

## Differential response of antioxidant defense system of *In-vitro* regenerated *Colocasia esculenta* (L.) Schott to Salinity Stress

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World Journal of Biology Pharmacy and Health Sciences, 2024, 20(02), 747-759

Publication history: Received on 20 October 2024; revised on 28 November 2024; accepted on 30 November 2024

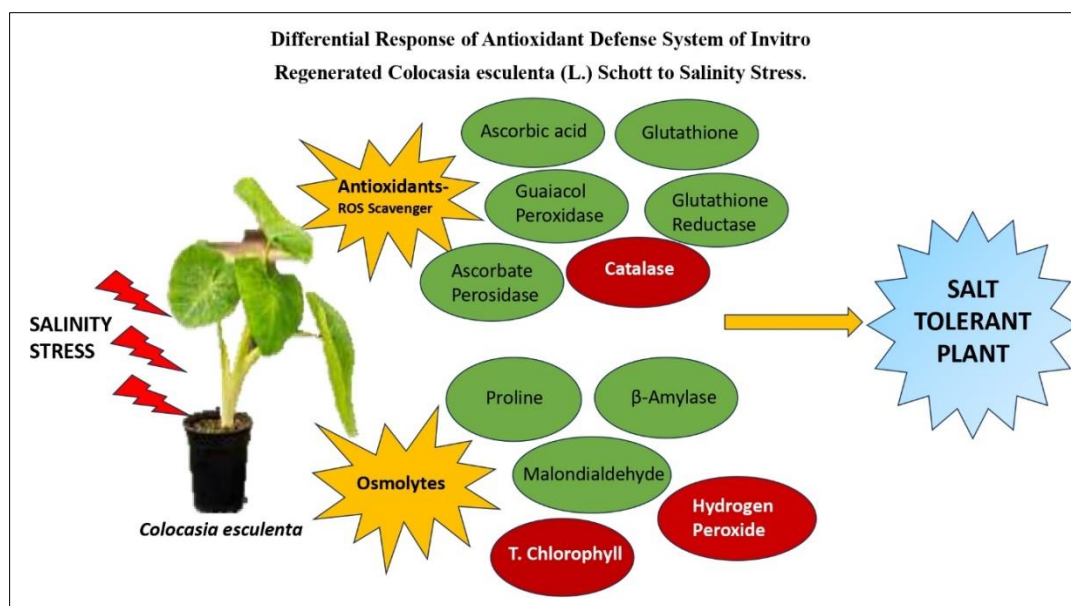
Article DOI: <https://doi.org/10.30574/wjbphs.2024.20.2.0953>

### Abstract

Soil salinity threat is escalating throughout the globe as it negatively impacts plant growth. The accumulation of sodium ions (Na<sup>+</sup>), disrupt ion balance and interfere with the acquisition of potassium (K<sup>+</sup>). The maintenance of a high K<sup>+</sup>/Na<sup>+</sup> ratio is crucial for plant survival under saline conditions. The effects of salinity include osmotic stress, ion toxicity, and oxidative stress caused by an overproduction of reactive oxygen species (ROS), leading to cellular damage such as lipid peroxidation and protein denaturation. To mitigate these effects, plants employ defense mechanisms like antioxidant systems, ion homeostasis, and osmotic adaptation. In this study, the salt tolerance of *Colocasia esculenta* (Taro), a plant known for its moderate salinity tolerance, was investigated. Using a hydroponic system, a tissue culture-regenerated variety of *C. esculenta* was exposed to varying levels of salt stress. The study focused on evaluating growth, antioxidant activity, and the expression of stress-specific components and enzymes. These findings aim to deepen understanding the response to salinity of *C. esculenta* and enhance strategies for its cultivation in saline-prone agricultural regions.

**Keywords:** *Colocasia esculenta*; Salinity Stress; Antioxidants; Salt tolerance; Catalase; Glutathione

### Graphical Abstract



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## 1. Introduction

Soil salinity is one of the major ecological factors that limit crop productivity, mainly in arid and semiarid regions [1]. The problem of soil salinity has been aggravated during the last decades as a consequence of irrigation and poor drainage systems. Approximately 20 % of the irrigated land in the world is affected by salinity, and it is expected that the increased salinization in agricultural fields will reduce the available land for cultivation by 30% in the next 25 years and up to 50% by the year 2050 [2]. Salinity exerts an unfavorable effect on the growth of plant mainly due to increased accumulation of sodium ions [3]. The alteration of ion ratios in plants is due to the influx of Na<sup>+</sup> through pathways that function in the acquisition of K<sup>+</sup>. Maintenance of high cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio is a key requirement for plant growth under high concentration of salt [4]. Ion-excess effects have been reported in a number of plants including mungbean [5], wheat [6], rice [7], brinjal [8] and *Sesuvium portulacastrum* [9] under salt stress. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutritional imbalance, specific ion effect, or a combination of these factors [10]. The sensing of these signals promotes signaling events that activate ion channels, kinase cascades, production of reactive oxygen species, and accumulation of hormones [11] and ultimately induced expression of specific genes that lead to the assembly of the overall defense reaction. Along with these interrelated and co-existing impacts, salinity results in an oxidative stress due to rapid and transient accumulation of reactive oxygen species (ROS) like superoxide and/or hydroxyl radical and singlet oxygen [12].

Salinity affects all the major processes; viz., growth, photosynthesis, protein synthesis, and lipid metabolism. Plants have to opt for a specific protective mechanism to alleviate the negative effect of ROS. Increase in ROS level causes pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and/or DNA mutation [13]. The common mechanisms include antioxidant defense system, ion homeostasis, osmotic adaptation, signal transduction and regulation of gene expression [14]. Measuring the antioxidant activity becomes a preliminary method for *in vitro* selection [15] of salt-tolerant cell-lines and plants viz., tomato [16] wheat, [17], rice [18], potato [19], sunflower [20]. The effect of salinity stress was investigated in *Colocasia esculenta* in terms of growth, antioxidant, stress-specific components and stress-specific enzymes

*Colocasia esculenta* has been reported to be moderately tolerant to salinity [21]. Keeping in mind its commercial cultivation employing extensive irrigation, possible salinity under flooding condition, it was proposed to evaluate the antioxidant status of the plant under salt stress. The experimental set up involving tissue culture regenerated variety of *C. esculenta* was exposed to salt stress in hydroponic system.

## 2. Material and methods

### 2.1. Plant Source

Tubers of Sree Reshmi cultivar of *Colocasia esculenta* were procured from CTCRI, Tiruvanthapuram, India. Tubers were used as explant for *in vitro* propagation. Regenerated plants were obtained from MS media supplemented with suitable auxin and cytokinin following the protocol of Anupama [22]. The regenerated plants were acclimatized and hardened for studying responses under salinity stress.

### 2.2. Stress Treatment and Experimental Design

30 days old regenerated plants were subjected to salt stress. Plants were grown in hydroponic system irrigated with half strength Hoagland media [23] containing different concentrations of NaCl (100 mM, 200 mM and 300 mM) and CaCl<sub>2</sub> solution. Plants grown with half strength Hoagland media without NaCl and CaCl<sub>2</sub> solutions served as control. The experimental design used was random factorial scheme with 3 evaluation points (24, 48, 72 h) and 4 media regimes (Control, 100 mM, 200 mM and 300 mM NaCl). Each experiment was done in triplicate. All the analyses for evaluating enzymatic and non-enzymatic antioxidants followed the procedures as mentioned in Anupama et al., [24].

### 2.3. Statistical Analysis

Statistical Analysis of all data are expressed in the study are means of triplicate experiments. The mean differences were compared by lowest standard deviations test using GraphPad prism 5.0 software. Differences in mean values were considered significant at  $p \leq 0.05$ .

### 3. Results and discussion

Under salt stress, one of the strategies that plants probably have to adopt is to slow down their growth and metabolism [25]

Plant survival under stress and the extent of salt tolerance often appears to be inversely related to growth rate [26]. The reduction in growth not only helps the plants to save energy for defense purposes but also limits the risk of further damage. Salinity results in the accumulation of Na<sup>+</sup> ions which decreases the shoot growth in terms of number of shoots per culture, shoot length and fresh and dry weights of plants [27]

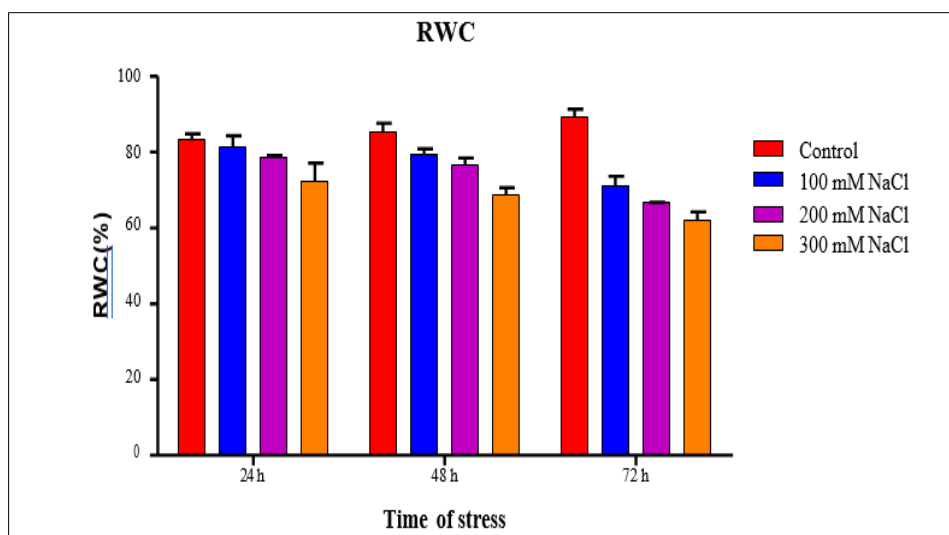
*Colocasia esculenta*, under salt stress showed relatively reduced plant height, fresh and dry weights with increasing time of exposure and concentration of salt (Table 1). Similar results were recorded in *Bacopa monnieri* [28] *Citrus aurantium* L. [29]. Evaluating the genotoxicity of salinity stress and secondary products gene manipulation in lime, *Citrus aurantifolia*, plants [30]. Since both Na<sup>+</sup> and Cl<sup>-</sup> ions accumulated during salinity stress are toxic, metabolic activities, hormonal synthesis [31] and hence, may contribute to growth reduction.

**Table 1** Effect of salinity on Plant height, Fresh weight (FW) and Dry weight (DW) of *C.esculenta*

NaCl Concentration	Time Interval	Plant Height (cm)	FW (g)	DW (g)
Control	24h	7.32±0.19	1.5±0.04	0.16±0.05
	48h	7.28±0.21	1.9±0.04	0.17±0.02
	72h	7.20±0.18	3.1±0.08	0.18±0.04
100 mM	24h	7.31±0.03	1.5±0.02	0.14±0.03
	48h	7.26±0.28	1.6±0.09	0.18±0.08
	72h	7.19±0.18	2.6±0.04	0.18±0.00
200 mM	24h	7.24±0.04	1.3±0.10	0.17±0.00
	48h	7.16±0.27	1.3±0.05	0.16±0.05
	72h	7.02±0.29	2.2±0.08	0.16±0.02
300 mM	24h	7.16±0.18	1.1±0.03	0.15±0.06
	48h	7.06±0.31	0.7±0.10	0.16±0.03
	72h	6.88±0.34	0.9±0.20	0.16±0.01

#### 3.1. Effect of Salinity stress on RWC

The relative water content is a primary parameter that reflects osmotic stress. Increase in salt concentration causes the reduction of osmotic potential of the medium. This could be explained due to the requirement for maintaining turgor of the growing cells, which consumes energy, and eventually leads to decreased growth. Increase in osmotic stress by NaCl application was accompanied by gradual decline in RWC in *C. esculenta* (Fig 1). Likewise, Yadav and Prasad [32], studied decrease in RWC in *in vitro* cucumber. *In vitro* salinity stress was shown to decrease RWC of *Capsicum annuum* [33].



**Figure 1** Relative water content of *Colocasia esculenta* after salt treatment. Ten plantlets from each treatment were analyzed. Data plotted are mean  $\pm$  SE obtained from three replicates.

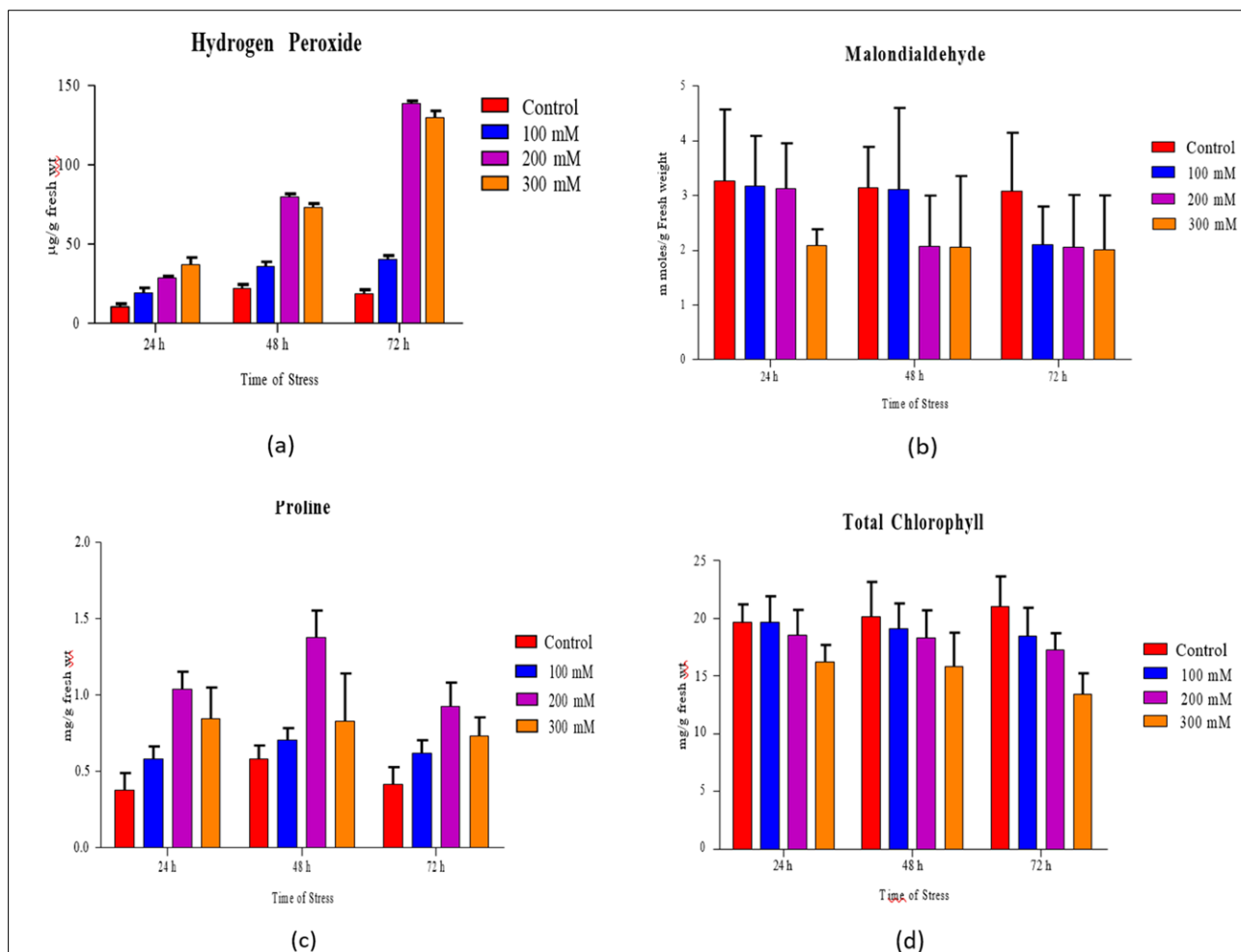
### 3.2. Stress Markers

Salt stress has been shown to cause stomatal closure, leading to reduced  $\text{CO}_2$  availability in the leaves and inhibit carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn, could increase the generation of ROS and induce oxidative stress [34]. Accumulation of  $\text{H}_2\text{O}_2$ , a stable ROS among reactive oxygen intermediates, is part of an immediate response of plants to salt stress. The levels of  $\text{H}_2\text{O}_2$  in *C. esculenta* increased more than 3-fold under salt concentration of upto 200 mM. But further increasing the stress caused decline in  $\text{H}_2\text{O}_2$  level (Fig 2a). Such progressive increase in  $\text{H}_2\text{O}_2$  was also observed in wheat [35] subjected to salt stress. Increase in  $\text{H}_2\text{O}_2$  level is a good indicator of generation of  $\text{O}_2^{\cdot-}$  which suggests the onset of enzymatic and non-enzymatic antioxidative defense system to control oxidative stress. Hence, it is essential for plants to keep the levels of both  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  to the minimum [36].

Reaction of  $\text{H}_2\text{O}_2$  with  $\text{O}_2^{\cdot-}$  leads to the formation of highly reactive hydroxyl radical ( $\text{OH}\cdot$ ) causing peroxidative damage of membranes [37]. The level of lipid peroxidation product, malondialdehyde is often used as an indicator of oxidative damage [38]. Malondialdehyde increased upto 2-fold under 200 mM NaCl (Fig 2b), but a gradual decrease was observed on further increase in salt concentration in *C. esculenta*. Similar results with soybean and rice [39, 40] have been reported to cause altered membrane permeability due to salinity stress. These results suggested that reaction of ROS with unsaturated fatty acids in membranes can result in loss of respiratory capacity in mitochondria and carbon fixing ability in chloroplasts [41]. The other reason for altered membrane permeability due to lipid peroxidation may be related to the ionic balance of the cell. Certain concentrations of  $\text{K}^+$  and  $\text{Ca}^{2+}$  ions are required to maintain the membranes intact. But at high salt concentrations,  $\text{Na}^+$  ions may substitute  $\text{Ca}^{2+}$  ions in the membrane causing a change in its permeability and leakage of  $\text{K}^+$  ions [42].

The amino acid proline, an osmolyte is known to accumulate in large quantities in higher plants in response to salt stress [43, 44]. Also, proline serves as a storage sink for carbon and nitrogen and as a free-radical scavenger [45]. It is believed to stabilize sub-cellular structures (membranes and proteins) and buffer cellular redox potential under stress. Proline in *C. esculenta* showed 2-fold increase with extended time of exposure (Fig 2c), suggesting its protective role to maintain cell turgor, osmotic adjustment, stabilization of membranes, protecting photosynthetic activity and free radicals scavenging and chaperoning through direct stabilization of proteins [46]. The osmoprotectant role of proline has been verified in transgenic potato crops by overexpressing genes involved in proline synthesis [47].

Plants exposed to salinity usually exhibit lower photosynthesis rate. Photosynthetic capacity depends on physiological characteristics such as; chlorophyll content, Rubisco activity and photosystem efficiency. Reduction in the chlorophyll content of the plant is accompanied by a lower efficiency of PS II and senescence [48]. A gradual decrease in total chlorophyll content with exposure to salt stress was observed in *C. esculenta* (Fig 2d) indicating loss of chlorophyll due to membrane damage. Similar result was observed in tomato and rice [49, 50].

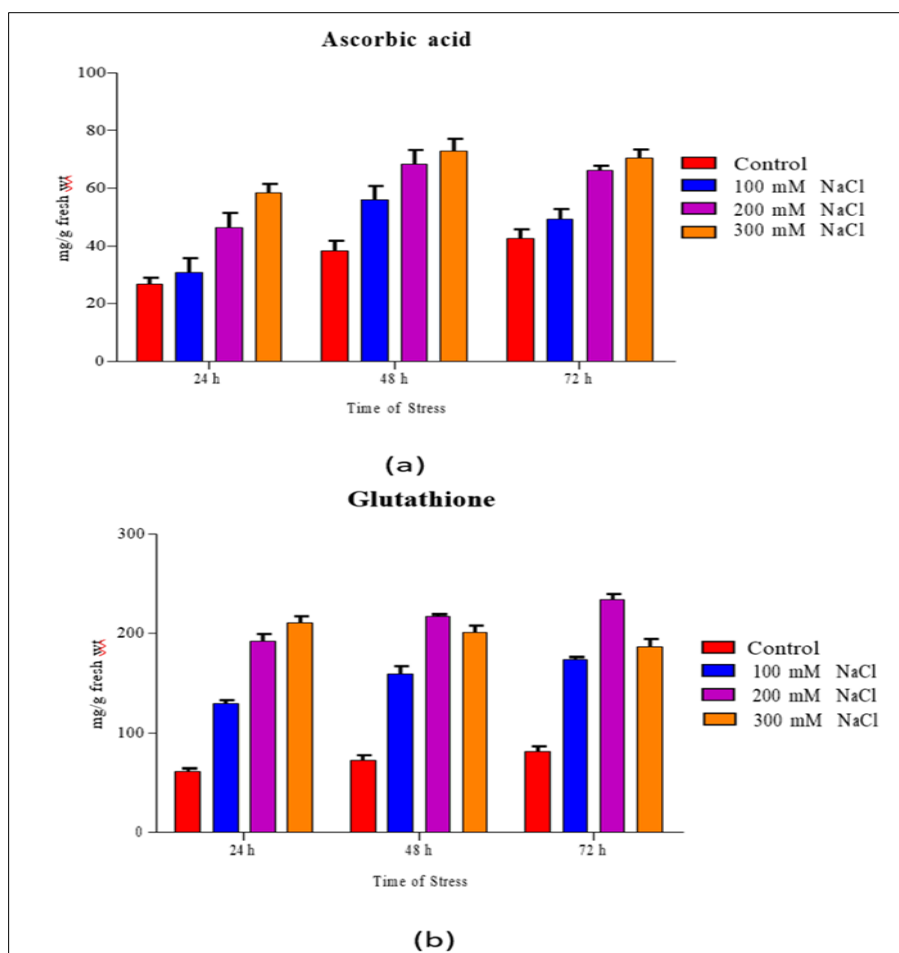


**Figure 2** (a) Levels of Hydrogen Peroxide (b) Malondialdehyde (c) Proline and (d) Total Chlorophyll in salinity stressed *C. esculenta*. Data plotted are mean  $\pm$  SE obtained from three replicates.

### 3.3. Antioxidants and Antioxidant enzymes

Ascorbic acid (ASC) is one of the non-enzymatic antioxidants having potential of not only for scavenging ROS, but also for modulating a number of fundamental functions in plants, both under stress and non-stress conditions. Due to the regenerative nature of ascorbate, it is one of the most powerful antioxidant molecules [51]. The ascorbate-glutathione pair is involved in regulation of plant development processes through the manipulation of oxidative metabolism [52]. Apart from this, APX, heme containing enzymes that are known to dismutate  $H_2O_2$  to water and molecular oxygen use ASC as an electron source [53]. ASC level in salt treated *C. esculenta* was found to increase significantly with increasing time of exposure and concentration of salt (Fig 3a).

Glutathione is a low molecular weight, ubiquitous tripeptide found at high concentration in aerobic organisms [54]. GSH can aid in maintaining cellular redox balance and perform signaling functions in plants under salt stress [55]. Higher GSH level is attributed to reduced stomatal aperture, transpiration rate and better vegetative development. Role of GSH in preventing the loss of photosynthetic pigments under salt stress has been reported [56]. GSH is used to maintain reduced state of ASC-GSH pathway by converting oxidized ascorbic acid (DHA) [57]; GSH is a substrate for GPX when converting the lipid hydrogen peroxide into a non-toxic form or water. GSH enhances salt tolerance by stabilizing plasma membrane to reduce passive  $Na^+$  influx [58]. Upto 2.5-fold increase in GSH level was recorded in salt stressed *C. esculenta* (Fig 3b). Parallel increase in ASC and GSH level at extended exposure to salt stress in *C. esculenta* suggested positive role in Ascorbate-Glutathione cycle. These results are in consonance with those observed in salt treated wheat and barley [59].

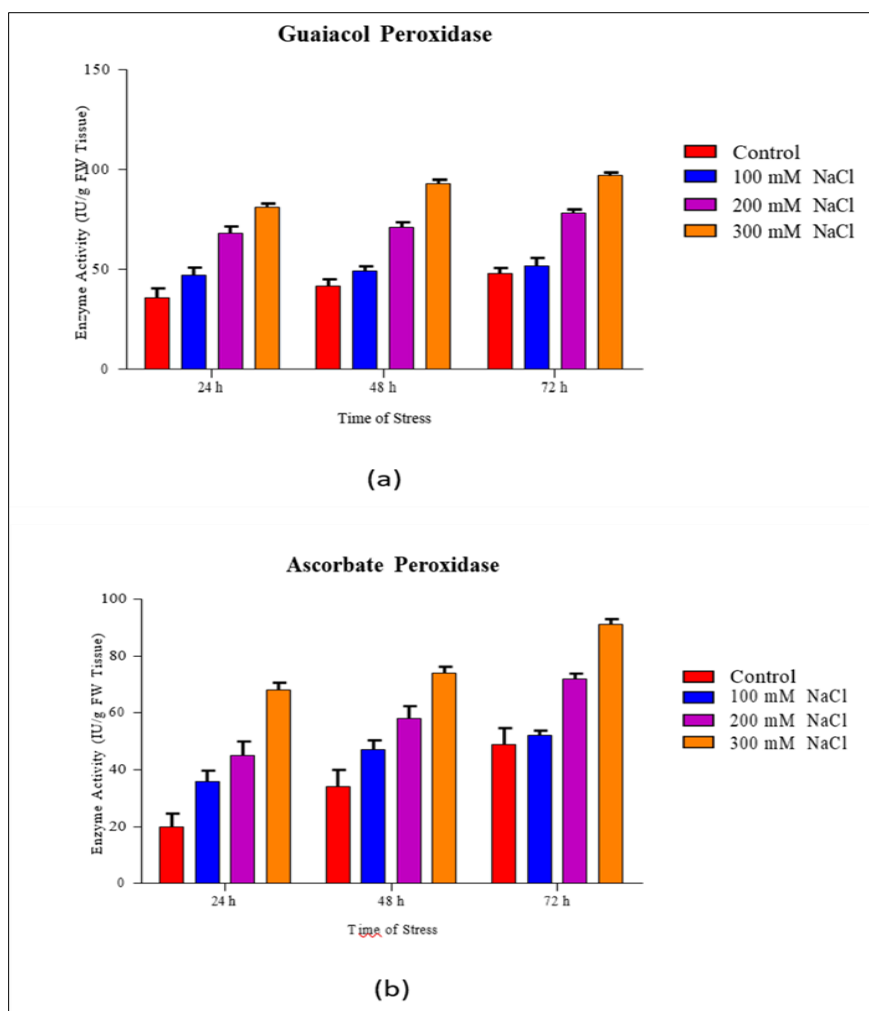


**Figure 3** (a) Levels of Ascorbic acid and (b) Glutathione in salinity stressed *C. esculenta*. Data plotted are mean  $\pm$  SE obtained from three replicates.

### 3.4. Antioxidant Enzymes

Antioxidant defense system is positively associated with salt tolerance to alleviate the oxidative damage in plants, this includes low-molecular-mass antioxidants, ASC and GSH as well as antioxidative enzymes like CAT, APX, GPX and GR [60].

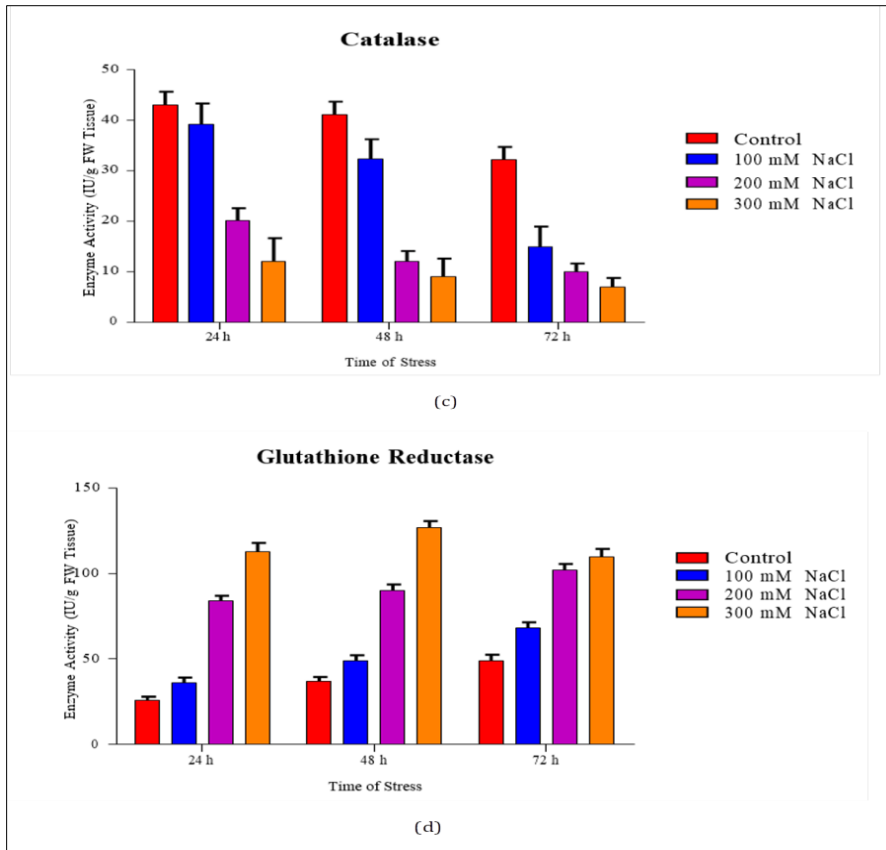
The peroxidases (POX) are associated with such biochemical and physiological processes as growth, cell formation, fruit development, ethylene biosynthesis, as well as the response to various stresses. APX catalyzes the detoxification of  $H_2O_2$  to water using ascorbate as reducing substrate through the ascorbate–glutathione cycle [61]. Salt-induced high APX activity was observed in tomato [62]. GPX, characterized by their broad specificity with respect to an electron donor, participate in a number of physiological processes like biosynthesis of cell wall, oxidation of toxic compounds, growth and development processes [63]. Both guaiacol peroxidase and ascorbate peroxidases exhibited time and concentration dependent elevations under salt stress reaching maximum level at 300mM NaCl and 72 h of exposure (Fig 4a, b). Further, in-gel assay of POX showed enhanced intensities and more than one isozymes under 200mM NaCl at 48h of exposure (Fig 5b). Appearance of additional isoforms under stress indicated the expression of different set of enzymes under salt stress, thus contributing to improved detoxification. The countervailing regulation of POX and CAT implies that CAT has low substrate affinity when compared to POX, and the latter has greater role in stress tolerance in *C. esculenta*. In line with these observations, higher activity of POX was recorded in salt tolerant genotypes of maize and rice plants compared to salt-sensitive ones [64].



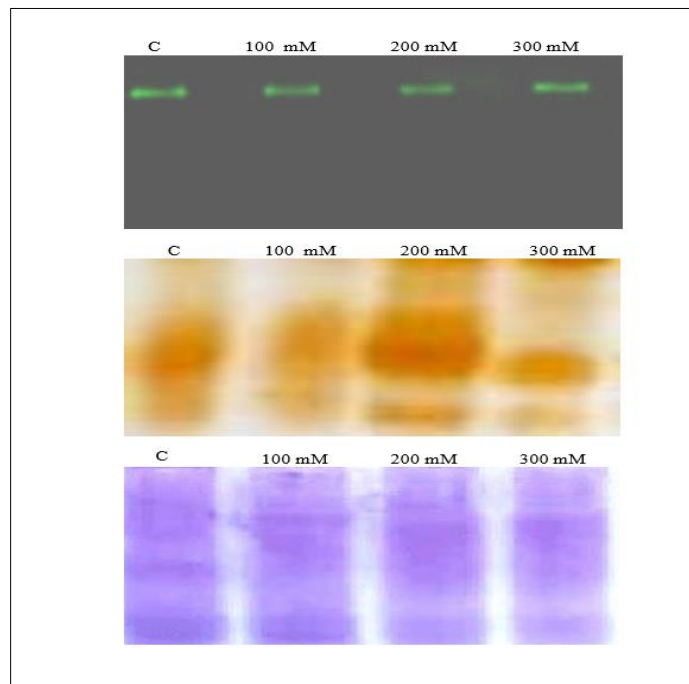
**Figure 4** Activity of Guaiacol peroxidase (a) and Ascorbate peroxidase (b) in *C. esculenta* after salt treatment for 24, 48 and 72 h with NaCl (0-300 mM). Data plotted are mean  $\pm$  SE obtained from triplicates

Catalases, located mostly in peroxisomes and glyoxisomes, are tetrameric heme proteins that are mainly associated with direct decomposition of  $H_2O_2$  produced during photorespiration and lipid decomposition under normal conditions are known to be accumulated during stress conditions. A positive correlation exists between levels of CAT and salt tolerance in plants [65]. Salinity in *C. esculenta* caused significant decline in catalase activity, which was also reflected in reduced intensity in in-gel assay (Fig 5a), thus suggesting that it is not the primary scavenger of ROS in *C. esculenta* (Fig 4c). Such observations have been made in salt-sensitive *Phaseolus vulgaris* plants which showed decreased enzyme activity under salinization [66]. Whereas elevated catalase activity was seen in rice seedlings at higher NaCl concentration [67]. CAT and POX maintain the steady state levels of cellular hydrogen peroxide to alleviate the oxidative stress.

Glutathione reductase (GR) is found to play crucial role in ascorbate- glutathione cycle by maintaining the GSH/GSSG ratio favorable to ascorbate reduction. Elevated levels of GR activity could increase the ratio of  $NADP^+$ /NADPH thereby ensuring the availability of  $NADP^+$  to accept electrons from photosynthetic electron transport chain, and minimize the reduction of oxygen and formation of superoxide radicals [68]. GR activity increased by 1.5- fold in time and concentration dependent manner in *C. esculenta* (Fig 4d), correlating with increased level of glutathione under stress condition. Higher levels of GSH, GR and ASC, coincided with enhanced levels of POX, which indicated that GR provided the reduced ASC, necessary for POX to reduce  $H_2O_2$  under salinity stress. The isozymes intensity (Fig 5c) indicated an enhanced expression of the same genes which are expressed in control rather than induction of new isozymes. Similar response were found in salt stress induced maize [69].



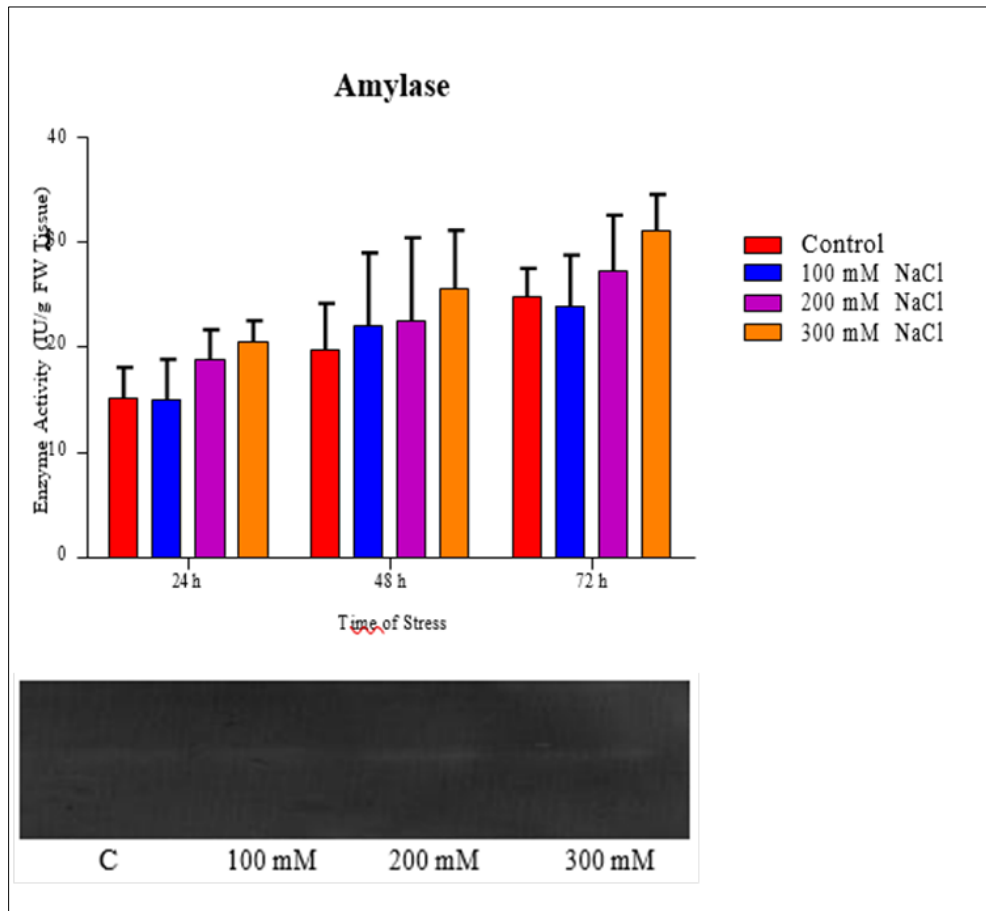
**Figure 5** Activity of Catalase (c) and Glutathione reductase (d) in *C. esculenta* after salt treatment for 24, 48 and 72 h with NaCl (0- 300 mM). Data plotted are mean  $\pm$  SE obtained from triplicates



**Figure 6** Zymograms of CAT (a), POX (b) and GR (c) of *C. esculenta* during salt stress. 100  $\mu$ g protein of each sample (48 h control and NaCl treated) was separated on non -denaturing PAGE (12%) and stained for enzyme activity.

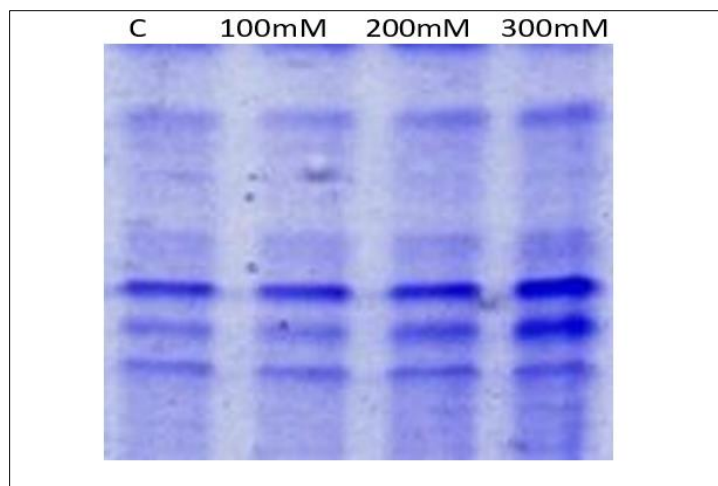


Apart from antioxidant enzymes, which are involved in stress response, there are few reports of induction of metabolite enzyme,  $\beta$ -AMY under abiotic stress.  $\beta$ -Amylases are localized to the stroma of mesophyll cell chloroplasts, the vacuole, and the cytoplasm. The primary function of  $\beta$ -amylase is starch breakdown in plants. The regulation of  $\beta$ -amylase activity by osmotic stress appears to be a general response for several plant species [70].  $\beta$ -amylase activity was found to be increased upto 1.5 fold till 200 mM NaCl (Fig 3.5 e). But a gradual decrease was noted on further exposure of stress. The isozyme intensities also showed similar pattern of enhancement and a gradual decline in band intensity. The induction of  $\beta$ -amylase in NaCl-induced osmotic stress, upto 200mM may lead to transitory starch degradation and protect structural integrity and turgidity of the cell. However, the higher NaCl concentration seem to be detrimental to plant. Similar findings were reported in abiotically stressed *Arabidopsis* [71].  $\beta$ -AMY activity increased in both leaves and roots of drought stressed seedlings and the enhancement of isozyme intensity were also correlated with *in vitro* levels. Elevated levels of  $\beta$ -amylase under these three conditions suggested a common mode of induction of the gene due to dehydration which entails in these conditions.



**Figure 7 (e)** Upper panel: Activity of  $\beta$ -amylase of *C. esculenta* after treatment with NaCl (0-300mM) for 24, 48 and 72 h. Data plotted are mean  $\pm$  SE of triplicates

Lower Panel: Zymogram of  $\beta$ -amylase during salinity stress. 100 $\mu$ g protein of each sample was separated on non-denaturing PAGE (10%) and stained for enzyme activity.



**Figure 7** SDS-PAGE pattern of 48 h salinity stressed *C.esculenta*. 100 µg soluble protein extracted from control (Con) and stressed plantlets were resolved on a 12 % gel and stained with Coomassie brilliant blue R – 250

The SDS-PAGE pattern of control and stressed sample exposed to varying concentration of salt for 24, 48 and 72 h showed no remarkable differences in band pattern (Fig 7). Control samples showed relatively low intensity bands compared to stressed samples. Intensity of each band showed increased with increasing stress level suggesting relatively higher protein contents in stressed seedlings. However, the higher band intensities could also be partly due to concentration of proteins due to dehydration. Similar level of protein content was recorded in chickpea [72] under salinity stress.

#### 4. Conclusion

In conclusion, *Colocasia esculenta* found to be tolerant to salinity stress upto 200 mM NaCl. Efficient detoxification of ROS requires potent antioxidant machinery which involves non enzymatic components like GSH, ASC, proline, total chlorophyll and enzymatic components like POX, CAT, GR. Efficient operation of ASC-GSH cycle was evident with enhanced levels of antioxidant ASC, GSH and antioxidant enzyme GR. The countervailing actions of POX and CAT showed apparent decrease in H<sub>2</sub>O<sub>2</sub> levels. Hence the combined effects of these antioxidant components and physiological factors serve as markers of salt stress tolerance in *Colocasia esculenta*.

#### Compliance with ethical standards

##### Acknowledgments

I would extend sincere gratitude to my supervisors who guided me with their valuable inputs.

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