Accumulation of Cadmium in Transplanted Lichen *Pyxine cocoes* (Sw.) Nyl., with Reference to Physiochemical Variation and Kinetics of Cadmium Biosorption

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Abstract

The present study aims to signify the role of *Pyxine cocoes* (Sw.) Nyl. (*P. cocoes*) as cadmium (Cd) biomonitor in atmosphere. This was achieved by quantifying the amount of Cd accumulated in transplanted *P. cocoes*, when stimulated with known concentrations of Cd (5μM, 50μM, 100μM, 150μM and 200μM) at increasing intervals of time up-to 40 days. All the five concentrations exhibited increasing trend of accumulation with time. As depicted by Pearson's Correlation (at $p < 0.001$), anti-oxidative enzymes (superoxide dismutase r= -0.812, ascorbate peroxidase r= -0.802, catalase r= -0.757) and electrical conductivity (r = 0.693) were the most efficient parameters to depict increased Cd presence in atmosphere. In the current study, accumulation of Cd by transplanted lichen has been first time analyzed by biosorption kinetics. The uptake of Cd by *P. cocoes* followed pseudo-second-order kinetics (range of R_2^2 value was 0.969–0.998). The marker parameters in combination with the ability to accrue Cd fortifies *P. cocoes's* role as a biomonitor.

Introduction

The role of lichens as biomonitor was reported as early as in the year 1866 by Nylander. Over the years lichens biomonitoring has gained popularity among scientists as a better mode of investigating noxious waste in atmosphere, since it is an ideal technology that utilizes inexpensive and easily available wide variety of lichens, which are relatively easier to implement as it requires lesser labor, equipment and minimal maintenance (Lopez Berdonces et al. 2016; Sari et al. 2007; Garty 2001). Lichen is a natural alliance of two organisms, i.e., (i) photobiont- cynobacteriae or chlorophyceae and (ii) mycobiont- ascomycetes (Calcott et al. 2018).

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Lack of waxy cuticle and roots, high surface to volume ratio, wide intercellular space and slow growth rate of lichens along with dependency on atmosphere to acquire nutrients makes it a remarkable biomonitor of heavy metals (Seminara et al. 2018). Cadmium (Cd) belongs to group I carcinogen and is added in the atmosphere by natural processes and anthropogenic sources (IARC 2012; WHO 2010). Long ranged transport of Cd by wind and its persistence in environment due to non-degradable nature increases its threat quotient multifold (Orlowski and Piotrowski 2003; Bernanrd 2008). Since Cd cannot be neutralized by metabolic degradation and has no essential biological role, it becomes toxic to humans and is also responsible for hepatitis, pulmonary edema, hemorrhage, lung cancer and renal cancer leading to mortality (ATSDR 2012). First step to pollution abatement is its monitoring and assessment.

Most of the previous studies on monitoring of pollutants using lichen were conducted by immersing the plant in the heavy metal solution which affected physiochemical parameters (Bačkor et al. 2007a; Bačkor et al. 2007b; Bačkor et al. 2010). To determine the biosorption capacity of biosorbents, the kinetic study is an integral part. For the liquid-solid adsorption system, Lagergren's kinetics equation describes

the adsorption of liquid-solid systems based on solid capacity (Lagergren 1898). The Lagergren's kinetics has been used to find the kinetic study in lichens for biosorption of methylene blue (Koyuncu and Kul 2020), fluoride (Mondal and Kundu 2016), antimony (Ulouzlu et al. 2010) and nickel and lead (Sari et al. 2007).

All previous studies on metals uptake kinetics by lichen is based on immersing lichen in solution, however, in our study different concentrations of Cd are sprayed on *Pyxine cocoes* (Sw.) Nyl. (*P. cocoes*), thus measuring its ability to accumulate Cd at varying concentrations and its usability as Cd monitor from atmosphere. The accumulation mechanism was then analyzed by applying biosorption kinetics. The present research work is also conducted with an aim to gauge the physiochemical response (chlorophyll content, cell membrane integrity, protein, enzymatic activity of superoxide dismutase, ascorbate peroxidase and catalase) that was augmented by spraying increasing concentration of Cd on transplanted *P. cocoes* at 10, 20, 30 and 40 days of interval.

Materials and Methods

Pyxine cocoes (Sw.) Nyl., collected from bark of *Mangifera indica* trees growing in Tokhaiedih area of Bahraich district of Uttar Pradesh (27°54'47.2"N 81°40'01.7"E) was chosen for current study. The collection site was situated in a mostly pollution free area away from any of the direct pollution sources and thought to be clean with respect to air pollution. Lichen samples collected from Tokhaiedih, Bahraich district, were analyzed for Cd to represent the background metal concentration, where the Cd was recorded as 0.173 ± 0.037 μg g^{-1}. The healthy thalli of *P. cocoes* along with barks were fixed on cardboard and were hung on trees in the garden of the Institute of Engineering and Technology, Lucknow to provide natural environmental conditions to lichens. In order to study the impact of different concentration of Cd, thalli were sprayed with 5μM, 50μM, 100μM, 150μM and 200μM CdSO$_4$ treatment solution. The quantity of sprayed solution per treatment per day was equivalent to 2 ml. The volume was based on the quantity to evenly moist the surface area (25×35 cm^2) of the lichen. Control sample were sprayed with HPLC grade water (deionized water). The solution was sprayed on transplanted lichen for 40 days. Further, the harvesting of the sample was done on 10th, 20th, 30th and 40th day for the analysis.

The cadmium concentration in lichen was estimated following the method of Bajpai et al. (2015). In brief, the lichen thalli (0.50 g) were oven (60 °C) dried to a constant weight. After drying, the lichen samples (triplicates) were digested in a mixture of concentrated HNO$_3$ and HClO$_4$

(9:1 v/v). The digested samples were filtered using Whatman filter paper no. 42 and diluted with MilliQ water. For quality control and assurance of the digestion method, one set of sample (n=3) was spiked with certified reference material of mix metals containing Cd (CRM, Periodic table mix 1 for ICP, Supelco, USA. Product No. 92,091, Lot No. BCCF2111). The metals concentrations were analyzed using ICP-MS (Perkin Elmer SCIEX ELAN DRCe). The Quality control of ICP-MS were maintained as per the requirements of the National Accreditation Board for Testing and Calibration Laboratories (NABL; CSIR-NBRI certification no. T-1381). The calibration and quality assurance for each analytical batch were ensured by repeated analysis (n=3) of CRMs. The recovery of the method was estimated and the difference was added in the final calculation of the samples.

The cell membrane integrity was estimated following the method of Marques et al. (2005). The thalli were soaked in deionized water (10ml) for 1 h. The electrolyte conductance was measured using conductivity meter (Crison Basic 30) to measure electrolyte leakage into the solution. Catalase (CAT) activity inside the thalli of the treated lichen were estimated following the method of Cakmak and Marschner (1992). Superoxide dismutase (SOD) activity, was measured using the method formulated by Beyer and Fridovich (1987). Ascorbate peroxidase (APX) activity, was detected spectrophotometrically at 290 nm (Nakano and Asada 1981). Chlorophyll a, b and total chlorophyll was quantified using method developed by Arnon (1949), and total carotenoid content was measured by Parsons et al. (1984) method. Protein was estimated by Lowry's method (Lowry et al. 1951).

The data was expressed as mean with standard deviation. One-way ANOVA followed by Tukey test was applied at $p \leq 0.05$ to evaluate significant difference between duration and treatment effects. Pearson's correlation was applied between Cd accumulation and physiological parameters.

Results and Discussion

Estimation of Total Cd in Treated Thalli

During the study period Cd got increasingly accumulated in thalli in dose and time dependent manner (Fig. 1), with no visible symptom on its external thalli (Supplementary Fig. 1). The deposition ranged from 1.87μgg^{-1} to 207.68 μgg^{-1}dry weight (DW). Since the accumulation was most pronounced at 150μM concentration, it indicated threshold limit. The ability of *P. cocoes* to accumulate Cd on broad range makes it an ideal lichen for biomonitoring of atmospheric Cd contaminant. Cd accumulation by *P. cocoes* at 200μM of treatment showed increasing trend but the amount

Fig. 1 Accumulation of Cd ($\mu g g^{-1}$ DW) by *Pyxine cocoes* at 10, 20, 30 and 40 days when sprayed with different concentrations of Cd. Significant difference due to concentration of Cd treatment (uppercase alphabets) and duration of treatment (lowercase alphabets) is denoted by different alphabets ($p \leq 0.05$)
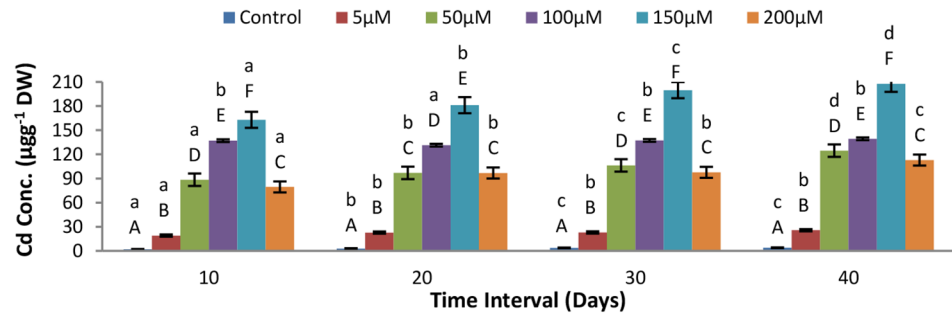
Table 1 Pseudo-first and second-order kinetics parameters obtained from biosorption of Cd onto *Pyxine cocoes* at different treatment concentrations

Conc.	Pseudo-first-order			Pseudo-second-order		
(μM)	$k_1 (days^{-1})$	$Q_e (\mu g g^{-1})$	R_1^2	$k_2 (g/(\mu g\ days))$	$Q_e (\mu g g^{-1})$	R_2^2
Control	-0.0351	3.84	0.2401	0.008	6.04	0.9910
5	-0.0574	25.67	0.9093	0.007	28.33	0.9899
50	-0.1117	124.60	0.7551	0.001	142.86	0.9696
100	-0.0384	139.31	0.3347	0.009	140.85	0.9984
150	-0.1262	207.68	0.9303	0.001	232.56	0.9981
200	-0.1058	112.89	0.7823	0.001	126.58	0.9823

stored was less than the uptake reported at 50µM. Reduction in Cd uptake at 200µM can be attributed to re-release of the trace metal into the environment instead of permeation into the cells. Reis et al. (1999) suggested that accumulation in lichen is a dynamic process, which involves uptake and release of heavy metal in environment. Loppi et al. (2020) observed that samples of *Evernia prunastri* treated with higher concentration (100µM of copper) released copper in significant quantity from intracellular site which reduced the total accumulation. Reduced uptake of Cd at 200µM treatment could be due to damage of plasma membrane during the initial Cd treatment which is a site for extracellular uptake, so the accumulation recorded at this concentration was only of intracellular uptake (Beckett and Brown 1984). This can be backed by maximum reported electrolyte conductivity at this concentration which suggests damage of cell membrane integrity.

Kinetics of Cd Biosorption by Pyxine Cocoes

The biosorption kinetics is most frequently used for controlled aqueous systems; however, an attempt has been made to apply the biosorption kinetic model in the present *in-vivo* study.

Two kinetic models, i.e., Lagergren's pseudo-first-order and pseudo-second-order models, were applied to elucidate the biosorption kinetics followed by *P. cocoes* to accumulate Cd. The validity of kinetic models was assessed by regression coefficient (R^2). The pseudo-first-order rate equation is represented in its linearized form (Eq. 1) as follows (Lagergren 1898):

$$\ln (q_e - q_t) = ln\ q_e - k_1 t \tag{1}$$

The plot between $\ln(q_e - q_t)$ versus t was used to determine biosorption rate constants (k_1, $days^{-1}$) of pseudo-first-order equation, where q_e (µg/g) is the amounts of the Cd ions biosorbed at equilibrium, q_t (µg/g) is the quantity of the Cd biosorbed at t (days).

The linearized form of pseudo-second-order kinetic model (Eq. 2) was also employed to investigate the results (Ho and McKay 1999):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \tag{2}$$

Where, q_e is the quantity of Cd ions biosorbed at equilibrium, q_t (µg/g) is the amount of Cd ions biosorbed at time t (days) and k_2 [g/(µg days)] is pseudo-second-order equation's rate constant for the biosorption of Cd ions onto *P. cocoes* thallus. To determine k_2, a graph was plotted between t/qt and t. The values of k_1, k_2, R_1^2 and R_2^2 is summarized in Table 1.

Kinetic model with higher regression coefficient (R^2) value is considered the best fitting model, implying that the adsorption mechanism is correlating highest with that biosoption kinetics. The range of R_1^2 (for pseudo first order kinetics) in this study was 0.240–0.930 and R_2^2 (for pseudo second order kinetics) value was 0.969–0.998 (Table 1), this advocates that biosorption of Cd on *P. cocoes* predominantly follows pseudo-second-order kinetic model. Chemisorption is the rate limiting step in pseudo-second-order kinetic model, which means that adsorption rate does not depend on Cd concentration but depends on adsorption

capacity of the lichen. Furthermore, in this study, increase in electrical conductivity can be attributed to chemisorption and decrease in enzyme activity is caused by effect exerted by the chemisorption of Cd. This outcome is similar to the result obtained by Tay et al. (2009), where biosorption of Cd on *Ramalina Fraxinea* followed pseudo-second-order kinetic model. Similarly, biosorption of lead and nickel ions onto *Cladonia furcate* followed pseudo-second-order kinetic model (Sari et al. 2007).

Cell Membrane Integrity

In the present study, electrolyte conductivity significantly increased ($p<0.05$) when exposed to increasing concentration of Cd, and highest value obtained was $39.25 \mu Scm^{-1} g^{-1}$ (Fig. 2a). Cell wall being the first line of defense (Sanità di Toppi et al. 2008) can intercept trace metals on it (Di Toppi et al. 2005). Damage to cell membrane integrity in the present study was observed to be the earliest indicator of trace metal tension, this is similar to study conducted by Yemets et al. (2015) on *Parmelia sulcata*, *Lobaria pulmonaria* and *Xanthoria aureola*, where electrolyte leakage increased at higher concentrations of heavy metals, without significant increase in photo system two efficiency (F_v/F_m).

The study showed that there was strong positive correlation ($R^2 = 0.693$, $P < 0.001$) between Cd uptake and electrical conductivity (Table 2). Similarly, Osyczka and Rola (2019) discussed a positive correlation between intracellular heavy metal uptake, cell membrane integrity and electrical conductivity. From our observation it is clear that electrical conductivity method is a sensitive parameter for detecting Cd stress in *P. cocoes* and can consequently serve as an early warning parameter.

Photosynthetic Pigments, Carotenoids and Protein

Photosynthetic pigments (chlorophyll a, chlorophyll b and total chlorophyll) are classical parameters that are invaluable indicator of stress in photoautotrophs. Exposure to different concentrations of Cd revealed that photosynthetic machinery of *P. cocoes* was stressed by Cd, and depicted a negative Pearson's correlation with Cd treatment (Table 2). However, the effect of Cd on photosynthetic machinery was less pronounced compared to anti-oxidative enzymes and cell membrane integrity. According to Gonzalez and Pignata (1994) lichen thalli subjected to pollution experienced chlorophyll degradation at slower pace than other physiological damage, which could be due to the fact that maximum amount of heavy metal (Cd) is impeded by cell wall. Similarly, *Xanthoria parietina* defended against Cd stress by accumulating most quantity of Cd at cell wall and penetrated amount of Cd got internally stored in concentric

bodies and vacuoles of mycobiont and chloroplasts of photobiont (Di Toppi et al. 2005; Sanità di Toppi et al. 2005).

Carotenoid, an accessory pigment for photosynthesis, provides membrane stability and protection against photo oxidative damage being a natural antioxidant. Carotenoid content had a positive Pearson's correlation with photosynthetic pigment, Cd treatment and protein (Table 2). Carotenoid content reduced when exposed to lesser heavy metal pollution or for shorter period, and it increased when the exposure period was prolonged or pollution increased (Bajpai et al. 2015; Garty 2001). Increase in carotenoid content can also be due to rainfall, as it has been reported that β-Carotene concentrations increased in thalli of *Lobaria pulmonaria* when subjected to rehydration (Kranner et al. 2003).

Similar to photosynthetic pigments, protein content on 10th day was more in control and it decreased from 10th to 20th day (Supplementary Table 1). The initial decline in protein suggests that *P. cocoes* suffers from Cd stress. Quantity of protein increased after 30th day. When first line of defense was no longer effective in presence of highest Cd concentration after long exposure, *Trebouxia impressa* cell elevated stress protein production as second defense mechanism (Sanità di Toppi et al. 2008). *P. cocoes* in response to heavy metal displayed increase in protein content; and highest value of chromium and arsenic corresponded to highest value of protein (Bajpai et al. 2015, 2012), which is analogous to present observation. Studies have shown that Cd exposure to lichen invokes protein production to detoxify heavy metal stress (Bačkor et al. 2006). Positive Pearson correlation ($p \leq 0.001$) was observed between protein, carotenoid, chlorophyll a and b (Table 2). Protein also had a positive correlation with Cd treatment, with which we can infer that toxic effect of Cd was overcome by production of stress protein and ROS generated was combated by carotenoid. During treatment, there was rainfall after 20th day, which could have improved metabolism and hence amplified protein production in the process.

Enzyme Activity

Superoxide Dismutase activity declined with increase in Cd concentration as shown in Fig. 2(b), and negative correlation was observed between Cd accumulation and superoxide dismutase activity at $p \leq 0.001$ (Table 2). The highest SOD activity of $70.23 \mu gg^{-1}$ fresh weight (FW)/protein was observed in control on 10th day and lowest activity of $19.09 \mu gg^{-1}$ FW/protein was recorded in $200 \mu M$ on 10th day.

Primary enzyme of anti-oxidative mechanism is SOD (metallo-isoenzymes) that dismutase O^{2-} to H_2O_2 which is an initial step in protection from ROS. Cadmium has a

strong affinity for sulfhydryl group, and plays an indirect role in the production of Reactive Oxygen Species (ROS) since it is not a Fenton-like metal. Similar to the observation carried out by Cardinaels et al. (1984) and Zhang et al. (2007), the present study also showed inhibition of SOD associated with Cd accumulation, which indicates that Cd has inhibitory effect on anti-oxidative mechanism, and can be attributed to enzyme damage due to the replacement of metals from their catalytic sites or the excessive production of free radicals and peroxides. Similarly, SOD depleted in response to Cd exposure in *Xanthoria parietina* (Di Toppi et al. 2005).

Ascorbate peroxidase activity decreased as the concentration dose of Cd increased, shown in Fig. 2(c). APX had a negative correlation with Cd concentration dose and a positive correlation with SOD ($r = 0.967$) and CAT ($r = 0.902$) at $p \leq 0.001$ as shown in Table 2. Similar to our results, APX activity reduced for increasing arsenic concentrations and duration of treatment in transplanted *P. cocoes* (Bajpai et al. 2012). Cd binds to thiol groups and inactivates APX since it's a thiol-containing enzyme and inhibits the enzymatic systems involved in H_2O_2 removal.

Catalase activity shared a negative correlation with Cd uptake as shown in Table 2 ($p \leq 0.001$). Comparable to APX and SOD, CAT activity declined with Cd dose (Fig. 2d). According to Bajpai et al. (2012), CAT activity in *P. cocoes* deteriorated as soon as the thalli were exposed to arsenic treatment. CAT activity also declined at higher copper concentrations in *Dermatocarpon luridum* and the deterioration was pronounced when exposure time increased to 48 h (Monnet et al. 2006). The decrease in CAT activity can also be due to inhibition of its synthesis in presence of heavy metal (Dat et al. 2000).

H_2O_2, a free radical, generated as a result of SOD activity acts as a signal to activate enzymatic defense of APX and CAT (Prasad et al. 1994; Karpinski et al. 1999). This ties well with our result, since H_2O_2 production declines with decrease in SOD activity, hence reducing the induction of APX and CAT.

In the present study, accumulation of Cd affected physiological and biochemical line of defense in *P. cocoes* as damage to cell membrane and stress on enzymatic machinery was observed. *P. cocoes* can be used for Cd air monitoring
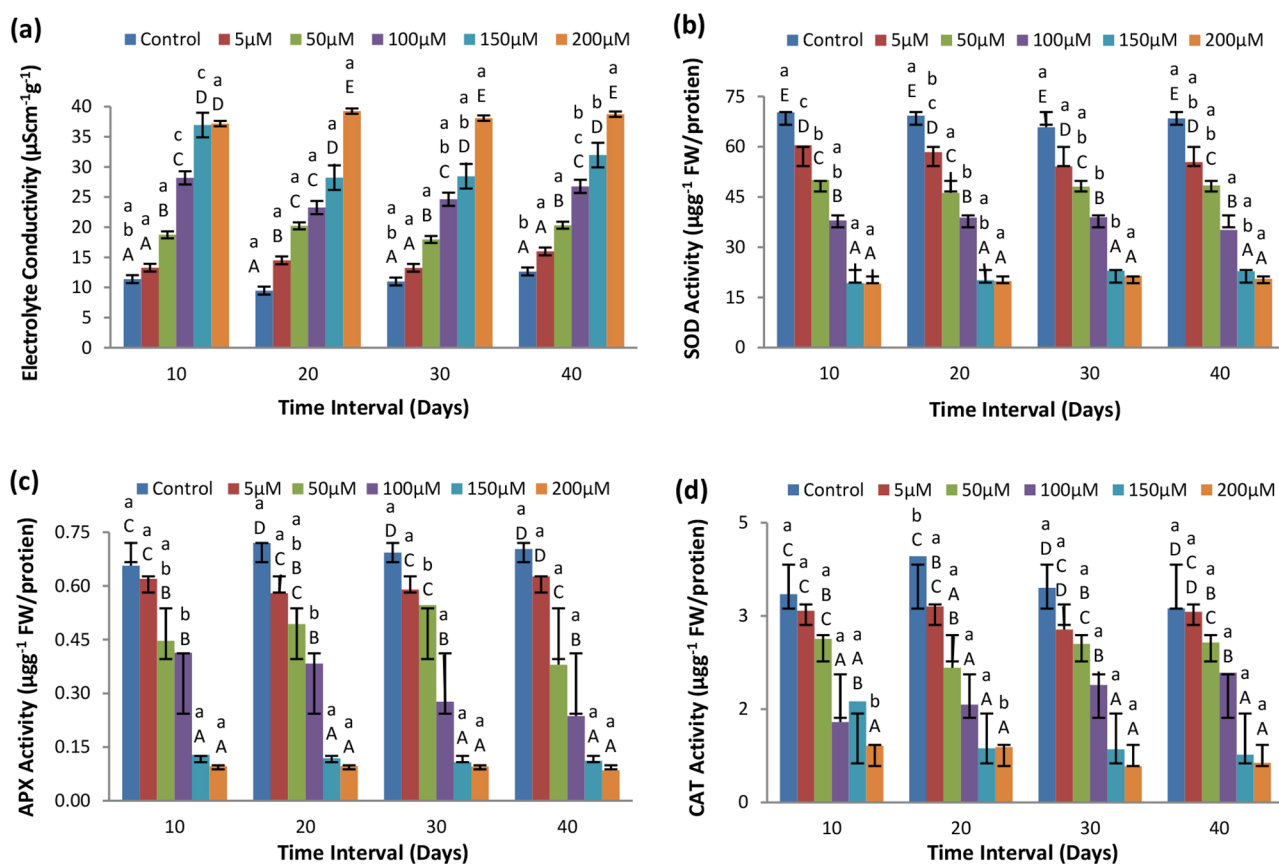
Fig. 2 Cell membrane integrity (a) and enzyme activity of SOD (b), APX (c) and CAT (d) measured in *Pyxine cocoes* thalli exposed to different concentration of Cd for 40 days. Significant difference due to concentration of Cd treatment (uppercase alphabets) and duration of treatment (lowercase alphabets) is denoted by different alphabets ($p \leq 0.05$)

Table 2 Matrix representing Pearson's correlation (r) between photosynthetic pigments, protein, cell membrane integrity, enzymes and Cd uptake in *Pyxine cocoes*

	Chl a	Chl b	Tot Chl	Caro	Pro	CMI	SOD	APX	CAT	Cd
Chl a										
Chl b	0.769***									
Tot Chl	0.958***	0.920***								
Caro	0.612***	0.944***	0.798***							
Pro	0.505***	0.872***	0.700***	0.953***						
CMI	-0.340**	0.109	-0.160	0.305**	0.335**					
SOD	0.313**	-0.141	0.130	-0.330**	-0.345**	-0.952***				
APX	0.307**	-0.128	0.132	-0.321**	-0.341**	-0.935***	0.967***			
CAT	0.271*	-0.167	0.092	-0.327**	-0.362**	-0.889***	0.919***	0.902***		
Cd	-0.366**	-0.038	-0.242*	0.117	0.205	0.693***	-0.812***	-0.802***	-0.757***	

Statistical significance of correlation at: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Chlorophyll a- Chl a; Chlorophyll b- Chl b; Total Chlorophyll- Tot Chl; Carotenoid- Caro; Protein- Pro; Cell Membrane Integrity- CMI; Superoxide dismutase- SOD; Ascorbate peroxidase- APX; Catalase- CAT; Cadmium- Cd

since it was able to accumulate different levels of Cd with detectable effects on its physiological parameters.

Conclusion

It is well evident from the present study that *P. cocoes* accumulated Cd in a dose and time dependent fashion. Our novel attempt to analyze increasing Cd accumulation in transplanted lichen by biosorption kinetics provided a beneficial insight on the accumulation pattern by *P. cocoes*. The findings indicated that the uptake of Cd by *P. cocoes* followed pseudo-second-order kinetics. Cell membrane being the first barrier against Cd suffered damage, and proved to be a potent physiological indicator parameter. Enzyme activities, portraying a negative trend at all concentrations, can be used as a marker for Cd presence in *P. cocoes*. Pigments and protein followed a similar trend in same treatment range, which indicates interdependency among them. Initial decline in pigments and protein quantity followed by an increase after rain suggests that even though lichen experienced Cd stress, it was able to persevere under favorable environmental conditions. The study carried out strengthens the hypothesis that *P. cocoes* can be used as a tool to biomonitor airborne Cd.

Declarations

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