et al., 2012). But now the question arises that whether these adaptations are stable are not? To answer this question the present study was aimed at adapting cell lines of *Oryza sativa* L. cv. Swat-1 to osmotic (poly ethylene glycol) and ion specific (LiCl) stress to elucidate the stability of adapted lines and to find which component of stress exerts greater inhibitory effects on the regeneration capability of rice cell lines. All these insights might contribute to the development of superior, stress-tolerant crops for changing environment.

Results

Relative growth rate (RGR)

Data for relative growth rate (RGR) of the rice cell lines on different concentrations of PEG is given as Fig. 1. Analysis of variance showed significant (P=0.05) effect of PEG concentrations on RGR. Maximum relative growth of 0.99 % was observed in control (0% PEG) followed by 0.61, 0.51, 0.15, and 0.034 % at 5, 10, 15 and 20 % PEG respectively. However, there was no growth at 25 % and 30 % PEG stress (Fig. 1). Similarly relative growth rate of all the cell lines was also adversely and significantly (P=0.05) affected by LiCl stress (Fig.2). There was a gradual reduction in relative growth rate with increasing LiCl concentrations in the medium. Highest growth was recorded at 0 mM LiCl (control) while minimum growth was observed at 20 mM LiCl concentrations. On the other hand relative growth of all the rice cell lines was completely inhibited at 25 and 30 mM LiCl (Fig. 2).

Stability test of adapted cell lines

The stability of adapted lines was double checked by growing these lines on MS medium without PEG and LiCl for 3 and 6 successive passages and growing them back on 20 % PEG and 20 mM LiCl containing medium (Fig. 3). It is evident that both lines grew well without showing any decrease in relative growth rates after growing for three to six generations without stress.

Effect of adaptation on Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) ions

Data regarding calcium (Ca^{2+}) and Magnesium (Mg^{2+}) contents of adapted (PEG and LiCl) and unadapted cell lines of rice are presented in Fig. 4. Data showed that PEG adapted line accumulated significantly higher contents of Ca^{2+} at their respective medium than unadapted and LiCl adapted lines. Moreover, Mg^{2+} contents of unadapted and LiCl adapted cell lines were almost similar at their respective media in contrast, PEG adapted line had significantly lower level of Mg^{2+}.

Major polypeptide differences between adapted and unadapted cell lines

The protein quantification did not detect any significant difference between adapted and unadapted cell lines. When an equal amount of protein approximately 30 µg was applied in each lane, SDS PAGE analyses revealed several new or enhanced polypeptide bands in case of adapted lines. Among

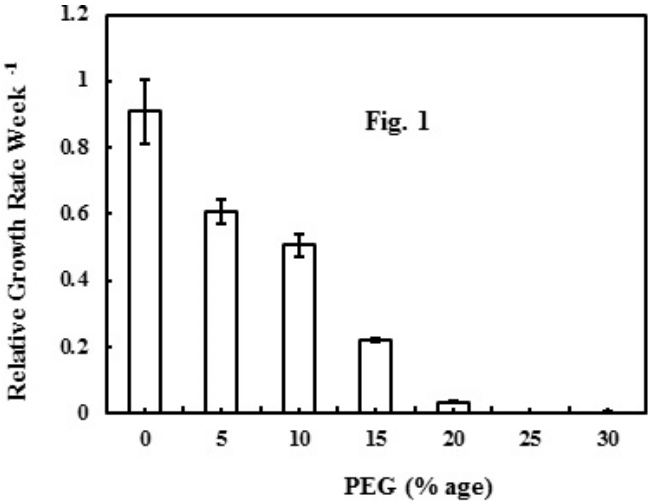

Fig 1. The effect of different concentrations of PEG on RGR of unadapted cells line of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE.

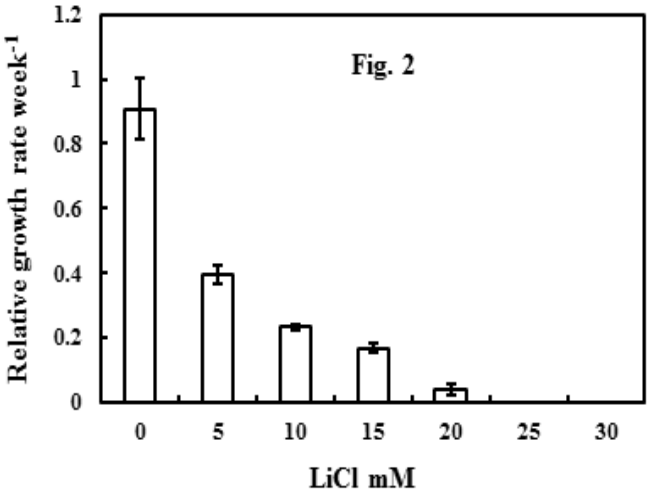

Fig 2. The effect of LiCl on RGR of unadapted cells line of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE.

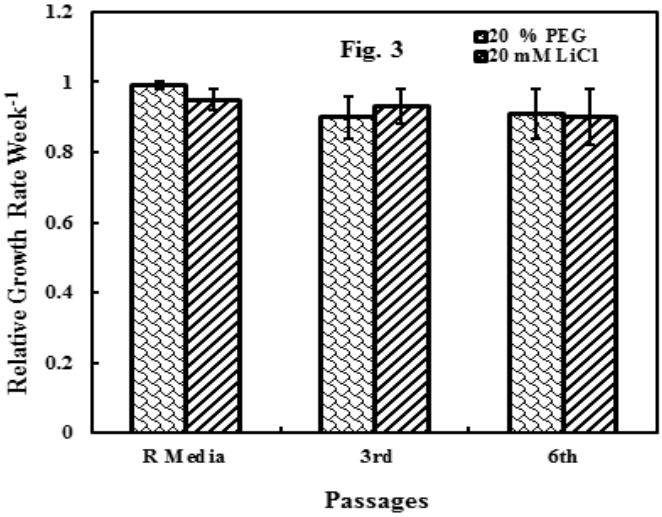

Fig 3. The stability test of PEG and LiCl adapted cell lines after 3rd and 6th consective passages on stress free medium. The bars represent mean values ± SE.

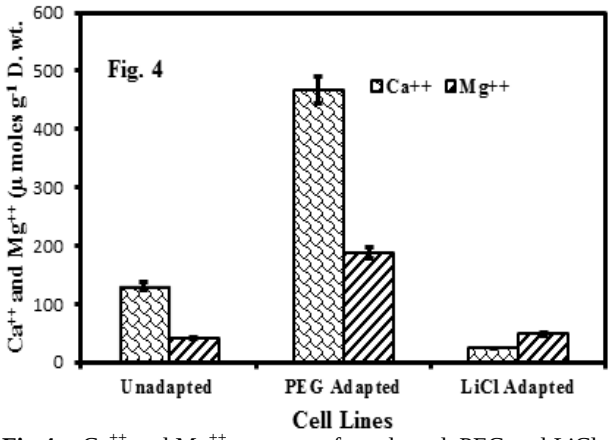

Fig 4. Ca^{++} and Mg^{++} contents of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1 for. The bars represent mean values ± SE.

Fig. 5

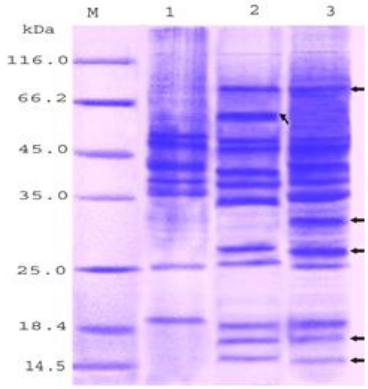

Fig 5. Proteins profile of unadapted, PEG adapted and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1 using SDS-PAGE, M = Molecular weight marker, 1= unadapted line, 2 = PEG adapted line, 3 = LiCl adapted line. Arrows indicate polypeptides which showed major changes (up-regulated, down regulated and/ or newly appeared) during PEG and LiCl adaptation. Molecular weight (in kDa) is given on left.

Fig. 6a

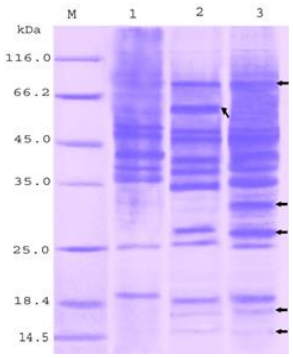

Fig. 6b

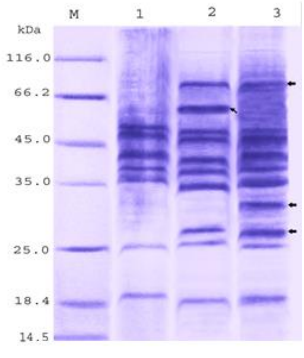

Fig 6a – 6b. Changes in the proteins profile of PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1 using SDS-PAGE after three (Fig. 6a) and six (Fig. 6b) passages recovery from PEG and LiCl stresses respectively. M = Molecular weight marker, 1= unadapted line, 2 = PEG adapted line, 3 = LiCl adapted line. Arrows indicate polypeptides which showed major changes (up-regulated, down-regulated and/ or newly appeared). Molecular weight (in kDa) is given on left.

these polypeptides four newly appeared polypeptide bands (15, 17, 26 and 68 kDa) were more abundant and common in both the adapted cell lines. While other two proteins of 60 kDa and 32 kDa were unique to the PEG and LiCl adapted cell lines, respectively. None of these polypeptide bands were detectable in unadapted cell lines on a stained gel. LiCl adapted cells line showed enhanced levels of polypeptide band of 26 kDa compared to PEG adapted cell line (Fig. 5). However, when adapted cell lines were transferred to control medium for three and six consecutive passages, a dramatic change in the expression of two proteins of 15 and 17 kDa was observed. After 3rd passage on a control medium the intensity of these polypeptide bands decreased and after six passages these bands disappeared completely. While another two proteins (60 kDa and 32 kDa) specific to the PEG and LiCl adapted cell lines, respectively remained stable (Fig. 6a and 6b).

Regeneration

Suspension cultures were adapted to 20 % PEG and 20 mM LiCl over a period of about 28 months (55 passages). Following adaptation, adapted and unadapted cell lines were compared for their regeneration frequency. The regeneration frequency of unadapted and LiCl adapted cell lines decreased 70 % and 90 % respectively after 28 passages. Interestingly, in PEG adapted cells line 80 % regeneration was recorded (Fig. 7)

Discussion

A substantial reduction in growth was observed in unadapted cell lines at 20 mM LiCl (Fig. 2). This reveal that adverse effects of LiCl on the growth is ion specific (cation specific), whereas, cations have shown co-tolerance towards cations of other alkali metals (Hodson et al., 1981; Shah et al., 2002). Similarly the impact of 20 % PEG on growth appeared to be drought specific rather ionic in nature because PEG is a non-ionic and non-penetrating polymer (Mexal et al., 1975; Shah et al., 2012). For osmotic or ion specific tolerance cells were incrementally adapted to either 20 % PEG and or 20 mM LiCl. In incremental adaptation there are gradually increasing requirements to the plant or tissues or cells to adjust itself to the environment and this is initiated by disturbance of normal metabolic processes, when organisms are able to restore their normal function due to increased tolerance, it is called adaptation (Alexieva et al., 2003). The parallel relative growth rates of adapted cell lines to unadapted cells line at their respective media manifest that 20 % PEG and 20 mM LiCl is no more a stressful environment for PEG and LiCl adapted lines, which otherwise, have resulted about 95 % reduction in the growth of unadapted line (Fig. 1 and 2). This indicated that adaptation of cell lines to osmotic and ion toxicity stresses was not at the cost of growth, because reduced/slow growth rate is considered as one of the strategy to cope with the stress (Ghars et al., 2007). Further exploration of the adaptive responses by studying the accumulation of inorganic cytosolutes, it appeared that adaptation to osmotic stress led to a substantial increase in Ca^{++} levels and significant reduction in Mg^{++} contents. At the cellular level tolerance to osmotic stress operates either through (i) dehydration tolerance that is slow growth at decreased turgor. (ii) avoiding dehydration through osmotic adjustment by ionic and organic solutes (Torello and Rice, 2006). In our case former strategy does not hold true because RGR of PEG adapted line was parallel to the RGR of unadapted line at their respective media. High concentration

of calcium is required for maintaining the membrane integrity and stability under stress, through bridging phosphate and corboxylate groups of phospholipids and proteins at the membrane surface (Leggs et al., 1982). It is widely accepted that Ca^{2++} increase the rigidity of the plant cell walls by complexing with matrix polysaccharides. Calcium also functions as a second messenger coupling a wide range of extracelluar stimuli to intracellular responses (Snedden and Fromm, 1998). The elemental characteristics of our PEG adapted line exhibited contrast with the findings of Hussain et al., (2004). In their studies PEG tolerant clones of chilli pepper and sun flowers accumulated more K^+ contents and Na^+ and less Ca^{++} contents than non-selected lines. The adaptation of cells line to ion specific stress (LiCl) did not exhibit any prominent alteration in organic and inorganic cytosolutes compared to the unadapted cells line. At the cellular level mechanisms controlling ion specific effects of salts are also of two types (i) those reducing the entry of salt at the plasmalemma (ii) those minimizing the entry of salt into cytoplasm by compartmentation (Munns, 2005). Since a little higher Ca^{++} level was observed in adapted line as compared to unadapted line. Therefore, tolerance of LiCl adapted line was speculated as an effective compartmentation of Li^+ ions within the cell (Fig. 4).

At the biochemical level protein profiling of adapted and unadapted lines were analyzed by SDS-PAGE. The protein analysis provides a direct assessment of genes involved in stress response and pathways because changes in proteins are attributed to changes in the gene expression (Jyothsnakumari et al., 2009). A total of six newly synthesized polypeptides were observed in adapted lines. Among these four polypeptides with an estimated molecular weight of 15, 18, 26 and 68 kDa were common in both adapted lines, while other two 60 kDa and 32 kDa were unique to PEG and LiCl adapted lines respectively. The appearance of common polypeptides could be interpreted as the result of gene expression for general adaptive response to stress and the presence of novel polypeptides of 60 kDa and 32 kDa may be the results of specific gene expression associated with drought and ionic stresses, respectively. The absence of proteins in the control cells line under normal conditions and their presence in adapted cell lines revealed that alteration in protein pattern are the consequence of an adaptive response that confers resistance to osmotic and ionic stresses. It is generally known that stress induced alterations in proteins of eukaryotes involve binding of an activated (transcriptional/translational) factor protein to the regulatory site on the DNA/gene and can be regarded as password that open multiple locks giving RNA polymerase to express specific gene (Tudge, 1993; Kim et al., 2003). Since, we developed cell lines over a period of about 28 months, therefore, changes were considered to be the results of long term adaptations, this raised the question, whether these adaptations/changes were stable or not? To answer this question, the adapted lines were grown in control medium (stress free) for six consecutive generations and proteins were extracted after 3rd and 6th cultures. Levels of two proteins of 15 and 18-kilo Dalton decreased markedly after 3rd passage and were undetectable after 6th passage, this revealed that in adaptation the possible role of these two stress inducible proteins (15 and 18-kDa) requires stress. While the level of other four polypeptides (26, 32, 60 and 68 kDa) remained stable during this experiment, an evidence of stable adaptation (Fig. 6a and 6b). Totipotency of unadapted and LiCl adapted cell lines decreased 70% and 90%, respectively. The decrease in totipotency with the passage of time is parallel with the general trend that regeneration frequency of cultured cells decline after few subcultures (Binh *et al*., 1992). While highly significant reduction in the totipotency of LiCl adapted line might be related with toxic effects of Li ions. On the other hand about 80% increase in regeneration frequency of PEG adapted might have increased intracellular attachment with balanced shear forces that have allowed the cells to enhanced the totipotency. Based on these results it can be concluded that culture media should be supplemented with PEG to maintain totipotency and to reduce regeneration time of undifferentiated cells (Fig. 7).

Materials and Methods

Plant material

Rice variety indica, cv. Swat-1 was used for experimentation due to its high regeneration capabilities and higher cultivation rate in the area. The rice seeds were collected from Agricultural Research Station North (Swat). Research was conducted at the Institute of Biotechnology and Genetic Engineering (IBGE), NWFP, Agricultural University Peshawar during 2006 - 2009. Calli were induced from mature seed of rice (*Oryza sativa* L.) cv. Swat-1. Cell lines tolerant to Polyethylene Glycol (PEG) and Lithium Chloride (LiCl) were developed in cell suspension culture from friable calli following Shah et al., (2002) a multistep procedure. The cell suspension lines of unadapted (control), PEG and LiCl adapted clones were used for experimentation.

Establishment of callus and suspension culture

Calli were induced from mature seeds of *Oryza sativa* Swat-1 by inoculating onto MS medium, supplemented with 2 mg^{-1} , 2. 4 -D, 0.25 mg^{-1} kinetin, 2 g^{-1} casin hydrolysate, 30 g^{-1} sucrose and solidified with 9 g^{-1} agar. Mature rice seeds were dehulled in Petri dishes. About 10 seeds were placed in a universal bottle. To soak the seeds poured on sufficient 70 % alcohol with a pasture pipette. The excess alcohol was immediately removed with the help of pipette. Seventy percent ordinary bleach was quickly poured on until the universal bottle containing the seed was about 2/3 full. Bottle was caped and shaked gently at intervals for exactly 15 minutes. Bleach was removed from the seeds and sterile distilled water (remembering to flamed the neck of the bottle etc.) was poured on. Shaked gently, pipetted off the water with a pasture pipette. We repeated the washing process 5 times. With flamed forceps, we transferred one or two sterile seeds into the universal bottle containing MS medium. Care was taken that seeds must not be burnt with hot forceps. Forceps was cooled by touching it against the wet inside the bottle containing the sterile seeds. Sterile practices were observed all the time. Test tubes/bottles were labelled and the capes were made loose fit. For each passage, callus cultures were incubated in the dark at 27 ± 2 ^oC for 15 days.

Suspension cultures

Following the 5th passage (each passage contain15 days), rapidly growing friable calli were used to establish suspension cultures by inoculating approximately 1 gram of calli into 250 ml conical flasks having 50 ml liquid MS medium. The flasks were incubated in the shaking incubator at 100 rpm in dark at 27 ± 2^oC. First and second culture of suspensions was sub-cultured after 21 days. After second subculture the suspensions were regularly transferred to the fresh medium with an interval of 15 days. For normal suspension maintenance, sub-culturing was done by

decanting, while for stress experiments a calibrated wire mesh scoop was used.

Selection of stress treatments

To select suitable level of stress treatment for osmotic (polyethylene glycol) and ion specific (lithium chloride) components of stress pilot experiments were conducted with 0, 5, 10, 15, 20, 25 and 30 % concentrations of polyethylene glycol (PEG) and 0, 5, 10, 15, 20, 25 and 30 mM concentrations of lithium chloride (LiCl) each treatment had 8 replicates. Those treatments from each stress were selected which substantially reduced the growth about 80 – 95 % but not totally inhibited the growth. Therefore, following the pilot study 20 % PEG and 20 mM LiCl was selected as a selection level for development of stress tolerant lines.

Adaptation procedure for cell lines tolerant to PEG (osmotic) and LiCl (ionic) stresses

According to Shah et al., (2002) a multi-step procedure was used to raise adapted lines. Cell lines were subjected to an incremental increase in PEG and LiCl concentrations in cell suspension cultures in order to develop resistant cell lines. Approximately 50 ml of suspension was inoculated into 50 ml of medium containing 5 % PEG and or 5 mM LiCl. Five replicates were used for each stress. After 15 days, the flasks containing the best growth of suspensions were subculture into medium of higher stress. The cell lines eventually developed follwed the sequence of increasing stress of the medium for coresponding passage. After adapting lines to a selection pressure of 20 % PEG and 20 mM LiCl, when concentration was raised to 25 % and 25 mM LiCl stresses, with exception of some cells in few flasks, all the other cultures turnned brown and no growth was observed in remaining flasks. The sequence of increasing PEG and LiCl concentrations were 5 % PEG and 5 mM LiCl (5 Passages), 10 % PEG and 10 mM LiCl (10 passages), 15 % PEG and 15 mM LiCl (15 Passages) and 20 % PEG and 20 mM LiCl for 25 passages. Concurrently, control lines were maintained in the absence of PEG and LiCl.

Regeneration of plantlets

The modified MS medium (Murashige and Skoog 1962) was used to regenerate plantlets from suspension culture. The medium was modified by omitting 2. 4 – D and kinetin and supplemented with NAA- $1mg^{-1}$ + BAP $1mg^{-1}$ + D sorbitol 30000 mg^{-1} + agar 9000 mg^{-1}. Explants/cells from suspension culture were transfered to regeneration medium. The cultures were incubated at 27 $^{0}C \pm 2^{o}C$ with a 16 hours photoperiod. After 28 days calli with embryiods or without embryiods were subcultured on to the fresh medium, which is necessary to promote conversion of embryiods into plants (Bingham et al, 1975). Further subcultures were made depending upon the conditions of the plantlets.

Measurement of growth

Growth of cell suspension was measured by the method of Shah et al., (1990) by following formula; RGR/Week = [ln (final weight)-ln (initial weight)] / 2

Extraction and measurements of inorganic ions

Oven dried cells were extracted and inorganic ions were determined by the method of Hodson et al., 1982.

Protein estimation and expressions

Standard protocols of Sambrook et al., (1989) for water soluble total calli protein extraction/separation was used

Experimental design and data analyses

The experiments were laid out in Randomized Complete Block Design (RCBD) having three (03) rice cell lines (unadapted, PEG adapted and LiCl adapted), eight (08) replications and seven (07) treatments in which 0% PEG and 0 mM LiCl concentrations was used as control.
For analysis of variance "Analysis ToolPak" of Excel and statistix 8.1 were used. Statistical analysis of data was carried out at 0.05 level of significance.

Conclusion

The present study concludes that incremental adaptation against osmotic (20% PEG) and toxic (20mM LiCl) stresses are very stable. However adaptation to LiCl reduced the totipotency of the cells and adaptation to PEG enhanced the regeneration capabilities of the cell so culture media should be supplemented with PEG to maintain totipotency and to reduce regeneration time of undifferentiated cells.

Acknowledgement

technical assistance of Mr. Sajjad Ahmad and Mr. Junaid are highly acknowledged

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