

Evaluation of Free Radical Scavenging Activities Against Multiple Heavy Metals Stress in *Avicennia marina* (Forsk.) and *Rhizophora mucronata* Lamk

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Abstract: The tolerance mechanism of mangrove species (*Avicennia marina* (Forsk.) and *Rhizophora mucronata* Lamk) was studied by inducing multiple heavy metal stress in roots and leaves of the plants. Mangrove seedlings were treated with different concentrations of heavy metal solution (Cr, Cd, Pb and Hg) for a period of 2 months. Scavenging antioxidant enzymes like Polyphenol Oxidase (PPO), Glutathione-S-transferase (GSTs) and Guaiacol Peroxidase (POD) were analyzed in both species after appropriate intervals of 15 days. Results revealed that activities of these antioxidative enzymes were altered due to multiple heavy metals exposure in both mangrove species, whereas leaves exhibited the higher values as compared to the roots. In the leaves of both mangrove plants GSTs exhibited an increased trend throughout the investigated period whereas, PPO activity initially showed an increasing trend, but gradually decreased with the passage of time in response to heavy metal stress. Furthermore, an increased level of antioxidants was also observed in *A. marina* than *R. mucronata* which proves that the former is a strong candidate for heavy metals phyto-remediation with its viable survival strategies.

Keywords: Heavy metals, mangroves, antioxidant, ROS, PPO, GSTs.

Introduction

Heavy metals are a group of heterogeneous elements that comprises a core group of marine pollutants. It is due to expansion of industries through which these toxic metals are slowly and frequently becoming a part of marine water and affecting mangroves (Caregnato et al., 2008). Heavy metals cannot be biologically or chemically degraded and thus remain persistent within the environment (Lu et al., 2007). Few essential heavy metals are vital for plant metabolic activities, whereas non-essential heavy metals including chromium (Cr), lead (Pb), cadmium (Cd), are the most lethal contaminants for various plant metabolic activities (Hall and Williams 2003; Ali et al., 2019).

Normal vital bio-functioning activities of a plant are adversely inhibited by stress hence, the existence of toxic metals (non-essential) in plant tissues show their activity for the most part in disruption of water potential, inappropriate nutrient accumulation, disturbing chloroplasts and finally causing the serious damage to photosynthetic activity (Boussama et al., 1999, Patra and Sharma 2000). The lethal effect of heavy metals brings an assortment of harmful impact on plant cells, because of inactivation of basic functional proteins (compounds) by direct bonding with unavoidable conditions, as plants cause an oxidative stress. This oxidative harm frequently initiates the generation of reactive oxygen species (ROS) like $\cdot\text{OH}$ (hydroxyl radicals) from H_2O_2 (hydrogen peroxide) and superoxide ($\cdot\text{O}_2^-$).

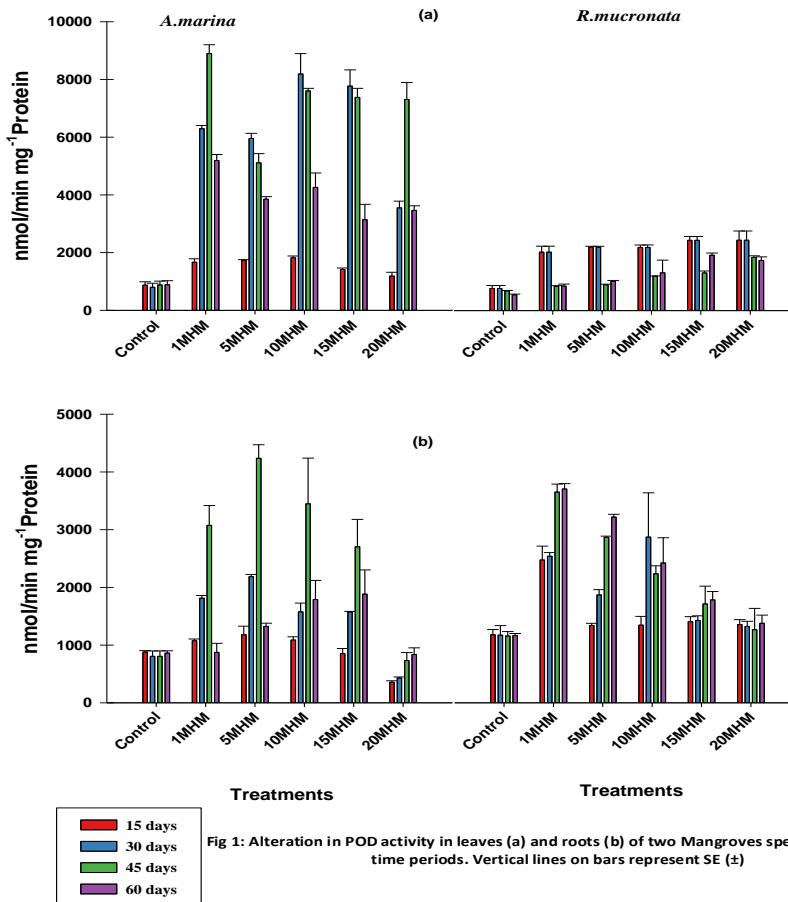
Higher plants develop an active and passive defense mechanism against high capacity for ROS generation

which triggers effective detoxification frame strategy and damages the profoundly toxic ROS or modify into non-dangerous particles (Larkindale and Huang, 2004). Among the defence system of antioxidants, high levels of Polyphenol oxidases (PPO), Glutathione-s-transferase (GSTs) Superoxide dismutase (SOD), Peroxidase (POX) etc are important enzymes (Zhang et al., 2007; Choo et al., 2003; Pukacka and Ratajczak, 2006).

Detoxification of phyto-toxic compounds from tissues is necessary and the process is mediated by three groups of enzymes. Phase I) Enzymes introduce functional groups to substrates. Phase II) Enzymes are used to conjugate this functional group for further conjugation. Phase III) Compartmentation enzymes identify pumps, and transfer conjugates across ATP-dependent membranes for excretion. GSTs is a strong, ubiquitous class of antioxidant and used to inactivate these toxic compounds and categorized in phase III enzymes.

PPO is typically well-known potential antioxidant, acts as chelating agent and performs antioxidative activity against ROS (Halliwell and Gutteridge, 1989; Rice and Miller, 1996). A main factor in enzymatic discoloration is the presence of copper proteins in nature and oxygen catalyzes the oxidation mono, di and polyhydric phenolic substrates to quinines (Mayer, 1987).

Indus delta approximately covers the area of 41, 440 km² extending from Sindh to Balochistan (SUPARCO, 2006). The coastline of Sindh is densely populated with thick forest of mangroves including few coastal locales of Karachi. Mangroves have their economic



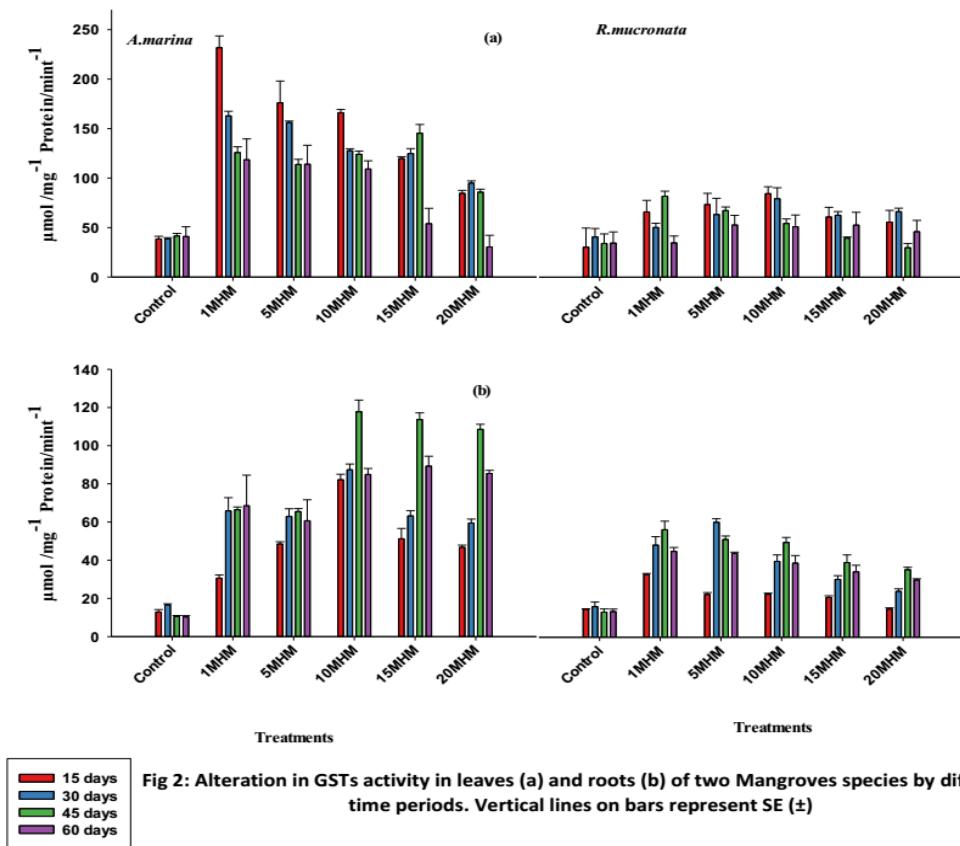
importance like providing timber, fuel wood, shelter of aquatic animals, barrier of coastal storm surges, tsunamis and cyclones. (Alongi and Dixon, 2000). Besides these benefits mangrove ecosystems is converting into source of pollution rather than sink of biochemical pollutants (Romanach et al., 2018)

Karachi is the largest municipality and massive industrial city. Over the last 50 years, toxic heavy metals which are primarily used in various industries like paints, tanning, paper mills, thermal power plants, textile mills, pesticides etc. have been directly dumped in marine water body. These industries discharge high proportion of chemicals, and solid spouts directly which ultimately has caused high damage to Indus river delta (especially districts of Karachi and Thatta). Industrial effluent containing heavy metals higher in the Karachi coastal region ranges between 0.21ppm-986ppm (Ismail et al., 2014). Similarly, in the sediments, the level of heavy metals like Cd (1.43ppm), Pb (59.99ppm) and Cu (52.23ppm) is classified into the serious polluted area (Ismail et al., 2014). Presence of toxic heavy metals is one of the limiting factors for mangrove forest in circum-tropical region. Dissolved heavy metals could be biologically accumulated at cellular level of mangroves which have tremendous tolerance against high levels of heavy metal concentration (Tam and Wong, 1997; Yim and Tam, 1999) but a very little work has been done on the effect of heavy metals in estuarine angiosperm in this

respect so far (Peters et al., 1997; MacFarlane and Burchett , 2001; Saenger 2002; Zhang et al., 2007; Huang et al., 2010). Therefore, there is a dire need to conduct the study to find out the phyto-remediation strategies adapted by mangrove plants to combat heavy metal pollution at the cellular level. In this regard, the present study was planned to determine the effect of induced MHM treatment on mangrove species (*A.marina* and *R.mucronata*).The aims is to carry out comparative analysis of free radical scavenging activities of PPO,GST and POD in mangrove plants against multiple heavy metals stress.

Materials and Methods

Healthy and complete viviparous seeds of *A.marina* and *R.mucronata* were collected from the forest of Indus delta. The propagules of both mangroves shifted to pots stuffed with water washed beach soil (three propagules used for every treatment). Using the sub-irrigation method weekly, each pot was irrigated by two liters of Hoagland solution (½ strength) with 10‰ NaCl (Hoagland and Arnon, 1950). After emergence of two pairs of leaves, the pots of each species were divided into six groups. Five groups treated with multiple heavy metals (inorganic salt of CdCl₂, HgCl₂, Pb (CH₃COO)₂ and K₂Cr₂O₇) at five levels namely 1MHM, 5MHM, 10MHM, 15MHM, 20MHM.The 6th group was treated as control (without multiple heavy metals). 1MHM containing 1.0 ppm Pb²⁺, 0.1ppm Cr²⁺,



0.1 ppm Cd²⁺ and 0.1 ppm Hg²⁺. 5MHM, 10MHM, 15MHM and 20MHM contained heavy metal concentrations that were $\times 5$, $\times 10 \times 15$ and $\times 20$ higher than 1MHM, respectively.

Periodically at 15 days interval, the leaves and roots were harvested from each group to investigate the effect of multiple heavy metals on antioxidative enzyme activities response of PPO, GPx and GSHs enzymes in both the species.

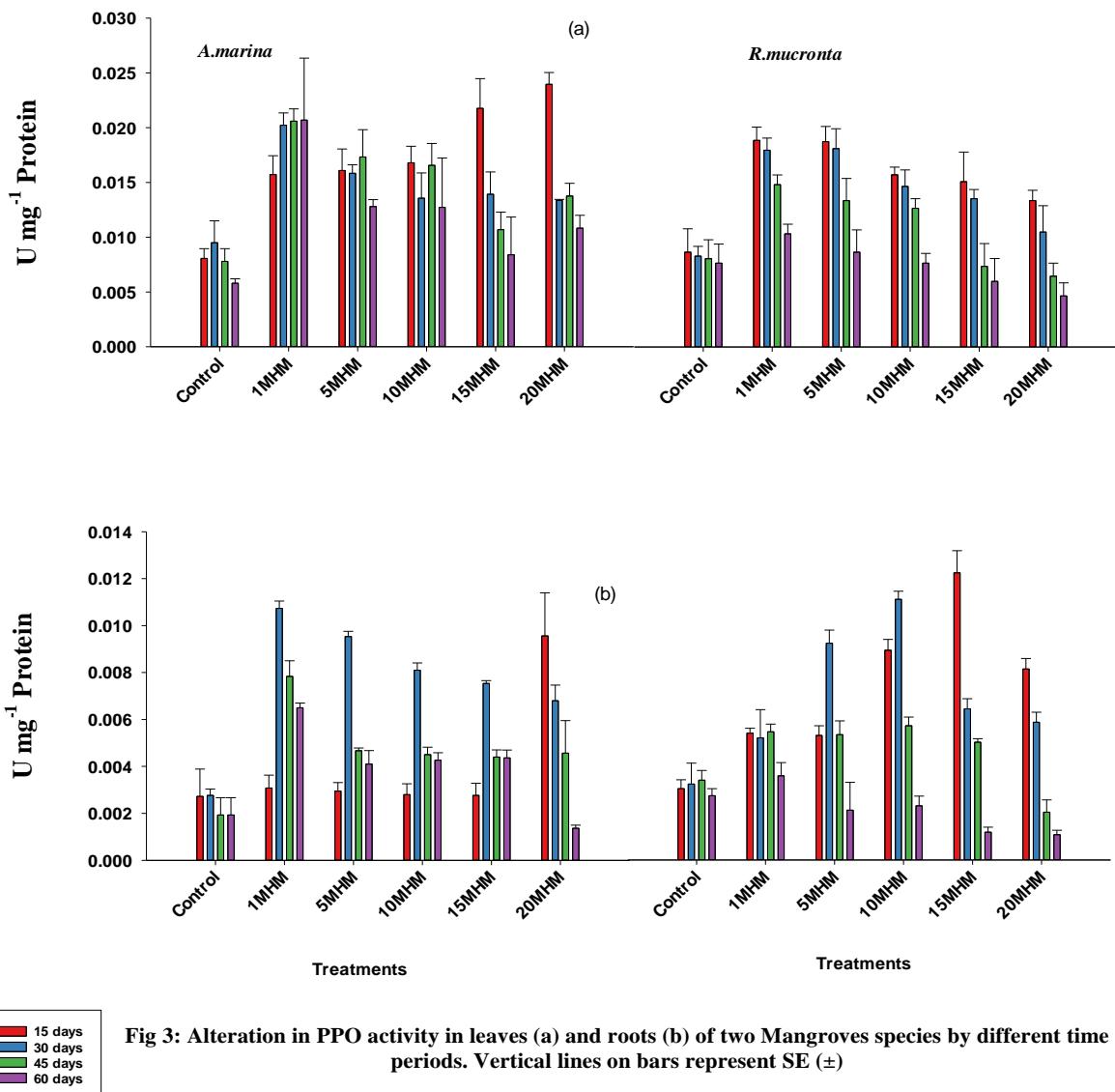
Fresh leaves and root tissues were homogenized in an ice-cold Potassium phosphate buffer (0.05M, pH7.0) with EDTA (0.05M) & 2% Polyvinyl polypyrolidone (PVP). The homogenate was centrifuged at 15,000rpm

for 15 min (4°C) and the clear supernatant taken for the source of enzyme assay. Bradford (1976) methods was used for estimation of Protein. Leaves and roots are estimated spectro-photo-metrically the change in absorbance as per the method of Putter, (1974). Analysis of GSTs activity in leaves and root sample was estimated as described by Habig et al., (1974). PPO was assayed by estimating the change in absorbance at 495 nm followed by Mayer et al., (1966). Data were analyzed by two-way analysis of variance (ANOVA). The Duncan's Multiple Range Test was used to determine the statistically significant difference between treatments at $p < 0.05$ using statistical software (Sokal and Rohlf, 1995).

Table 1 Two-way ANOVA (Duncan's multiple range test) indicating effect of different multiple heavy metal concentration (C), time duration (T) and their interactions (C \times T), from GSTs (Glutathione-s-transferase), POD (Guaiacol peroxidase), PPO (Polyphenol oxidase), of *A.marina* and *R.mucronata* df = Degree of freedom.

<i>A. marina</i>							
FACTORS	DF	Leaves			Roots		
		GSTs	POD	PPO	GSTs	POD	PPO
Concentration(C)	5	193.2 ***	8.4 ***	12.9***	25.4***	28.4***	2.80*
Time duration (T)	3	120.1 ***	14.4 ***	6.14**	21.5 ***	47.2 ***	5.66**
C×T	15	18.4 ***	1.44 ^{ns}	2.57**	5.3 ***	6.07***	1.26 ^{ns}
Error	48						
<i>R. mucronata</i>							
FACTORS	DF	Leaves			Roots		
		GSTs	POD	PPO	GSTs	POD	PPO
Concentration(C)	5	13.8***	16.9***	11.43***	7.40***	35.1***	6.09***
Time duration (T)	3	290.4 ***	61.6 ***	20.95***	272.4 ***	11.3 ***	25.5 ***
C×T	15	9.9***	10.4***	0.891 ^{ns}	4.64***	3.25 ***	3.67 ***
Error	48						

F-value *** = significant at <0.001 , ** = significant at <0.01 & * = significant at <0.05 ns = non-significant



Results and Discussion

The multiple heavy metals effects on POD enzyme activities were assayed after appropriate interval (15 days) for a specific period of 2 months of exposure. POD activity in the leaves and roots of both species remained unaltered at all level of treatments after exposure of 15 days, but leaves of *A.marina* showed marked increase after exposure of 30-45 days at other multiple metal stress levels. Whereas, leaves of *R.mucronata* showed unaltered entire periods of investigated time in comparison with their respective controls (Fig.1). Multiple heavy metals significantly enhanced the activity of POD in *A.marina* leaves at all stress levels versus the control.

The effect of MHM on the activity of POD enzyme was examined, and results revealed that in roots of *A.marina* POD activity increased at 5MHM (4238.1 nmol /min mg⁻¹ Protein) after 45 days of treatment but sharply decreased after 60 days of exposure. It is also observed that POD activity in roots of

R.mucronata was peaked at 1MHM (3703.9 nmol /min mg⁻¹ Protein) while at the highest MHM concentration (20MHM) lower enzyme activity 1377.05 nmol /min mg⁻¹ Protein was found(Fig 1). Statistical analysis shows that concentration (C), time duration (T) and their interaction (C x T) showed significant ($p<0.001$) variance in roots and leaves of both species (Table 1). POD is one of the principal enzymes involved in the elimination of ROS and mostly hydrogen peroxide dependent oxidations of substrate are catalyzed by it. POD is also involved in the synthesis of physical barrier against heavy metals by active participation in lignin synthesis (Hegedus et al., 2001). Previous investigation in other angiosperm plants have reported increases, decreases and no alterations in POD activity in response to heavy metal exposure (Shaw, 1995; Schützendübel et al., 2001, Schützendübel et al., 2002; Zhang and Kirkham, 1994; Chawla et al., 2014; Głowacka et al., 2019) In the present study the POD increases during entire period 15-60 days showing its tremendous effect in defending plants against oxidative

damage. Thus, increased POD activity shows the generation of heavy amount of H₂O₂ could release enzyme from membrane structure and there was significant difference in POD activity between *A.marina* and *R.mucronata* which also indicated that scavenging H₂O₂ ability in *A.marina* plays more prominent role than it does in *R.mucronata*.

A.marina and *R.mucronata* when treated with multiple heavy metals showed higher values of mean in GSTs activity over the control (Fig. 2). Overall, higher activity of GSTs is found in 1 MHM in the leaves and 15MHM in the roots tissues of *A.marina*. Between the species, the highest activity was observed in the leaves of *A.marina* $231.8 \pm 11.7 \mu\text{mol/mg}^{-1}\text{protein}$ after 15 days at 1MHM and the lowest activity $29.7 \pm 4.4 \mu\text{mol/mg}^{-1}\text{protein}$ was observed in *R.mucronata* at 20MHM after exposure of 45 days. In *A.marina* the highest activity is observed in roots at 15MHM ($117.7 \pm 6.10 \mu\text{mol/mg}^{-1}\text{Protein}$) in 45 days of treatment whereas in *R.mucronata* the highest activity of enzyme ($60.19 \pm 1.88 \mu\text{mol/mg}^{-1}\text{Protein}$) was observed in roots after 30 days at 10MHM. Analysis of variance specified highly significant ($P < 0.001$) effect of concentration (C), time duration (T) on PPO and GSTs (Table 1). The conjugation of the tri-peptide glutathione (GSH) to a number of hydrophobic, electrophilic, and cytotoxic substrates are catalyzed by the enzymes like GSTs (Mannervik and Danielson, 1988; Pickett and Lu, 1989). GSTs are responsive toward different heavy metals. Studies suggest that heavy metal exposure induces the generation of Reactive Oxygen Species (ROS) leading to oxidative stress and in response GSTs have been recognized and analyzed in various plant species (Wei et al., 2010; Shukla et al., 2018).

Present investigation showed that GSTs enzyme was able to balances the free radical at all treated levels in leaves and roots throughout the investigated period of stress (15-60 days). In leaves of *A.marina*, high GSTs activity was observed in 15 days of interval whereas an opposite trend was observed in the leaves of *R.mucronata* (Fig. 1). Overall decline was only observed in GSTs activity with high concentration of multiple heavy metals and their prolonged exposure, suggesting that the scavenging function of GSTs was impaired. These findings are in agreement with the results of *Arabidopsis thaliana* L and *Hordeum vulgare* L (Halusková et al., 2009; Srivastava et al., 2018; Shukla et al., 2018). In roots of *A.marina* GSTs activity is peaked at higher metal concentrations than in *R.mucronata* and also *A.marina* showed more pronounced response, indicating that increase in GSTs has better defence against oxidant cellular damage (Bowler et al., 1992; Takemura et al., 2000).

PPO activity in leaves and roots of *A.marina* and *R.mucronata* is significantly enhanced by multiple heavy metals (MHM) exposure compared to control. Overall, progressive increase in the activity of PPO was observed with the increase of MHM concentration and

exposure time in both the plants with some variation (Fig. 3). However, response of *A.marina* and *R.mucronata* was different in terms of different concentrations and exposure duration. *A.marina* showed highest activity of PPO in 20 MHM ($0.0240 \text{ U mg}^{-1} \text{ Protein}$) after 15 days of exposure in leaf tissues whereas its roots showed highest activity after 30 days of exposure in 1 MHM ($0.0107 \text{ U mg}^{-1} \text{ Protein}$). At highest concentration (20MHM), the PPO activity was also higher in leaves of *A.marina*, whereas leaves of *R.mucronata* showed maximum extent of decline in activity after 60 days of exposure while no significant loss in PPO activity was observed in control plants during these time periods. Statistical analysis stated that concentration (C), time duration (T) and their interaction (C x T) showed significant ($p < 0.001$) variance in roots and leaves of both species (Table 1). PPO is one of the major enzymes, consisting of catechol oxidase and laccase. The polyphenol oxidase (PPO) enzyme as the name indicates that it catalyzes the oxidation of one particular phenol or phenolic compound more rapidly than others (Steffens et al., 1994). It also provides defensive mechanism against stressful environment. The polyphenol oxidase (PPO) enzyme indicates that it catalyzes the oxidation of one particular phenol or phenolic compound more rapidly than others. Usually it works as enzyme, but also affect on formation and development of roots (Yilmaz and Parlak, 2011). In this study, by raising the phenolic compounds in the mangroves, increased activity of the peroxidase and enzyme Phenol oxidase is also observed. Similar results are also found by Aibibu et al., 2010 and Aria, et al., 2017 in *Vetiveria zizianoides* during the cadmium exposure.

In leaves of both species, an increase in POD, GSTs and PPO activities were noticed even at the lower MHM concentrations. In roots, this effect was more prominent as they responded to low MHM stimulation more rapidly because roots are the first direct organs that get in touch with MHM. Additionally, the activities of different enzymes in leaves may be due to the lower MHM concentration in the aerial as compared to the lower part.

The dynamic tendency of POD, GSTs, and PPO activities in leaves and roots of MHM-stressed plants almost all raised and then declined in contrast with the non-stress plants. The increase in enzymatic activities demonstrated that *A.marina* is tolerant to multiple heavy metals in certain concentration. The result significantly endorses the hypothesis that for detoxification of ROS, antioxidant enzyme plays a central and vital protective role in mangroves species (Liang et al., 2003; Georgiadou et al., 2018).

Conclusion

Hence, the current investigation confirms that *A.marina* and *R.mucronata* show distinct variation in antioxidant levels whereas, leaves and roots of both species, showed a synchronized increase in POD,

GSTs and PPO activities for scavenging the induced ROS against increased MHM concentration during the prolonged exposure. The decrease in the activities of scavenging enzymes in *R.mucronata* as compare to *A.marina* at the higher concentration of induced MHM treatments indicate that *A.marina* has more tolerance characteristics which help them to fight against the oxidative stress caused by the higher MHM treatment for a longer period.

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