COMPARATIVE ANALYSIS OF DIFFERENT ABIOTIC STRESS TOLERANCE MECHANISMS UNDER SALT STRESS IN RICE (*ORYZA SATIVA L.*,)

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Abstract

Salinity is a major threat to global rice production. This study investigated salinity stress tolerance mechanisms, *in vitro* in six rice genotypes – TRY 4, TRY 3, CR 1009 Sub 1, CO 53, CO 55 and ADT 37. Callus induction frequency, relative growth rate, regeneration percentage and biochemical markers (proline, catalase, superoxide dismutase and peroxidase) were analysed under 0-100 mM NaCl treatments. Callus induction frequency, growth rate and regeneration declined with increasing salinity in all genotypes, however TRY 3 and TRY 4 maintained comparatively higher growth and regeneration up to 100 mM NaCl indicating superior tolerance. Proline, catalase, superoxide dismutase and peroxidase levels increased with salinity levels in most genotypes as protective responses. TRY3 and TRY4 exhibited highest increases in these biochemical markers, conferring tolerance against salinity-induced osmotic and oxidative stresses. ADT 37 and CR 1009 Sub 1 showed reduced growth parameters and lower biochemical defence responses, marking them as salt-sensitive. Principal component analysis clearly distinguished control and salt-treated groups based on measured parameters. Overall, the study revealed varying salinity tolerance mechanisms in rice genotypes, with TRY 3 and TRY 4 as promising salinity-tolerant lines. These findings provide insights to aid breeding salt-tolerant rice varieties to ensure food security against rising soil salinity.

Keywords: Antioxidant enzymes, Tissue culture, Salinity tolerance, Rice

Abbreviations: SOD, Superoxide dismutase; **CAT,** Catalase; **POX**, Peroxidase; **CIP**, Callus induction frequency; **RF**, Regeneration frequency; **RGR**, Relative growth rate; **ROS**, Reactive oxygen species.

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Introduction

Soil salinity poses a significant challenge to global agriculture, leading to substantial land degradation and estimated annual losses of USD 27.3 billion in irrigated areas due to decreased crop production (FAO, 2022). This escalating salinity crisis has profound implications for food security, with a staggering loss of approximately 124 trillion kilocalories annually, enough to sustain over 170 million people each day. In India alone, 6.74 million hectares of land are affected by salinity, and this area is projected to grow at a rate of nearly 10% each year, potentially rendering 50% of arable land salt-affected by 2050 (Shrivastava & Kumar, 2015). The prevalence of saline and sodic soils covers substantial portions of India, impacting 12 states and one Union Territory for saline soils and 11 states for sodic soils. Among them, Tamil Nadu grapples with 0.0427 million hectares of salt-affected land, with Ramanathapuram (16,200 ha) and Trichy (11,165 ha) being the most affected regions in the state (Thamodharan *et al.*, 2023).

Rice is a crucial staple crop, providing sustenance to billions worldwide, with India being the world's second-largest producer, contributing 20% of the global output (Mahajan *et al.*, 2017). However, rice is the most salt-sensitive cereal crop and is significantly affected by salinity stress, with cultivated varieties typically having a threshold of 3 dS/m. Soil is deemed saline when its electrical conductivity of saturation extract exceeds 4 dS/m (IRRI Rice Knowledge Bank). Salt stress disrupts various morphological, physiological, and biochemical processes in plants, leading to membrane instability, imbalanced ion homeostasis, reduced turgor, and increased accumulation of reactive oxygen species (ROS) (Munns & Tester, 2008). Salinity also impacts on respiration, photosynthesis, impairs biological N_2 fixation and soil nitrogen mineralization, and results in reduced germination rates, stunted plant growth, poor root development, and increased spikelet sterility. The quality of rice is also impacted as the imbalance of ions in saline soil affects the nutrient content (Zhang *et al.*, 2012). Hence, addressing soil salinity's impact on rice necessitates the implementation of sustainable land management practices and the development of salt-tolerant crop varieties. To combat salt stress, plants have evolved survival strategies, including osmoprotectant biosynthesis, activation of antioxidant enzymes like Superoxide Dismutase (SOD), Catalase (CAT), and Peroxidase (POX), synthesis of antioxidant compounds, ion homeostasis, transport and uptake, as well as polyamine and nitric oxide synthesis, and hormone modulation (Saberi Riseh *et al.*, 2021). Elevated levels of Proline, SOD, CAT, and POX in salt-affected cells have been associated with improved salinity resistance in various crop plants, including rice (El-Beltagi *et al.*, 2020; Gupta & Huang, 2014; Nefissi Ouertani *et al.*, 2022; Shrivastava & Kumar, 2015). Additionally, *in vitro* selection through tissue culture techniques has proven valuable in enhancing biotic and abiotic stress tolerance in rice, which is susceptible to drought and salt stress (Sahu *et al.*, 2023).

The objective of this study is to investigate the morpho-physiological and biochemical responses of multiple abiotic stress tolerant genotypes of rice *(Oryza sativa L.)* under salinity stress conditions. By analysing data on callus induction frequency, regeneration percentage, and the activity of antioxidant enzymes and proline levels, we aim to gain deeper insights into the mechanisms governing salinity stress tolerance in rice.

Material and Methods

Plant material

To investigate the impact of salt stress on various rice genotypes, this experiment was conducted using six *indica* rice varieties: TRY 4, TRY 3, CR 1009 Sub 1, CO 53, CO 55, and ADT 37. These genotypes were specifically chosen to represent a range of abiotic stress tolerances. TRY 4 and TRY 3 are salt-tolerant, CR 1009 Sub 1 is submergence-tolerant, CO 53 is drought-tolerant, ADT 37 is salt-susceptible, and CO 55 is moderately tolerant to salt. The seeds for all genotypes were obtained from the Paddy Breeding Station at Tamil Nadu Agricultural University (TNAU) in Coimbatore, Tamil Nadu. The experiment was carried out in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding at TNAU in Coimbatore, Tamil Nadu.

Explant Surface sterilization

The mature rice seeds were manually dehusked. For surface sterilization, the seeds were first immersed in 70% ethanol (v/v) for 2 minutes, followed by 0.1% mercuric chloride treatment for 5 minutes, and finally 2% sodium hypochlorite containing a drop of Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. The seeds were then rinsed several times with double distilled sterile water and blot dried with filter paper.

Callus induction experiment

Seeds from six rice varieties were cultured in modified MS media (Murashige & Skoog, 1962)

supplemented with optimal combinations of growth regulators. The callus induction media was prepared by adding optimized doses of plant growth regulators (ADT 37: 2,4-D (2.0 mg/L) + Kn (0.25 mg/L), CO 53: 2,4-D (2.5 mg/L) + Kinetin (0.5 mg/L), CO 55: 2,4-D (2.0 mg/L) + Kinetin (1.0 mg/L), CR 1009 Sub 1: 2,4-D (2.0 mg/L) + Kinetin (0.5 mg/L), TRY 3: 2,4-D (2.5 mg/L) + Kinetin (0.5 mg/L), TRY 4: 2,4-D (2.0 mg/L) + Kinetin (0.5 mg/L)). The callus induction medium was then supplemented with different concentrations of NaCl (0, 20, 40, 60, 80, 100 mM) to impose the salinity. The experiment was conducted in CRD with three replicates. Following surface sterilization, seeds were inoculated into the various treatments, including a control, with three seeds per test tube. The test tubes were then incubated at 23 ± 2ºC in the dark. Callus growth was observed after 10 days of inoculation, and calli developed from the scutellum region were excised and sub-cultured once every two weeks onto fresh media containing the same basal salts and plant growth regulator combinations to promote callus growth and proliferation.

Morpho, Physio and biochemical analysis

Callus Induction Frequency (%)

Number of seeds that showed callus induction were noted after 15 days of inoculation and the callus induction frequency (CIF) was calculated as:

$$\mathbf{CIF} = \frac{\text{Number of seeds showing callus induction}}{\text{Total number of seeds inoculated}} \text{ x } 100$$

Relative growth rate

Initial fresh weight (FW) of the callus tissue was measured after 2 weeks of culture initiation. Final fresh weight of the callus was measured after 8 weeks of culture. The relative growth rate (RGR) was calculated using the formula,

$$\mathbf{RGR} = \frac{\text{Final weight (FW)} - \text{Initial weight (FW)}}{8} \text{ x } 100$$

Regeneration frequency

Embryogenic calli with creamy white, friable nature were selected from each treatment, and were transferred to regeneration media containing optimal growth regulator concentrations and previous NaCl concentrations. The genotypes and their respective NAA, BAP, and KN values in mg/L are as follows: ADT 37 with 0.5 NAA mg/L + 1 BAP mg/L, CO 53 with 0.5 NAA mg/L + 0.5 BAP mg/L, CO 55 with 0.5 NAA mg/L + 0.5 BAP mg/L, CR 1009 Sub 1 with 0.5 NAA mg/L + 1 BAP mg/L, TRY 3 with 0.5 NAA mg/L + 1 BAP mg/L, and TRY 4 with 0.5 NAA mg/L + 0.5 BAP mg/L. Callus was incubated at 25 ± 2°C with a 16/8 h light/dark cycle. The regeneration frequency (RF) was calculated using the formula:

$$\mathbf{RF} = \frac{\text{Number of calli with green spots}}{\text{Total number of calli inoculated}} \text{ x } 100$$

Biochemical assay

Proline content in callus

In this study, we examined the proline content in fresh callus samples from six different rice varieties using a modified method based on (Ábrahám *et al.*, 2010). First, 0.5 g of fresh callus samples from each rice variety was collected and separately homogenized in 3% aqueous sulfosalicylic acid. The homogenates were then subjected to centrifugation at 10,000 rpm for 30 minutes to obtain the supernatant. For proline estimation, 2 ml of the supernatant was mixed with equal volumes of glacial acetic acid and acidic ninhydrin solution. To prepare the acidic ninhydrin solution, 1.25 g of ninhydrin was warmed in 30 ml of glacial acetic acid and 20 ml of 6 M ortho phosphoric acid, and the mixture was agitated until the ninhydrin dissolved completely. The reaction mixture containing the supernatant and acidic ninhydrin solution was placed in a water bath and incubated at 100°C for 1 hour. After incubation, the tubes were immediately cooled in an ice bath to terminate the reaction. To quantify the total proline content, the chromophore-containing toluene layer was separated by adding 4 ml of toluene to the reaction mixture and vigorously mixing. The absorbance of the resulting toluene layer was measured at 520 nm using a microplate reader (Spectramax® i3X), with toluene serving as a blank. To establish a standard curve, l-Proline standards ranging from 20 to 100 $\mu g ml^{-1}$ were prepared. The absorbance values obtained from the standards were used to calculate the total proline content in the rice callus samples. The results were expressed as milligrams of proline per gram of fresh weight (mg. g^{-1} FW).

Soluble Protein content in Callus

For each genotype, 500 mg of callus per genotype was collected and promptly homogenized into a fine powder using liquid nitrogen. The homogenization was carried out in 5 ml of ice-cold buffer, comprising 50 mM potassium phosphate buffer at pH 7.0, 1 mM EDTA, and 1% (w/v) PVP (polyvinyl pyrrolidone). After homogenization, the leaf extracts were centrifuged at 10,000 g for 30 min at 4°C, effectively separating soluble components from the cell debris. The resultant supernatant was stored at 0–4 °C for subsequent enzyme assays. The Bradford method, utilizing

bovine serum albumin as the standard (Bradford, 1976), was employed to accurately determine the soluble protein content.

Superoxide Dismutase (SOD) activity

The activity of SOD was determined using the method described by Beauchamp and Fridovich (1971), which measures its ability to inhibit the photochemical reduction of Nitro blue Tetrazolium (NBT). To obtain the enzyme extract, 1 g of fresh callus sample was homogenized in 1 ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4 °C. After centrifugation, the supernatant was collected as the enzyme source. The assay mixture contained 3 ml of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, and 100 µl of enzyme source. The reaction mixture was incubated for 15–30 min at 28 ± 2 °C and exposed to a 40 W fluorescent lamp for 20 min at 25 °C. The increase in absorbance at 560 nm, resulting from NBT photoreduction, was measured against non-illuminated tubes. SOD activity was expressed as $\mu mol^{-1}mg^{-1}$ protein.

Catalase (CAT) activity

CAT activity was estimated using the UV-spectrophotometric method as described by (Chaparro-Giraldo *et al.*, 2000). A 3.0 ml assay mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 2.5 mM freshly prepared H^2O^2 was used, and 100 µl of enzyme extract was added. CAT activity was measured by monitoring the degradation of H^2O^2 at 240 nm over 1 min, using a microplate reader (Spectramax® i3X), with a plant extract-free blank as the reference. CAT activity was calculated using the extinction coefficient (ε240nm = 40/mM/cm) for H^2O^2 and expressed in units g^{-1} FW min^{-1}.

Peroxidase (POX) Assay

POX activity was determined using a reaction mixture containing 100 µl of the homogenate, 2.9 ml of 0.01 M sodium phosphate buffer (pH 6.0) with 0.25% v/v guaiacol, and 0.1 M H^2O^2. The absorbance of the colored product was measured at 470 nm against a heat-boiled enzyme extract used as a blank. POX activity was represented as $\mu mol^{-1}mg^{-1}$ protein (Hammerschmidt *et al.*, 1982).

Statistical Analysis:

All experimental data were subjected to the analysis of variance (ANOVA) to evaluate significant differences among the genotypes and treatments. To determine specific variations between groups, Duncan's Multiple Range Test (DMRT) was applied at a significance level of $p < 0.05$. Principal Component Analysis (PCA) was employed to assess the cumulative variance of observed parameters under both control and stress treatments. Bar plots and line plots offering a visual representation of the pattern of variation among the different genotypes were also created. All the analyses were done using R statistical package and shiny packages like PB – Perfect (Allan, 2023) for data analysis and *ggplot2* (Wickham *et al.*, 2016) for data visualization.

Results

Physiological Responses of callus to Salinity Stress

The study investigated the effects of salinity stress on six rice genotypes using callus induction frequency (CIF), relative growth rate (RGR), and regeneration percentage (RP) as evaluation parameters. The results showed that the genotypes had varying degrees of tolerance and sensitivity to salt stress. Table 1 shows the callus induction frequency, relative growth rate, and regeneration percentage of the six rice genotypes under different salinity treatments (Figure 1).

Callus Induction Frequency

Callus induction frequency (CIF) refers to the ability of rice seeds to produce callus tissue under varying salinity conditions. As depicted in Table 1, the callus induction frequency generally decreased with increasing salinity levels for all genotypes. Among the genotypes, TRY 4 exhibited the highest callus induction frequency, with 84% at the control level, which declined to 69.2% at 100 mM NaCl. Similarly, TRY 3 (82% in control level, 79.3% in 20 mM, 75.2% in 40 mM, 70.5% in 60 mM, 68.3% in 80 mM and 65.7% in 100 mM) and CO 53 (81.1% in control level,75.2% in 20 mM NaCl, 63.2 %in 40 mM NaCl, 55.2 % in 60 mM NaCl, 47.5 % in 80 mM NaCl and 43.2 % at 100 mM NaCl) displayed relatively high callus induction frequencies, even at elevated salinity concentrations, indicating their resilience to salt stress. On the other hand, ADT 37 (80% in control level, 69.1% in 20 mM NaCl ,53.5 % in 40 mM NaCl, 44.5 % in 60 mM NaCl, 43.2 % in 80 mM NaCl and 31.5 % at 100 mM NaCl) and CR 1009 Sub1 (74.3% in control level, 65.3% in 20 mM NaCl, 47.5 % in 40 mM NaCl ,41.2 % in 60 mM NaCl, 38.5 % in 80 mM NaCl and 27.5 % at 100 mM NaCl) known as salt-susceptible and submergence-tolerant genotypes respectively showed reduced callus induction frequency as salinity levels increased indicating their sensitivity to salt stress.

The observed decline in callus induction frequency (CIF) with increasing salinity levels aligns with previous studies (Ahmad *et al.*, 2008; Summart *et al.*, 2010). Higher callus induction frequency at control levels and moderate salinity levels in some genotypes, such as TRY 4, suggests their initial resilience to salinity stress. On the other hand, the reduced callus induction frequency in salt-susceptible genotypes ADT 37 and CR 1009 Sub 1 indicates their sensitivity to salt stress. This decrease in CIF with salinity is likely due to the negative effects of high salt concentrations on the mineral nutrient uptake and cell division processes, as reported in previous research (Ahmad *et al.*, 2008; Summart *et al.*, 2010).

Relative Growth Rate (RGR)

The relative growth rate (RGR) measures the growth performance and the ability to maintain cell division in callus tissue under different salinity conditions. As observed in Table 1, the RGR consistently decreased with increasing salinity levels for all genotypes. Among the cultivars, CO 53 exhibited the highest initial RGR at the control level (2.3), which progressively declined to 0.6 at 100 mM NaCl, indicating its relatively moderate tolerance to salinity stress compared to the susceptible genotypes. However, it's important to note that both TRY 3 and TRY 4 showed interesting responses. While the relative growth rate (RGR) of TRY 3 and TRY 4 decreased with increasing salinity, they demonstrated a relatively higher RGR at 100 mM NaCl (1.22% for TRY 3 and 0.90% for TRY 4) compared to other cultivars possessing other abiotic stress tolerances. This suggests that these genotypes may possess specific mechanisms or genetic traits that allow them to better cope with extreme salinity conditions, enabling them to maintain a certain level of growth and osmotic potential even under high salt stress. On the other hand, ADT 37, known as a salt-susceptible genotype, displayed the lowest RGR at all salinity concentrations (2.0 at control level and 0.09 at 100 mM NaCl). This consistent decline in RGR indicates that ADT 37 is highly sensitive to salinity stress, lacks genetic mechanisms and struggles to maintain its growth under elevated salt conditions.

The consistent decline in relative growth rate (RGR) with increasing salinity levels across all genotypes is in line with the findings of (Alhasnawi *et al.*, 2017) and (Sarker & Oba, 2018) in rice and other plant species. The higher RGR at 100 mM NaCl in TRY 3 and TRY 4 suggests that these genotypes possess specific mechanisms that allow them to cope with extreme salinity conditions and maintain growth and osmotic potential. This observation is supported by the findings of Alhasnawi *et al.* (2017), who reported the biochemical responses of rice callus treated with salt stress.

Regeneration frequency:

The regeneration percentage represents the ability of calli to form green spots and undergo regeneration into plantlets under different salinity treatments. The regeneration frequency of the rice genotypes exhibited distinct responses to varying salinity levels (Table 1). Under the control conditions, CR 1009 Sub 1 and ADT 37 displayed relatively higher regenerative abilities (27.2% and 22.7%, respectively. Conversely, TRY 4 exhibited the lowest regeneration frequency (17.4%) under the control condition. As salinity levels increased, regeneration frequencies in most genotypes showed a declining trend. At 20 mM salinity, all genotypes experienced a significant decrease in regeneration frequency compared to the control. This decline in regeneration frequency intensified as salinity levels further increased. Among the tested genotypes, TRY 3 and TRY 4 demonstrated relatively higher regenerative abilities under moderate salt stress (40 mM and 60 mM). Notably, TRY 3 displayed a regeneration frequency of 17.2% at 20 mM salinity, which was significantly higher than the other genotypes under the same conditions, even though it shows lower regeneration that other genotypes in control conditions. On the other hand, CO 55, ADT 37 and CR 1009 Sub 1 were found to be more salt-susceptible, as evidenced by their significant reduction in regeneration frequencies at 20 mM salinity (7.5%, 8.2% and 17.5%, respectively). At higher concentration of NaCl these genotypes failed to produce green spots and callus turned brown.

Regarding the decline in regeneration frequency with increasing salinity levels, the results are consistent with (Ramesh *et al.*, 2004). The higher regeneration ability of TRY 3 and TRY 4 under moderate salt stress (40 mM and 60 mM) suggests their higher tolerance to this stress level, as also observed by (Alhasnawi *et al.*, 2017) in saline tolerant rice cultivars. The reduced regeneration frequency in salt-susceptible genotypes, such as CO 55, ADT 37, and CR 1009 Sub 1, indicates their lower capacity to undergo regeneration under salinity stress conditions.

Biochemical Responses to Salinity Stress

The study evaluated the biochemical responses of rice callus tissues under varying salinity treatments. Proline content, catalase activity, superoxide dismutase (SOD) activity, and peroxidase (POX) activity were measured in response to NaCl concentrations ranging from 20 mM to 100 mM, along with a control group. The results showed distinct biochemical variations among the genotypes, indicating different mechanisms of salinity tolerance. PCA (Principal Component Analysis) (Figure 3) further confirmed the separation of treatments based on their biochemical responses to NaCl, with PC1 being the most influential in distinguishing control and salt-treated groups. The first two principal components (PC1 and PC2) explained 96.28% of the total variance, and they clearly separated the treatments into different groups (Table 3). PC1, which had the highest contribution of all the traits, separated the control treatments towards the left, while the treatments with increasing salinity were aggregated to the right. The control treatments were characterized by lower levels of proline, CAT, SOD, and POX, while the treatments with increasing salinity were characterized by higher levels of these biochemical markers. The proline content, catalase activity, superoxide dismutase (SOD) activity, and peroxidase activity in the callus tissue of different genotypes were measured under various salinity treatments (Table 2) (Figure 2).

Proline

Proline content was assessed in callus tissues subjected to different NaCl concentrations, ranging from 20 mM to 100 mM, alongside a control group without NaCl exposure. The results are presented in Table 2. Among the tested genotypes, TRY 4 maintained a increase level of proline in all treatments, exhibiting significant respone as the NaCl levels rose (Control: 7.93 ± 0.33 µg. g^{-1} FW; 100 mM: 18.75 ± 0.22 µg. g^{-1} FW). Likewise, TRY 3 also displayed notable proline accumulation with increasing salt concentrations, reaching a peak at 17.61 ± 0.23 µg. g^{-1} FW under 80 mM NaCl.

Conversely, CO 53 and CO 55 exhibited relatively moderate proline content, with fluctuations under different salt treatments. CO 53 showed a steady rise in proline accumulation with increasing salt concentrations, reaching a peak at 17.44 ± 0.15 µg. g^{-1} FW under 60 mM NaCl. Similarly, CO 55 displayed a gradual increase in proline content with higher salt concentrations, peaking at 11.94 ± 0.36 µg. g^{-1} FW under 80 mM NaCl.

Notably, CR 1009 Sub 1 and ADT 37 demonstrated the lowest proline content among all genotypes tested. CR 1009 Sub 1 exhibited a relatively stable proline content throughout the salt treatments, with a slight increase at 100 mM NaCl (9.46 ± 0.01 µg. g^{-1} FW). ADT 37 displayed a similar trend, with proline levels ranging from 4.24 ± 0.17 µg. g^{-1} FW (control) to 6.71 ± 0.09 µg. g^{-1} FW under 60 mM NaCl and a gradual decrease to 4.68 ± 0.06 at 100 mM.

Proline is a well-known osmolyte and plays a crucial role in osmotic adjustment under stressful conditions, including salinity. The observed increase in proline content with higher NaCl concentrations is consistent with previous studies (Alhasnawi *et al.*, 2017; Summart *et al.*, 2010). TRY 4 and TRY 3 exhibited the highest proline accumulation at all salt treatments, indicating their strong ability to synthesize and accumulate proline as a response to salinity stress. This enhanced proline content may contribute to the osmotic adjustment and protection of cellular structures in these genotypes, enabling them to better cope with high salt concentrations. Conversely, ADT 37 and CR 1009 Sub 1 displayed lower proline content, suggesting their limited capacity to synthesize proline and possibly contributing to their higher sensitivity to salinity stress (Alhasnawi *et al.*, 2017).

Catalase

Among the tested genotypes, TRY 4 and TRY 3 exhibited the highest Catalase (CAT) activity under all salt treatments, with significant increases in activity as NaCl concentrations escalated (TRY 4, Control: 12.96 ± 0.02 U. g^{-1} FW min^{-1}; 100 mM: 21.25 ± 0.83 U. g^{-1} FW min^{-1}; TRY 3, Control: 12.04 ± 0.44 U. g^{-1} FW min^{-1}; 100 mM: 19.82 ± 0.38 U. g^{-1} FW min^{-1}).

Similarly, CO 53 and CO 55 displayed significant increases in Catalase activity with higher salt concentrations. CO 53 exhibited a steady rise in CAT activity, reaching its peak at 21.61 ± 0.98 U. g^{-1} FW min^{-1} under 100 mM NaCl. Likewise, CO 55 demonstrated a gradual increase in CAT activity, peaking at 18.21 ± 0.42 U. g^{-1} FW min^{-1} under 100 mM NaCl.

Conversely, ADT 37 and CR 1009 Sub 1 displayed relatively lower CAT activity throughout the salt treatments. ADT 37 showed a mild increase in CAT activity with increasing salt concentrations, reaching 17.22 ± 0.75 U. g^{-1} FW min^{-1} under 100 mM NaCl. CR 1009 Sub 1 exhibited a similar trend, with CAT activity ranging from 10.5 ± 0.16 U. g^{-1} FW min^{-1} (control) to 17.07 ± 0.26 U. g^{-1} FW min^{-1} under 100 mM NaCl.

Catalase (CAT) is a key enzyme involved in scavenging reactive oxygen species (ROS) generated under stress conditions. The observed increase in CAT activity with higher salinity levels suggested its role in combating oxidative stress induced by salt (Sarker & Oba, 2018). TRY 4 and TRY 3 exhibited the highest CAT activity at all salt concentrations, indicating their efficient ROS scavenging capacity and superior defence against oxidative damage. Similarly, CO 53 and CO 55 displayed increased CAT activity, implying their ability to cope with salinity-induced ROS. In contrast, ADT 37 and CR 1009 Sub 1 showed lower CAT activity, suggesting their reduced ability to detoxify ROS, which might have contributed to their higher vulnerability to oxidative damage under salinity stress.

Superoxide Dismutase

Among the tested genotypes, TRY 4 displayed the highest Superoxide Dismutase (SOD) activity under all salt treatments, with a significant increase in activity as NaCl concentrations escalated (Control: 72.86 ± 3.62 $\mu mol^{-1}mg^{-1}$ protein; 100 mM: 120 ± 0.7 $\mu mol^{-1}mg^{-1}$ protein). TRY 3 also exhibited notable SOD activity, with a gradual increase as salt concentrations increased (Control: 78.57 ± 3.28 $\mu mol^{-1}mg^{-1}$ protein; 100 mM: 105.71 ± 1.12 $\mu mol^{-1}mg^{-1}$ protein).

Similarly, CR 1009 Sub 1, CO 53 and CO 55 demonstrated significant increases in SOD activity with higher salt concentrations. CO 53 exhibited a steady rise in SOD activity, reaching its peak at 103.57 ± 4.19 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl. Likewise, CO 55 displayed a gradual increase in SOD activity, peaking at 96.43 ± 2.31 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl. CR 1009 Sub 1 displayed a similar trend, with SOD activity ranging from 67.14 ± 2.09 $\mu mol^{-1}mg^{-1}$ protein (control) to 97.43 ± 0.04 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl.

Conversely, ADT 37 exhibited relatively lower SOD activity throughout the salt treatments. ADT 37 showed a mild increase in SOD activity with increasing salt concentrations, reaching 101.14 ± 0.35 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl.

Superoxide dismutase (SOD) is the crucial enzyme involved in ROS detoxification. The significant increase in SOD activity with higher salinity concentrations aligns with previous findings (Ramesh *et al.*, 2004; Sarker & Oba, 2018). TRY 4 displayed the highest SOD activity at all salt treatments, indicating its efficient ROS scavenging ability and enhanced protection against oxidative stress. TRY 3 also exhibited notable SOD activity, contributing to its salinity tolerance. CO 53 and CO 55 demonstrated increased SOD activity, implying their ability to cope with ROS generated under salinity stress. Surprisingly CR 1009 Sub 1 showed moderate SOD activity under salt stress. Conversely, ADT 37 exhibited lower SOD activity, suggesting their reduced ROS scavenging capacity and possibly contributing to their higher susceptibility to oxidative damage under salinity stress.

Peroxidase

Among the tested genotypes, TRY 3 exhibited the highest Peroxidase (POX) activity under all salt treatments, with a gradual increase in activity as NaCl concentrations increased (Control: 6.69 ± 0.13 $\mu mol^{-1}mg^{-1}$ protein; 100 mM: 10.67 ± 0.35 $\mu mol^{-1}mg^{-1}$ protein). TRY 4 also displayed significant POX activity, with a similar increasing trend (Control: 6.65 ± 0.07 $\mu mol^{-1}mg^{-1}$ protein; 100 mM: 10.25 ± 0.42 $\mu mol^{-1}mg^{-1}$ protein).

Similarly, CO 53 and CO 55 demonstrated notable increases in POX activity with higher salt concentrations. CO 53 exhibited a steady rise in POX activity, reaching its peak at 11.87 ± 0.23^{a} $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl. Likewise, CO 55 displayed a gradual increase in POX activity, peaking at 10.00 ± 0.03 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl.

Conversely, ADT 37 and CR 1009 Sub 1 exhibited relatively lower POX activity throughout the salt treatments. ADT 37 showed a mild increase in POX activity with increasing salt concentrations, reaching 9.75 ± 0.44 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl. CR 1009 Sub 1 demonstrated a similar trend, with POX activity ranging from 6.53 ± 0.08 $\mu mol^{-1}mg^{-1}$ protein (control) to 9.88 ± 0.1 $\mu mol^{-1}mg^{-1}$ protein under 100 mM NaCl.

Peroxidase (POX) also plays a crucial role in ROS detoxification. The significant increase in POX activity with higher salinity concentrations aligns with previous findings (Ramesh *et al.*, 2004; Sarker & Oba, 2018), TRY 3 exhibited the highest POX activity at all salt treatments, indicating its efficient ROS detoxification capacity, which could have contributed to its higher tolerance to salinity-induced oxidative stress. TRY 4 also displayed significant POX activity, supporting its salinity tolerance. CO 53 and CO 55 demonstrated increased POX activity, suggesting their ability to cope with oxidative stress under high salinity conditions. ADT 37 and CR 1009 Sub 1 exhibited lower POX activity, likely contributing to their higher vulnerability to oxidative damage under salinity stress.

The integrated analysis of biochemical and morpho-physiological studies offers a

comprehensive understanding of the adaptive mechanisms employed by the genotypes to cope with salinity stress, shedding light on potential strategies for developing salt-tolerant rice varieties. TRY 4 stood out as a promising genotype, exhibiting higher callus induction frequency and relative growth rate at 100 mM NaCl, indicating its resilience to extreme salt stress conditions. Similarly, TRY 3 displayed a relatively higher regenerative ability at moderate salt stress, indicating its better tolerance to this specific stress level. TRY 4 and TRY 3 exhibited the highest levels of proline accumulation, catalase activity, SOD activity, and POX activity under salinity stress, indicating their superior ability to adapt and counteract the detrimental effects of oxidative stress induced by high salt concentrations. Hence, these results suggest that TRY 3 and TRY 4 may possess specific genetic traits or mechanisms that allow them to better cope with salinity stress, leading to their enhanced morpho-physiological performance under challenging conditions. In contrast, ADT 37 and CR 1009 Sub 1 demonstrated a reduced morpho-physiological performance and lower levels of these biochemical responses, suggesting their reduced capacity to manage ROS and osmotic stress under salinity conditions.

In conclusion, the integrated analysis of biochemical and morpho-physiological responses in different rice genotypes provides a comprehensive understanding of salinity stress tolerance. The knowledge gained from these studies contributes to the development of novel strategies for breeding salt-tolerant rice varieties, thus addressing the challenges of salinity stress and ensuring food security in salt-affected regions.

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Tables

Table 1. Physiological responses of callus to salinity stress in under different rice genotypes.

S.No	Genotypes	Callus induction frequency (%)					
		Control	**20 mM**	**40 mM**	**60 mM**	**80 mM**	**100 mM**
1	**ADT 37**	80 ± 3.53^{c}	69.1 ± 2.46^{bc}	53.5 ± 0.58^{c}	44.5 ± 0.1^{cd}	43.2 ± 0.16^{b}	31.5 ± 1.54^{c}
2	**CO 53**	81.1 ± 3.36^{ab}	75.2 ± 2.34^{ab}	63.2 ± 1.21^{b}	55.2 ± 2.87^{bd}	47.5 ± 0.03^{b}	43.2 ± 0.03^{b}
3	**CO 55**	72 ± 0.09^{cb}	62.3 ± 1.1^{cb}	55.2 ± 2.1^{c}	47.5 ± 1.18^{cd}	45.3 ± 0.8^{b}	41.5 ± 1.14^{b}
4	**CR 1009 Sub 1**	74.3 ± 1.34^{bc}	65.3 ± 1.71^{cb}	47.5 ± 1.95^{d}	41.2 ± 0.45^{dd}	38.5 ± 1.35^{c}	27.5 ± 0.58^{c}
5	**TRY 3**	82 ± 2.79^{ab}	79.3 ± 2.06^{ab}	75.2 ± 1.47^{a}	70.5 ± 2.08^{ad}	68.3 ± 3.14^{a}	65.7 ± 1.49^{a}
6	**TRY 4**	84 ± 3.33^{ab}	82.4 ± 3.69^{ab}	77.5 ± 2.98^{a}	73.5 ± 0.17^{ad}	71.3 ± 0.88^{a}	69.2 ± 2.09^{a}
		Relative Growth Rate					
1	**ADT 37**	2.0 ± 0.03^{d}	1.3 ± 0.03^{e}	0.5 ± 0.02^{d}	0.2 ± 0^{d}	0.2 ± 0.01^{f}	0.09 ± 0^{d}
2	**CO 53**	2.3 ± 0.11^{a}	2.1 ± 0.03^{a}	1.8 ± 0.02^{a}	1.3 ± 0.03^{b}	0.95 ± 0.03^{c}	0.6 ± 0.03^{b}
3	**CO 55**	1.9 ± 0.02^{c}	1.5 ± 0.06^{cd}	1.1 ± 0^{c}	0.96 ± 0.03^{c}	0.75 ± 0.01^{d}	0.65 ± 0.02^{b}
4	**CR 1009 Sub 1**	2.2 ± 0.1^{ab}	1.4 ± 0.05^{de}	1.2 ± 0.06^{c}	0.95 ± 0.03^{c}	0.6 ± 0.02^{e}	0.42 ± 0.02^{c}
5	**TRY 3**	2 ± 0.07^{bc}	1.92 ± 0.03^{be}	1.75 ± 0.09^{ab}	1.6 ± 0.02^{a}	1.42 ± 0.07^{b}	1.22 ± 0.05^{a}
6	**TRY 4**	1.8 ± 0^{cc}	1.65 ± 0.08^{ce}	1.6 ± 0.05^{bb}	1.61 ± 0.04^{a}	1.55 ± 0.01^{a}	0.90 ± 0.04^{a}
		Regeneration frequency (%)					
1	**ADT 37**	22.7 ± 0.28^{c}	8.2 ± 0.41^{c}	-	-	-	-
2	**CO 53**	23.9 ± 0.47^{b}	15.2 ± 0.67^{b}	8.7 ± 0.13^{c}	1.2 ± 0.04^{c}	-	-
3	**CO 55**	18.2 ± 0.8^{cd}	7.5 ± 0.15^{c}	-	-	-	-
4	**CR 1009 Sub 1**	27.2 ± 0.13^{ad}	17.5 ± 0.67^{a}	9.5 ± 0.34^{bc}	-	-	-
5	**TRY 3**	22.3 ± 1.06^{bd}	17.2 ± 0.1^{a}	10.5 ± 0.31^{ac}	5.2 ± 0.27^{b}	5.3 ± 0.15^{b}	4.2 ± 0.02^{a}
6	**TRY 4**	17.4 ± 0.44^{dd}	15.2 ± 0.26^{b}	9.7 ± 0.46^{ab}	9 ± 0.01^{a}	8.2 ± 0.13^{a}	3.5 ± 0.04^{b}

Table 2. Biochemical responses of rice callus under different salinity treatments.

S.No	Genotypes	Proline (µg. g^{-1}FW)					
		Control	20 mM	40 mM	60 mM	80 mM	100 mM
1	ADT 37	4.24 ± 0.17^{d}	5.95 ± 0.12^{e}	6.68 ± 0.31^{d}	6.71 ± 0.09^{d}	5.68 ± 0.1^{e}	4.68 ± 0.06^{e}
2	CO 53	7.85 ± 0.25^{b}	12.34 ± 0.19^{a}	13.55 ± 0.58^{b}	17.44 ± 0.15^{a}	14.23 ± 0.44^{b}	12.67 ± 0.5^{c}
3	CO 55	5.98 ± 0.18^{c}	7.83 ± 0.37^{c}	9.93 ± 0.23^{c}	10.88 ± 0.45^{c}	11.94 ± 0.36^{c}	10.12 ± 0.14^{d}
4	CR 1009 Sub 1	4.54 ± 0.07^{d}	6.77 ± 0.33^{d}	7.95 ± 0.26^{d}	7.22 ± 0.23^{d}	8.2 ± 0.37^{d}	9.46 ± 0.01^{d}
5	TRY 3	9.84 ± 0.36^{a}	9.92 ± 0.21^{b}	10.29 ± 0.53^{c}	14.55 ± 0.15^{b}	17.61 ± 0.23^{a}	16.72 ± 0.48^{b}
6	TRY 4	7.93 ± 0.33^{b}	12.46 ± 0.14^{a}	15.67 ± 0.61^{a}	14.97 ± 0.3^{b}	17.42 ± 0.19^{a}	18.75 ± 0.22^{a}
		Catalase (U. g^{-1} FW min^{-1})					
1	ADT 37	10.18 ± 0.2^{d}	12.32 ± 0.54^{ab}	13.92 ± 0.17^{bc}	13.86 ± 0.47^{c}	16.34 ± 0.26^{b}	17.22 ± 0.75^{c}
2	CO 53	11.18 ± 0.27^{c}	13.54 ± 0.58^{ab}	14.36 ± 0.12^{abc}	17.86 ± 0.52^{a}	18.93 ± 0.41^{a}	21.61 ± 0.98^{a}
3	CO 55	10.04 ± 0.36^{d}	13.14 ± 0.46^{ab}	13.5 ± 0.17^{cdc}	16.25 ± 0.03^{b}	17.07 ± 0.65^{b}	18.21 ± 0.42^{bc}
4	CR 1009 Sub 1	10.5 ± 0.16^{cd}	11.18 ± 0.25^{bb}	12.61 ± 0.21^{ddc}	13.18 ± 0.64^{c}	15.36 ± 0.21^{b}	17.07 ± 0.26^{cc}
5	TRY 3	12.04 ± 0.44^{bd}	12.89 ± 0.37^{ab}	14.75 ± 0.33^{abc}	17.5 ± 0.16^{ab}	19.04 ± 0.79^{a}	19.82 ± 0.38^{ab}
6	TRY 4	12.96 ± 0.02^{ad}	13.14 ± 0.18^{ab}	15.39 ± 0.64^{abc}	18.21 ± 0.32^{ab}	20.39 ± 0.84^{a}	21.25 ± 0.83^{ab}
		Superoxide dismutase (µmol^{-1}mg^{-1} protein)					
1	ADT 37	70 ± 1.9^{bc}	71.43 ± 2.01^{d}	72.86 ± 2.08^{c}	87.71 ± 3.68^{ab}	92.43 ± 1.06^{bc}	101.14 ± 0.35^{bc}
2	CO 53	82.5 ± 0.13^{ac}	87.86 ± 0.52^{a}	94.29 ± 0.25^{a}	96.43 ± 4.98^{ab}	95.57 ± 1.47^{bc}	103.57 ± 4.19^{bc}
3	CO 55	72.86 ± 3.02^{bc}	80 ± 2.76^{bc}	81.43 ± 3.71^{bc}	83.57 ± 1.76^{bb}	90 ± 3.08^{cd}	96.43 ± 2.31^{cd}
4	CR 1009 Sub 1	67.14 ± 2.09^{cc}	73.57 ± 3.21^{cd}	75.71 ± 3.82^{cc}	92.86 ± 0.8^{bb}	91.1 ± 3.91^{dd}	97.43 ± 0.04^{dd}
5	TRY 3	78.57 ± 3.28^{ab}	87.14 ± 1.01^{ad}	89.29 ± 3.83^{ab}	94.29 ± 2.79^{ab}	98.57 ± 0.26^{bd}	105.71 ± 1.12^{bd}
6	TRY 4	72.86 ± 3.62^{bc}	83.57 ± 2.12^{ab}	91.43 ± 3.67^{ab}	96.43 ± 2.55^{ab}	106.43 ± 0.51^{ad}	120 ± 0.7^{ad}
		Peroxidase (µmol^{-1}mg^{-1} protein 1)					
1	ADT 37	6.5 ± 0.29^{a}	6.87 ± 0.08^{a}	8.1 ± 0.3^{a}	8 ± 0.1^{b}	8.75 ± 0.14^{a}	9.75 ± 0.44^{b}
2	CO 53	6.62 ± 0.31^{a}	7.25 ± 0.14^{a}	7.5 ± 0.27^{a}	9 ± 0.4^{a}	9.87 ± 0.4^{a}	11.87 ± 0.23^{a}
3	CO 55	6.55 ± 0.25^{a}	6.88 ± 0.13^{a}	7.75 ± 0.01^{a}	8.75 ± 0.16^{a}	9.75 ± 0.49^{a}	10 ± 0.03^{b}
4	CR 1009 Sub 1	6.53 ± 0.08^{a}	6.82 ± 0.05^{a}	7.38 ± 0.16^{a}	8 ± 0.01^{b}	9 ± 0.32^{a}	9.88 ± 0.1^{b}
5	TRY 3	6.69 ± 0.13^{a}	7.38 ± 0.02^{a}	8 ± 0.38^{a}	8.5 ± 0.17^{ab}	9.5 ± 0.37^{a}	10.67 ± 0.35^{b}
6	TRY 4	6.65 ± 0.07^{a}	7.25 ± 0.37^{a}	7.88 ± 0.06^{a}	9 ± 0.1^{ab}	9.88 ± 0.44^{a}	10.25 ± 0.42^{b}

Table 3. Eigen values and Eigenvectors of PCs for four biochemical traits studied in rice callus of six genotypes under five salinity treatments

	PC1	PC2	PC3	PC4
Eigen values and Variance %				
Eigen Value	3.37	0.48	0.13	0.02
Variance %	84.26	12.02	3.15	0.57
Cumulative Variance %	84.26	96.28	99.43	100.00
Eigenvectors				
Proline	-0.45	0.81	-0.33	0.19
CAT	-0.53	-0.20	-0.22	-0.79
SOD	-0.52	0.03	0.85	0.11
POX	-0.50	-0.55	-0.36	0.57

Figure 1. Barplot of callus induction frequency, relative growth rate and regeneration percentage of six rice genotypes under five different salinity stress

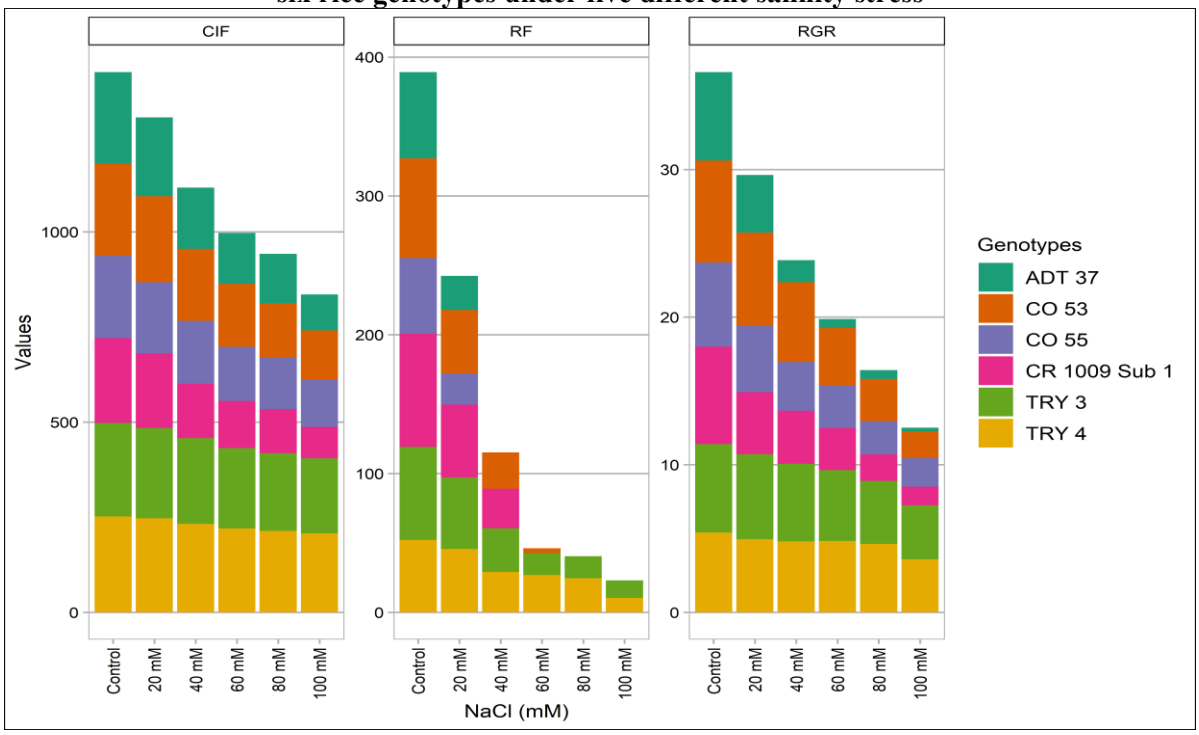

Figure 2. Line plot with error bars of biochemical responses of rice callus to NaCl in six rice genotypes

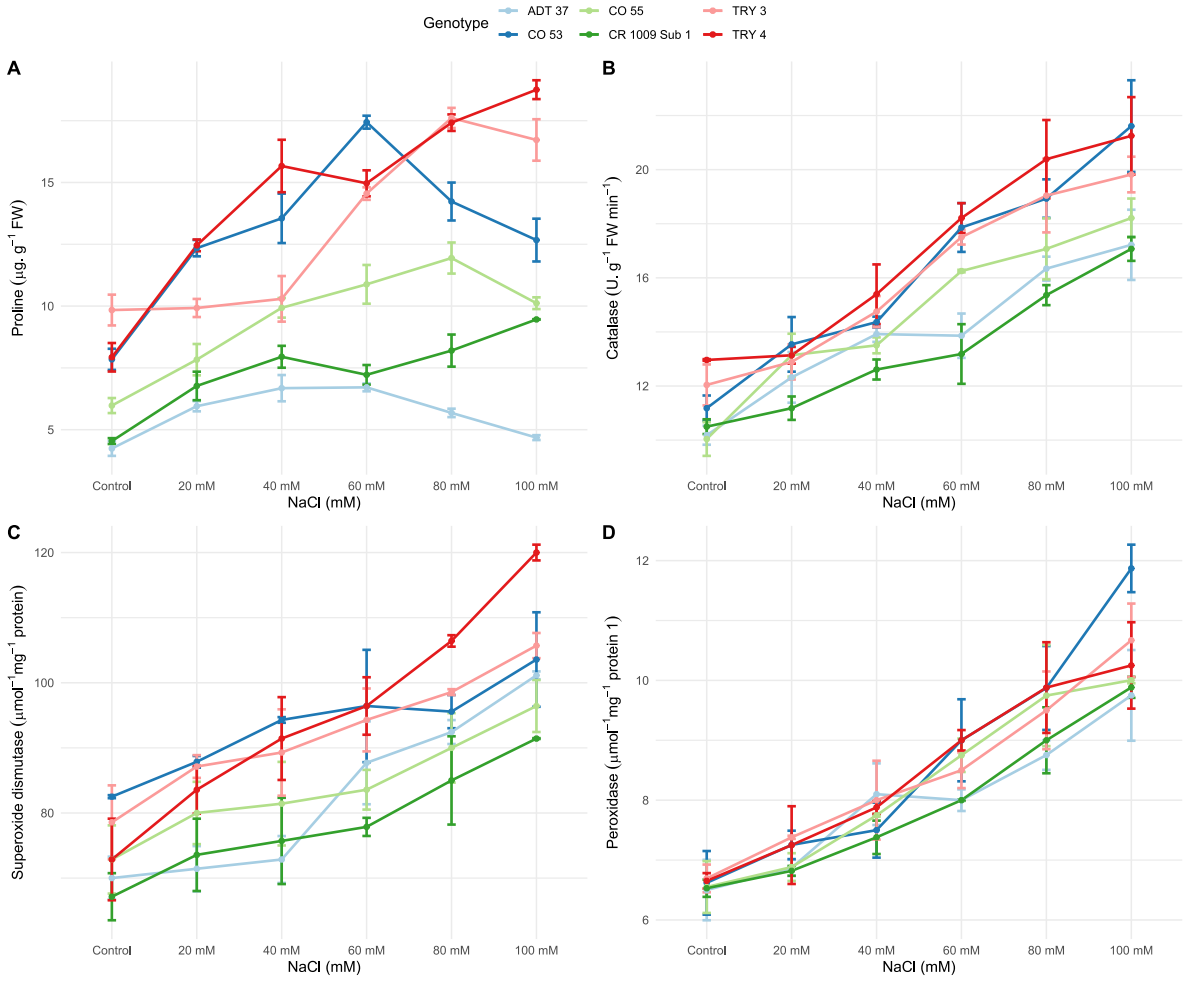

Figure 3. PCA Biplot of biochemical responses of rice callus to NaCl in six rice genotypes