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# Effect of abiotic stress on polyamine oxidase activities in rice

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**Abstract**
Polyamines are small aliphatic amines present in plants which help in abiotic stress acclimation. The cellular concentration of polyamines are maintained by a delicate balance between their synthesis and degradation. The principal enzyme responsible for polyamine degradation are polyamine oxidases. Polyamine oxidases have been reported from multiple plant species, however, their behavior in response to abiotic stresses remains obscure. In this paper, we have investigated the polyamine oxidase activity in model plant rice (*Oryza sativa*) in response to various abiotic stresses, namely salinity, dehydration, cold and heat stress.

**Keywords:** abiotic stress, polyamine oxidase, rice

## Introduction

Polyamines (PA) are small nitrogen containing compounds present in all the organisms starting from prokaryotes to eukaryotes. The commonest PAs in higher plants are putrescine (Put, a di-amine), spermidine (Spd, a tri-amine) and spermine (Spm, a tetra-amine) (Alca´zar *et al.,* 2006; Groppa *et al.,* 2007; Kusano *et al.,* 2008) [1, 8, 10]. Physiologically these PAs are grouped together assuming that they carry out similar functions, (Groppa *et al.,* 2007; Takahashi *et al.,* 2010, 2018), [8, 18, 19] however, their amplitudes may differ. In lower plants such as in algae and mosses (bryophytes) some unusual PAs namely norspermidine (norspd) and norspermine (norspm) have been reported. In lower plants these unusual PAs constitute the bulk of the PA population (Sagor *et al.,* 2015) [16]. The structure of norspermidine and norspermine are similar to triamine spermidine (spd) and tetramine spermine (spm) respectively (Fincato *et al.,* 2011) [7], suggesting that these unusual PAs may be the lower plant analog of spermidine and spermine. Recently, these unusual PAs have also been reported in higher plants such as alfalfa (Sagor *et al.,* 2015) [16] and maize (Takano *et al.,* 2012) [21]. However, the unusual PAs in higher plants are present in very low amounts. PAs have been known to be involved in a wide array of functions starting from embryogenesis to senescence (Alcázar *et al.,* 2010; Ono *et al.,* 2012) [1, 13]. Their involvement in stress tolerance have also been documented (Yu *et al.,* 2019) [25].

The PA homeostasis within the cell is strictly regulated by a well-crafted balance between PA anabolism and catabolism. The PA anabolic pathway is well described in literature (Bagni *et al.,* 2001, Wallace *et al.,* 2003) [3, 21], however, the catabolic pathway still needs attention. Newer genes, gene products are being identified and annotated (Federico *et al.,* 1999, Bordenave *et al.,* 2019) [6, 4]. The PA catabolic pathway principally has two kinds of enzymes. A copper-dependent diamine oxidase (DAO) and another flavin adenine dinucleotide (FAD) dependent polyamine oxidase (PAO). PAOs act upon spermidine and spermine to produce 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively with concomitant production of hydrogen peroxide ($H_2O_2$). The $H_2O_2$ produced may further act as a signaling molecule as well. These types of PAOs are called terminal catabolism (TC) type PAO. The other group of PAOs referred to as back conversion (BC) type converts the tetramine to triamine and triamin to diamine (Kusano *et al.,* 2015, Ono *et al.,* 2012, Liu *et al.,* 2014, Sagor *et al.,* 2016, Sagor *et al.,* 2017) [10, 13, 11, 17].

PAOs have been found to be localized in apoplastic regions, cell wall and cytosol (Yoda *et al.,* 2006) [24], however, lately they have been reported to be localized in subcellular organelle peroxisomes. The peroxisomal localization of PAOs have been documented in *Arabidopsis thaliana* (Fincato *et al.,* 2011) [7] and *Oryza sativa* (Ono *et al.,* 2012) [13]. In total rice contains seven PAOs out of which three, namely OsPAO3 (*Oryza sativa* polyamine oxidase), OsPAO4 and OsPAO5 have been shown to be localized in peroxisome (Ono *et al.,* 2012) [13]. Peroxisomes are the subcellular organelle which do not contain any DNA of its own, hence all the peroxisomal proteins are encoded by nuclear DNA, synthesized on cytosolic ribosomes and imported in a signal dependent manner. There are two types of signal present in peroxisomal matrix proteins - peroxisome targeting signal (PTS) type one and PTS type 2. PTS1 is represented by the last three amino acids present at C-terminus while that of PTS2 is a nona-peptide present at N-terminus (Reviewed in Reumann *et al.,* 2018) [14]. The PAOs are PTS type 1 (Ono *et al.,* 2012, Bordenave *et al.,* 2019) [13, 4]. PAOs, being a crucial player in regulating the cellular level of PAs, which being an important osmolyte, plays a crucial role in abiotic stress tolerance (Yamaguchi *et al.,* 2006) [23]. The reduced heterologous expression of *Zea mays* PAO (ZmPAO) has been reported to enhance thermotolerance in *Nicotiana tabacum* (Mellidou *et al.,* 2017) [12]. In *Arabidopsis thaliana* the role of AtPAO3 has been implicated in cellular ROS homeostasis (Andronis *et al.,* 2014) [2]. In rice it is predicted that OsPAO7 might be imparting abiotic stress tolerance via $H_2O_2$ production (Liu *et al.,* 2014) [11]. In this paper we have studied the PAO enzymatic assays in responses to salinity, cold, heat and dehydration stress under various time points, in order to decipher their involvement in abiotic stress tolerance.

## Materials and Methods

### Plant material and growth conditions

Rice seeds were sterilized by washing with distilled water 2-3 times, followed by washing in 70% ethanol for 1 min. Finally the seeds were treated with 0.1% sodium hypochlorite for 5 min in an orbital shaker with very slow shaking. The traces of sodium hypochlorite were removed by washing with distilled water 2-3 times and put onto germinated paper. The seeds were incubated under controlled conditions at 28 ± 2 ºC, 60% humidity and light/dark cycle 16/8 h. For all the experiments, 9 -11 days old seedlings of *Oryza sativa* IR 64 variety were used. The seeds of *Oryza sativa* IR 64 were obtained from National Rice Research Institute, Cuttack, Odisha, India.

### Abiotic stress treatments to rice seedlings

To induce saline stress, rice seedlings were treated with 50, 100, 150, 200 and 250 mM of sodium chloride. For cold, dehydration and heat stress, rice seedlings were treated at 4 ± 2ºC, PEG6000 and at 45ºC respectively. All the treatments were given at a time interval of 0.5, 2, 4 and 8 h. The untreated seedlings were used as control and the data obtained from the treated seedlings were compared with untreated ones to obtain the fold changes. After treatment, the seedlings were frozen in liquid nitrogen followed by preparation of enzyme extraction for PAO assay. All the experiments were performed a minimum of three times. The data was plotted in excel and standard deviation was calculated.

### Protein extraction and polyamine oxidase enzyme assay

For Polyamine oxidase (PAO) assay, 100 mg of rice seedlings were crushed in 100 mM phosphate buffer pH 8.0 containing 20 mM sodium ascorbate, 1 mM pyridoxal-5-phosphate, 10 mM Dithiothreitol, 0.1 mM sodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 15000 g for 60 min at 4˚C. The supernatant was used for enzyme assay. The reaction was carried out by adding 100 mM sodium phosphate buffer (pH 8.0), N, N-dimethylaniline and Horseradish Peroxidase (POX; 250 U/mL). The reaction was initiated by adding spermidine. The absorbance change was measured at 515 nm. A change in 0.01 in absorbance was calculated as 1 unit of enzyme activity (Ono *et al.*, 2012) [13].

## Results and Discussions

As explained earlier in the introduction section, PAOs have been implicated in abiotic stress tolerance in plants, hence here we have carried out a detailed investigation on rice PAO enzymatic activities in response to various abiotic stresses.

### Saline Stress

For salinity stress, rice seedlings were treated with sodium chloride. Initially varied concentrations of sodium chloride were used ranging from 50, 100, 150, 200 to 250 mM for 2 h, in order to determine the appropriate concentration of sodium chloride for further experimentations. Upon the assay the enzyme activity was calculated in units. Under control conditions the PAO activity was found to be 4.4 units, which increased to 5.82, 7.42, 11.45, 16.27 and 6.05 at 50, 100, 150, 200 and 250 mM respectively (Fig. 1a). The PAO activity at 50 and 100 mM sodium chloride did not show significant increase as compared to control as the fold changes were found to be 1.3 and 1.68 respectively. However, activity increased with increasing sodium chloride to 150 and 200 mM with fold changes of 2.6 and 3.69 respectively. Further, at 250 mM sodium chloride concentration the enzyme activity declined and the fold change reduced to 1.37. The data here showed a steady increase in PAO activity upon increase in sodium chloride concentration until 200 mM. The 250 mM sodium chloride was found to be detrimental for the survival of the rice seedling and hence, the disintegration of cellular activities might have started leading to reduced enzyme activity. Hence, for further salinity related experimentations 200 mM sodium chloride concentration was used.

Further, a time course assay was performed. The rice seedlings were treated with sodium chloride at 200 mM for 0.5, 2, 4 and 8 h. Upon the treatment the PAO assay was performed, enzyme activity and fold changes were calculated. With increase in time duration of sodium chloride treatment, the enzyme activity increased and reached its peak of 19 units at 4 h (Fig 1 b). At 8 h of sodium chloride treatment the PAO enzyme activity reduced below the activity observed under control conditions.

### Dehydration stress

Dehydration stress was provided by treating the seedling with PEG6000, which led to withdrawal of water. Dehydration stress led to sharp increase in PAO enzyme activity at 0.5 h reaching to 10.1 units, which remained static till 2 h of treatment. The enzyme activity reached its peak of 14.3 units at 4 h of treatment (Fig. 2). Further increasing the dehydration stress treatment time led to decline in PAO enzyme activity which fell below the enzyme activity under control conditions.

Both in case of salinity and dehydration stress the prolonged treatment of 8 h leads to decline in enzyme activity below the control level. This could be due the sensitive nature of *Oryza sativa* IR64 variety to abiotic stresses. In the initial time points the plant tries to cope up with stress conditions, however, with prolonged time duration it succumbs to stress conditions leading to disintegration of cellular activities and reduced enzyme activity

### Cold stress

For inducing cold stress the seedlings were incubated in the cold room maintained at 4 ± 2ºC for the assigned time points. In case of cold stress the enzyme activity increased to 10.5 units at 0.5 h, which reduced to 6.9 units upon increasing the stress treatment duration to 2 h. The enzyme activity peaked to 20.5 units at 4 h of stress treatment. Upon further increasing the stress treatment duration till 8 h the enzyme activity showed a drop, however, it remained higher than the enzyme activity under control conditions (Fig. 3).

### Heat stress

The seedlings were given heat stress treatment by incubating in an incubator maintained at 45°C for the assigned time points. The enzyme activity increased to 9.1 units at 0.5 h of treatment, which further reduced to 6.3 and 4.6 units at 2 and 4 h of treatment respectively. The peak of enzyme activity in case of heat stress was delayed and observed at 8 h of treatment with 22.7 units of activity (Fig. 4). In case of salinity, dehydration and cold, the peak of enzyme activity was observed at 4 h of stress treatment, while that in case of heat stress was delayed and observed at

8 h of treatment. In case of heat stress the further time points need to be verified to see whether the enzymes activity increases or falls back to normal level. Salinity and dehydration stress showed a similar pattern of enzyme activity where it increased with time, reach to peak followed by reduction in enzyme activity. In case of cold and heat stress it showed a dual surge in enzyme activity; the stress led to increase in enzyme activity followed by a dip then again rise in enzyme activity (Fig. 5). PAO have been reported to be involved in abiotic stress tolerance in plants, however, no PAO enzymatic activity assays are available with respect to abiotic stresses to the best of author's knowledge. However, PAO transcript data is available on a limited scale. Wang and Liu (2015) [22] demonstrated the upregulation of *Citrus sinensis* PAO (CsPAO) upon cold, sodium chloride and dehydration stress. *Solanum lycopersicon* PAO (SlPAO) have been shown to be induced upon salinity, heat, cold and dehydration stress (Hao *et al.*, 2018) [9].

A correlation between PAO transcript and protein expression would be required in future. This will be helpful in understanding the role of PAO in mediating abiotic stress tolerance in plants.

**Figures**

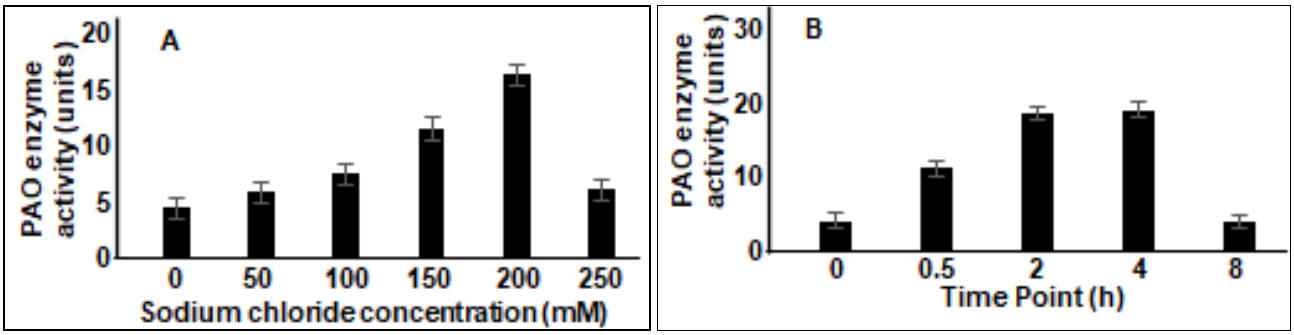


**Fig 1:** PAO enzyme activity in rice seedlings upon salinity stress: The 9 - 11 day old rice seedlings were treated with sodium chloride, followed by preparation of crude enzyme extract and PAO assay. Fig 1A represents the various sodium chloride concentrations used for treatment on the x-axis. The treatment was given for 2 h. Fig B shows the rice seedlings treated at 200 mM sodium chloride for various time points on the x -axis. The y axis in both fig A and B represents enzyme activity in units. The data presented here is the mean of three replicates plotted with error bars.

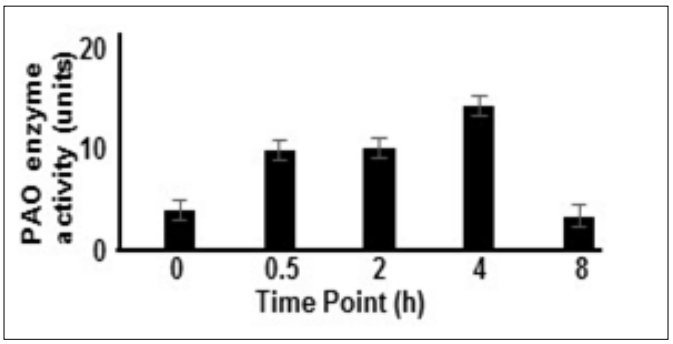


**Fig 2:** PAO enzyme activity in rice seedlings upon dehydration stress: The 9 - 11 day old rice seedlings were treated with PEG6000 to withdraw water, followed by preparation of crude enzyme extract and PAO assay. On the axis time point of treatments are mentioned while the y-axis shows the enzyme activity. The data presented here is the mean of three replicates plotted with standard error bars.

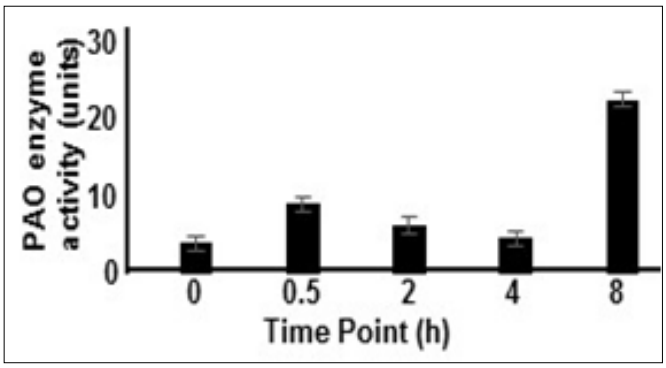


**Fig 3:** PAO enzyme activity in rice seedlings upon cold stress: The 9 - 11 day old rice seedlings were incubated at cold room maintained at 4+2°C to induce cold stress, followed by preparation of crude enzyme extract and PAO assay. On the axis time point of treatments are mentioned while the y-axis shows the enzyme activity. The data presented here is the mean of three replicates plotted with standard error bars.

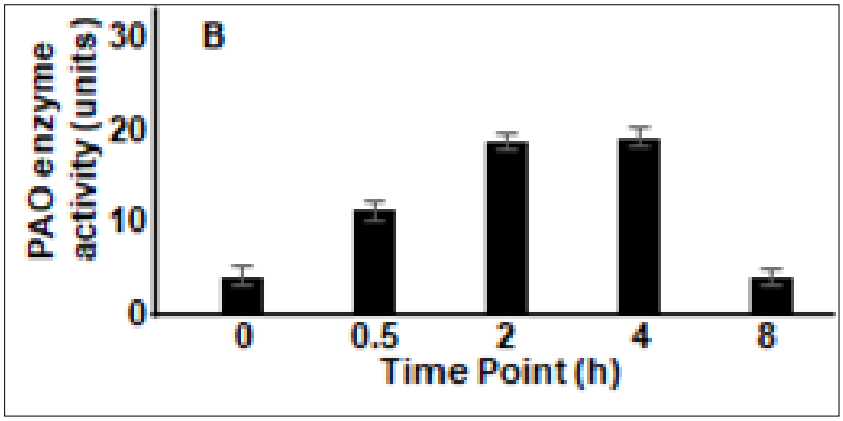


**Fig 4:** PAO enzyme activity in rice seedlings upon heat stress: The 9 - 11 day old rice seedlings were incubated at incubator maintained at 45°C to induce heat stress, followed by preparation of crude enzyme extract and PAO assay. On the axis time point of treatments are mentioned while the y-axis shows the enzyme activity. The data presented here is the mean of three replicates plotted with standard error bars.

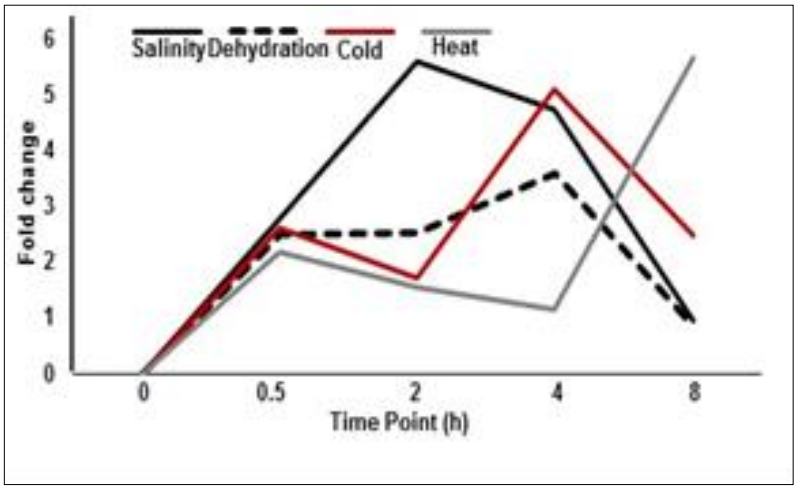


**Fig 5:** Fold changes in the PAO enzyme activity in rice seedlings upon abiotic stress: The 9 - 11 day old rice seedlings were given saline, dehydration, cold and heat stress treatment and enzyme activity was determined (Fig. 1, 2, 3 and 4). The fold changes in enzyme activity was determined and plotted in excel. On the axis time point of treatments are mentioned while the y-axis shows the Fold changes.

## Conclusion

In nature abiotic stresses do not act in isolation rather they act in combinations. Hence it is imperative to study abiotic stress in mutual combination rather individually. In order to do this the target candidates which are affected by multiple abiotic stresses would be suitable. Here, the PAO from rice was demonstrated to be induced by salinity, dehydration, cold and heat stress, hence it seems to be a universal player in abiotic stress tolerance in rice. This could be a suitable candidate and could be used to impart abiotic stress tolerance. However, further studies in this regard are required.

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## Conflict of Interest

Authors declare no conflict of interest.

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