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Impact of Heavy Metal Stress on Antioxidant Mechanisms of *Avicennia marina* (Forsk.) and *Rhizophora mucronata* Lamk.

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Abstract. Mangrove species are growing in exposed areas which have heavy metal contamination. The safeguard the mangrove ecosystem, it is important to understand their antioxidant responses to heavy metal toxicity. The goal of this study was to determine the effect of multi-heavy metals i.e. (Pb, Cd, Cr and Hg) on two mangrove plants *Avicennia marina* and *Rhizophora mucronata* of Indus delta *via* investigating their antioxidative defence mechanism of leaves and roots. In this regard mangrove seedlings of both species were treated with five different concentrations of four heavy metals and different time durations (15, 30, 45 and 60 days) for ascorbate peroxidase, catalase and superoxide dismutase in leaves and root tissues. The findings indicate that the heavy metals have significantly altered the antioxidant enzyme activities with respect of metals concentration and duration of exposure. With extended exposure higher antioxidant activities was observed in metal treated roots and leaves at higher concentrations. A pronounced stimulation ($P<0.001$) of CAT activity in both roots and leaves of *A. marina* occurred after 15 days of stress (38.3 and 26.6 µmol/mg protein/min) at 1 MHM. Our analysis also found that roots have shown greater activity in protecting against reactive oxygen species (ROS). Among the roots of two mangroves SOD activity in *A. marina* showed better tolerance towards metals stress (9.26 U/mg protein at 15 MHM) compare to *R. mucronata* (6.09 U/mg protein at 10 MHM). APX showed maximum stimulation at 20 MHM in leaves (19.130 µmol/mg protein/min) and at 10 MHM in roots (19.02 µmol/mg protein/min) of *A. marina* after 30 days metals treated plant. Hence, it confirms that the antioxidative defence system plays a critical role in *A. marina* and *R. mucronata* to tolerate the multiple heavy metals stress. However, *A. marina* showed greater antioxidant activity especially catalyst enzyme activity as compared to *R. mucronata*, which is well evident by its dominancy in the region.

Keywords: mangroves, multiple heavy metals, oxidative stress, antioxidants

Introduction

The mangrove is the planet's most important and organically dynamic ecosystem because it provides human society seaside living spaces and coastal ecosystem supplies and administrations (Giri *et al*., 2010). These forests are the source of living for a large number of human beings around the world by providing wood for fuel, charcoal and timber as well as areas for fishing. They act as nurseries for the marine fauna, protect and stabilize shoreline and perform protective mechanism against Tsunamis, erosion and flooding and storm surges in the coastal zones (Ismail *et al*., 2014). Despite playing a crucial role, this ecosystem is continuously subjected to severe degradation due to many factors like industrialization, urbanization, overharvesting and grazing, over exploitation for fuel and fodder, tourism, aquaculture and pollution.

Mangrove ecosystems act as a sink of bio-chemical pollutants, but now have converted into a source of pollution. Heavy metals serve as most lethal contaminants in the mangrove biological ecosystem due to its toxicity, bioaccumulation problems and long resistance time in food chain (Hagibi *et al*., 2018). They are non-biodegradable as a result they continue to persist in the environment (Haoliang *et al*., 2007). According to researcher tolerance against heavy metals varies among mangrove species even in the same species the behaviours of metals absorption and enrichment differ at different locations (Ismail *et al*., 2014; Zhang *et al*., 2007). Excessive uptake of heavy metals by plants and its accumulation in tissues, may trigger various morphological, physiological and biochemical response (Banu and Atmaca, 2011) and may also causes a variety

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of sub-cellular activities, i.e., metabolic response that are offended at cellular phases or may prompt more extensive phototoxic reactions (Zhang *et al*., 2007).

In order to minimise the harmful effects of reactive oxygen species (ROS), the plants have developed their own protection mechanism, which includes non-enzymatic and enzymatic antioxidants, that play a role in scavenging ROS, (Choo *et al*., 2004; Ukaszewicz and Arcin, 2004). Among the antioxidants defence system ascorbate peroxidase (APX), catalyst (CAT), superoxide dismutase (SOD) and other enzymes play an important role either directly or indirectly in scavenging ROS from the plant cells (Aravind *et al*., 2005; Noctor, 1998). These enzymes exist in essentially all sub-cellular compartments. Normally, a cell organelle has multible enzymes that are capable to rummage a single ROS (Scandalios, 2005; Mittler *et al*., 2004b; 2002a). SOD is one of the widespread plant chemicals that play a crucial role in cell hindrance towards receptive oxygen organisms (ROS). Its activity changes the general proportions of free O_2^- and H_2O_2, the two Haber-Weiss reaction substrates, and decreases the risk of OH radical arrangement, which exceptionally opens and may make extreme disruption effect on protein and DNA (Pandey and Gaurav, 2012; Zhang *et al*., 2007). Stunted plant growth (shoot and root) is a very common morphological outcome when the roots systems of plants interact with soil sap including toxic heavy metals (Dalcorso *et al*., 2013; Omae *et al*., 2012). Metal toxicity result in a decrease of fresh and dry mass and fruit productivity of a plant whereas, (Rahman *et al*., 2010) reported reduction in a number of leaves and stunted stem heights in *Kandelia candel* when treated with Cd. Similarly, along with the alteration in morpho-physiological aspect; a decrease in the rate of photosynthesis and respiration process also occurred (Giri *et al*., 2010). Production of a lower amount of energy in the plant due to heavy metal stress consequently, causes lower rate of metabolism (Ying *et al*., 2010; Burton and Morgan, 1984).

However, researchers concluded that mangroves have remarkable ability to with stand high levels of heavy metal concentration, but very little evidence has been published previously regarding the physiological and biochemical processes in mangrove plants under multiple heavy metal stress (Dudani *et al*., 2017; Mahadavi *et al*., 2012; Dang *et al*., 2006). Therefore, it is imperative to study the tolerance strategy of mangrove plants against heavy metal for an understanding of physio-logical and biochemical response. Thus, this study was undertaken to address the following objectives:

To investigate the effect of multiple heavy metal stress on;

1) Antioxidative enzymes (ascorbate peroxidase, catalyst and superoxide dismutase) in *A. marina* and *R. mucronata*, leaves and roots.
2) To assess the effect of multiple heavy metals toxicity on young *A. marina* and *R. mucronata* seedlings.

Materials and Methods

Collection of propagule. *R. mucronata* propagules were collected from plants, grown at the mangrove forest site of Miani Hor stand (670 9N, 260 25E), Balochistan, Pakistan. The region is subtropical with annual rainfall (150-250 mm) during July-September. Propagules of *A. marina* were collected from the Indus delta forest near Karachi, Pakistan. The average annual temperature during winter season ranges from 11.8 to 27.8 °C. For planting, only complete, undamaged propagation with unbroken testa was without emerging hypocotyls or radicles.

Rate of germination. For germination four propagules randomly planted in pots were filled with washed sand. Propagules were kept in a greenhouse under natural sunlight. Each pot was irrigated weekly using two litres of ½ strength Hoagland solutions (Hoagland, 1950) with 10% NaCl through sub-irrigation. The water level for submerged condition in each container was adjusted daily with distilled water. Seedling establishment requires the period of one month. Four months old young seedlings were treated multiple heavy metals Cd, Pb, Cr and Hg with ½ Hoagland's nutrient medium, (pH~6.0). The multiple heavy metals containing nutrient solution were refilled after every 3 days.

Study design. A one-way complete randomized design (CRD) with three replicates per treatment was used in this study. The random samples with five independent replicates were assigned to different treatments of multiple heavy metals concentrations, namely 1MHM, 5MHM, 10MHM, 15MHM, 20MHM.

In 1MHM represented 0.1 mg/L Hg^{2+}, 1 mg/L Pb^{2+}, 0.1 mg/L Cd^{2+} and 0.1 mg/L Cr^{2+} containing Hoagland's solution 0.5MHM, contained ×5, 10MHM contained ×10, 15MHM contained ×15 and 20MHM contained ×20 time higher concentration of multiple heavy metals as compare to 1MHM, respectively. Whereas, six sets

of group considered as control, treated with ½ strength of Hoagland's nutrient solution only (Hoagland and Arnon, 1950). Distilled water was added in the plastic container up to the mark to compensate the concentration of metal ions in order to balance for evapouration losses. Control (C) plants were supplemented with 2000 mL of ½ strength of normal Hoagland's solution (Hoagland and Arnon, 1950).

Antioxidant enzyme extraction and assay. To measure the effect of multiple heavy metals on antioxidative enzymes activities in leaves and roots of both mangrove plants, samples were collected after every 15 days' interval till 60 days, whole plants harvested to observe SOD, CAT and APX in leaves and roots.

Sample extraction. To investigate the effect of heavy metals on enzymatic antioxidant (CAT, APX, and SOD) in leaf and root tissue was obtained from all seedlings and control seedlings of mangrove. Grind 500 mg fresh leave and root in ice-cold potassium phosphate buffer (0.05M, pH7.0) with EDTA (0.05M) and 2% polyvinyl polypyrolidone (PVP). The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was used for the enzyme assay. Bradford methods used for estimation of protein. For standard using bovine serum albumin (Bradford, 1976).

Estimation of superoxide dismutase (SOD). Analysis of SOD activity in plant sample was estimated described by Fridovich. Set two tubes in which added 3 mL reaction mixture contained potassium phosphate buffer (50 mM, pH7.0 with 2 mM EDTA), Triton × 100 (0.22%), L-methionine (9.98 mM), NBT(0.058 mM), enzyme extract and lastly added riboflavin (0.1168 mM). Place one of the sample tube in dark (used as blank), while other sample tube were exposed to light under two 20W fluorescent tubes to initiate reaction. After 7 min, incubation recorded optical density at 560 nm against blank.

Estimation of catalyst (CAT). In cell, hydrogen peroxide decomposed into water and oxygen due to common enzyme catalase. The activity of CAT was assayed the method of Aebi. Aebi studied in (1984) that 0.3 mL of reaction mixture consisted of 40 µL supernatant, potassium phosphate buffer (50 mM, pH7.0). The reaction was initiated by addition of the H_2O_2 (2 mM in phosphate buffer). The reduction of H_2O_2 was recorded at 240 nm. APX and catalyst activity was expressed as Unit/mg protein.

Estimation of ascorbate peroxidase (APX). To record the APX activity was previously determined Nakano and Asada (1981) by monitored the optical density at 290 nm. 100 µL supernatant was added to reaction mixture contained 600 µL potassium phosphate buffer (50 mM, pH7.0), ascorbic acid (0.5 mM), disodium EDTA (0.1 mM), H_2O_2 (0.1 mM).

Statistical analysis. All statistical analysis was done by two-way analysis of variance (ANOVA). The Duncan's multiple range test was using to determine the statistically significant difference between treatments at $P<0.05$. All data are showed as means ± std. error of mean (SEM) of three independent replicates of every treatment using a completely randomized design.

Results and Discussion

Operation of SOD in leaves and roots of *A. marina* and *R. mucronata* is significantly enhanced by multiple heavy metals (MHM) relative to control. Overall, progressive increase in the activity of SOD was observed with the increase of MHM concentration and exposure time in both plants with some variations (Fig. 1). However, response of *A. marina* and *R. mucronata* was different in terms of different concentrations and exposure duration. *A. marina* showed highest activity of SOD in 20MHM (6.87 U/mg protein) after 45 days of exposure in leaf tissues, whereas its roots showed highest activity after 30 days of exposure in 15MHM (9.26 U/mg protein). At highest concentration (20MHM), the SOD activity was also higher in leaves of *A. marina* and in leaves of *R. mucronata* showed maximum extent of decline in activity after 60 days of exposure, while no significant loss in SOD activity was observed in control plants during these time periods. Statistical analysis stated that concentration (C), time duration (T) and their interaction (C×T) showed significant ($P<0.001$) variance in roots and leaves of both species (Table 1).

A. marina and *R. mucronata* when treated with multiple heavy metals showed higher value of mean in CAT activity (Fig. 2) over the control. Overall, higher activity of CAT is found in 1MHM in the leaves and roots tissues of *A. marina*.

Between the species, the highest activity was observed in the roots of *A. marina* 38.3±1.52 µmol/mg protein after 15 days at 1MHM and the lowest activity 11.6 ± 0.57 µmol/mg protein was observed in *R. mucronata* at 20MHM after exposure of 60 days. In *A. marina* the highest activity is observed in leaves at 1MHM (28.33±0.5774 µmol/mg protein) in 45 days of treatment, whereas in *R. mucronata* the highest activity of catalase (25.3±2.08 µmol/mg protein) was observed in leaves after 15 days at 1MHM.

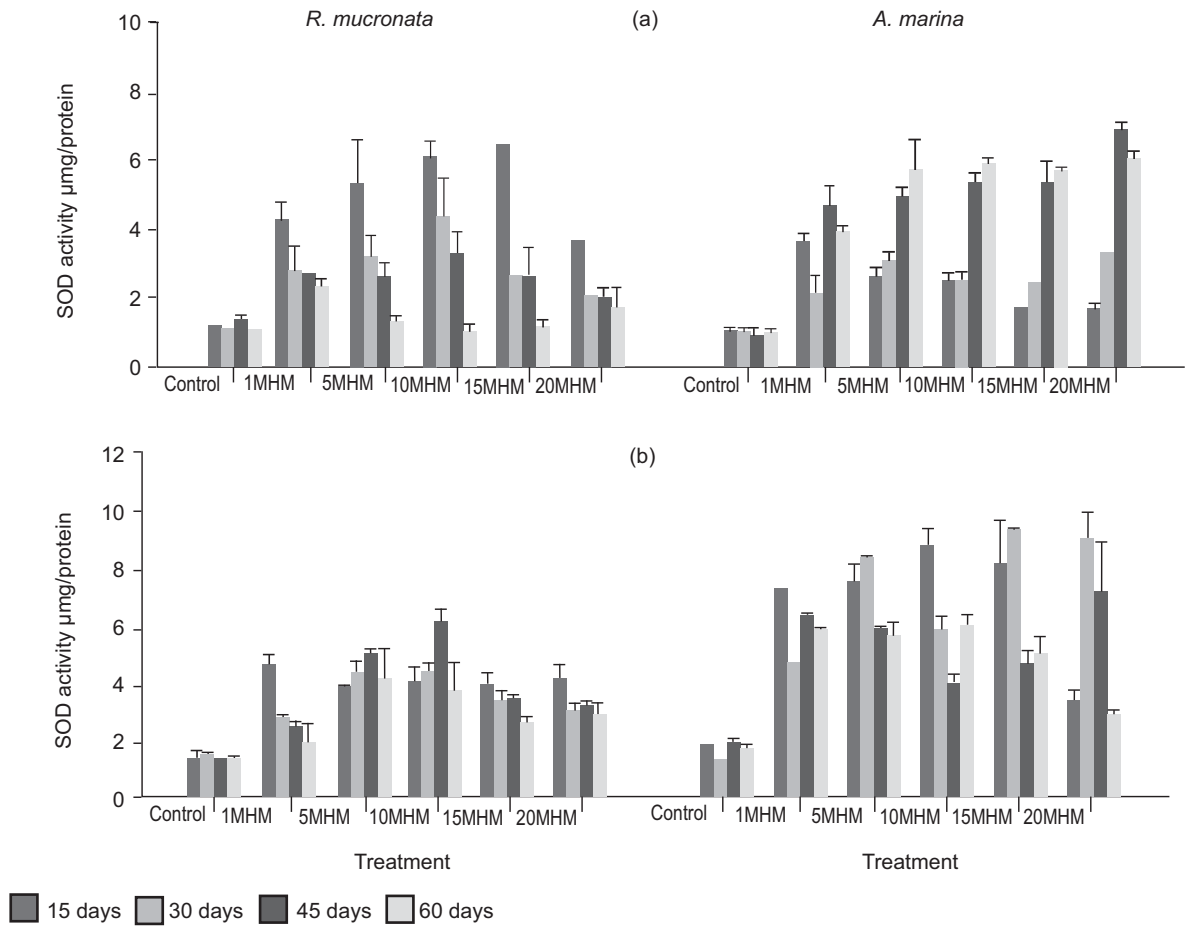

Fig. 1. Alteration in SOD activity in leaves (a) and roots (b) of two Mangroves species by different time periods (15, 30, 45 and 60 days) multiple heavy metals (n = 3, vertical bars represent ± SE.

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×T), from catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) of *A. marina* and *R. mucronata* and df = Degree of freedom

A. marina		Leaves			Roots		
Factors	DF	CAT	APX	SOD	CAT	APX	SOD
Concentration (C)	5	199***	85***	146***	132***	220***	125***
Time duration (T)	3	13.9***	55***	258***	44.1***	45.3***	43.4***
C×T	15	3.99***	9.43***	23.01***	3.96***	14.66***	27.23***
Error	48						

R. mucronata		Leaves			Roots		
Factors	DF	CAT	APX	SOD	CAT	APX	SOD
Concentration (C)	5	90.36***	36.73***	38.69***	33.54***	41.54***	140.22***
Time duration (T)	3	128***	58.77***	126.75***	29.31***	84***	27.11***
C×T	15	8.63***	5.57***	10.11***	1.239^{ns}	3.37***	13.16***
Error	48						

*** = significant at <0.001; ns = non-significant confidence levels, respectively.

The highest value that was 31±1.0 µmol/mg protein was observed in roots of *R. mucronata* at 1MHM after 30 days of metals treatment. Statistical analysis expressed that concentration (C), time duration (T) and their interaction (C × T) were significant (P<0.001) differences in CAT at both parts (roots and leaves) of *A. marina* and in roots did not show significant variance in roots of *R. mucronata* (Table 1).

The overall APX activity in both species showed an increasing trend due to MHM stress in reference of control (Fig. 3). Comparatively, *A. marina* showed higher APX activity than *R. mucronata* in both leaves and roots. The highest activity in leaves was recorded 19.130±0.19 µmol/mg protein/min in 20MHM after 30 days of multiple metals stress, where as in roots, it was recorded 19.02±0.087 µmol/mg protein/min in 10MHM after 30 days' period. In *R. mucronata*, the highest values in leaves found 9.35±0.92 µl/mg protein/min after 15 days at 5MHM and in roots the higher activity was recorded 12.7±0.49 µmol/mg protein/min at 10MHM after 15 days of treatment. Statistical analysis expressed that concentration (C), time duration (T) and their interaction (C × T) indicated highly significant (P<0.001) differences in APX at both parts (roots and leaves) of mangrove (Table 1).

In this research, response of *A. marina* and *R. mucronata* were evaluated against induced multiple heavy metals stress such as cadmium, chromium, lead and mercury. The results show increased antioxidant activities in both species, with the exposure of heavy metals reflecting a damage response to stress factors in mangrove species. The activation of CAT, APX and SOD are an essential protective mechanism to reduce oxidative injury in Mangrove plants when exposed to multiple heavy

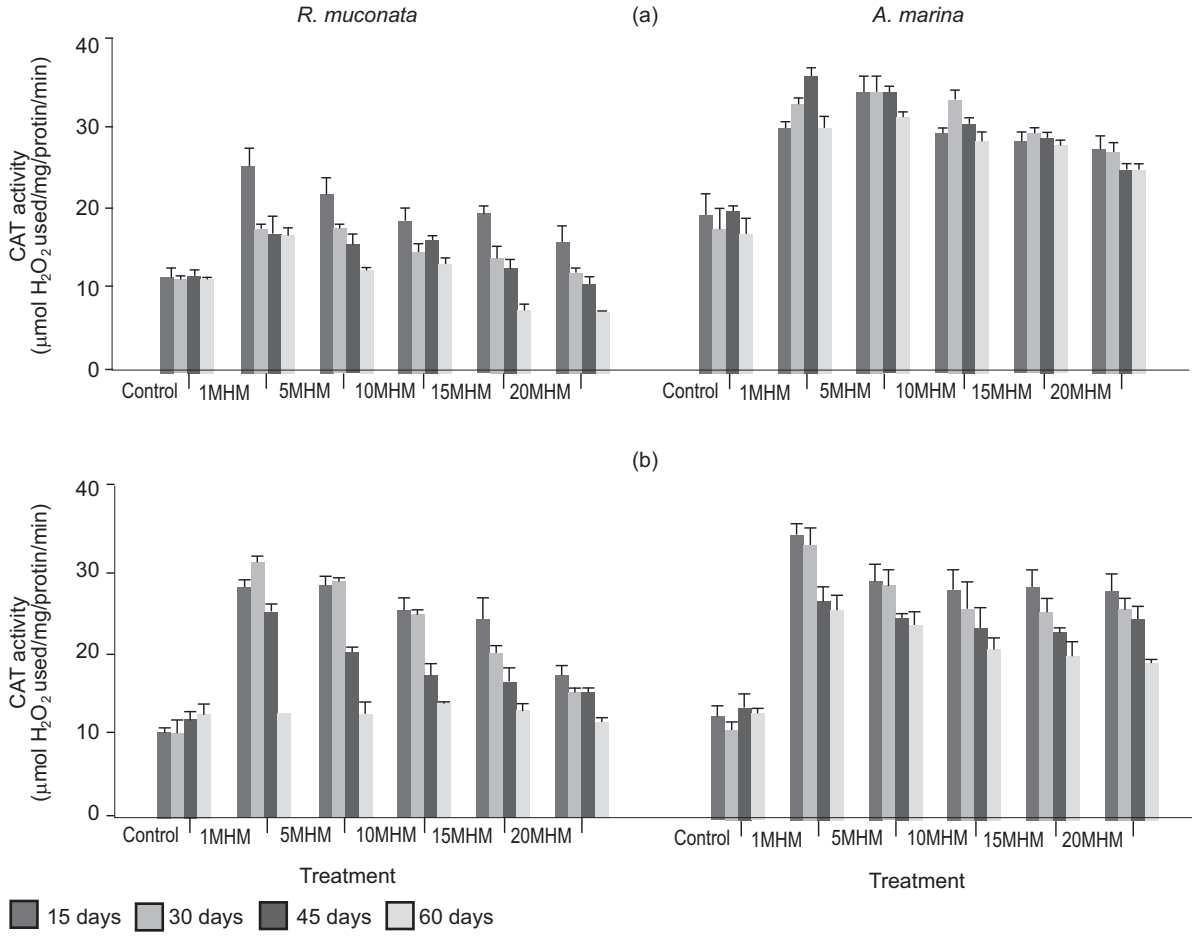

Fig. 2. Alteration in CAT activity in leaves (a) and roots (b) of two Mangroves species by different time periods (15, 30, 45 and 60 days) multiple heavy metals (n = 3, vertical bars represent ± SE).

metals. All three antioxidant enzymes (SOD, APX and CAT) were significantly higher in *A. marina* compared to *R. mucronata*. There is also a marked difference in the antioxidants activities in root and leaves tissues of both the species, however, the disparity was more distinct in *A. marina* indicating that grey mangrove has an efficient antioxidant system.

SOD is the primary antioxidant enzyme that performing in living organisms, as a superoxide radical dismutation into oxygen and H_2O_2 (Zayneb *et al*., 2015). Our investigation showed that SOD enzyme balances the ROS in all concentration levels in leaves and roots throughout the time period of stress from 15 days to 60 days. In leaves of *A. marina*, high SOD activity was observed in prolonged exposure that is 45 and 60 days of interval, whereas an opposite trend was observed in the leaves of *R. mucronata* (Fig. 1). In SOD activity with high concentration of multiple heavy metals and their prolonged exposure, indicating that the oxygen scavenging function of SOD was impaired. These findings are in agreement with the results of *Alyssum* species and *Allium sativum* (Zhang *et al*., 2007; Schckler and Caspi, 1999). SOD activity in roots of *A. marina* peaked at higher metal concentrations than in of *R. mucronata* and the response of *A. marina* was more pronounced, suggesting that increase in SOD has better protection against oxidant damage (Takemura *et al*., 2000; Bowler *et al*., 1992).

For most living cells catalase is ubiquitous, which plays an oxidative catalyst and serves as a main enzyme necessary for ROS detoxification in plants. CAT removes H_2O_2 by decomposing it, into water and oxygen (Lin and Kao, 2000). According to our results, there is enhancement of CAT activity in both species of

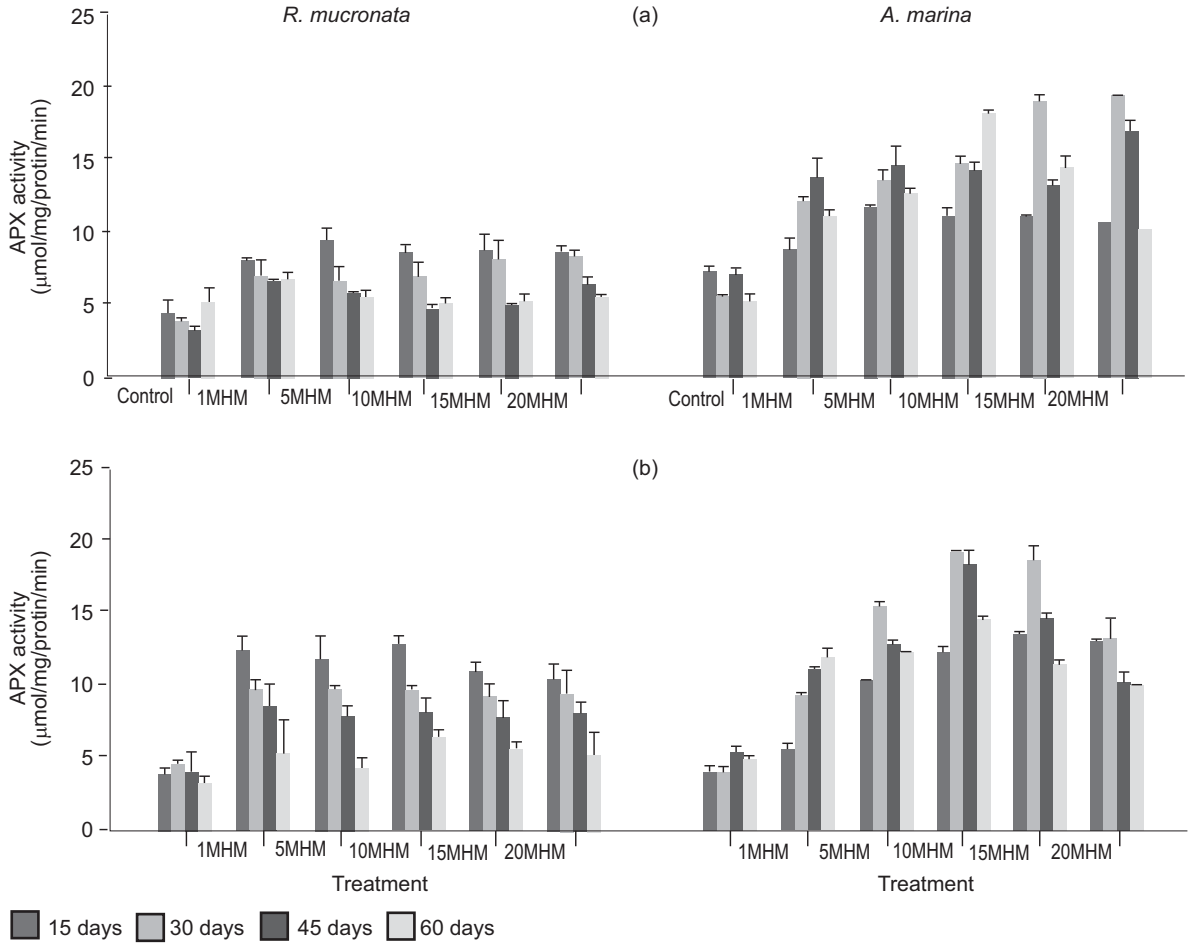

Fig. 3. Alteration in APX activity in leaves (a) and roots (b) of two *Mangroves* species by different time periods (15, 30, 45 and 60 days) multiple heavy metals (n = 3, vertical bars represent ± SE).

mangroves and the catalase activity is a good symbol for reducing cellular damage *via* lower H_2O_2 level (Sakmen *et al*., 2007; Witlekens *et al*., 1995). Similar trend were reported under multiple heavy metals stress in two months old *Kandelia candel* (Huang *et al*., 2010). Different researchers have also confirmed that various antioxidants defences in mangroves are elicited comprising SOD, APX and CAT under abiotic stress. (Hossain *et al*., 2010; Jithesh *et al*., 2006; Takemura *et al*., 2000). Same result was also observed at different concentrations (control, 100 mM, 200 mM, 300 mM) of induced Zn in finger millet at different days of seedling growth (Gonr and Srivastava, 2018).

In *R. mucronata*, CAT activity was higher in roots then leaves, and higher activity (38.3±1.52 µmol/mg proteins were observed after 15 days' interval. In *A. marina*, CAT activity was overall increased in both leaves and roots tissues under heavy metals stress in different interval of time. The higher SOD and CAT activities in *A. marina* signifies that the H_2O_2 scavenging strategy is more efficient than in *R. mucronata*, since CAT activity synchronized with SOD activity play a very important protective role in the O_2 and H_2O_2 scavenging process (Badawi *et al*., 2004; Liang *et al*., 2003). Hence, our results imply that *A. marina* may be more tolerant to heavy metals.

APX is one of the main enzymes for scavenging the poisonous H_2O_2 from the cell, which is widely, distributed antioxidant found in plants (Huang *et al*., 2010; Noctor, 1998). In the current study, APX depicts a trend with initial increase and following decline in response to increment in metal dose level (Fig.3). This shows conformity with previous studies about the APX activities in different plants including mangroves. Enhancement of APX activities were also observed in roots and leaves tissues of both the species which advocates an augment in H_2O_2 amount and their involvement in its removal from the two species *via* AsA-GSH cycle (Yamane *et al*., 2010; Liu, 2007; Karam and Teixeira, 2006; Singh *et al*., 2006) and this shows that induction of defence mechanism adapted by plants in stress environment (Yamane *et al*., 2010; Zhang *et al*., 2007). Whereas (Kisa, 2018; Aghaei *et al*., 2009; Gallego *et al*., 1996) recorded decreased APX activity in plants under various development stage. APX activities in the roots of *A. marina* and *R. mucronata*, increased to their respective control levels which shows similarity with *Kandelia candel* and dis-similarity with *Bruguiera gymnorrhiza* (Zhang *et al*., 2007).

It is worth mentioning that the SOD, CAT and APX to most response towards the treatments against MHM stress also varies with a dose-exposure period. In the case of a dose-exposure experiment under MHM tension, results (Fig 1, 2, 3 and Table 1) reveal that both of the species behaved differently. *A. marina* appeared to with hold the MHM stress for longer period of time then *R. mucronata* at different time periods. It may be a plant endured at one period may be sensitive at other period. It is also explained by (Maksymiec, 2007) that plant varies in response against exposure time of heavy metals and this variation may depict that H_2O_2 accrual developed in a different way during a long stress action, however exact mechanism is not yet known. On the other hand, studies reveal that CAT, SOD and APX activity increases as a first effect of heavy metals due to the increased NADPH oxidase activity and after a longer time decrease in the activity was observed which is connected with attenuation of the enzymatic antioxidative system and increment in the peroxidation of lipids (Maksymiec, 2007; Sandalio *et al*., 2001).

In any case, oxidative stress was established to be obligatory in every heavy metal-treated samples manifest in the course of rising amounts of H_2O_2, with biochemical verification for this induction being supply *via* up regulated action of main ROS metabolizing enzymes such as SOD, CAT and APX. Dismutation of superoxide radicals to H_2O_2 is done by SOD, while CAT and APX act as H_2O_2 scavengers (Gill and Tuteja, 2010). Current findings in mangroves (*A. marina* and *R. mucronata*) are line with numerous other reports which confirmed up-regulated enzymatic activity of SOD, CAT and APX, in tomato, watercress, sunflower (Duman and Fatma, 2010; Chaboute *et al*., 2009; Choudhary *et al*., 2007) signifying the occurrence of a ROS scavenging mechanism in an endeavour of the plants to guard themselves (Georgiadou, 2018). However, in the present study the combined effect of MHM investigated in two different mangroves species which are dominant in Pakistan that ultimately signifies the scavenging activity of ROS.

Conclusion

The outcome of the present investigation clearly concluded that two mangroves species *Avicennia marina* & *Rhizophora mucronata* exhibit distinct variation of

enzymatic antioxidant level when exposed to multiple heavy metals stress during different periods. Nevertheless, in leaves and roots of both the plants, there is synchronised increase in SOD, CAT, APX activities for scavenging the toxic metals. *A. marina* compared to *R. mucronata*, has more tendency as antioxidative defence, to adapt to the oxidative stress induced by heavy metal toxicity as a metal tolerant mangrove species.

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Conflict of Interest. The authors declare no conflict of interest.

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