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**ORIGINAL ARTICLE**

# Co-tolerance Mechanism of Rice Cell Lines for Heavy Metal

**[1]Nagina, [1]Azhar Hussain Shah, [2]Safdar Hussain Shah, [3]Khalid Khanand [4]Habib Ahmad**
[1]Department of Botany, Hazara University, Mansehra Pakistan
[2]IBGE, The University of Agriculture Peshawar, Pakistan
[3]Swabi Women University Swabi, Pakistan
[4]Department of Genetics, Hazara University, Mansehra Pakistan
Email: drahshu@gmail.com

**ABSTRACT**
*Cell lines of Oryza sativa cv. Swat-1 incrementally adapted to ion toxicity (25 mM LiCl) stress were compared with unadapted line to investigate the mechanism of co-tolerance towards Zinc (a heavy metal). Physiological characterization of the lines showed substantial increase in the $K^+$ and sugar contents on adaptation to LiCl. While no prominent differences in accumulating proline and $Ca^{++}$ contents were observed between adapted and unadapted cell lines. When unadapted and adapted lines were subjected to Zinc stress, there was a substantial increase in $Zn^{++}$ contents and significant decrease in the growth of unadapted line. The reduction in the growth of unadapted cell line was 47.6%, 60% and 96% at 10, 15 and 25 mM Zinc stress respectively. On the other hand LiCl adapted line accumulated significantly less $Zn^{++}$ contents at all the stresses, showing its more restricted uptake than unadapted line. Though it was restricted uptake even then LiCl adapted line accumulated considerable amount of $Zn^{++}$ ions at 10 mM Zn stress which could inhibit the metabolic functions in cytoplasm and reduce growth. But significantly higher growth of LiCl adapted line at all the stress levels reveals that this line has developed a cross adaptation/tolerance mechanism for ionic discrimination (selective ion uptake) and ions compartmentalization (sequestering of ions into vacuole). Based on these results we conclude that more resistant/tolerant plants can be produced by adapting cells/tissues or whole plants to higher concentrations of heavy metals. Such plants will help in phytoremediation of heavy metals polluted sites.*
***Keywords;*** *cross tolerance, LiCl, heavy metal, phytoremediation*

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## INTRODUCTION

Recently the environment polluted by various heavy metals has created threat for plants and animals. Heavy metals are toxic to plants either at all concentrations or above certain threshold levels and biological magnification of these metals takes place through food chain. They infect the environment by affecting the properties of soil, its fertility, biomass and finally crop yields [1].

Toxic concentrations of heavy metals can result in the formation of superoxide radicals ($O^{2-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH^-$), etc., and can cause severe oxidative damage to biomolecules like lipids, proteins and nucleic acids and ultimately human health. The accumulation of heavy metals in soils due to industrial effluents and atmospheric emissions like paper mill, fertilizers, glasses and mining wastes is a big issue [1].

Due to high cost and scarcity of chemical fertilizers, the land disposal of agricultural, municipal and industrial wastes is widely practiced as a major and economic source of nutrients and organic matter for growing cereal crops by poor farmers in developing countries [2]. The most reported heavy metals in waste amended agricultural soils are Cu, Pb and Zn [2]. Zinc is one of the micronutrients essential for plant growth but is toxic to plants at higher concentrations and can retard plant growth and disrupt various essential physiological processes either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient [3].

Plants respond to heavy metals by adapting different strategies. While adaptation is considered as comparatively fast inheritable, biochemical, physiological and / or morphological changes for improving plant's resistance to the impact of a stress factor and enabling it to survive in modified/stressed environment [4]. However, adaptation of a plant to one stress factor can result in its increase tolerance to other stress requiring the same physiological and / or morphological modifications; this phenomenon is called cross-adaptation or cross-tolerance [4, 5, 6].
A number of investigations have shown that cross adaptation is not only possible in case of subsequent impact of heavy metals, but a much more general event. Cross adaptation is attributed to the fact that at a cellular level the effects of different stressors are the same [7]. Keeping this in view in current study an attempt has been made to investigate the co-tolerance mechanism of 25 mM Lithium Chloride adapted cells line of rice (*Oryza sativa* L.) towards Zn, a heavy metal.

## MATERIALS AND METHODS

The experiments were conducted at the Institute of Biotechnology and Genetic Engineering (IBGE), Agricultural University Peshawar. Unadapted (control) and LiCl adapted clones of *Oryza sativa* L. cv.swat-1 cells lines were used for experimentation. For heavy metal stress $ZnSO_4.7H_2O$ was used as Zn source.

### MEDIA FOR CALLUS CULTURING

Calli were induced from mature seed of rice (*O. sativa*L.) cv. Swat-1. Dehulled seeds were surface sterilized in 70% ethanol for 30 s followed by a 15 min washing with 70% bleach. After five washes with sterilized distilled water, seeds were incubated onto Murashige and Skoog (MS) medium [8] supplemented with 2 mg/l 2,4 -D, 0.25 mg-l kinetin, 2 g-l casein hydrolysate, 30 g-l sucrose and pH was adjusted to 5.8 and solidified with 9 g-l agar. All the cultures were incubated in the dark at 27 ± 2°C.

### Selection procedure

A multi-step procedure was used to raise adapted lines [9]. Cell lines were subjected to an incremental increase of LiCl stress. The sequence of increasing LiCl concentrations were 5 mM LiCl (5 passages), 10 mM LiCl (10 passages), 15 mM LiCl (15 passages), 20 mM LiCl for 20 passages and 25 mM LiCl for 20 passages while each passage consist of 15 days. Concurrently, control lines were maintained in the absence of LiCl.

### Sterilization of the media

Dispensed media in flasks was sterilized by autoclaving at 15 p.s.i. at 121 $^0$C for 20 minutes. Other equipments (Scalpel, forceps, and spatulas) were also sterilized with media.

### Stress treatment

To select a concentration of Zn that would have inhibitory effect on growth of callus yet not lethal, pilot experiment was conducted with 0, 15, 25, and 50 µm/l treatments of $ZnSO_4$. $7H_2O$ with 4 replicates each and reduction in RGR/week of unadapted line was recorded. Based on this data concentrations of 0, 10, 15 and 25 mM were selected for further studies of adapted and unadapted cells line.

**Measurement of growth** The relative growth rate (RGR) of the callus was calculated by the method of Shah *et al*.[10].

RGR/Week = [ln (final weight)-ln (initial weight)] / 4

### Determination of Proline

Proline was determined by the method of Singh *et al*.[11] as follows.

### Determination of total sugars

Total sugars were measured by using the method of Duboius *et al*. [12].

### Determination of Ionic Content

For determination of ionic content ($K^+$, $Ca^{+2}$, $Zn^{+2}$), the method of Chase [13] was used.

### Statistical Analysis

"Analysis Toolpak" of Excel 2003 was used for analysis of variance and graphs, the significance level was found at a $P < 0.05$

RM = Respective Medium

## RESULTS

### Relative Growth Rate (RGR)

Data regarding RGR week$^{-1}$ of un-adapted and LiCl adapted calli lines showed non significant difference at their respective medium. But, with increasing concentration of stress in the medium and compared to respective medium, RGR week$^{-1}$ of unadapted lines reduced gradually and significantly. While reduction in adapted line at 0, 10, 15, and 25 mM was non significant. RGR week$^{-1}$ of adapted lines was significantly higher than unadapted line at all concentrations of stress in the medium (Fig 1). ANOVA showed overall highly significant effect of $ZnSO_4$ stress and also showed highly significant difference between the calli lines.

**Divalent cation (Zinc ion ($Zn^{++}$)**

The $Zn^{++}$ contents of unadapted and LiCl adapted calli lines were almost similar at their respective medium. While at 10 mM of $ZnSO_4$stress,content of $Zn^{++}$ was increased significantly in both calli lines compared to their respective medium(Fig. 2). But, in unadapted lines it was significantly higher than adapted ones. At 15 and 25 mM of stress, $Zn^{++}$ contents of unadapted lines further increased gradually and significantly but, in case of adapted lines $Zn^{++}$ contents were almost same as noted at 10 mM of stress. ANOVA showed overall significant effect of $Zn^{++}$ stress on increase in $Zn^{++}$ content and also significant difference in response of both calli lines.

**Calcium ion ($Ca^{++}$)**

At respective medium difference between $Ca^{++}$ content of unadapted and adapted rice calli lines was non-significant (Fig. 3). Both calli lines showed significant increase in $Ca^{++}$ content at 10 mM of $ZnSO_4$ stress and the difference between their $Ca^{++}$ content was non significant. Upon exposure to 15 mM of stress unadapted lines maintained $Ca^{++}$ concentrations while, in case of adapted line $Ca^{++}$ content increased significantly. However, when 25 mM of stress was given $Ca^{++}$ content of both the lines increased significantly and the difference between Ca++ content was non significant. Maximum ions were accumulated at 25 mM of stress in both calli lines. ANOVA showed over all significant effect of stress on $Ca^{++}$ accumulation and non significant difference in response of calli lines.

**Potassium ion (K+)**

At respective medium $K^+$ content of adapted line was significantly higher than unadapted one (Fig. 4). Upon exposure to 10, 15 and 25 mM of stress $K^+$ content of adapted line decreased significantly while in unadapted line significant increase was noted. When 15 mM of stress was given, unadapted line maintained $K^+$ content but in adapted lines it was increased none significantly. At 25 mM of stress in the medium $K^+$ content of unadapted lines increased significantly while in case of adapted line the increase was non significant. The $K^+$ content was significantly higher in adapted line than unadapted one at all concentrations of stress in the medium. ANOVA showed overall significant effect of stress on $K^+$ accumulation and significant difference in response of calli lines.

**Soluble Sugar**

Soluble sugar content was significantly higher in adapted lines than unadapted one at respective medium (Fig. 5). When exposed to 10 mM of $ZnSO_4$ stress, sugar content of both the calli lines increased significantly but this increase was significantly higher in unadapted lines compared to adapted one. At 15 mM of stress content of soluble sugar further increased significantly in both calli lines but the difference was non significant between the lines. While at 25 mM stress sugar content of unadapted lines increased none significantly. While, in adapted lines non significant decrease was noted and the difference between sugar accumulation in both the calli lines was also non significant. ANOVA showed significant effect of $ZnSO_4$ stress on soluble sugar accumulation and non significant calli lines difference in response to $ZnSO_4$ stress.

**Proline Content**

At respective medium the difference between Proline content of adapted and unadapted calli lines was non significant (Fig. 6). The figure shows significant increase of proline level in both calli lines at 10 mM of ZnSO4 stress compared to respective medium. But, the level of proline was significantly higher in unadapted line than adapted one. Upon exposure to stress of 15 mM stress, proline level was maintained in adapted line (same as noted at 10 mM) while, in unadapted line it was reduced non significantly and the difference between proline content of calli lines was also non significant. However, at 25 mM of stress proline content of unadapted line reduced significantly and that of adapted line increased significantly. The difference between accumulated proline levels was also significant in both lines at 25 mM stress. ANOVA showed non significant effect of $ZnSO_4$ stress on proline accumulation and non significant calli lines difference in response to $ZnSO_4$ stress.

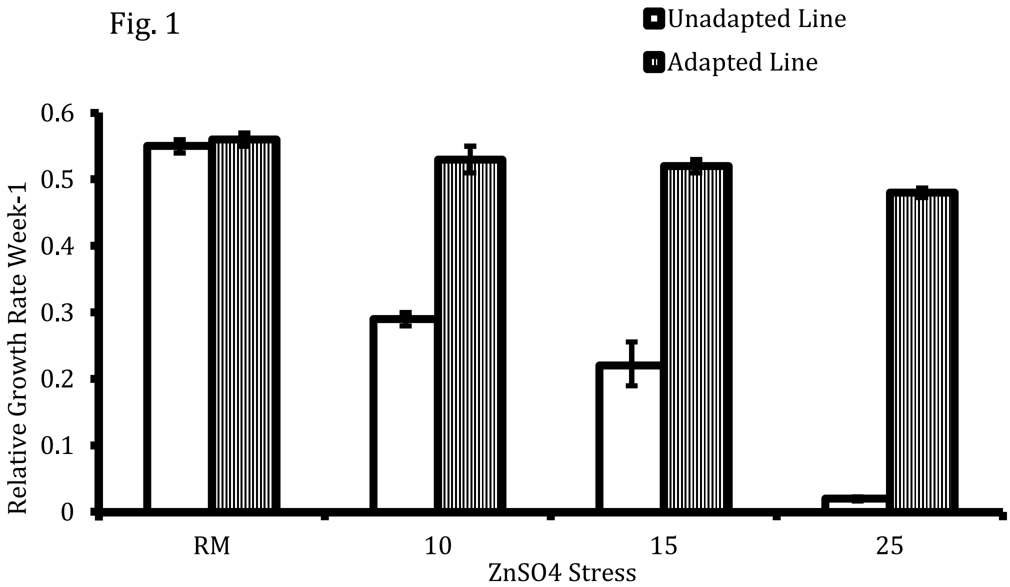


**Fig. 1.**The effect of $ZnSO_4$ on Relative Growth Rate of the calli lines. The data presented in the graph is means of five replicates ± SE.

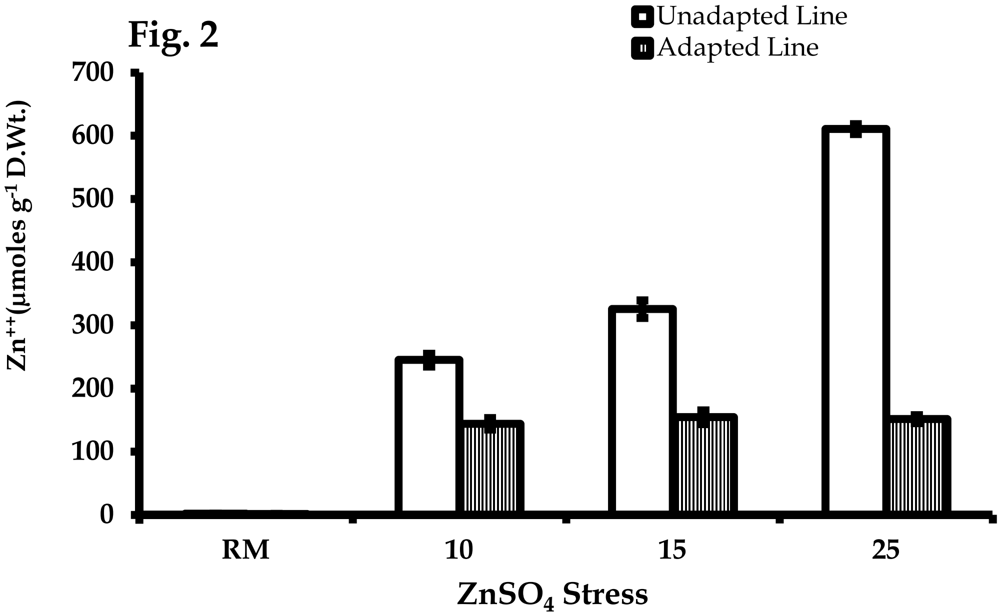


**Fig. 2.**The effect of different concentrations of $ZnSO_4$ on $Zn^{++}$ ion content of the calli lines. The data presented in the graph are the means of five replicates ± SE

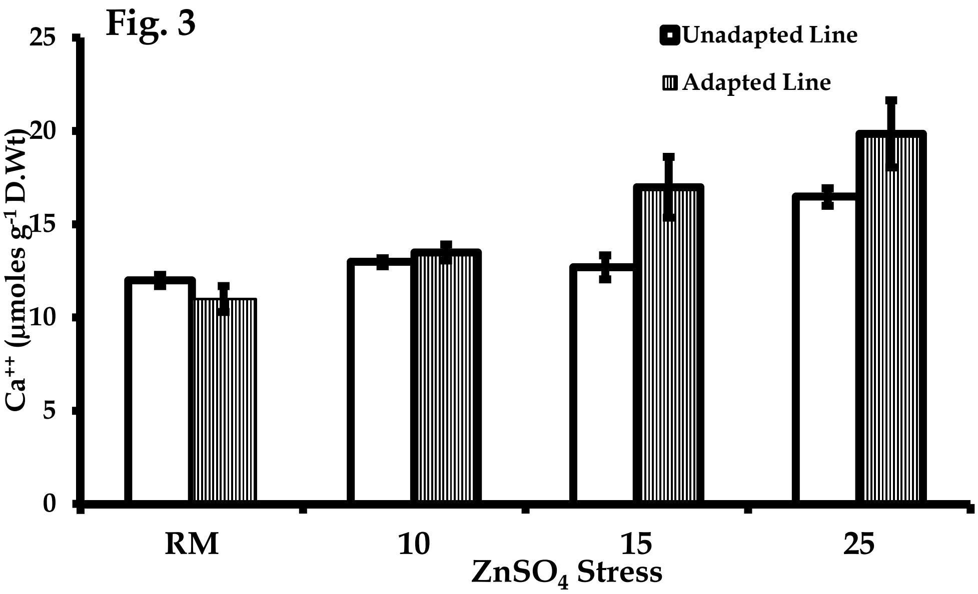


**Fig. 3.**The effects of different concentrations of $ZnSO_4$ on $Ca^{++}$ ion content of the calli lines. The data presented in the graph are the means of five replicates ± SE.

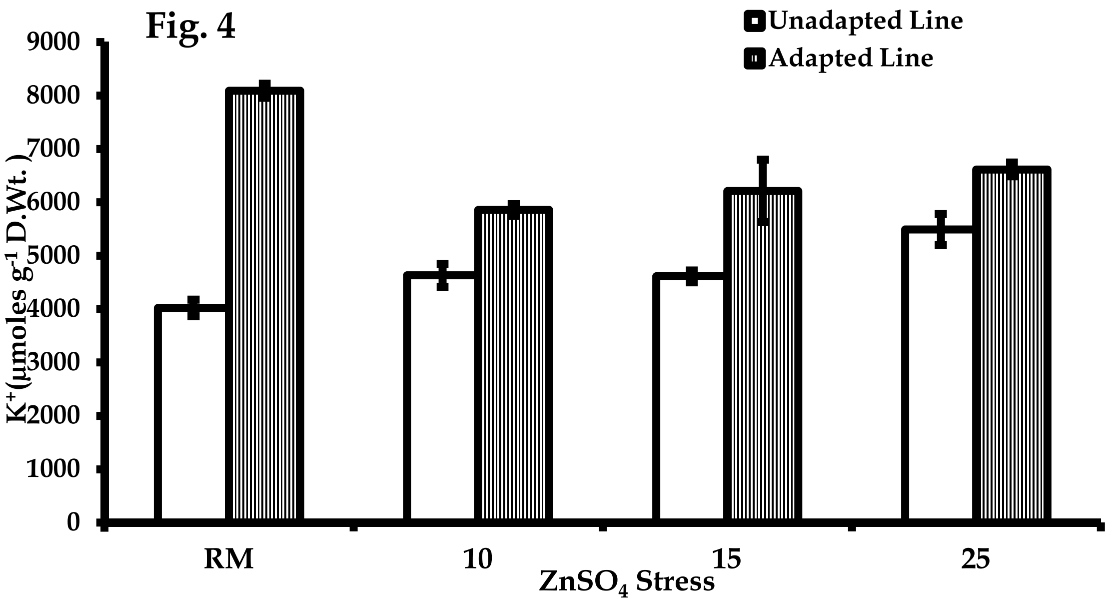


**Fig. 4.**The effect of different concentrations of $ZnSO_4$ on $K^+$ ion content of the calli lines. The data presented in the graph are the means of five replicates ± SE.

 

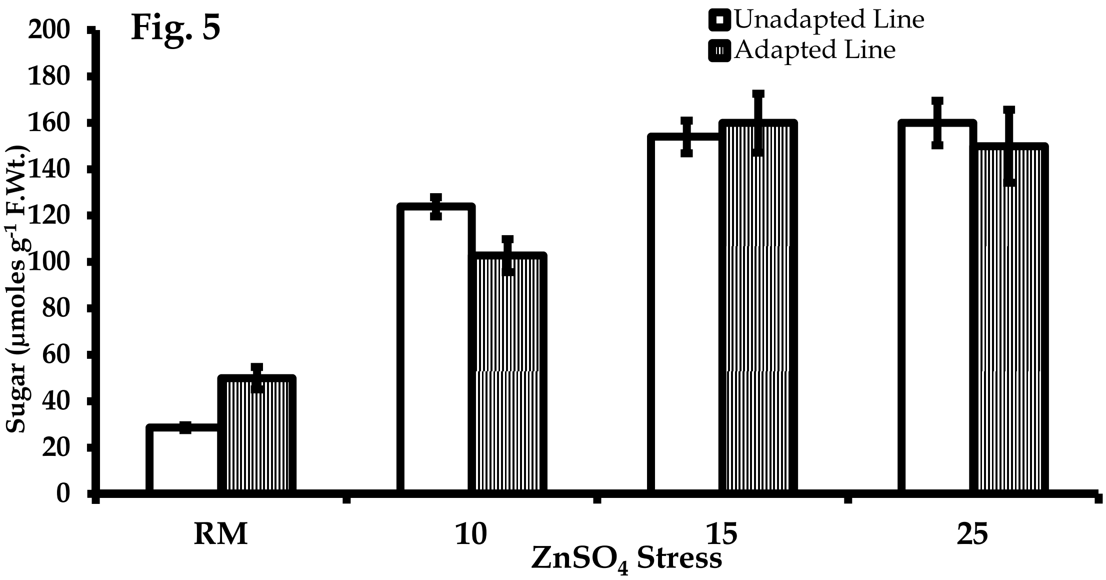


**Fig .5.**The effect of different concentrations of $ZnSO_4$ on total soluble sugar of calli lines. The data presented in the graph are means of five replicates ± SE.

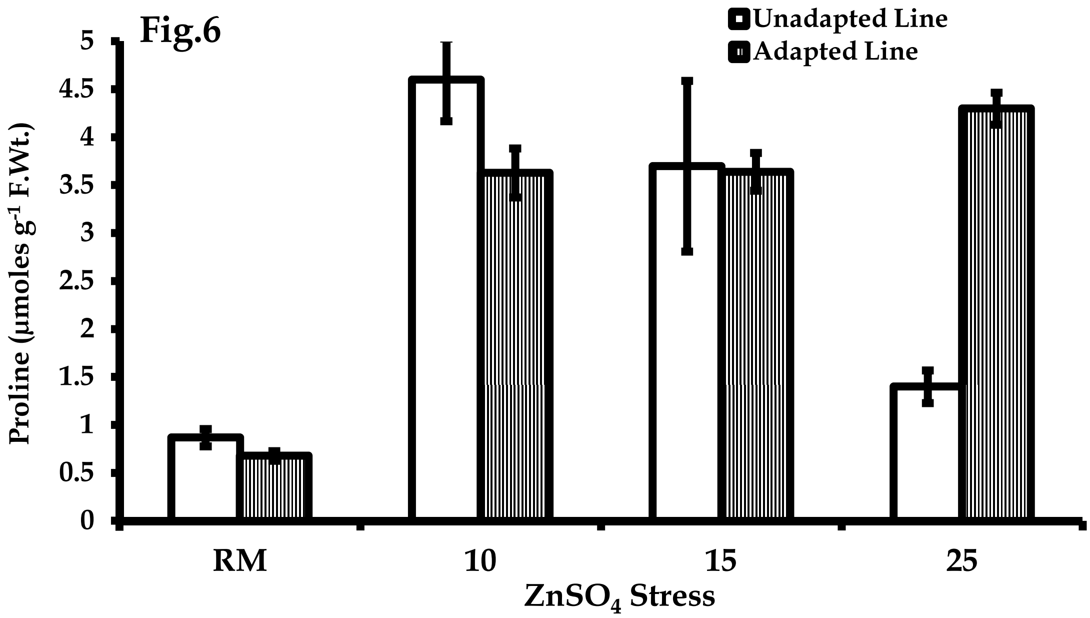


**Fig. 6.**The effect of different concentration of $ZnSO_4$ stress on proline content of calli lines. The data presented in the graph are the means of five replicates ± SE

**DISCUSSION**

Zinc is a component of bigeochemical cycles, essential for plants in small amounts but its high concentration is extremely toxic to plant tissues/cells as it interferes with the essential ions uptake, transport and regulation, thus affecting the ionic homeostatic systems of plants. Its excess results in disturbance of metabolic processes such as metabolic enzyme activity photosynthesis and transpiration [14]. When present in high concentrations, it can inhibit competitively the functions of metal requiring proteins and decrease the stability of DNA and RNA [15]. Excessive amounts of zinc can be present in soil due to the use of chemicals and sewage sludge in agriculture and metal mining. Therefore, plant's genotypes more resistant/tolerant to high concentration of zinc is needed [16].

In the present study developed callus line of *Oryza sativa* L. cv. Swat -1 tolerant to 25 mM LiCl (ionic stress) was used to investigate its cross adaptation /tolerance against elevated levels of $ZnSO_4$ stress. As, adaptation to one specific stress allows plants/cells to adapt a range of different stresses [4, 5]. It is the first report probably in which we investigated cross adaptation of developed callus line precisely tolerant to ionic ( 25 mM LiCl) stress, against different concentrations of $ZnSO_4$ (a heavy metal) stress. Variation in response of LiCl adapted and unadapted lines involved peculiar abilities to cope with $ZnSO_4$ toxicity stress.

Compared to respective medium, significant reduction in growth was observed in unadapted callus line with increasing concentration of $ZnSO_4$ stress in the medium. While, equimolarconcentration of stress have shown no significant reduction in RGR of adapted callus line (Fig. 1). The RGR of unadapted lines was reduced 47.6%, 60.3%, and 96% at 10, 15, and 25 mM of stress respectively compared to respective medium. However, reduction at 10, 15, and 25 mM in adapted line was 5.4%, 7.5% and 14.3% respectively. The data showed that unadapted line was more sensitive to Zn stress. Our results suggested negative response of unadapted calli line of rice to higher Zn stress. Because high concentration of zinc inhibits growth, by disturbing normal metabolic processes of cells, leading to visible injuries and physiological disorder. Similar results were shown by Luo *et al*. [17] for Jatropa seedlings, possibly by enhancing the production of ROS which cause oxidative damage to plant cells that reduce growth.

LiCl adapted lines were significantly more tolerant to $ZnSO_4$ stress than unadapted line. These results are in agreement with the findings of Alexieva *et al*.[5] who reported that a mild treatment of short duration with one stress agent may counteract the toxic effects of the succeeding stress – possibly because of the induction of defensive mechanism(s) by the earlier stress which increased resistance against the later stress.

Significantly higher content of $Zn^{++}$ was accumulated in unadapted lines at all stress conditions as compared to respective medium and adapted lines, and the increase in $Zn^{++}$ content with increasing stress in the medium was also significant. These results support the findings of Wei *et al.,* 2012 who reported significant increase in $Zn^{++}$ content of brown rice when concentration of external Zn was increased from 25 to 250 mg $kg^{-1}$. Similarly Sharma *et al*. [18] observed increased uptake of $Zn^{++}$ with increasing concentration of Zn by the seedlings of *Brassica juncea* at different concentrations of Zn. Toxicity level of zinc ranges from 64 μg $L^{-1}$ zinc for sorghum to 2000 μg$L^{-1}$ for cotton [19].

On the other hand in LiCl adapted line significantly higher $Zn^{++}$ content was accumulated only at 10 mM stress compared to respective medium, but no further increase was noted as stress was increased to 15 and 25 mM.Though LiCl adapted line accumulated substantially greater $Zn^{++}$ contents at 10 mM stress but overall concentration of $Zn^{++}$ in adapted line was significantly low than unadapted line at all the stresses (10, 15 and 25 mM). Therefore, the adaptive mechanism(s) acquired by LiCl adapted line seems to be selective uptake, exclusion of ions at plasma membrane and then compartmentalization/sequestration of toxic ions into vacuoles. The controlled/limited uptake of zinc protects the plant /cells against the injurious effects of excessive Zn these results support the findings of Styanova and Doncheva[20] who stated that this protection could be the result of Zn binding to the wall of root cells or due to a tolerance mechanism within the cell. Similar conclusion were drawn by Mathys[21] that tolerance against excessive zinc could be the result of increased ability of transporting Zn into vacuole away from metabolically active site of the cell.

Content of calcium was significantly higher in unadapted line at 10 mM stressnon significant increase was noted at 15 mM however, at 25 mM it was increased significantly as compared to respective medium. On the other hand LiCl adapted line showed significant and gradual increase in $Ca^{++}$ content at 10, 15 and 25 mM of stress compared to respective medium. This may be adaptive strategy of LiCl adaptive line against Zn stress because under stress, concentration of calcium in cell increases to maintain integrity and stability of membrane, by bridging corboxylate and phosphate groups of phospholipids and proteins at the surface of membrane as reported by Leggs *et al*.[22]. Generally it is accepted that by making complexes with the polysaccharides of matrix $Ca^{++}$ add to the rigidity of cell wall in plants. Calcium also play role as second messenger as it pairs a wide range of extracellular stimuli to intracellular responses [23]. Our results also support the findings of Yang and Poovaiah[24]who reported that high concentration of heavy metals changes Ca channels stability and increase calcium flux into the cell. Cellular $Ca^{++}$ act as second messenger , interacting with calmodulin for signal propagation and finally adjusting downstream genes concerned with transport, metabolism, and enhance tolerance of heavy metals

$K^+$ content of unadapted line significantly lower than adapted line at respective medium, increased significantly at 10 mM. This was maintained at 15 mM and then further increased at 25 mM. However, in adapted line $K^+$ content decreased significantly at 10 mM , which was maintained at 15 mM, as increase at 15mM was non significant . Upon exposure to 25 mM stress $K^+$ content further increased significantly. Aktas, *et al.*[25] have reported increase in $K^+$ concentration of shoot when zinc application was increased from 2 to 10 mg $kg^{-1}$soil. Higher concentration of zinc have shown no significant affect on $K^+$ level of stems and leaves in pea plant while in roots $K^+$ content increased continuously at zinc stress of 350 µM onwards [20].

Soluble sugar content of unadapted line significantly less than adapted line at respective medium, but they were increased gradually and significantly in both the calli lines at 10mM and 15 mM stress compared to respective medium. However, no further significant increase was noted at 25 mM of stress. Though unadapted and adapted lines accumulated similar levels of soluble sugars at 10, 15 and 25 mM stress but significantly higher growth rate of adapted line than unadapted line on the same stress indicates that increased production and accumulation of sugars and proline is a strategy of LiCl adapted line to tolerate stress. Because increased contents of soluble sugar could be the result of starch synthesis inhibition, degradation of starch and invertase inhibition. High content of total soluble sugars is essential for the production of energy, cellular membrane stabilization, turgor maintenance and signaling which possibly give plants/cells tolerance against heavy metal stress. Our results are very similar to the findings of D'souza[16] who stated that total soluble sugar and proline appear to protect hyacinth bean from osmotic and ionic stress induced by Zn stress.

At respective medium proline content of unadapted line was significantly higher than adapted one, at 10 mM stress it was increased significantly in both the lines and these lines maintained their proline content at 15 mM stress. But there was a significant increase in the proline contents at 25 mM Zn stress in adapted line. While, in unadapted line the level of proline accumulation was significantly lowered (representing probable death of callus). Increased production and accumulation of proline is one of the plant's strategies for tolerance against abiotic stress. D'souza[16] reported increase in proline level with increasing exposure time to Zn stress in hyacinth bean. Accumulation of proline in cell provides help to chelate metal ions, sustain the structural integrity of cytoplasmic proteins, maintain pH of cytosole, NAD(P)+/NAD(P)H ratio, protects enzymes from denaturation and also serves as nitrogen and carbon source [26]. Similar reports were presented by Nagoor[27] who stated that amount of proline is increased by various stress factors and proline plays role in alleviating stress act as carbon and nitrogen source, stabilize synthesis of proteins, act as an antioxidant and regulates pH. Ferreira *et al.*[28]reported proline accumulation caused by mercury in maize. Cd application also results in accumulation of proline in maize plant [27, 29].

Significantly higher growth rate, proline contents and considerable less contents of $Zn^{++}$ in LiCl adapted line as compared to unadapted line reveals co-tolerance (resistant/ tolerant) mechanism towards heavy metal ($ZnSO_4$).

Based on our findings we recommend that i. more highly resistant plants should be produced by adapting cells/tissues or whole plants to elevated levels of heavy metals, which can survive as well as accumulate heavy metals. Such plants will help in phytoremediation of heavy metals polluted sites. ii. Cross adaptation should be used for genetic modification in programs of crop improvement.

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