Biochemical and Physiological Response of *Brassica Juncea* and *Nephrolepis Exaltata* in Mercury Spiked Soil*

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Abstract. The current study sought to investigate the variations in the physiological functions such as Photosynthetic rate, Stomata conductance, Transpiration rate, Total Chlorophyll and the significant role of enzymatic and non-enzymatic antioxidants in eliminating the Reactive Oxygen Species (ROS) generated in response to varying concentration of mercury viz., 0 , 2.5, 5, 10 and 20 mg kg^{-1} in Indian mustard (*Brassica juncea*) and fern *(Nephrolepis exaltata)*. Results revealed a 17.3 and 10.4 per cent reduction in chlorophyll content of Indian Mustard and Boston Fern between the 20 mg kg^{-1} treated plants and the control suggesting reduction in photosynthetic rate of the plant Albeit these parameters were affected, plants tolerated 20 mg kg^{-1} without any visual phytotoxicity symptoms. Gaseous parameters were inversely proportional to the mercury concentration whereas oxidative stress indicators and antioxidant enzymes exhibited a positive correlation. An average increase of 38 per cent Proline was observed in both plants. In *B.juncea* and *N.exaltata*, Average catalase activity and peroxidase activity ascended from 2.35 to 5.12 min^{-1} g^{-1} and 3.26 to 6.80 min^{-1} g^{-1}, and 0.23 to 1.17 min^{-1} g^{-1} and 0.30 to 1.27 min^{-1} g^{-1}, respectively which assures the phytoremediation potential of these plants in mercury contaminated soils.

Index Terms: Phytoremediation, Total Chlorophyll, Gaseous Exchange parameters, Oxidative stress, Enzymatic and Non-enzymatic Antioxidants

I. INTRODUCTION

Entry of heavy metals and metalloids into the environment and their escalating toxicity threatens the stability of the ecosystem. Increased anthropogenic activities have resulted in an uncontrolled and unmonitored release of these pollutants in the ecosystem. With the advancements in the field of Science and technology, several physical and chemical technologies were employed in the remediation of contaminated sites. Unlike the application of physical and chemical approaches currently used in the remediation process, phytoremediation is less expensive, less harmful and efficient in eliminating pollutants which switched the focus of scientific community towards phytoremediation (1). The process efficiency is determined by soil and plant factors. Plant biomass and heavy metal content in various parts of plants are the key factors that influence phytoremediation. Low environmental consequences, simple to operate and can be implemented on a broad scale are the key benefits of this process (2-4).Mercury is a ubiquitous environmental toxin which could pose major health risk. It is easily oxidized to other forms of mercury. High solubility in water and the versatility with which Hg shifts to the gaseous phase reflect the capacity and efficacy of Hg to travel in different environmental matrices and persists in the environment for long periods of time, eventually being deposited in soil or water (5-7). Plants may remove a range of metal ions, including Hg, from their growing substrates. Mercury exposure causes significant phytotoxicity, which is preceded by lipid peroxidation, Proline, and rapid hydrogen peroxide (H_2O_2) build up, as well as the activation of enzymatic and non-enzymatic defence mechanisms (8,9). The level of understanding about the mechanism and extent of Hg phytotoxicity is limited. It is essential to understand and define the magnitude of Hg-induced phytotoxicity because of the recurrence of Hg contamination and also the lack of expertise about the effects of this heavy metal in plants.

The primary response of the plants is to generate reactive oxygen species (2) under any oxidative stress leading to plant growth destruction, inhibition of photosynthesis and biochemical processes. Photosynthetic pigments (chlorophylls and carotenoids) has been affected (10) by the interference of Hg through direct enzyme inhibition (11). As a coping mechanism, plants tend to adopt suited defense such as ligand formation, activation of stress enzyme, proteins and osmolytes etc (12) which entails Catalase, Peroxidase, Polyphenol oxidase, Super oxide dismutase, Glutathione peroxidase and heat shock proteins. Heavy metal toxicity causes a variety of host defensive responses in plants, and their effectiveness varies depending on dosage, plant species, and other factors (13).

In India, Indian mustard is a significant oil seed crop that belongs to the Brassicaceae family. There are currently 400 plant species in the Asteraceae, Caryophyllaceae,

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Brassicaceae, Poaceae, Violaceae, and Fabaceae families that can tolerate extremely high heavy metal levels in the soil. Various literatures have reported Indian mustard as a potential candidate for Mercury and other heavy metal remediation because of its dry matter production and translocation of heavy metals (14-15). Variations in the membrane's lipid composition, combined with increased biomass, make it appropriate for phytoextraction of Hg and other heavy metals like Pb, Ni, Cd, As, and Se with improved removal efficiency (16 ,1 & 17). Mustard has developed a unique defence mechanism in response to heavy metal stress (18). Binding of metal to cell wall, Efflux (17), storage in apoplast (19), conjugation of ionic species and subcellular localization onto the vacuole, Volatilization and Storage in intracellular location (20), release of protective enzymes (21). Pteridophytes or Ferns are non-flowering vascular plants which have been speculated with high potential in remediating heavy metal polluted soils due to their inherent biological characteristics and also add aesthetic value to the site (22). *Pteris vittata* with 2.8% of arsenic in its biomass has been identified as the first arsenic hyperaccumulator. Other ferns were also found to remediate heavy metals, such as *Nephrolepis cordefolia, Hypolepis muelleri, Pteris umbrosa, Pteris cretica,*etc. Ferns are efficient in adapting to metal stress conditions by generation of ROS which resulted in the accumulation of H_2O_2 preceded by scavenging of H_2O_2 by antioxidant enzymes (23). The current study was attempted to learn the ecological response of Indian mustard and Boston Fern under Mercury stress by physiological and biochemical changes.

II. MATERIALS AND METHODS

The current experiment was done in Factorial Completely Randomized Design with two factors (Factor 1 – Plant (P_1,P_2) and Factor 2 – Mercury dosage(T_1, T_2, T_3,T_4,T_5)) which embraces a total of 10 variants. Each treatment was provided in four replicates. Uncontaminated soil collected from Kodaikanal was used for the pot culture experiment and it is spiked with different known concentration of mercury *viz.,* 0 , 2.5, 5, 10 and 20 mg kg^{-1} as mercuric chloride salt on weight basis. The disease-free seeds of *Brassica juncea* var. pusa tarak and 3 months old Boston Fern (*Nephrolepis exaltata*) were obtained from IARI, New Delhi and Grass rootz nursery, Coimbatore, India, respectively. The pots contained soil of 2 kg each. The experiment was carried out for 45 days. Plant samples were collected at definite intervals such as 15th day, 30th day and 45th day after mercury treatment and were analysed for physiological and biochemical parameters. Total chlorophyll in *B. juncea and N. exaltata* was measured using chlorophyll content meter or SPAD meter. Gaseous exchange parameters of plants like photosynthetic rate, vapour pressure deficit, intercellular CO_2 concentration were measured with the help of Portable photosynthetic system, LC pro-SD. The measurement was performed within the time period 10.00-12.00 h maintaining the air temperature and air relative humidity at 25°C and 80-90%, respectively.

The content of Proline was estimated in the sample as defined by Bates et al (1973) at 520 nm. Lipid peroxidation and Hydrogen peroxide was quantified by at 532 nm (Heath and Packer (1968) and 390 nm as per the procedure alluded by Velikova et al. (2000). Catalase and Peroxidase activity was determined at 240 and 420 nm according to the method given by Aebi (1974) and Kar and Mishra (1976), respectively. Experimental results were recorded and statistically analyzed as suggested by Panse and Sukhatame (29). The standard analysis of variance test was performed to compare the treatment means at 5% level of significance using Least significant difference. Pearson correlation and Linear regression analysis was used to assess the influence of mercury concentration on physiological and biochemical parameters.

III. RESULTS

Chlorophyll is an important indicator of photosynthetic potential and sensitive to oxidative stress. Photosynthetic rate and Chlorophyll levels in the leaves of *B. juncea* and *N. exaltata* significantly decreased with increasing Hg concentration compared to control ($F=52.71$,$P<0.05$ and $F=19.41$, $P<0.05$). However, it does not show any visual toxicity symptoms. Average Total Chlorophyll content significantly reduced from 17.70 (T_1) to 16.17 (T_5) and 4.43 (T_5) to 5.47(T_1) and Average photosynthetic rate declined from 9.63 (T_1) to 8.38 (T_5) and 3.12 (T_1) to 2.84 (T_5) in *B. juncea* and *N. exaltata*, respectively (Table 1). Highest chlorophyll content was observed in control plants (26.5 in *B.juncea* and 6.5 in *N.exalata*) and the least recorded in plants treated with 20 mg kg^{-1} treated plants. As far as gaseous exchange parameters are concerned, they are inversely proportional to the increasing mercury concentration except for intercellular CO_2 concentration. Mean Transpiration rate decreased from 3.45 (T_1) to 2.65 (T_5) and 1.14 (T_1) to 1.03 (T_5) and Mean Stomatal conductance recorded from 0.38 (T_1) to 0.41 (T_5) and 0.36 (T_1) to 0.40 (T_5) in *B. juncea* and *N. exaltata*, respectively (Table 2).

Table 1. Effect of increasing mercury concentration on Total Chlorophyll and Photosynthetic rate *B. juncea* and *N. exaltata*

Plant Species	Treatments	Total Chlorophyll				Photosynthetic rate (μ mol CO_2 m^{-2} s^{-1})			
		15 DMT	30 DMT	45 DMT	Mean	15 DMT	30 DMT	45 DMT	Mean
P_1	T_1	10.6	16.0	26.5	17.70	3.76	9.53	15.60	9.63
	T_2	10.4	15.9	25.4	17.23	3.70	8.63	14.80	9.04
	T_3	10.2	16.1	24.8	17.03	3.06	8.73	15.20	9.00
	T_4	9.90	15.2	23.4	16.17	3.45	8.43	14.40	8.76
	T_5	9.90	14.8	23.8	16.17	2.90	8.63	13.60	8.38
	Mean	10.2	15.6	24.8		3.37	8.79	14.72	
P_2	T_1	4.70	5.20	6.50	5.47	1.24	3.52	4.61	3.12
	T_2	4.40	4.60	5.80	4.93	1.16	3.38	4.42	2.99
	T_3	4.50	4.70	6.10	5.10	1.21	3.28	4.31	2.93
	T_4	3.80	4.30	5.50	4.53	1.22	3.21	4.26	2.90
	T_5	3.70	4.40	5.20	4.43	1.18	3.13	4.22	2.84
	Mean	4.22	4.64	5.82		1.20	3.30	4.36	
P	SE(d)	0.039	0.078	0.084		0.016	0.046	0.040	
	CD	0.080	0.161	0.172		0.033	0.094	0.082	
T	SE(d)	0.062	0.124	0.133		0.025	0.072	0.063	
	CD	0.126	0.255	0.273		0.052	0.149	0.130	
PXT	SE(d)	0.087	0.175	0.189		0.036	0.102	0.089	
	CD	NS	0.358	0.386		0.073	0.21	0.183	

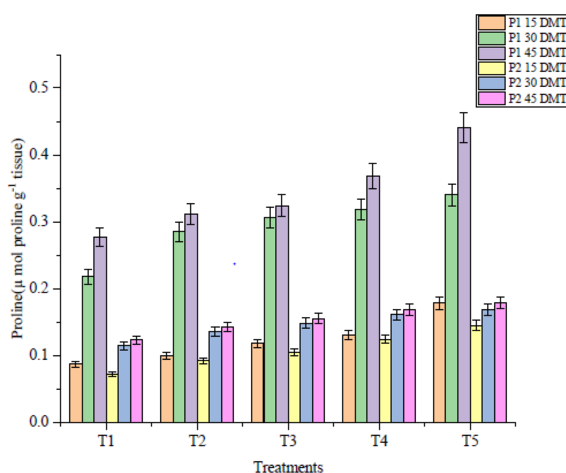
Table 2. Effect of increasing mercury concentration on Transpiration rate and Stomatal Conductance *B. juncea* and *N. exaltata*

Plant Species	Treatments	Transpiration rate (m mol m^{-2} s^{-1})				Stomatal conductance (mol m^{-2} s^{-1})			
		15 DMT	30 DMT	45 DMT	Mean	15 DMT	30 DMT	45 DMT	Mean
P_1	T_1	3.34	3.2	3.81	3.45	0.07	0.45	0.62	0.38
	T_2	3.2	3.15	3.25	3.20	0.07	0.46	0.63	0.39
	T_3	3.12	2.74	2.97	2.94	0.08	0.46	0.64	0.39
	T_4	2.84	2.16	2.38	2.46	0.08	0.45	0.65	0.39
	T_5	2.98	2.36	2.6	2.65	0.09	0.47	0.67	0.41
	Mean	3.10	2.72	3.00		0.08	0.46	0.64	
P_2	T_1	0.98	1.2	1.24	1.14	0.07	0.42	0.6	0.36
	T_2	0.95	1.18	1.21	1.11	0.08	0.43	0.61	0.37
	T_3	0.97	1.24	1.18	1.13	0.07	0.44	0.63	0.38
	T_4	0.94	1.16	1.1	1.07	0.08	0.45	0.65	0.39
	T_5	0.92	1.12	1.04	1.03	0.09	0.45	0.67	0.40
	Mean	0.95	1.18	1.15		0.08	0.44	0.63	
P	SE(d)	0.020	0.015	0.018		0.000	0.002	0.004	
	CD	0.042	0.039	0.036		0.001	0.004	0.009	
T	SE(d)	0.032	0.023	0.028		0.001	0.003	0.007	
	CD	0.066	0.048	0.058		0.001	0.006	0.014	
PXT	SE(d)	0.046	0.033	0.04		0.001	0.004	0.010	
	CD	0.094	0.068	0.082		0.002	0.009	NS	

Plants : P_1 - Indian Mustard , P_2 – Boston Fern
Treatments: T_1 - 0 mg kg^{-1}Hg, T_2 - 2.5 mg kg^{-1} Hg, T_3 - 5 mg kg^{-1} Hg, T_4 - 10 mg kg^{-1} Hg , T_5 - 20 mg kg^{-1} Hg

Average intercellular CO_2 concentration varied from 472 (T_1) to 578 (T_5) ppm and 472 (T_4) to 484 (T_3) was recorded in *B. juncea* and *N. exaltata*, respectively . The ratio of photosynthetic rate to intercellular CO2 concentration is used to calculate carboxylation efficiency. Carboxylation efficiency exhibited a gradual decline ranging from 2.04 (T_1) to 1.41 (T_5) and 0.62 (T_1) to 0.45 (T_5) in *B. juncea* and *N. exaltata*, respectively. However an increasing trend was observed in the analyzed parameters with respect to days after mercury treatment. Proline is generally referred as stress enzyme and a sensitive plant marker of oxidative stress caused by biotic or abiotic factors. Significant difference was observed in the production of proline after 15 days

(F=61.13, P<0.05), 30 days (F=82.76, P<0.05) and 45 days (F=86.83, P<0.05) in response to mercury treatment with highest content of 0.441 µ mol proline g^{-1} tissue in T_5 and the least in T_1 with 0.277 µ mol proline g^{-1} tissue. Mean Proline content, Mean Lipid peroxidation and Mean Hydrogen Peroxide content of 0.19 (T_1) to 0.32 (T_5) and 0.10 (T_1) to 0.16 (T_5) µ mol proline g^{-1} tissue, 0.39 (T_1) to 0.62 (T_5) and 0.13 (T_1) to 0.22 (T_5) µmol g^{-1} fresh weight, 4.31 to 5.79 and 0.44 to 0.54 µmol g^{-1} fresh weight was recorded in *B. juncea* and *N. exaltata*, respectively (Figure 4). Significant parallel changes were observed in antioxidant enzymatic activity between mercury treated *B. juncea* and *N. exaltata* and control (Catalase: After 15 day F=20.61, p<0.05, 30 day F=86.60, p<0.05, 45 day F= 10.70, p<0.05 and Peroxidase after 45 days F=119.96, p<0.05). In *B. juncea* and *N. exaltata*, Mean catalase activity accelerated from 2.35 (T_1) to 5.12 (T_5) min^{-1} g^{-1} and 3.26 (T_1) to 6.80 (T_5) min^{-1} g^{-1}, respectively while mean peroxidase activity increased 0.23 (T_1) to 1.17 (T_5) min^{-1} g^{-1} and 0.30 (T_1) to 1.27 (T_5) min^{-1} g^{-1}, respectively. There was no significant difference in peroxidase generation was observed up to 30 days but 45 days after mercury treatment marked a significant difference. The results of the simple linear regression analysis are listed in Table 3 which reveals the relationship between Hg and the attributes and the per cent variation whereas Table 4 depicts the correlationship among all the variables and reveals the inter relationship among the variables.



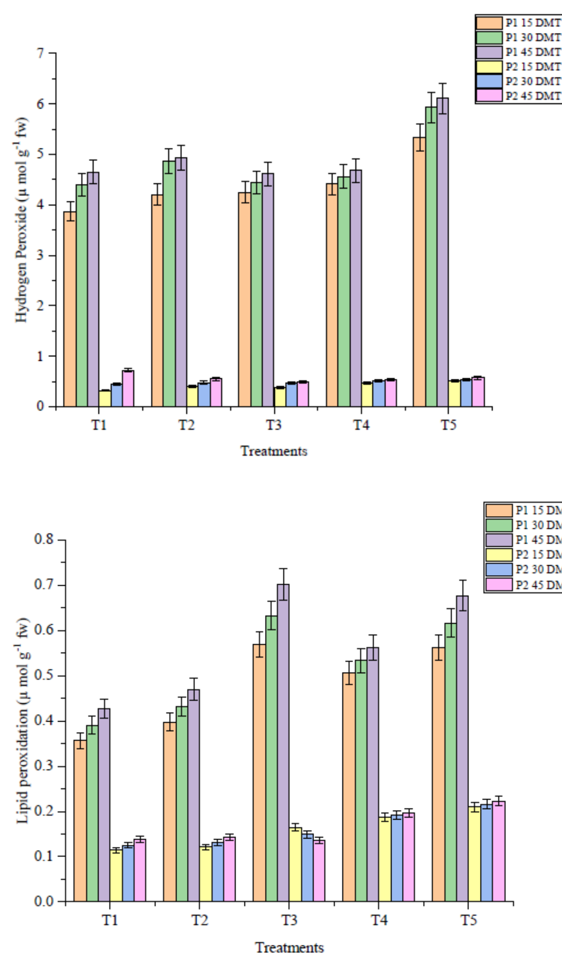


Figure 4. Effect of increasing mercury concentration on Proline, Hydrogen peroxide and Lipid peroxidation in *B. juncea* and *N. exaltata*

Table 3. Linear Regression Model to assess the influence of Hg on Physiological and Biochemical parameters of *B. juncea* and *N. exaltata*

Parameter	Regression Equation		Standard Error		Coefficient R^2	
	Indian Mustard	Boston Fern	Indian Mustard	Boston Fern	Indian Mustard	Boston Fern
Total Chlorophyll	17.46 - 0.086 Hg	5.244 - 0.047 Hg	0.314	0.240	0.86	0.76
Photosynthetic rate	9.360 - 0.053 Hg	3.044 - 0.012 Hg	0.207	0.061	0.84	0.75
Transpiration rate	3.239 - 0.040 Hg	1.137 - 0.006 Hg	0.286	0.016	0.62	0.90
Stomatal Conductance	0.382 + 0.001 Hg	0.366 + 0.002 Hg	0.004	0.006	0.88	0.90

Intercellular CO_2 concentration	570.70-5.664 Hg	485.023 – 0.57 Hg	21.95	1.268	0.84	0.94
Proline	0.208 + 0.006 Hg	0.114 + 0.003 Hg	0.014	0.013	0.93	0.78
Lipid Peroxidation	0.447+ 0.010 Hg	0.127 + 0.005 Hg	0.089	0.010	0.49	0.95
Hydrogen Peroxide	4.243 + 0.068 Hg	0.470 + 0.003 Hg	0.289	0.032	0.81	0.42
Catalase	2.701 + 0.131 Hg	3.730 + 0.167 Hg	0.315	0.445	0.93	0.92
Peroxidase	0.249 + 0.043 Hg	0.338 + 0.048 Hg	0.070	0.053	0.98	0.98

Table 4. Pearson Correlation matrix illustrating the relationship among the variables

	Hg	**TC**	**PR**	**TR**	**SC**	**ICC**	**CE**	**CAT**	**POX**	**PRO**	**LP**	**HP**
Hg	1											
TC	-0.08	1										
PR	-0.08	0.98	1									
TR	-0.16	0.98	0.98	1								
SC	0.83	0.38	0.38	0.32	1							
ICC	0.47	0.55	0.54	0.44	0.55	1						
CE	-0.23	0.97	0.97	0.98	0.25	0.35	1					
CAT	0.82	-0.57	-0.57	-0.62	0.52	0.07	-0.69	1				
POX	0.98	-0.23	-0.23	-0.31	0.76	0.40	-0.38	0.91	1			
PRO	0.45	0.82	0.81	0.74	0.76	0.84	0.68	-0.06	0.32	1		
LP	0.27	0.9	0.89	0.85	0.63	0.82	0.78	-0.23	0.13	0.96	1	
HP	0.30	0.98	0.98	0.96	0.43	0.63	0.93	-0.50	-0.17	0.85	0.95	1

IV. DISCUSSION

Plants use a variety of mechanisms to regulate heavy metal levels in accordance with changes in trace metals in the environment, including phytohormones, osmolytes, and antioxidant enzymes. (30). When the detoxification potential of the plants is less than the accumulation, then it is toxic to plants (31 & 32). With increasing Hg doses, photosynthesis impairment and fall in gaseous exchange measurements were observed. When garden cress was exposed to heavy metal, similar results were recorded (33). It could be because Mercury inhibits Fe and induces chlorosis in leaves, which has a deleterious effect on chlorophyll metabolism. Heavy metal toxicity reduces micronutrients, which are necessary for plant growth and development. As a result of the metal stress, the pigment level decreases which is one of the primary causes of photosynthesis impairment. These findings are consistent with those of Januskaitiene (34), who found that with heavy metal stress, physiological functions got reduced in pea plants. Hg, both organic and inorganic, has been shown to inflict potassium, magnesium, and manganese depletion, as well as iron accumulation (6). In certain cases, parts of chlorophyll can be transformed to pheophytin. Sanmartin *et al.* (35) reported that chlorophyll degradation results in the formation of pheophytins by the loss of magnesium ions. Pheophytin build up and oxidative stress have been seen in plants subjected to high quantities of trace elements (36 & 37). Heavy metal toxicity resulted in a decreased carbon assimilation due to disruption of chloroplast structure and reduced Photosytem II photochemical efficiency, which affects plant development (38 & 39).

The production of reactive oxygen species is the basic mechanism of plants exposed to stress. Reduced forms of atmospheric oxygen are ROS intermediates (O_2). Excitation of oxygen results in singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical, and hydroxyl radical (40-42). With higher Hg dosages, the level of H_2O_2 likewise increased in the current study. This could be mostly due to membrane instability in plants subjected to increasing metal stress. Oxidative stress or Haber-Weiss processes produce reactive oxygen species (ROS). In plant cells, ROS formed as a result of oxidative stress induces a range of negative effects, including photosynthetic inhibition, ATP inhibition, lipid peroxidation, and DNA damage (31 & 43). Inordinate accretion of free radicals has been linked to mercury-induced plant cellular oxidative damage. As a signal molecule, H_2O_2 is essential for plant development and resilience, but excessively H_2O_2 with ROS damages membrane lipids. TBARS can be utilised as a marker of lipid peroxidation in tissues since they are generated

when certain primary and secondary lipid peroxidation products breakdown. Mercury exposure resulted in a substantial accumulation of H_2O_2 but had no effect on TBARS, according to statistical analysis (44-45). Plants possess both enzymatic and non enzymatic defense mechanism to tolerate any abiotic stress. Free radicals are scavenged by a variety of antioxidative enzymes. Stress protecting proteins, such as heat shock proteins, also protect plants from oxidative damage. (46). Plants develop a variety of defence responses in response to heavy metal toxicity, but their effectiveness is dependent on doses, plant species, and other factors. Plants' ability to mitigate heavy metal toxicity or to endure stress helps them to thrive under such environments (47,48). Similarly, metal treatment induced increased activities of catalase and peroxidase enzymes, which aided in the scavenging of free radicals in the current study. These findings align with those of Doganlar *et al.* (49).The plant's antioxidant capacity was increased in a dose-dependent manner. Catalase directly scavenges H_2O_2 and converts it to H_2O and O_2. Peroxidase enzymes scavenge H_2O_2 by combining it with antioxidants such as ascorbate (50,51), lignin precursors, or secondary metabolites. (52). As the concentration of Hg in the plant tend to increases, plant cells generate greater amounts of those enzymes (53,12).

V. CONCLUSION

Since mercury is a critical pollutant, several studies has been carried out to get insights into the ecotoxicity of mercury. This study documents a reduction in the physiological functions (Photosynthetic and Gaseous exchange parameters) in *B. juncea* and *N. exaltata* with increasing Hg concentration leading to slower metabolism in association with various factors and development of antioxidant defense system against ROS generation. Even though ROS has an indispensable role in plant system (For instance, as signal molecules for stomatal closure), generation of larger quantity would result in phytotoxicity. However *B. juncea* and *N. exaltata* exhibited tolerance up to 20 mg kg^{-1} without any toxic symptoms which might be due to the antioxidant defense system. In addition, Proline significantly increased from 0.27 (control) to 0.44 (20 mg kg^{-1}) and 0.12 (control) to 0.18 (20 mg kg^{-1}) μ mol proline g^{-1} tissue in *B. juncea* and *N. exaltata* which acts as an osmoprotectants. While comparing, Proline, Catalase and Peroxidase was higher in *B. juncea* than *N. exaltata* which highlight the ability of *B. juncea* to tolerate the Hg contaminated Soil.

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