Research Article

Assessment of Adaptation, Regeneration Capability and Fatty Acid Profiles of LiCl Adapted and Unadapted Cell Lines of *Oryza sativa* L. Cv. Swat-1

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Abstract | In the current studies calli of *Oryza sativa*. L. cv. Swat-1 were incrementally adapted to ionic stress (25 mM LiCl) to asses some of the adaptation mechanism(s). Adapted calli line was obtained on MS medium supplemented with 2,4-D (2 mg/l) and kinetin (0.25 mg/l). For regeneration MS medium with 1.0mg/l NAA and BAP + 30g/l D-Sorbitol was used. LiCl adapted line showed significantly higher indices of tolerance than unadapted line at 100, 150, 200 and 250 mM NaCl stress. Adaptation resulted in enhanced accumulation of saturated fatty acids (myristic and stearic acids), while unsaturated fatty acids exhibited variable profiles with enhanced oleic acid and significantly reduced linoleic acid. The regeneration capability of adapted line decreased 10 times (20% to 2%), while that of unadapted line kept on increasing (26% to 38%) at each subsequent subculture on regeneration medium. Addition of 10 mM LiCl resulted in enhanced and earlier regeneration in adapted line. Complete plantlets were obtained in about ten days while normally plantlets were obtained after 28 days.

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Introduction

Rice belongs to glycophytes and is the second major crop in terms of food intake worldwide. It is cultivated mostly on coastal /irrigated areas due to larger water demand. It is severely affected by salinity stress due to mismanagement of irrigation water and intrusion of brackish water in coastal agricultural lands. It is estimated that almost 20% of the irrigated land of the world is affected by salinity presently excluding the arid and desert lands which is 25% of the total land of our planet (Yeo, 1999).

In Pakistan approximately 265880 hectares of land is saline and 95420 hectares is sodic and each year this effected land is increasing at an exponential rate (FAO, 2006). This salinity problem severely affects overall production of the crops. Pakistan is ranked eighth largest salinity affected country in the world (FAO, 2006). Concomitantly stresses are the backbone of evolution but in case of plants these stresses are of more crucial importance because plants are sessile organisms and they are more frequently exposed to the adversities of nature thus effecting their survival, development and productivity (Serrano et al., 1999).

Plants use diverse mechanisms to cope with the salinity. Various techniques and technologies are being employed to develop salt tolerant/resistant plants: (a) old selective breeding technique, this is really hampered due to the multigenic nature of the trait,

its interaction with the environmental factors and with growth stages of plants. (b) Biotechnological approaches. This approach help in targeting specific character by simplifying the complexity of salt stress trait on one side and to minimize the interaction with environment on the side. This helps in searching and identification of genes responsible/associated with specific stress. More importantly techniques of plant tissue culture are used to awaken the genes already present in the plant machinery via software adaptation (Zhang, 1991; Hasegawa et al., 1994; Kiegele et al., 2000; Iwasaki and Paszkowski, 2014).

Plants, while adapting to salinity stress compromise on some of their normally occurring mechanisms in order to channalize the energy towards adaptive mechanisms. Salt stress affects the plants by creating ionic and osmotic potential across the cellular membranes that disturb the transport across the membranes. In response the plants start accumulating osmolytes to balance the potentials for normal transport.

As the osmotic potentials are dealt at cell membrane, the membrane integrity has to be maintained. This mainly depends upon deposition of fatty acids at plasmamembrane and ratio of saturated and unsaturated fatty acids (Mushtaq et al 2013). The outcome of any biological technique, that involves cell cultures and lines depend on the regeneration capacity of the culture material.

The present project was undertaken in the context to understand the mechanism(s) of software adaptation by developing rice calli lines tolerant to LiCl (ion specific) stress and their characterization (physiological and biochemical) under NaCl stress and regeneration capability of these cell lines.

Materials and Methods

Experiments were conducted at the Institute of Biotechnology and Genetic Engineering Peshawar during 2010-2014.

Sterilization of seeds

Mature seeds of *Oryza sativa* L (Swat-1) were dehulled in sterilized petri plates. Around 15 seeds were placed in universal bottle and poured with 70% ethanol with a pasteur pipette. Excess alcohol was immediately sucked back. Seeds were then washed with 80% bleach for 10 minutes by inverting the tightly capped universal bottle at short intervals. Then seeds were washed with sterilized distilled water for 5 minutes.

Establishment of callus cultures: Sterilized seeds of *Oryza sativa* L (Swat-1) were inoculated with sterilized forceps on the MS medium, supplemented with 2, 4-D (2 mg/l), kinetin (0.25 mg/l) and 30 g sucrose. Cultures were incubated for 28 days in the dark at 28±2°C. After 6th subculture, the rapidly growing calli were used for adaptation to ionic stress (LiCl).

Selection of tolerant cell lines for ionic stress: By adopting a multistep technique the tolerant cell lines to ionic stress (LiCl) were selected (Shah et al., 2002). The stress was raised upto 25 mM LiCl in incremental manner (Azhar et al., 2012) and control calli were also maintained at the same time.

Adaptation procedure: At first the rice calli were subjected to the minimum stress concentration i.e. 5 mM LiCl. The developed calli were subcultured for 6 generations in the same stress for adaptation. Then it was shifted to the higher level of stress i.e.10 mM LiCl likewise calli were developed for up to 25 mM LiCl.

Response of unadapted and adapted line to NaCl stress: There were two calli lines, unadapted and adapted. To test the tolerance of adapted calli lines six NaCl treatments i.e. 0, 50, 100, 150, 200 and 250 mM were given each treatment with 5 replications.

Regeneration

For regeneration basic MS media (Murashige and Skoog, 1962) was supplemented with 1.0 mg/l NAA, 1.0 mg/l BAP and 30 g/l D-sorbitol. Embryogenic and non embryogenic calli were transferred to the regeneration medium. Cultured flasks were incubat ed for 28 days at 28±2°C with 16 hours of photoperiod. The data regarding regeneration was collected.

Measurement of growth

One year old calli of adapted and unadapted cell lines were used for this experiment. The method of Shah et al. (1990) was used for callus growth measurement. At first the wide necked flask containing 100ml medium was pre-weighted then weighted after inoculation. These flasks were incubated for 28 days at 28 C ±2 in the dark. The RGR (relative growth rate) of callus was calculated as:

RGR/WEEK= In [(Final weight) - In (Initial weight)]/4

Lipid extraction and analysis

Lipids were extracted and analyzed using recommended biochemical techniques (Morrison and Smith, 1964) at PCSIR Peshawar.

Fatty acid methyl ester (FAME) method

Fatty acid methyl esters were prepared by using the method of Morrison and Smith (1964). Dry samples (adapted and unadapted calli lines), 20µg each were taken in air tight glass tube and 1.5ml methanolic NaCl was added to them. Tubes were tightly closed and heated in the boiling water bath for 5min. After cooling 2.5ml of 10%methanolic boron fluoride (BF3) was added and heated in water bath for 30min to boil. Tubes were cooled and 5ml conc. NaCl and 1.5ml of Hexane were added and mixed. Upper hexane layer was transferred to a separate tube followed by addition of 1ml hexane to the tube containing sample and were vortexed. The Hexane layer was collected and added to the tubes containing the extract. The relative percentage of fatty acids determined from the peak areas of the methyl esters.

Gas chromatography/mass spectrometry

The fatty acid methyl esters were determined by GC on a GCMS-QP2010 plus, equipped with an electron ionizer (EI) detector. Chromatography was performed through TRB-FFAP (free fatty acid Phase) column (30m×0.32×0.25µM diameter). One µl of sample was injected with a 1:50 split ratio. The temperature of injection was 240°C, the ion source was kept at 250°C.and the interface at 240°C. Oven temperature was kept at 50°C for 1min, then to 150°C for 15min then to 175°C for 5min and at 220°C for 5min prior to next injection. Carrier gas was Helium. Compounds were identified by comparison of retention time with external standard and further confirmed by similarity search using mass spectral libraries. NIST database was used as external standard.

Results

Growth of undapted and adapteted calli lines under NaCl stress

Indices of Tolerance: The relative growth rates (RGR) of unadapted and adapted calli lines exhibited little (non-significant) difference at their respective media i.e. unadapted line on control medium and adapted line on medium supplemented with 25 mM LiCl. So, for precise comparison the indices of tolerance were calculated by keeping the RGR values of both lines as a reference value on their respective media Figure 1. The tolerance of both lines increased when 50 mM NaCl was added in the medium, followed by a gradual decrease in the indices of tolerance of unadapted cell line on increasing the stress which decreased to 0.12 at 250 mM. On the other hand the indices of tolerance of adapted line slightly increased at 100 mM NaCl followed by gradual decrease at 150, 200 and 250 mM NaCl stress respectively. Overall, the adapted showed significantly more tolerance than unadapted line on NaCl stress.

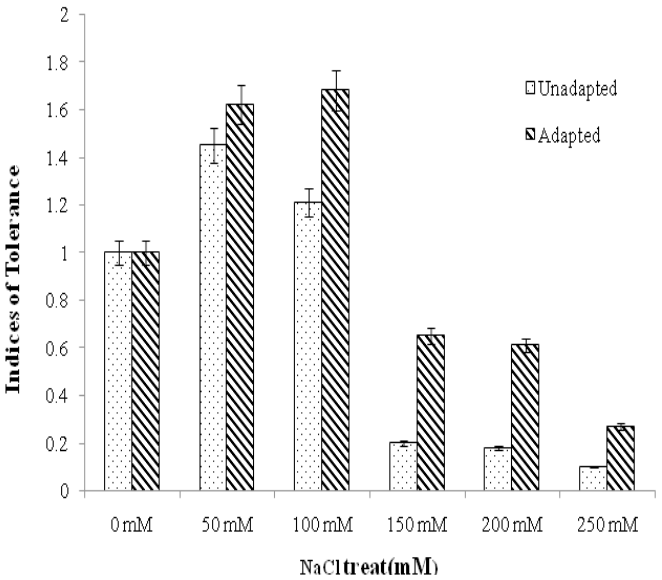

Figure 1: *Indices of tolerance of unadapted and adapted calli lines of rice (Oryza sativa.L.cv.Swat-1) under NaCl stress. RM=control line on control media and adapted line on 25 mM LiCl stressed media. The bars represent the mean values of 5 replicates ±SE.*

Fatty acid profile of adapted and unadapted cell lines

A total of five fatty acids were detected in calli lines among them three were saturated Myristic acid (14:0), Palmatic acid (16:0) and stearic acid (18:0) and two were unsaturated (Oleic acid (18:1c) and Linoleic acid (C 18:2c) in both the unadapted and LiCl adapted cell lines.

Saturated fatty acids: The data is presented in Figure 2a and Table 1. Which shows that mysteric acid (C14:0) contents of adapted line is significantly greater than the contents found in unadapted cells line. Whereas, Palmatic acid (C16 :0) was the most frequently saturated fatty acid in both the rice cell lines with non- significant difference for lines. Stearic acid (18:0) was second abundant saturated fatty acid in both the unadapted and LiCl adapted rice celli lines.

Unsaturated fatty acids: Oleic acid (C18:1c) was the

Table. 1: *Summary of analysis of variance (ANOVA) of unsaturated and saturated fatty acid of adapted and unadapted calli lines under NaCl stress*

Summary of ANOVA						
Unsaturated fatty acid				**Saturated fatty acid**		
Source of Variation	df	P value	F crit	df	F crit	P value
Unadapted line	2	1.24E-05	4.259677273	1	0.112957281	4.493998
Adapted line	2	1.24E-05	4.259677273	1	0.112957281	4.493998
Interaction	2	0.108618	3.402826105	2	0.001239055	4.493998

major unsaturated fatty acids (USFA) identified, about 14% in LiCl adapted line and 13% in control line. On the other hand percentage of Linoleic acid (C 18:2c) was significantly higher in unadapted cells line than in adapted cells line (Figure 2b and Table 1).

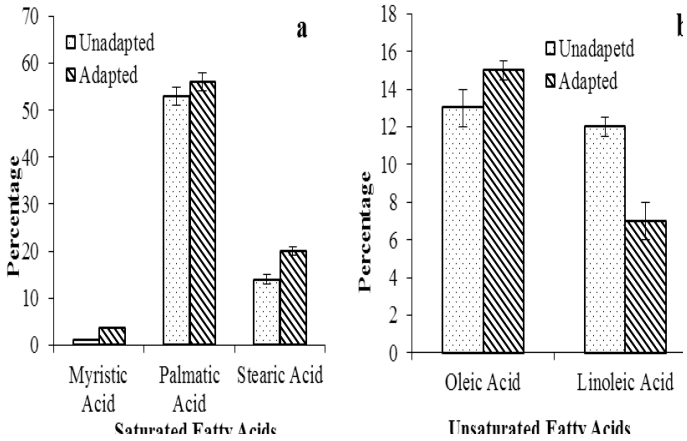

Figure 2*: Fatty Acid profile of unadapted and adapted cell lines of rice.* **(a)** *saturated fatty acids,* **(b)** *unsaturated fatty acids. The bars represent the mean values of 5 replicates ±SE.*

Regeneration: Regeneration efficiency of both the adapted and unadapted calli lines was calculated. Calli lines were cultured on regeneration medium. Regeneration events were recorded and regenerated plantlets were transferred to liquid medium (Figure 3).

When unadapted and LiCl adapted calli lines were grown on normal regeneration media, in unadapted ed 13 plantlets regenerated with about 26 percent regeneration percentage during first culture while in adapted line regeneration percentage was 20 percent. Upon subsequent 2nd and 3rd subculturing the regeneration percentage of unadapted line increased to 34

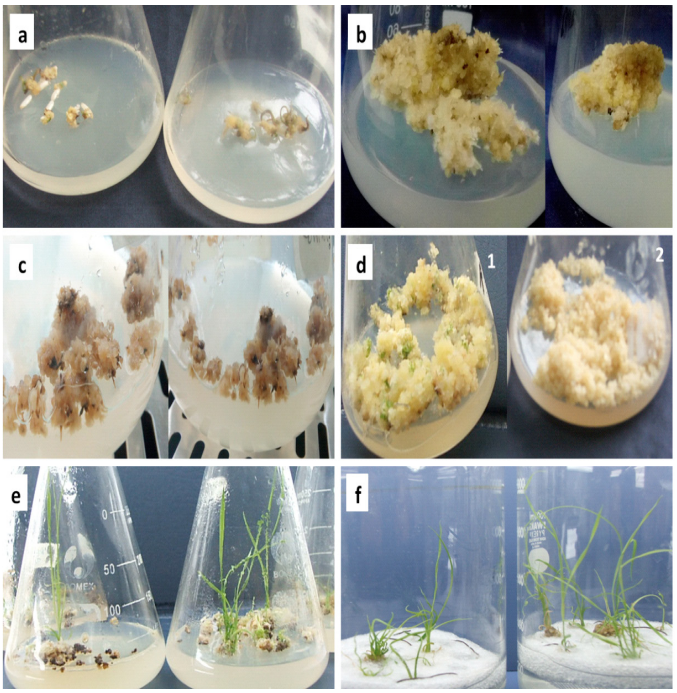

Figure 3: *Sequential steps in adaptation and regeneration process. a) Callus induction from rice seeds, b) unadapted calli line, c) LiCl adapted calli line, d) 1: embryogenic calli, 2: non embrugenic calli, e) regenerated plantlets and f) plantlets transferred to liquid medium.*

Table 2: *Regeneration percentages of unadapted and adapted lines of rice (Oryza sativa L) cv. Swat-1 on normal regeneration medium.*

Culture	**Lines**	**Total replicates**	**Plantlets regenerated**	**Percent regeneration**
1st Culture	Unadapted	50	13	26%
	Adapted	50	10	20%
2nd Subculture	Unadapted	50	17	34%
	Adapted	50	4	8%
3rd Subculture	Unadapted	50	19	38%
	Adapted	50	1	2%

and 38 % and of adapted line decreased to 8 and 2% respectively (Figure 4, 5 and Table 2).

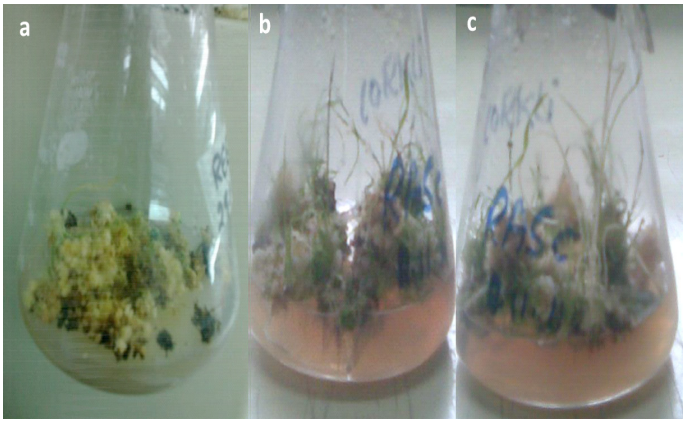

Figure 4: *Regeneration of unadapted line during different subcultures.* **a)**. *regeneration during first culture,* **b)** *regeneration during 2nd subculture,* **c)**; *regeneration during 3rd subculture.*

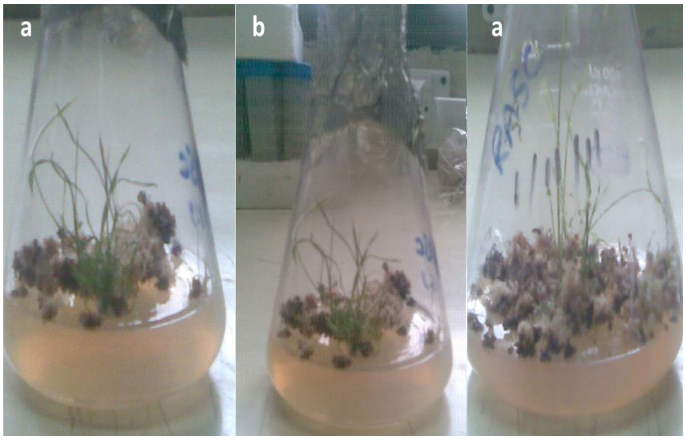

Figure 5: *Regeneration of adapted line during different subcultures a) regeneration during first culture b) regeneration during 2nd subculture c) regeneration during 3rd subculture.*

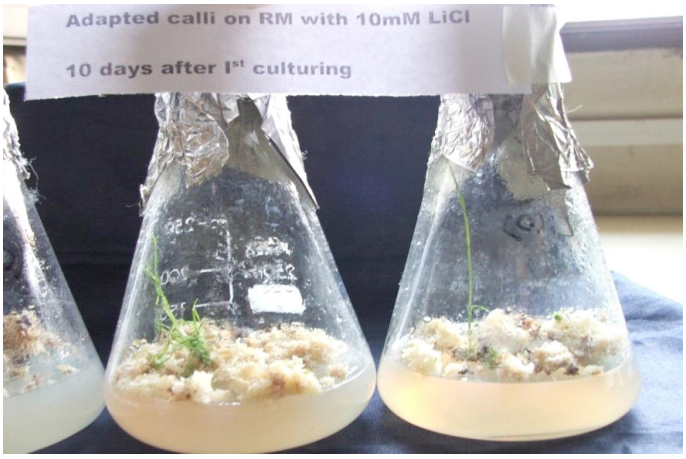

Figure 6: *Adapted calli on regeneration media with10 mM LiCl 10 days after first culturing.*

When regeneration frequency of unadapted and LiCl adapted lines were analyzed on medium supplemented with 10 mM LiCl the best results were found of adapted line. Surprisingly complete plant lets were developed after 10days of culture in both lines (Figure 6). The calli were white and many embryogenic green spots appeared at their surface. The regeneration percentage of adapted line rose to 40-45% while that of unadapted line not only decreased but plantlets were also very weak.

Discussion

For development of salt tolerant crops/plants a clear understanding of the adaptive mechanisms and their coordination is inevitable. A lot of research has been done in this area but there are many missing links left over needed to be addressed. With this perspective the current effort was made to understand better at least some of the aspects of adaptation working underneath.

In this study calli lines were adapted to LiCl, a stress that has only ionic component of stress not osmotic. Relative growth rate is known to be the best parameter for studying the acclimatization and level of tolerance, as all biochemical, physiological and molecular mechanisms are related with growth. The data pertaining to relative growth rates (RGR) of unadapted and adapted calli lines were similar on their respective media indicating that the 25 mM LiCl is no longer a stress for the adapted calli line. Which otherwise caused more than 90% reduction in growth. Secondly significantly higher indices of tolerance of adapted line than undapted line at almost all NaCl stresses (Figure 1) is indicative of the fact that adaptation to ionic stress (LiCl) has resulted development of tolerance in calli line towards a stress having both ionic and osmotic components of stress, a phenomenon of co-tolerance. This is in line with the findings of Munns and Tester (2008) and Shah et al. (2017) that when a salt sensitive plants/cells get ncremental exposure to different levels of ionic stress, this could lead the plant/cells to tolerate an enhanced level of ionic and osmotic stresses. This process of acclimatization work at the three levels, tolerance to osmotic stress, tolerance to ionic stress and tolerance to salt induced secondary effect.

Significant increase in saturated fatty acids (Myristic and Stearic acid) and unsaturated fatty acids (Oleic acid) while significantly lower level of linoleic acid in adapted line compared to the level found in unadapt-

ed cell line shows association with the mechanism of tolerance in adapted cells line under NaCl stress (Figure 2a and Table 1). The high concentrations of saturated fatty acids reduce the membrane fluidity which in turn reduces the ionic flux through the membrane. On the other hand unsaturated fatty acids significantly reduced (Figure 2b and Table 1), as they seem to be negative regulators of membrane fluidity. Increase in saturated fatty acid (Palmatic acid and Steric acid) has been found in *Bryophuyllum pinatum* and *Arabidopsis thaliana* (Mushraq et al., 2013; Falcone et al., 2004) under salt stress. Similarly decreased concentration of unsaturated fatty acids were recorded when *Bryophyllum pinatum* and *Atriplex lentifomis* were subjected to high temperature stress (Mushtaq et al., 2013; Peracy 1978). Despite differential nature of stress the significant increase (p= 0.05), in saturated fatty acid and significant decrease (p= 0.05) in unsaturated fatty acids in adapted cell line (Figure 2a, 2b and Table 1) under ionic stress in rice and heat stress in *Bryophyllum pinatum* and *A. lentiformis* reveals the fact that both ionic and heat stress trigger the stress induced common mechanisms which ultimately regulate fatty acid concentration, major component of cell membrane to sense and regulate the stress response at various facets. Overall the physiological growth response and biochemical data (fatty acid profiles) of adapted and unadapted lines under NaCl stress manifested that adaptation to ionic stress strengthened or activated tolerance mechanism of rice calli line to cope with the adverse effects of NaCl.

Regeneration percentage of unadapted line was high in first culture which increased upon subsequent culturing but in adapted line the regeneration percentage reduced with every subculturing. This is in line with the reports (Li and Heszky, 1986) that re- differentiation ability of adapted cells could be strongly reduced by stress (NaCl) on regeneration media. High rate of heterogeneity in all the developmental cells could be the main cause of reduction in regeneration rate due to NaCl/stress as observed in other cereal cultures (Binh and Heszky, 1992).

On the other hand the enhanced regeneration frequency of adapted line on media supplemented with 10 mM LiCl then of unadapted line and earlier regeneration and development of complete plantlets in both lines on 10th day of culture shows that ionic stress in regeneration medium signals for earlier regeneration. On the basis of these results it can be recommended that for quicker adaptation to salt stress calli/cell lines may be adapted to LiCl and for earlier regeneration 10 mM LiCl may be supplemented in the regeneration medium.

Author's Contribution

This research work is a part of Ph.D research project of Qudsia Khalid supervised by Dr. Safdar Hussain Shah. Faiza Zaeem carried out fatty acid analysis (part of her M. Phil thesis) while Saad Hussain Shah prepared graphical presentation of data and manuscript.

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