IN VITRO CHARACTERIZATION AND ANALYSIS OF RICE VARIETIES AGAINST VARIOUS LEVELS OF SALT STRESS

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ABSTRACT

Salinity is a significant environmental factor that greatly impacts plant yield, particularly in arid and semi-arid regions. The study examined the response of two rice varieties (Swat-1 and Pakhal) to salinity stress on the callus. Various salt concentrations were utilized. Among the two varieties, Swat-1 displayed the highest rate of callogenesis in the initial results. The Calli were exposed to varying concentrations of salt (50mM, 100mM, and 150mM), with each concentration tested in three replicates. Under the influence of stress, Swat-1 experienced a decrease in its Relative growth rate (RGR), whereas Pakhal remained steady at 50mM and 100mM. It was only at 150mM that Pakhal also exhibited a decline in RGR. Regarding water content, the stress caused a decrease in the content. At the highest stress level (150mM), the lowest water content was observed. The impact on soluble sugars was also noteworthy. As stress levels rose, concentrations of total sugars also increased. Similar findings were noted for proline, with stress leading to an increase in its concentration. Out of the two varieties, Pakhal appeared to have the highest proline accumulation. Similarly, there was a noticeable upward trend observed in catalase activity. There was a significant increase in catalase activity in both varieties after being exposed to stress for 30 and 60 days. The findings indicate that both varieties effectively developed strategies to combat salinity stress. They exhibited high concentrations of total soluble sugars and proline, as well as demonstrated significant catalase activity.

Keywords: *Oryza sativa*, sodium chloride, callus induction, salt stress, proline, rice, catalase, and salt tolerance

INTRODUCTION

Rice (*Oryza sativa L.*) is the main crop of the world, and the human race depends on rice for their daily diet. Rice is a monocot plant, a member of the family *Graminae* and subfamily *Oryzoidea*. Rice germinates well in waterlogged soil, moist environment, and in low radiation habitats (**Pandey *et al.,* 2014**). The genomic size of rice is 389 Mb with a chromosome number of 24 (**Goff *et al.,* 2002; Yu *et al.,* 2002**). Rice belongs to the genus Oryza having 27 species out of which wild species are 25 and 2 are cultivated. Cultivated species are *Oryza glaberrima* and *Oryza sativa*. For the last ~3500 years, *Oryza sativa* is grown all over the world while *Oryza globerrima* has been cultivated in West Africa (**Babaei *et al.,* 2010**). The rice embryo and pericarp contain 7% protein, 1.5% oils, 70-80% starch, Vit A, Vit B, Vit C, and some other important minerals. As compared to other cereals it produces more carbohydrates and calories per hectare than any other cereal (**Lu and chang, 1980**)

Globally, rice is one of the most important staple foods with an overall production of 718.34 million tons from 163.46 million hectares with an average yield of 4395 kg/ha. In the world, India stands first in rice area and second to China in rice production. During 2012-2013 in India rice was grown on an area of 43.65 m ha with a production of 104 mt (**Lu and chang, 1980**). Philippines, Indonesia, China, India, Japan, and Pakistan are the most important rice-growing countries. The productivity of many commercial bowls of cereal decreases by abiotic stresses including waterlogging, heat, salinity, and drought. Annual production of rice decreases as a result of several biotic and abiotic stresses worldwide. Pathogens, herbs, and insects are the most common biotic stresses which severely affect crops while major abiotic stresses are extreme temperatures, salinity, dehydration, chemical toxicity, and oxidative stresses (**GOI. 2013; Rodziewicz *et al.,* 2013**). It is predicted that due to increased salinization of arable land there is destroying global effects, resulting in 30% land loss within the next 25 years and it will increase and reach 50% by 2050 (**Wang *et al.,* 2003**). As a result of these stresses in plants molecular, physiological, biochemical, and morphological changes occur (**Wang *et al.,* 2001**).

Rice is rated as drought and salt-sensitive crop (**Mass *et al.,*1997**). Osmotic and ionic stresses are the general effects of salinity (**Greenway and Muuns, 1980**). Particularly in arid and semi-arid regions of the world salinity stress is one of the serious problems affecting various growth phases of the crops, production of food, the yield of the crop, and photosynthesis (**Jamil *et al.,* 2010; Osakabe *et al.,* 2011**). Around 800 million hectares of land are salt and sodicity affected globally (**Kumar**

et al., 2010; Munns *et al.,* 2008; Tavakkoli *et al.,* 2011; Munns, 2005**). Salinity is capable of reducing the optimum growth of crop plants by affecting water absorption as well as biochemical processes like Na+ assimilation and protein biosynthesis (**Dubey, 1994**).

Salinity disturbs the growth of plants by physiological and morphological (**Hasegawa *et al.,* 2000; Munns, 2002**). As a result of salinity plant growth, the rate of survival and development is reduced. Soil salinity affects the rice crop because of rice (**Winicov, 1996**). The response of rice to stress is to maintain a good balance of Na^+ K^+ in stem by a reduction in uptake of Na^+ and increased absorptions of K^+. Under salinity stress, proline accumulation is a significant factor that helps the plant to adapt (**Basu *et al.,* 2002**). To counteract the adverse effects of salt stress plants have evolved many molecular and biochemical mechanisms to protect from the harmful effects of salt stress. The main biochemical strategies are induction of ion homeostasis, antioxidative enzyme, and production of compatible organic solutes. The salt-tolerant plant development would be a practical solution to such a problem in soil having salt (**Yamaguchi and Blumwald, 2005**).

New techniques have been utilized worldwide for getting better plants. By using this technique, we get plants of our desired trait in a very short time then plant breeding (**Flowers, 2004**). Plant tissue culture technique can be used for micropropagation of clones after breeding, specifically in selection for stress tolerance such as salinity tolerance and cold tolerance in plants. A possible way was suggested by Flowers for getting salt-tolerant of crop plants by genetic variation within the presented crops by using recurrent selection (**Koe *et al.,* 2009**). *In vitro* selection is the fastest, simplest, and most widely used method for obtaining salt-tolerant plants (**Shekhawat and Kumar, 2006**). The *In vitro* technique for the selection of salt tolerance has been widely studied (**Lutts *et al.,* 1999**). In the successful *In vitro* selection technique, the requirement is a high variation of cells. Apart from that the response of rice calli culture and shoot regeneration under salt stress is a very important factor to improve salt tolerance (**Lestari, 2006**). In the present study, rice calli of Swat-1 (salt-sensitive) and Pakhal (salt-tolerant) were generated on callus induction media through tissue culture techniques, and then NaCl was applied in different concentrations to induce osmotic stress in these calli. Tolerant calli of Swat-1 and Pakhal were selected, then physiological and biochemical analysis of calli was conducted.

MATERIALS AND METHODS

Plant Material

Rice varieties Swat-1 and Pakhal were used in this study which were already grown in the field. The seeds were provided by Genomics and Proteomic lab, Institute of Biotechnology and Genetic engineering (IBGE), The University of Agriculture Peshawar.

Preparation of callus induction media

MS basal salt medium augmented with 1 mg/ml 2,4-D, and 0.25 mg/ml kinetin with a pH value of 5.2 was used for the preparation of callus induction. In 1-liter CIM, 4.43 gm MS media, 30 gm sucrose, 2 gm casein hydrolysate, 0.1 gm myoinositol, 4 ml 2,4-D, 2 ml kinetin, and 8 gm agar were added. At last, the pH was adjusted to 5.2 and then autoclaved.

Explant Preparation

Dehusking of rice seeds

Seeds of rice varieties Swat-1 and Pakhal were taken as explants sources for callus induction which was provided by the Institute of Biotechnology and Genetic engineering, the University of Agriculture Peshawar, Pakistan. Healthy and mature seeds were carefully selected by physical look and they were dehusked manually

Surface sterilization of rice seeds

For two minutes dehusked seeds in the falcon tube were washed two times with distilled water. 70% Ethanol was applied for thirty seconds for surface sterilization of seeds. Again, for two minutes seeds were washed with distilled water. 15% sodium hypochlorite was applied to seeds for twenty minutes. After washing with 15% sodium hypochlorite and 70% Ethanol, seeds were washed with double distilled water. Finally, autoclaved tissue paper was used to dry the seeds. Separate the seeds with the help of forceps. All the process was carried out in aseptic condition in a laminar airflow cabinet.

Callus initiation

In a laminar airflow cabinet, 20 seeds were placed in MS media. Seeds were evenly positioned to allow for growth. Petri plates were wrapped with parafilm and incubated at 28°C in the incubator.

Subculture

The calli were subcultured after two weeks and were transferred into a fresh callus induction medium for growth.

Stress Treatment

After four weeks calli were subjected to callus induction media augmented with different concentrations of NaCl (50mM, 100mM, and 150mM). Each concentration had three replicates. The treated calli were sampled for analysis after 30 days and 60 days. Calli grown on callus induction medium were used as control. The growth of calli was observed regularly after one week.

Selection of the calli on various NaCl concentrations

Relative growth rate (RGR)

RGR was determined based on the previously described method Shah et al 1990. After 30 days of culture, RGR was used to find callus growth (mg). To estimate the initial weight of callus Petri plates along with MS media were weighted by using weight balance before and after inoculation of callus. The callus was then incubated for 4 weeks at 28°C. For getting standard data the following formula was used to determine the Relative Growth Rate.
RGR Week-1 = (Final fresh weight (FW) - Initial FW)/4.

Relative water content

The water content of calli was determined based on previously described by **Patade et al 2011** method. Fresh calli of weight 500 mg were taken and placed in an oven for drying at 75°C for 50 hours. The dried calli were weighted again. Formula used for Water content (WC) were WC % = [(FW - DW)/FW] x100

Extraction of sample for sugar analysis and proline

For proline and sugar analysis Methanol, chloroform and water were used in the ratio of 12: 5:1 as extraction buffer. Calli weighted 500 mg was taken and frozen in liquid nitrogen. By using mortar and pestle calli were crushed to make a homogenate. Samples were added to test tubes with 5 ml Methanol, Chloroform, and H_2O in the ratio of 12:5:1. For 5 minutes homogenate was centrifuged at an rpm of 5000. In another test tube, the supernatant taken from the homogenate was kept. 5 ml Methanol, chloroform, and H_2O were poured into the remaining pellet and again centrifuged for 5 minutes at rpm of 5000. The new supernatant was added to the previously collected supernatant. Two ml of chloroform and three ml of dH_2O were added with pressure to the extract and vortex well. After mixing the extract was lifted for some time until two layers were formed upper methanol-water layer and the lower chloroform layer. The test tubes were covered by Aluminum foil and stored at 4 °C. The upper layer was used for the examination of proline and total soluble sugar.

Proline was determined based on the previously described method (**Matysik *et al.*, 2002**). The process was carried out in a laminar airflow hood. In test tubes, 0.5 ml sample was taken along with 0.5 ml of Methanol: DH_2O and 1 ml of acetic acid. After adding 1 ml solution of ninhydrin, the sample was heated in a water bath at boiling temperature for 45 minutes. Coldwater was used for cooling the test tubes. Five ml toluene was poured into each test tube. Vortex the tubes and left for a few minutes until two layers were formed. The upper layer of the sample was used for measuring the absorbance. The absorbance was checked at 520 nm with a UV-visible spectrophotometer by using toluene (blank).

Estimation of total soluble sugars

Sugar was determined based on the previously described method (**Munns, 2002**). In a test tube, a 1 ml sample was taken. After which 1 ml distilled water, 5 ml of concentrated sulphuric acid, and 1 ml of 5% phenol were added to the sample. In the fume hood, all the test tubes were placed for 3 minutes. At room temperature test tubes were cooled and were shaken for 12 minutes on a shaker. Then incubated in a water bath at 25-30ºC. The absorbance was measured at 490 nm against D-glucose (standard).

Catalase activity

In a pre-chilled mortar and pestle one gram of frozen callus was crushed with 1 ml of ice-cold 50mM phosphate buffer for 20 minutes the slurry was centrifuged at 4ºC at 12000 pm. After centrifugation, we took 900 µl of the reaction mixture (10mM Potassium phosphate buffer pH 7.0 + 10 mM H_2O_2) and added it to 100 µl of supernatant. For enzyme assay, this mixture was used. The samples were vortex. The absorbance of samples was measured at 290nm after 5 minutes. Catalase activity was shown in unit mg-1 protein. One unit showed the quantity of enzyme catalyzing the decomposition of 1µmol H_2O_2 per ml per mg protein.

Statistical Analysis

Collected data were subjected to the analysis of variation (2-way ANOVA) with the least significance difference (LSD) test.

RESULTS

The response of rice varieties was checked on optimized callus induction media towards callus induction. Callus induction response, morphology, and growth of each variety were compared on daily basis. Swat-1 showed the best response of callus induction, morphology, and growth. The color of calli was off-white, had a soft texture, and was large. Pakhal showed a poor response of callus initiation and growth. The appearance of calli was off-white, had a compact texture, and was small in size. Under suitable atmosphere, the varieties responded as follows as shown in Table 1 and 2: Fig 1 and 2.

Table 1 Callus induction response of two distinct types of rice cultivated under identical environmental conditions prior to stress treatment.

Variety name	Morphology	Color	Survival rate
Swat-1	Soft	Off white	95%
Pakhal	Compact	Off white	87%

Table 2 Callus induction response of two distinct rice types cultivated on callus induction media with salt stress.

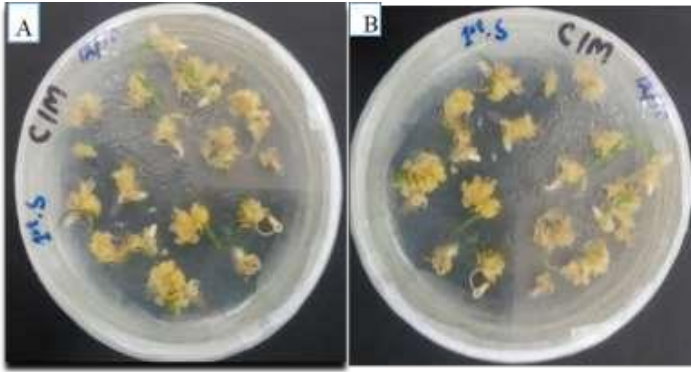| Variety name	Morphology	Color	Survival rate
Swat-1	Soft	Off white	76%
Pakhal	Compact	Off white	84%

Figure 1 (A) Callus induction response of Swat-1 **(B)** Pakhal on CIM before being subjected to stress.
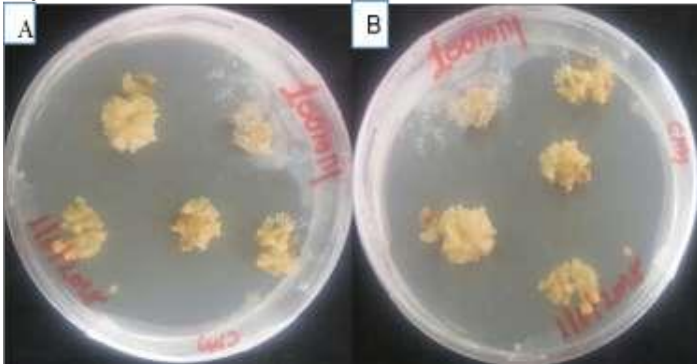

Figure 2 (A) Callus induction response of two different varieties of rice Swat-1 **(B)** Pakhal grown on salt stress containing callus induction media.

Salt stress evaluation

After four weeks of culture, callus was subjected to various increasing doses of salt stress that is 50mM, 100mM, and 150mM. The callus was exposed to NaCl stress for 30 and 60 days. After completion of 30 days, samples were collected, then physiological and biochemical parameters were analyzed. The same procedure was done for calli of 60 days and the same physiological and biochemical parameters were analyzed. (Fig 3 and 4)
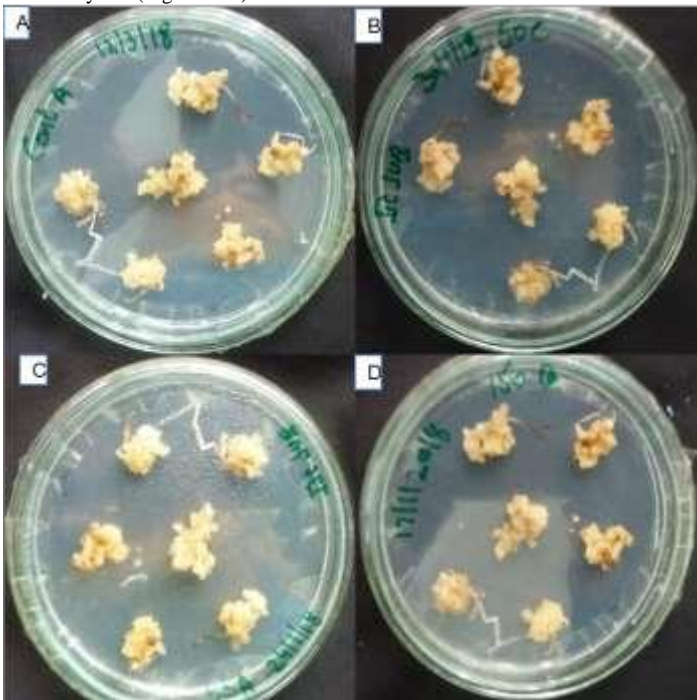
Figure 3 Pictorial representation of control and different stressed induced calli of Swat-1 (**A** = control, **B** = 50mM, **C** = 100mM and **D** = 150mM).
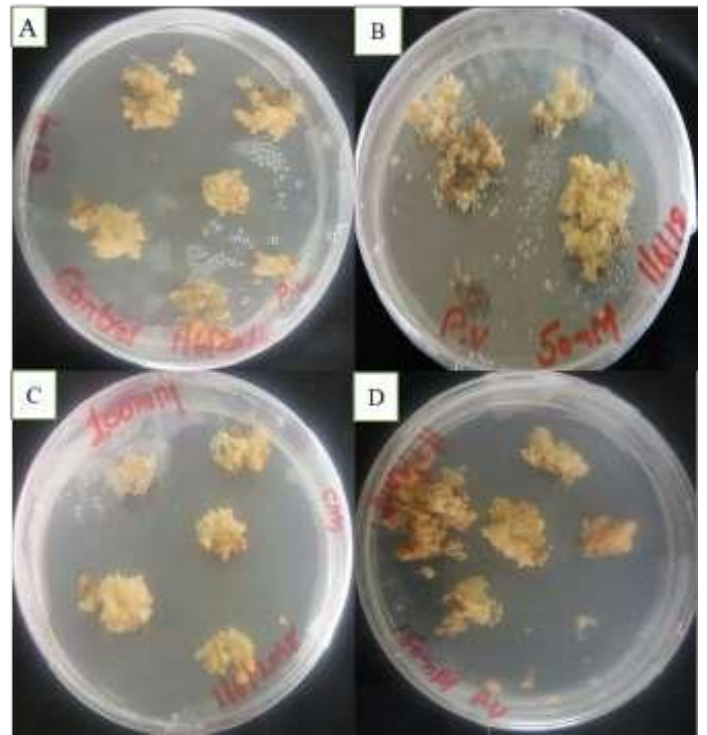

Figure 4 Pictorial representation of control and different stressed induced calli of Pakhal (**A** = control, **B** = 50 mM, **C** = 100 mM and **D** = 150 mM)

Effect of NaCl stress on Calli Relative Growth Rate

Upon exposure to salt stress, the growth of callus decreased as the concentration of salt increased as shown (Fig 5). Cultured seeds of Swat-1 and Pakhal showed callus formation from germ pore in the first week in some, while in others the base of initiating shoot swelled and formed callus in the second week.
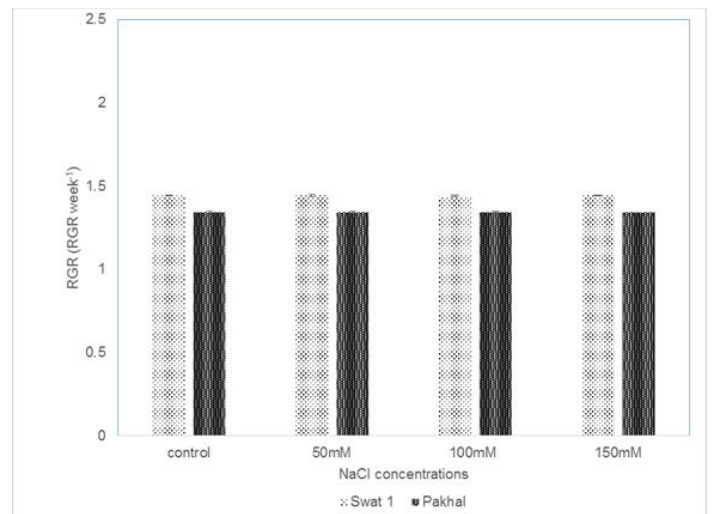

Figure 5 Relative growth rate of Swat-1 and Pakhal calli at a time when calli were subjected to salt stress after the second subculture. RGR after 14 days of NaCl stress

RGR when calli shifted to NaCl stress CIM after the second subculture

After completion of two subcultures, the callus was transferred to plates containing CIM having different concentrations of NaCl, the growth was almost the same for all concentrations. That is control, 50mM, 100mM and 150mM. The growth observed in control of Swat-1 and Pakhal was 1.4421 and 1.3409, at 50mM it was 1.4427 and 1.3412, at 100mM it was 1.4414 and 1.3403, at 150mM it was 1.4432 and 1.3407 respectively.

After 14 days of stress, the relative growth rate of both varieties increased at normal CIM and various concentrations of stress (Fig 6). Control calli of Swat-1 showed growth of 2.0128 and Pakhal showed 1.7809 whereas at 50mM growth showed in Swat-1 was 1.8719 and 1.4872 in Pakhal. At 100mM the Swat-1 showed a slight increase in growth with values of 1.4738 and Pakhal 1.4301. At 150mM growth noticed both in Swat-1 and Pakhal was 1.4519 and 1.3979 respectively. At 100mM and 150mM the RG was almost the same when results were checked individually for each variety.
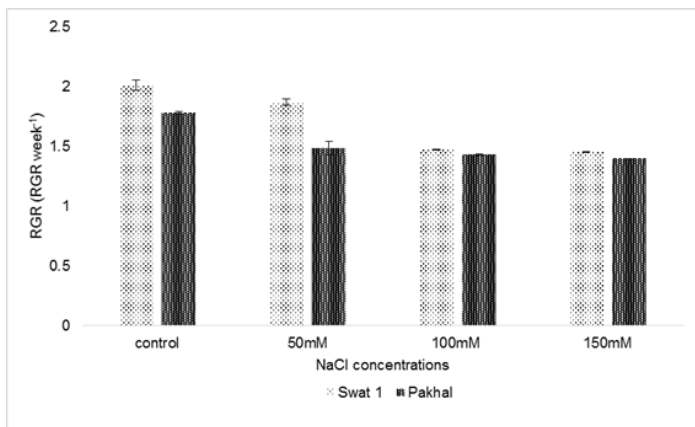
Figure 6 Relative growth rate of Swat-1 and Pakhal calli after 14 days of NaCl stress.

RGR after 30 days of NaCl stress

After 30 days of exposure, the growth noticed in Swat-1and Pakhal was shown in (Fig 7). Control calli of both Swat-1 and Pakhal showed a slight increase in growth that is 2.8905 and 2.6475. Compared to control 50mM showed a slight increase in growth of both varieties Swat-1 with values of 2.175 and Pakhal with 1.5513. At 150 mM concentration decreased growth rate was noticed in Swat -1 and Pakhal with values of 1.4085 and 1.3671. The decreased growth rate was noticed in Swat-1 at 100mM with the value of 1.4505 but at this concentration, the Pakhal was stable and showed an increase in growth with a value of 1.4738.
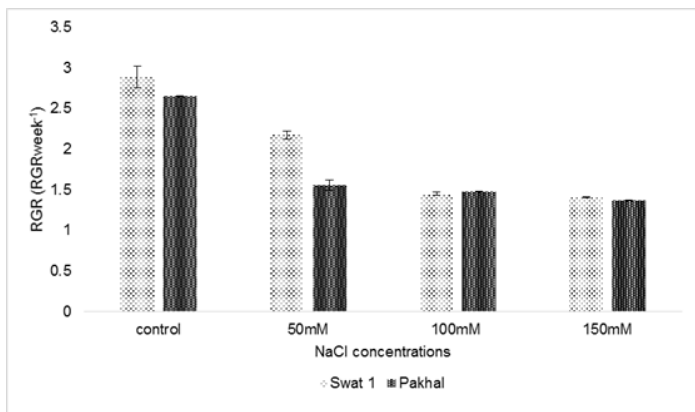

Figure 7 Relative growth rate of Swat-1 and Pakhal calli after 30 days of NaCl stress.

RGR after 60 days of NaCl stress

After 60 days control calli of both salt sensitive and salt tolerant variety showed increased growth with values of 3.0364 and 2.7928 respectively (Fig 8). As the concentration increases the growth rate of Swat-1 decreases but in Pakhal growth rate independently increased as the concentration of salt stress rises, but as the concentration reached 150mM the decrease in growth rate was noticed. The decreased growth rate was noticed at 150mM in both Swat-1 and Pakhal with values 1.3182 and 1.3349. At 100mM the growth rate decreased in Swat-1 with a value of 1.3862 but in Pakhal growth rate increased that is 1.5014 respectively. Second to control increased growth rate was noticed at 50mM in both Swat-1 and Pakhal with values 2.3177 and 1.5847.

Water Content

Water content of calli after 60 days of NaCl stress decreased significantly as the concentration of salt stress increased (Fig 9). The water content at cellular level of both varieties were almost same to each other, but at different levels of stress the water content were found to be different (Control = 20.5% and 18.5%, 50mM = 16.8% and 14.5%, 100mM = 11.81% and 9.04% and 150mM = 6.02% and 5.2%) respectively in Swat-1 and Pakhal. Overall effect of different levels of stress in both Swat-1 and Pakhal was non-significant, as the water content of both Swat-1 and Pakhal were non significantly different from each other that is Control = 20.5% and 18.5%, 50mM = 16.8% and 14.5%, 100mM = 11.81% and 9.04% and 150mM = 6.02% and 5.2%. When the results were checked individually for each variety significant difference was observed for each variety. For Swat-1 control showed 20.5%, 50mM (16.8%), 100mM (11.81%) and 150mM (6.02%) respectively

whereas for Pakhal control showed 18.5 %, 50mM (14.5%), 100mM (9.04%) and 150mM (5.2%) exponentially decreased in water content.
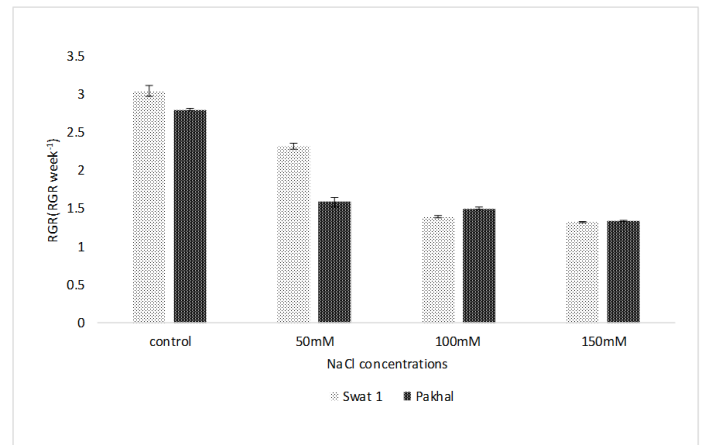

Figure 8 Relative growth rate of Swat-1 and Pakhal calli after 60 days of NaCl stress
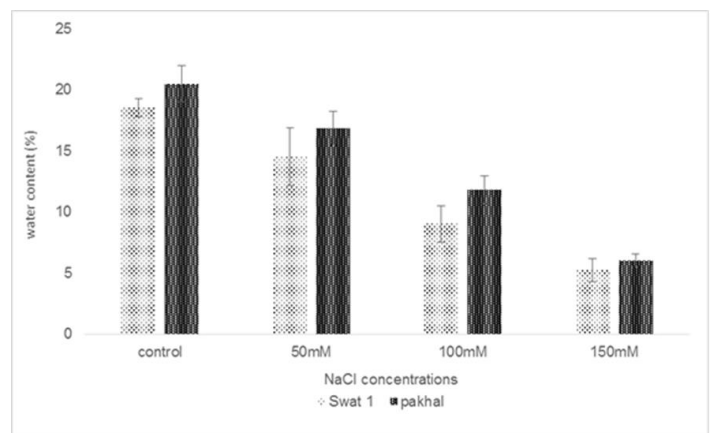

Figure 9 Water Content of NaCl stressed rice calli of Swat-1 and Pakhal after 60 days. The data are presented as a mean of 3 replicates ± SD.

Accumulation of free proline

To know about the effect of NaCl stress on the proline content of rice calli, two selected rice varieties were grown at the control, and three levels of NaCl stress that is 50mM, 100mM, and 150mM NaCl. With every degree rise in salt stress concentration the proline accumulation significantly increased (Figure 4.10). After 30 days of exposure at 150mM highest proline content was observed in Pakhal (31.869 µmoles mg-1) and Swat-1 (24.890 µmoles mg-1) whereas control calli of Pakhal and Swat-1 showed the least concentration of proline (13.035 µmoles mg-1) and (1.182 µmoles mg-1) respectively. The concentration of 100mM was second to 150mM in the accumulation of proline (25.080 µmoles mg-1) which was recorded for Pakhal as compared to Swat-1 (18.598 µmoles mg-1). Compared to control 50mM showed an increase in accumulation of proline (19.787 µmoles mg -1) in Pakhal and (11.0855 µmoles mg -1) in Swat-1. When the results were checked individually there was a significant increase in proline content. Among the two varieties, the Pakhal seemed to contain the maximum amount of proline. (Fig 10)

Proline content at the cellular level of both varieties after 60 days of exposure to stress significantly increased as the concentration of salt stress increased (Fig 11). At 150mM and 100mM NaCl significantly higher amount of proline was found in Pakhal (35.970 µmoles mg-1 and 27.837 µmoles mg-1)) and Swat-1 (28.525 µmoles mg-1 and 21.846 µmoles mg -1) as compared to control which contained less proline in Pakhal (15.204 µmoles mg-1) and Swat-1 (6.199 µmoles mg^{-1}). At 50mM the accumulation of proline noticed in Pakhal was (21.389 µmoles mg-1) as compared to Swat-1 which was (17.621 µmoles mg-1). Among the two varieties, Pakhal appeared good to accumulate more proline.
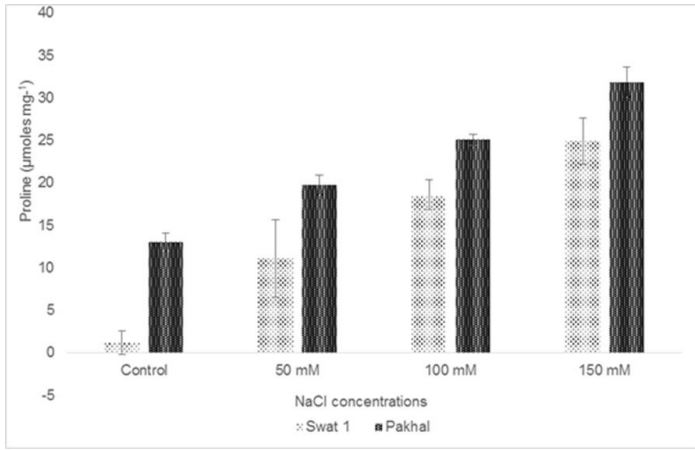
Figure 10 Free proline accumulation in NaCl stressed calli of Pakhal and Swat-1after 30 days. The data is represented as 3 replicates with ± SD.
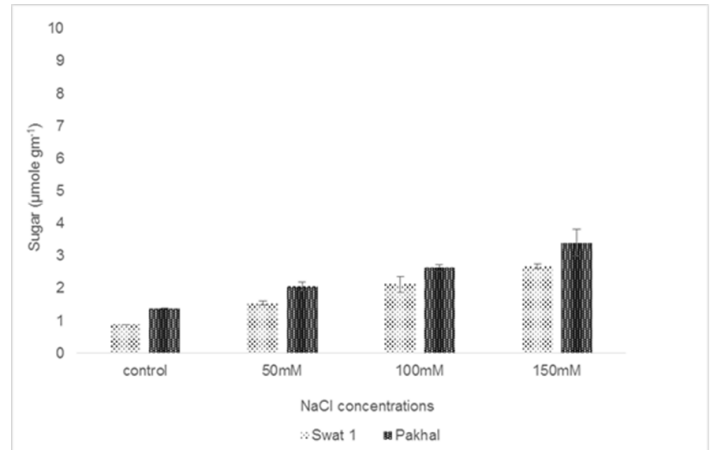

Figure 11 Free proline accumulation in NaCl stressed calli of Pakhal and Swat-1after 60 days. The data are represented as means of 3 replicates ± SD.

Accumulation of Total Soluble Sugar

With every degree increase in salt stress, the accumulation of total soluble sugar content increases but the degree of difference was not so much higher as proline. After 30 days major increase in total soluble sugar content was found in the cellular level of both varieties, exposed to a higher concentration of salt stress (Figure 4.12). Control calli of both Pakhal (1.3873 µmoles gm-1) and Swat1 (0.8861µmoles gm^{-1}) showed the least total soluble sugar content compared to 150mM which showed the highest content of total soluble sugar, Pakhal (2.0600 µmoles gm^{-1}) and Swat-1 (1.5435 µmoles gm^{-1}). Results showed that at 50mM the content of total soluble sugar was less as compared to 100mM and 150mM but in comparison to Swat-1 (2.1251 µmoles gm^{-1}) Pakhal had the maximum amount of total soluble sugar content (2.6320 µmoles gm^{-1}). A 100mM increase in total soluble sugar was found in Pakhal (3.4004 µmoles gm1) as compared to Swat-1 with total soluble sugar (2.6765 µmoles gm^{-1}). (Fig 12)

After 60 days of exposure, the overall effect of different levels of stress in both the varieties was significant, as the accumulation of total soluble sugar of both the varieties at different levels of stress were significantly different from each other which is shown in (Fig13). At 150mM highest amount of total soluble sugar was noticed in Pakhal that is (8.3792 µmoles gm^{-1}) and Swat-1 (6.8612 µmoles gm^{-1}). However, 100mM was second to 150mM having a greater amount of total soluble sugar (4.0030 µmoles gm^{-1}) in Swat-1 and (5.905 µmoles gm^{-1}) in Pakhal. Control calli of Swat-1 had the lowest quantity of total soluble sugar (1.6170 µmoles gm^{-1}) as compared to Pakhal (2.4864 µmoles gm^{-1}). Compared to 100mM and 150mM, at 50mM the content of total soluble sugar was less (2.5815 µmoles gm^{-1}) in Swat-1 and (3.8100 µmoles gm^{-1}) in Pakhal. Among Swat-1 and Pakhal, Pakhal contained high sugar content.

Figure 12 Total soluble sugar accumulation in NaCl stressed calli of rice varieties after 30 days. The data are presented as a mean of 3 replicates ± SD.
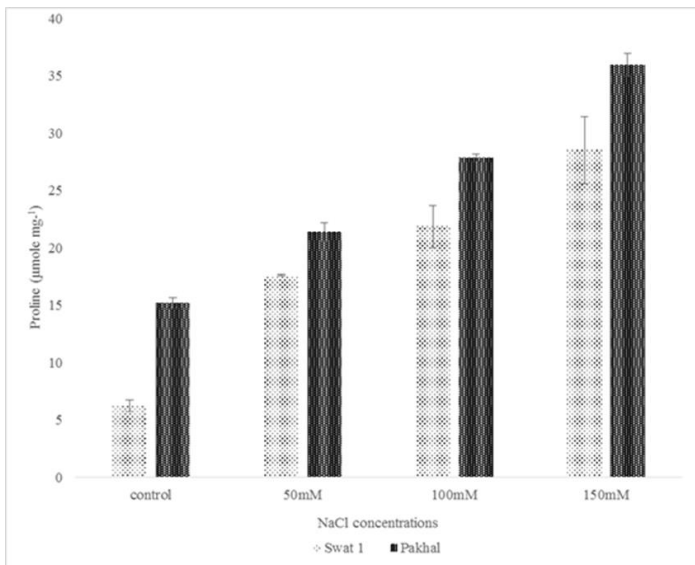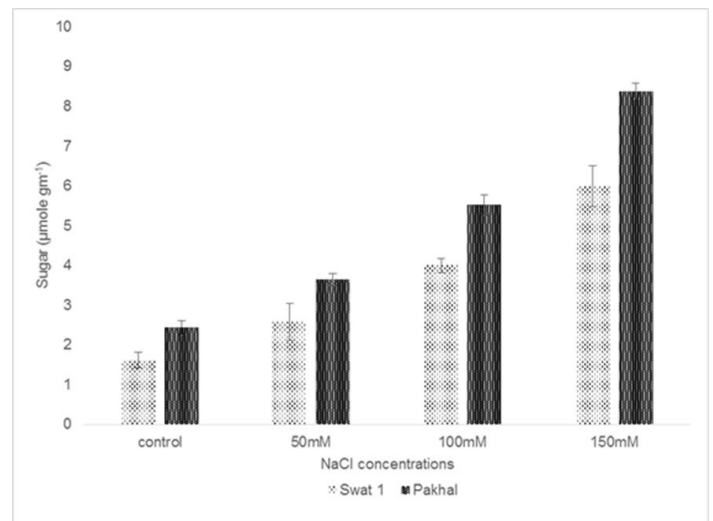

Figure 13 Total soluble sugar accumulation in NaCl stressed calli of rice varieties after 60 days. The data are presented as a mean of 3 replicates ± SD.

Catalase activity

The catalase activity of both the varieties at control was almost same to each other but at different levels of stress the activities were found to be different (at 50mM = 0.03737 µmole ml^{-1} min^{-1} and 0.0645 µmole ml^{-1} min^{-1}, at 100mM = 0.1367 µmole ml^{-1} min^{-1} and 0.0535 µmole ml^{-1} min^{-1}, at 150mM = 0.0954 µmole ml-1 min -1 and 0.1800 µmole ml^{-1} min^{-1}) for Swat-1 and Pakhal respectively. On the other hand, the overall effect of different levels of stress in both the varieties was non-significant as the catalase activity of both the varieties at different levels of stress (control, 50mM, 100mM, and 150mM) were non significantly different from each other (Fig 14). Where there was found significant difference for one variety, the other canceled its effect and made the overall effect non-significant. When the results were checked individually for each variety, all the levels of stress showed a significant effect on the catalase activity. For Swat-1 control and 50mM, NaCl showed the same activity but beyond 50mM the activity increased exponentially at 100mM and 150mM. Similar results were obtained for Pakhal where all the concentrations showed significant results including control vs 50mM. Beyond 50mM the same increasing trend was found (at 100mM = 0.1367 µmole ml -1min-1 and 0.0535 µmole ml-1 min-1 and at 150mM = 0.0954 µmole ml-1min -1 and 0.1800 µmole ml-1 min-1), as was observed for Swat-1.

After 60 days of exposure to salt stress, the catalase activity of both varieties non significantly increased (Fig 15). The catalase activity of Swat-1 and Pakhal at control and different levels of salt stress were found to be different that is (at control = 0.0675 µmoles ml^{-1} min^{-1} and 0.1085 µmoles ml^{-1} min^{-1}, at 50mM = 0.0812 µmoles ml-1 min-1 and 0.1838 µmoles ml-1 min-1, at 100mM = 0.0938 µmoles ml-1 min-1 and 0.2210 µmoles ml-1 min-1 and at 150mM 0.1345 µmoles ml-1 min^{-1} and 0.2538 µmoles ml^{-1} min^{-1}). When results were checked individually for each variety, both showed non-significant increased. For Swat-1 control (0.0675 µmoles ml^{-1} min^{-1}), at 50mM (0.0812 µmoles ml-1 min-1) and at 100mM (0.0938 µmoles ml-1 min-1) showed almost same activity but only increased occurred at 150mM (0.1345 µmoles ml-1 min-1). For Pakhal control and 50mM showed significance increased in catalase activity with values (0.1085 µmoles ml^{-1} min^{-1} and 0.1838 µmoles ml-1 min-1) but beyond 50mM NaCl non-significant

increase was noticed at 100mM and 150mM (0. 2210 µmoles ml^{-1} min^{-1} and 0.2538 µmoles ml^{-1} min^{-1}).
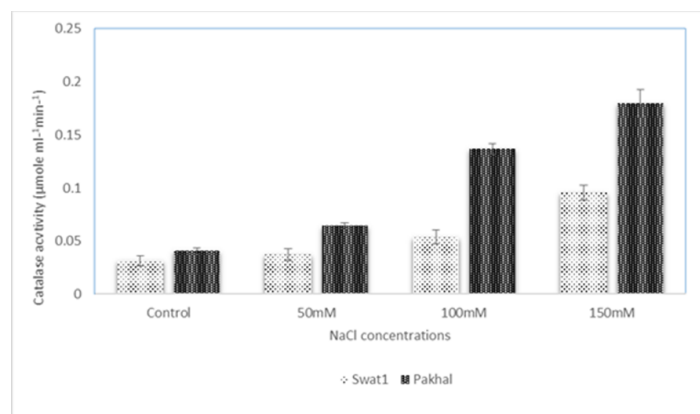

Figure 14 Catalase activity in NaCl stressed calli of Pakhal and Swat-1 after 30 days. The data are presented as a mean of 3 replicates ± S.D.
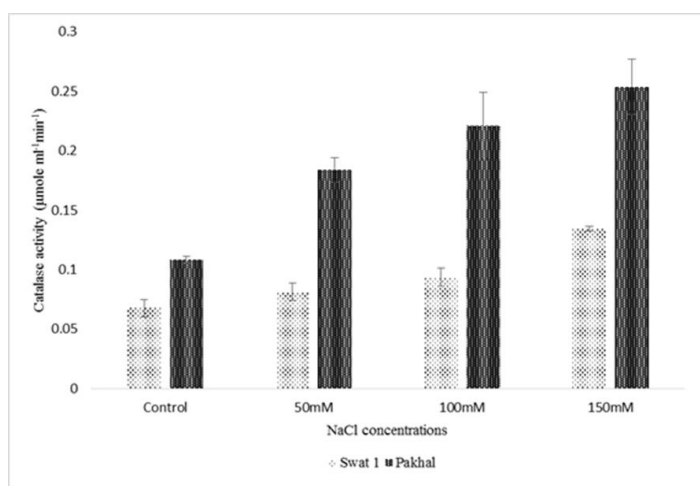

Figure 15 Catalase activity was measured in calli of Pakhal and Swat-1 subjected to NaCl stress for a duration of 60 days. The data are presented as the mean of 3 replicates ± S.D.

DISCUSSION

Around the world ecological stresses have been recognized as the most harmful issues principal to decreased plant productivity. In arid and semi-arid regions of the world, salinity is one of the major threats to crop yield, while NaCl is the most abundant source of salinity in the soil (**Flowers and Yeo, 1995**).

Calli is mainly composed of undifferentiated cells and is the best starting material for *In vitro* use. Calli induced from scutellar tissue of mature seeds are the best source of cells for callus induction and callus regeneration (**Hiei *et al.,* 1994; Ikram *et al.,* 2009**). The current study was planned to check the callogenesis response of Swat-1 and Pakhal to callus induction. Both showed a different response to callus induction. Swat-1 showed the best response to callus induction. Results of the current study agreed with the results reported by (**Seraj *et al.,* 1997; Peng and Hodge, 1989**) in rice. In the current study, the difference noted in callus capacity appears to be mainly due to the genotypic effect.

Calli of Swat-1 (salt-sensitive) and Pakhal (salt-tolerant) was subjected to numerous increasing stages of salt stress in direction to pattern the physiological and biochemical aspects of rice at the cellular level. Physiological and biochemical parameters were changed significantly after 30 and 60 days of stress from least to a higher concentration of salt stress. Calli of Swat-1 and Pakhal were exposed to iso-osmotic NaCl for 30 and 60 days. Salinity slows down the relative growth of calli of both Swat-1 and Pakhal, and less growth was observed at 150mM as compared to control. The results were in agreement with the reported work of **Smith and Mc Comb, 1981; Suenaga *et al.,* 1982** and **Mill, 1981** in rice genotypes CSR27 (salt-tolerant) and HBC19 (salt-sensitive). To avoid stress, less amount of water was used by Swat-1 and Pakhal due to decreased growth reported earlier by (**Wahid *et al.,* 2007**). The reduction of the relative growth is due to osmotic stress as well as cell injury (**Levitt, 1980**). In the present study, calli of Swat-1 showed a loss in growth at 150mM and 100mM NaCl stress as compared to control and 50mM whereas Pakhal was stable at 50mM and 100mM, only at 150mM showed a reduction in RGR.

NaCl stress also affects the relative water content like growth rate. In the present study relative water content of both Swat-1 and Pakhal decreased at 150mM, and 100 mM as compared to control and 50mM. The results were in agreement with

the reported work of (**Lutts *et al.,* 1996**) in Oryza sativa. Under stress conditions, the relative water content decreased as a result of water nonavailability and loss of turgor pressure in the cell of calli. **Errabi *et al.,* 2007** reported that due to the involvement of Na$^+$ and Cl$^-$ ions an uptake, translocation, and food alterations might be created, and the growth of callus is dropped.

Production of total soluble sugar is one of the main responses of calli to incremental salt stress. With every degree rise in stress, a significant increase occurred in sugar content. The current study showed an increase in sugar content at 150mM and 100mM than control and 50mM. The results about the increase in total soluble to increasing salinity were also observed by (**Yang *et al.,* 2014**) in Arabidopsis and (**Munns and Weir. 1981**) in wheat. Total sugar helps adjustment of osmotic homeostasis under salt stress (**Gupta and Kaur, 2005**).

Callus culture accumulates free proline inside the cells when subjected to salinity stress to protect it from dehydration and it was proved by (**Handa *et al.,* 1983**) also proline accumulation increases with every degree rise in salt stress. In callus culture increased level of proline may be due to alteration in the amino acids pool (**Yoshiba *et al.,* 1997**) and fresh production of proline is due to the breakdown of the protein-rich product (**Greenway and Munns 1980**). When calli of Swat -1 and Pakhal were exposed to salt stress, these calli accumulate free proline to resist the effect of stresses. Although the proline content of Pakhal calli was higher than that of Swat-1 calli. **Igarashi *et al.,* 1997** in rice and (**William Son & Slicum 1992**) in pea showed the same result of the accumulation of proline under salt stress. Proline was higher in 150mM & 100mM, and less in control and 50mM. Proline is known as an osmoprotectant and plays an important role in osmotic balancing, protection of subcellular structure and enzymes under salt stress conditions (**Matysik *et al.,* 2002; Sairam and Tyagi 2004**). Proline also adjusts osmotic homeostasis in plants under stress conditions (**Farkhondeh *et al.,* 2012**).

Antioxidant enzymes play an important role in conferring tolerance to drought and other abiotic stresses. During stress ROS are liberated which are toxic and react with metabolites and caused damage to the membrane of cells and organelles, degrade protein (**Davis, 1987**) and inactivate enzymes (**Fridovich, 1986**). To protect cellular and subcellular systems from the toxic effects of these radicals' plants developed endogenous mechanisms in the form of antioxidant enzymes (**Larson, 1988**). The activity of antioxidant enzymes increases when the callus is exposed to stress. As within varieties, the difference was significant which showed the variety can cater with the salinity and drought stress at the cellular level. In the present study at 150mM and 100mM, the calli of both Swat-1 and Pakhal showed an increase. The results were in agreement with the results reported by (**Li and Van Staden, 1998**) in maize cultivars.

CONCLUSION

It could be concluded from current findings that the Swat-1 showed the best callogenesis as compared to Pakhal. Sodium chloride (NaCl) induced stress affects the physiological and biochemical parameters of rice calli. Relative water content and relative growth rate decreased as the dose of NaCl concentration increased. To counteract the negative effects of increasing salt concentrations proline and sugar content increased. Catalase activity of rice calli was significantly increased as compared to control when exposed to NaCl. On the whole, increasing NaCl concentration negatively affected the physiological parameters of calli growth and induction. The effect of NaCl on calli was variety-dependent and dose-dependent. It is suggested that the callus phase plays a crucial role in determining the response of varieties to salt stress. This phase is of utmost importance in selecting both salt tolerant and salt sensitive varieties. It would be beneficial to expand the experiments to include higher doses of NaCl in order to examine the effects on growth, biochemical parameters, and molecular pathways in more detail.

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