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RESEARCH ARTICLE

Sensitivity of two mosses against short-term visible changes in ambient air quality using physiological parameters

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Abstract Recently, air pollution existed as a major affair across the globe due to expeditious development activity vide acute urbanization and industrialization. Increment in pollution level serves as a major factor in disturbing the air quality of surrounding areas, which thus necessitates the study of such changes on various groups of plants, to mark them as the best bio-monitors. The diversity of habitats coupled with their structural simplicity and ability to accumulate pollutants suggests the utilization of bryophytes as potential bioindicators of air pollution. In this work, we have evaluated the effect of changing levels of different gaseous pollutants in form of smog, on antioxidative enzyme activity and pigment system of two mosses: *Semibarbula orientalis* (Web.) Wijk. & Marg. and *Physcomitrium eurystomum* Sendtn., continuously for seven days. Results demonstrated that catalase and superoxide dismutase activity enhanced as the concentration of nitrogen oxides and particulate matter increased in the atmosphere, whereas the activity of peroxidase was greatly influenced by sulphur dioxide. Chlorophyll contents along with carotenoids were found to be damaged by pollutants like sulphur dioxide. Our results suggest that these mosses can act as day-to-day indicators as well as immediate reflectors of changes in ambient air quality and have the potential to be a reliable biomonitoring system.

Key words: Antioxidants, biochemical parameters, biomonitoring, environmental changes.

Introducation

Development-related activities of mankind have increased the level of industrialization, urbanization, population growth, and economic development followed by a consequent rise in vehicular traffic, which has led to elevated levels of pollutants in the atmosphere. In almost all the towns and cities in India, the environment has reached its carrying capacity in terms of air pollutants, posing a very serious problem for human health. According to the Central Pollution Control Board report (2017), changes in air quality due to various air pollutants also affects the functioning of the ecosystem and may lead to changes in the earth's climate in form of warming.

It has been considered that every living form shows responses against environmental changes, and naturally occurring bioindicators serve as an important tool for detecting changes in air quality. In relation to cryptogams, bryophytes are considered as one of the most victorious groups of cryptogamic plants in relation to angiosperms, as assessed by their number and geographical distribution. Being poikilohydric in nature, they are dependent on their environment for the continuous availability of moisture, as for water loss regulation, these plants don't have any alternates (Proctor 2011). Due to this continuum of water uptake, the bryophytes are most susceptible to changes in pollutant levels, which may be either dry, wet or aerosols. They are considered the most reliable and influential indicator species to monitor various alterations in the ambient environment (LeBlanc and Rao 1975, De Temmerman et al. 2004), and are more susceptible to pollutants like carbon monoxide (CO), hydrogen sulphide (H_2S), oxides of nitrogen (NOx), ozone (O_3), sulphur dioxide (SO_2), ammonia (NH_3), particulate matter ($PM_{2.5}$) and automobile exhausts (Tretiach *et al.* 2007, Adamo *et al.* 2011) in comparison to the

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vascular plants.

Among bryophytes, mosses are more widely distributed than the liverworts, occurring in almost every habitat, particularly in moist places on soil, rocks, branches, tree trunks and fallen logs etc., supporting life. There are several recent reports of sources of pollutants, mechanisms of pollutant accumulation and detoxification by mosses as well as their bioaccumulation capacity. Several mosses viz., *Fontinalis antipyretica, Thuidium cymbifolium, T. delicatulum, T. sparsifolium* and *Scleropodium purum* have been reported to be used as a potential biomarkers in biomonitoring systems for various heavy metals viz., copper, lead, zinc, cadmium, and nickel (Sharma and Kapila 2007, Shakya et al. 2008, Sun et al. 2009, Mariet et al. 2011). *Polytrichum commune*, *P. strictum*, and *Racomitrium* sp. have been reported to be tolerant to fluoride fumes (Glime 2007) while *Sphagnum* spp. is sensitive to ozone, showing extenuated growth, decreased photosynthesis, colour loss, and various characteristic features of drying (Gagnon and Karnosky 1992).

Though mosses have no true root system, and their leaves are not covered with a protective layer, therefore aerosols and gases present in the atmosphere are absorbed directly through the surface of the plants. A review of biological records shows that all the bryophytes present in nature, are not uniformly susceptible to all the pollutants (Pescott *et al*. 2015). Whenever changes occur in air quality in form of any imbalance in gaseous proportion, the generation of reactive oxygen species (ROS) is triggered, which can be harmful to the cellular constituents of mosses. To acquire tolerance to environmental changes, mosses developed an enzymatic and non-enzymatic defense system that can scavenge toxic species of oxygen and ameliorate oxidative stress. Enzymatic element of antioxidative defense method involves, catalase (CAT) and peroxidase (POD), which shattered hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O), whereas superoxide dismutase (SOD) catalyzes the dismutation of superoxides to O_2 and H_2O_2 (Ghorbanli et al. 2007). In addition, chlorophyll pigments (chl a, chl b, total chl), as well as non-enzymatic compounds (carotenoids) are also able to contribute in ROS scavenging (Mattos and Moretti 2015). Increasing and decreasing quantities of CAT, POD, SOD, photosynthetic pigments, and carotenoids in mosses, may be treated as biomarkers or early warning methods for detection of various natural as well as anthropogenically induced changes in the environment.

In the present study, we performed the experiment on two moss species namely, *Semibarbula orientalis* (Web.) Wijk. & Marg. and *Physcomitrium eurystomum* Sendtn. to assess the variability in enzymatic and non-enzymatic parameters over a period of continuous 7 days in response to changes in ambient air quality i.e. imbalance in the concentration of gaseous pollutants viz. sulphur dioxide (SO_2), oxides of nitrogen (NOx), particulate matter ($PM_{2.5}$), etc., which was visible as smog in the atmosphere. Changes in the level of different pollutants were confirmed by the Central Pollution Control Board report (2017). Thus, the purpose of this experiment was to explore quantitative alterations in antioxidative enzymes (CAT, POD, and SOD), chlorophyll pigments (chl a, chl b, total chl), and non-enzymatic pigments (car) against various air pollutants to develop early warning indicator systems.

Materials and methods

Study area and sampling

Two moss species *viz*. *S. orientalis* and *P. eurystomum* were used as representative species to evaluate their sensitivities to the pollution stress. Both were found to be growing in different areas of the University of Lucknow (26°86′33″ N, 80°93′6″ E with an altitude of 123 m above sea level). Fresh plants were collected in the month of November continuously for 7 days i.e. from 05.11.17 to 11.11.17 when the concentration of different pollutants increased in the atmosphere whereas control samples (C) were collected in the month of October (05.10.17) when air quality was reportedly not affected with pollutants. After collection, moss plants were cleaned with running tap water followed by sterile distilled water, to remove all attached substrate, litter and soil particles associated with them. After cleaning, the apical green part of the gametophyte was cut from each plant, and kept at –20º C in folds of sterilized filter paper. Assay of the activity of enzymes was done on a fresh weight (FW) basis.

Enzyme analysis

Catalase (CAT) activity was expressed as µmole H_2O_2 decomposed per 100 mg fresh weight of tissue and was estimated by a modified method of Euler and Josephson (1927). The reaction mixture for enzyme assay contained 0.005 M H_2O_2 in potassium phosphate buffer (pH 7.0). After 5 minutes, the reaction was ceased by adding 2 N H_2SO_4, and this was standardized against 0.1 N $KMnO_4$.

Extraction and assay of peroxidase (POD) were based upon the method of Luck (1963), and colour intensity was read at 485 nm. Moss samples (50 mg) were homogenized in distilled water (10 ml) and centrifuged at 12,000 rpm for 15 minutes. After that, 0.1 M phosphate buffer (pH 6.0), H_2O_2 (0.01%) and 0.5% (w/v) p–phenylenediamine were added. In the end to stop the reaction, 5 N H_2SO_4 was added. For expression of POD activity, the difference in optical density between blank and samples (per 100 mg fresh weight) was calculated.

SOD activity was measured by recording the reduction of nitro-blue tetrazolium (NBT) by superoxide radicals. The estimation was done according to the modified method of Beuchamp and Fridovich (1971). Solution of phosphate buffer (pH 7.0), PVP, EDTA and $MgCl_2$ was used to ground the fresh moss plants and then centrifuged for 10 minutes at 12,000 rpm. Reaction mixture contained phosphate buffer (50 mM), methionine (10 mM), riboflavin (1.17 mM), NBT (56 mM) and enzyme extract. Ultimately, for the development of colour, the samples were placed in light for 15 minutes, and blanks were incubated in dark. When the reaction was completed, the absorbance of the samples was recorded at 560 nm.

Pigment estimation

Chlorophyll content (chl a, chl b, total chl) and carotenoids (car) were quantified according to Arnon (1949). Green tissue (100 mg) was homogenized in 80% chilled acetone (1:10 w/v). The extract was centrifuged for 10 minutes at 5,000 rpm, and absorbance of the supernatant was recorded at 645, 652 and 663 nm for chlorophyll and 480, 510 nm for carotenoid estimation using a double beam UV-VIS spectrophotometer. The amount was expressed in mg/g fresh weight.

Statistical analysis

The data were recorded in three replicates and were subjected to statistical analysis. Value described as mean ± standard error, and SPSS ver. 15.0 for Windows (SPSS Inc., Chicago, Ill., USA) was used for the analysis of variance (ANOVA). Comparison of mean values was done with one-way ANOVA and Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$, 0.01 and 0.001.

Results

Physiological differences between the controls of *S. orientalis* and *P. eurystomum* and their counterparts growing under stress were estimated and the authors observed an increase in the activity of CAT, POD and SOD enzymes. Further, both the plants showed a reduction in photosynthetic pigments (chl) and non-enzymatic low molecular metabolites (car). The results demonstrated that changes in air quality caused significant variations in the physiological parameters as compared to control.

Catalase activity

CAT activity for *S. orientalis* was lower than that of *P. eurystomum,* and it showed an increment of 37.5 and 16%, respectively than their respective controls (C) on 1st day of evaluation (DOE). After that, the activity gradually increased by 50, 87.5, 143.8 and 156% in *S. orientalis* and 52, 84, 116 and 128% in *P. eurystomum* on 2nd to 5th DOE, but on 6th DOE, activity suddenly started decreasing, and was only 62.5% higher in *S. orientalis* and 64% in *P. eurystomum* than their respective controls. On 7th DOE, the activity showed only 19% increment in *S. orientalis* and 48% in *P. eurystomum* in relation to their control plants (Fig. 1a). It is apparent that, as pollutants concentration in the atmosphere increased, CAT activity also enhanced, becoming highest on 5th DOE, and after that, it started decreasing.

The correlation between CAT activity and NO_2, NO_x (Fig. 2a) was significant for both the mosses. It showed a positive correlation at $P \leq 0.05$, 0.01 for *S. orientalis* and at $P \leq 0.05$, 0.01, 0.001 for *P. eurystomum*. Besides, correlation between CAT and $PM_{2.5}$ was also positively significant at $P \leq 0.05$

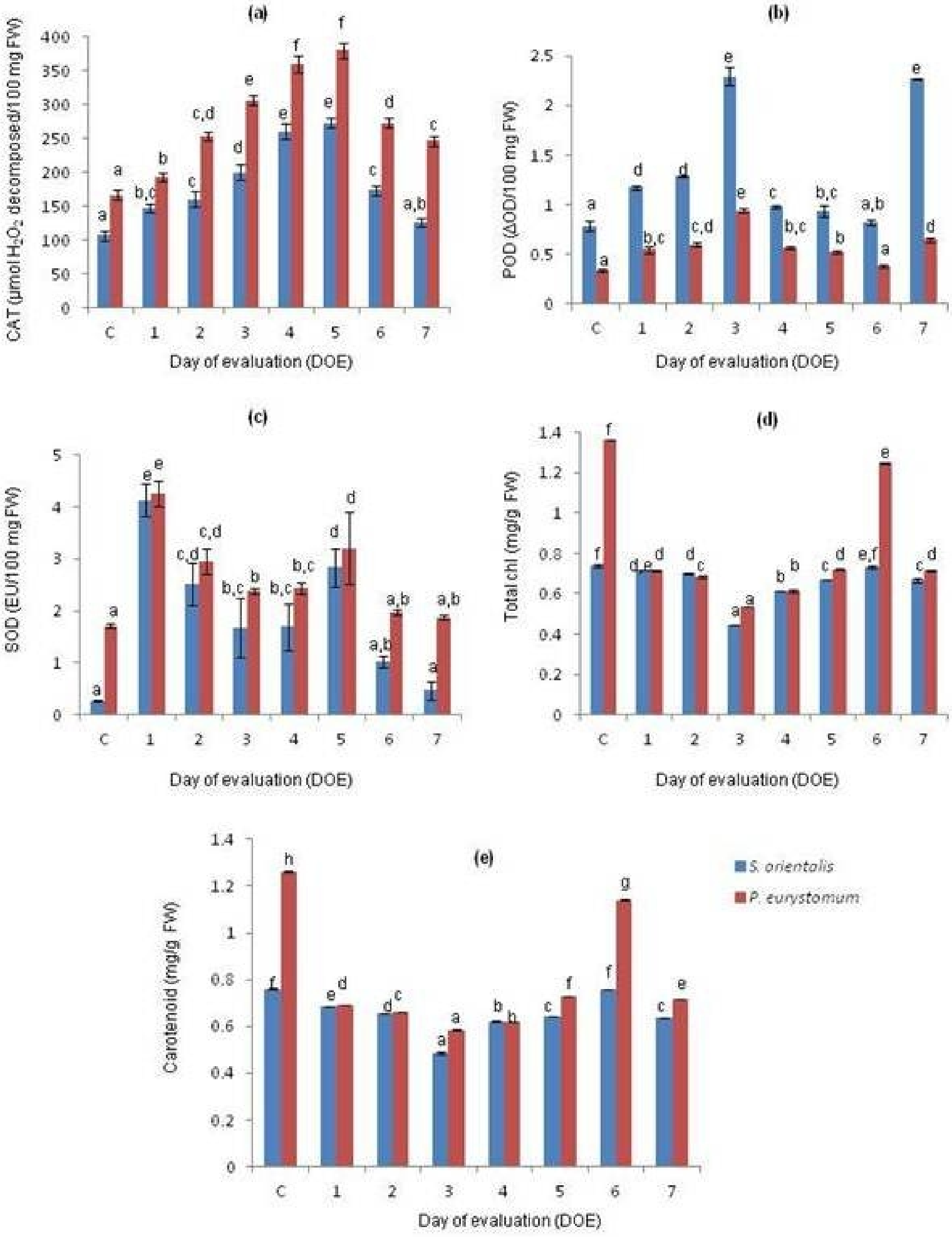

Figure 1: Activity of antioxidant enzymes and pigment contents of *Semibarbula orientalis* & *Physcomitrium eurystomum:* **(a)** Catalase (CAT), **(b)** Peroxidase (POD), **(c)** Superoxide dismutase **(**SOD), **(d)** Total Chlorophyll **(**Total chl), **(e)** Carotenoid **(**car). Data are presented as mean values ± S.E. of three replicates and data with different superscripts are significantly different at $P \leq 0.05$, as determined by analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT)

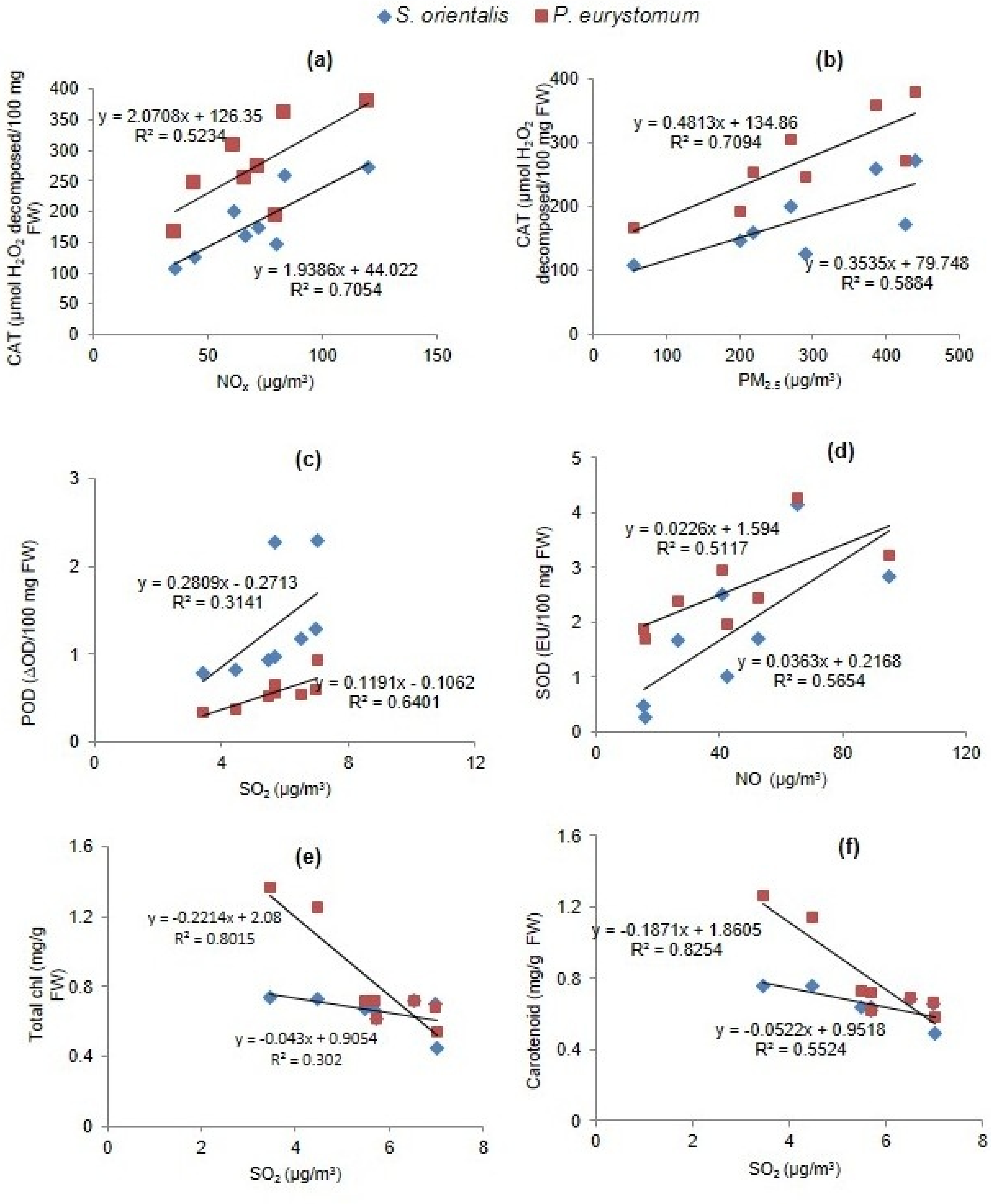

Figure 2: Graphical representation of correlation studies in *Semibarbula orientalis* and *Physcomitrium eurystomum* between **(a)** Catalase **(**CAT) and nitrogen oxides (NOx), **(b)** Catalase **(**CAT) and particulate matter ($PM_{2.5}$), **(c)** Peroxidase (POD) and sulphur dioxide (SO_2), **(d)** Superoxide dismutase (SOD) and nitric oxide (NO) showing significant differences and positive value at $P \leq 0.05$, 0.01 and 0.001. Correlation study between, **(e)** Total chlorophyll (Total chl) and sulphur dioxide (SO_2), **(f)** Carotenoid (car) and sulphur dioxide (SO_2) which was negatively significant at $P \leq 0.05$ in *S. orientalis* and at $P \leq 0.05$, 0.01 in *P. eurystomum*

in *S. orientalis* and at $P \leq 0.05, 0.01$ in *P. eurystomum* (Fig. 2b).

Peroxidase activity

Increment in the concentration of atmospheric pollutants increased POD activity on 1st DOE by 49 and 61% in *S. orientalis* and *P. eurystomum*, respectively than their controls. On 2nd DOE, activity was 64 and 76.5% higher but, on 3rd DOE still higher increment of 191.5 and 176.5% than that of control plants was observed. On 4th and 5th DOE, there was a decrement in POD activity in relation to 3rd DOE and activity increased by 24.6, 19.5% in *S. orientalis* and 66.7, 54.9% in *P. eurystomum* (Fig. 1b) as compared to controls. On 6th DOE the activity was minimum which on 7th DOE showed an abrupt increment by 189% in *S. orientalis* and 90.2% in *P. eurystomum* than their control.

In *P. eurystomum,* POD activity was correlated with SO_2 concentration (Fig. 2c) which was positively significant at $P \leq 0.05$ in *S. orientalis* (with an r value 0.80).

Superoxide dismutase activity

Contrary to oxidative stress, SOD acts as a primary barrier therefore, at control levels, both the species of moss maintained corroborated their SOD activity. For *P. eurystomum,* SOD activity was much greater (1.71 EU/100 mg FW) than *S. orientalis* (0.27 EU/100 mg FW) however, the value on 1st DOE was increased by 1434.4% (4.137418 EU/100 mg FW) in *S. orientalis* and 149.3% (4.256376 EU/100 mg FW) in *P. eurystomum*, respectively than their controls (Fig. 1c). During 2nd and 3rd DOE, SOD activity rapidly decreased and showed 833.9, 519.8% increment in *S. orientalis* and 73.3, 39.1% in *P. eurystomum*, respectively than their control, whereas increment of 527.5% in *S. orientalis* and 42.5% in *P. eurystomum* was observed on 4th DOE, which increased abruptly by 951.7% in *S. orientalis* and 87.8% in *P. eurystomum* on 5th DOE in respect to their controls. Thus maximum activity was observed on 1st DOE, which was about 15.3 folds higher in *S. orientalis* and 2.5 folds in *P. eurystomum* than that of control and minimum was observed during 7th DOE which reached near to their controls.

Statistical analysis revealed that SOD activity was positively correlated with NO and NOx (Fig. 2d) which was significant in both the moss species (at $P \leq 0.05$) with an r value 0.75 in *S. orientalis* and 0.71 in *P. eurystomum*.

Photosynthetic pigments

During the evaluation of photosynthetic pigments and carotenoids, control plants showed maximum chl a, chl b, total chl and car content in both the mosses but the values started decreasing during 1st DOE which became minimum during 3rd DOE and showed 39.4% lower value of total chl and 35.9% of car in *S. orientalis* and 60.5% of total chl (Fig. 1d) and 53.5% of car (Fig. 1e) in *P. eurystomum* than their respective controls. After that, the values gradually increased till 6th DOE, but slightly decreased on 7th DOE.

In case of *S. orientalis*, statistical analysis revealed a negative correlation between car and SO_2 at $P \leq 0.05$ (with *r* value -0.74). Also in *P. eurystomum*, a correlation was observed between total chl, car, and SO_2, which was negatively significant at the two probabilities $P \leq 0.05$ and 0.01 (Fig. 2e, f).

Discussion

Mosses are considered a valuable constituent of the ecosystem and have a stable impact on water cycling, energy, nutrients and carbon, etc. (Song *et al.* 2015). Owing to some of their morphological as well as physiological properties, such as the absence of cuticle, the presence of large cationic exchange quality inside the cell wall, these miniature plants are widely utilised as air pollution indicators in geological prospecting (Calzoni *et al.* 2007). Because of the presence of extreme climatic phases and stresses, caused due to air pollution, bryophytes outlive in two ways *viz*., by their inherent capability to live out and their efficiency to obtain tolerance to deathly environmental changes. A low level of ROS continuously results in an adaptation response, which involves enhancement in cellular activities of antioxidants, whereas a high level of these aggregated ROS, could affect membrane fluidity and protein, leading to oxidative damage (Esfandiari *et al.* 2007). `

Among gaseous pollutants, the combustion process leads to the production of nitrogen oxides and NOx is generally > 90% nitric oxide (NO), with the balance being composed of nitrogen dioxide (NO_2). NO_x effect on nitrate assimilation was studied by Morgan et al. (1992), who suggested that, although NO_2 and NO showed apparent impact on bryophyte's nitrogen metabolism at attainable atmospheric concentrations, these pollutants do not interrupt the plant growth strongly. In contrast, sulphur dioxide (SO_2) is considered as most natural and detrimental pollutant, and has been recognized as strong indicator in atmospheric environmental monitoring. Particulate matter ($PM_{2.5}$) also plays a significant role in interrupting the metabolic as well as physiological processes in mosses. The suspended $PM_{2.5}$ enters through the general body surface and hinders the metabolism thus causing a reduction in biochemical parameters. In the present study, we provide the perception of the competence of various air pollutants to enhance antioxidant responses in two moss species namely *S. orientalis* and *P. eurystomum*.

Corresponding to antioxidative enzymes, CAT is considered a free element of antioxidative metabolism, which directly eliminates H_2O_2 in absence of any electron donor, whereas POD oxidises hydrogen donor at expense of peroxides. The study reveals an increase in CAT and POD activity in response to nitric oxide (NO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and particulate matter ($PM_{2.5}$). Under normal growth conditions (control), plant cells experienced only mild oxidative stress, but during 1st DOE, as the amount of NO_2 (53.61 μg/m^3), $PM_{2.5}$ (200.92 μg/m^3) and SO_2 (6.52 μg/m^3) increased, the activity of CAT and POD also increased. After that during 2nd and 3rd DOE, the concentration of NO_2, $PM_{2.5}$ and SO_2 increased again (NO_2 64.03 μg/m^3, $PM_{2.5}$ 218.76 μg/m^3, SO_2 6.97 μg/m^3 during 2nd DOE and NO_2 74.75 μg/m^3, $PM_{2.5}$ 269.21 μg/m^3, SO_2 7.02 μg/m^3 during 3rd DOE, respectively) which must have triggered a rapid increment in O_2^- production, due to which CAT and POD activity enhanced in both the moss species. Additionally, the activity of CAT increased on 4th DOE (NO_2 78.78 μg/m^3, $PM_{2.5}$ 387.52 μg/m^3) and 5th DOE (NO_2 82.98 μg/m^3, $PM_{2.5}$ 439.76 μg/m^3) but after that as the concentration of NO (42.73 μg/m^3), NO_2 (71.54 μg/m^3) and $PM_{2.5}$ (426.5 μg/m^3) decreased during 6th DOE, the activity further decreased. However, during 7th DOE when the concentration of NO (15.58 μg/m^3) and NO_2 (59.58 μg/m^3) started decreasing in the atmosphere, the activity of CAT also decreased. Similarly, POD activity was greatly influenced by SO_2 and reached the highest level during 3rd DOE in both the mosses, when the concentration of SO_2 was maximum (7.02 μg/m^3) in the atmosphere.

It has been reported that in plants, CAT and POD are considered as elementary H_2O_2 scavenging enzymes. Therefore, CAT-POD are treated as efficient detoxifying enzymes to decrease the cellular level of H_2O_2 under NO, NO_2, SO_2 and $PM_{2.5}$ stress to keep the biochemical and physiological indices unchanged. Same results were attained by Vlahogianni et al. (2007), Das and Roychoudhury (2014) who have suggested that during changes in air quality, the activity of CAT and POD increased to regulate the production of ROS. Varshney and Varshney (1985) also observed enhancement in POD activity due to the influence of environmental pollutants like SO_2. Later, Sun et al. (2011) suggested that in *Brachythecium piligerum,* CAT-POD activities could be utilized as biomarkers, which helps in biomonitoring of pollution present in the atmosphere. The results of the present study are in harmony with those of Jia et al. (2017) and Chen et al. (2017) who have shown that changes in the ambient environment due to $PM_{2.5}$ had a profound influence on the physiological status of plants.

Similar to CAT-POD, SOD could also efficiently scavenge ROS generated due to oxides of nitrogen (NO_x). The control samples of both the mosses retained their SOD activity, but as NO_x concentration increased the activity also increased and was maximum during 5th DOE due to the highest concentration of NO (95.25 μg/m^3) and NO_x (120.24 ppb) in the environment. Afterwards as the concentration of NO and NO_x decreased, SOD activity also showed gradual decrement. Similar results were also obtained by Pukacka and Pukacki (2000) in *Pinus sylvestris* L., who suggested that SOD can respond against increased activity to signals from environmental pollution. Similar to our observations, Muneer *et al.* (2014) exposed strawberry plants for 24 hours to low, medium and high magnitude of NOx and SO_2 and observed that activity of antioxidative enzymes viz., ascorbate peroxidase, CAT and SOD were enhanced during pollution stress.

As far as pigments were considered, we observed that disparity in air quality induced the degradation of chlorophyll. Under stress conditions caused due to increment of SO_2 in the atmosphere, lower plants can endure various photochemical reactions viz., reduction, oxidation and reversible bleaching etc. Therefore, reduction in concentration of chlorophyll in plants grown in polluted areas, could be attributed due to damage in chloroplast or chlorophyll biosynthesis inhibition. According to Senser et al. (1990), at the time of stress, general protective mechanism may become overloaded, leading to destruction of cell as well as degradation of pigments. Furthermore, being a part of light harvesting complex for photosynthesis, carotenoids are essential components which also act as a non enzymatic chemical quenchers of singlet oxygen. Importance of carotenoid contents towards the protection of photosynthetic apparatus and antioxidant defense mechanism was also reported in *Timmiella barbuloides* and *Pleurochaete squarrosa* (Aydoğan *et al*. 2017).

The present study revealed that level of chlorophyll and carotenoids in control plants of both the mosses was maximum but the values were decreased, as level of SO_2 increased in the environment. These results are in harmony with those of Asada and Kiso (1973) and Awanish (2014) who suggested that, when plants are exposed to SO_2, their tissues absorb it and intracellularly converts it to sulphite and bisulfite ions, which causes denaturation of chloroplast and chlorophyll degradation by enhancing ionic species concentration and free radicals, which afterward displace the Mg^{+2} ions from the chlorophyll molecule. Similar decrement in the pigment contents were observed in *Orthotrichum obtusifoliu*m, *Platygyrium repens, Pylaisiella selwynii, Ulota crispa* (Rao and LeBlanc 1966), *Brachythecium* sp. and *Hypnum* sp. (Daly 1970). Gilbert (1968) also observed rapid breakdown of chlorophyll due to high level of SO_2 in *Camplothecium sericeum, Grimmia pulvinata, Hypnum cupressiforme* and *Tortuta muralis*. Later, Yu (1988) reported that during SO_2 metabolization, HSO_3 was formed which caused oxidation of carotenoid leading to chlorophyll oxidation, which results in reduction of photosynthetic capacity of plant. However, Surowka, et al. (2007) demonstrated that in the presence of light, SO_2 can easily penetrate into the action site of sulphite ions i.e. chloroplast, which shows disorganization and significant changes at ultrastructural level also. Hence, in nut shell, mosses which are grown in polluted sites, have been noticed with a reduction in chl a, chl b, total chl and car contents.

Conclusion

From the present study, it can be inferred that due to the simplicity of organization, mosses can act as immediate reflectors of changes in ambient air quality. The absence of true root system and lack of protective barrier such as a waxy cuticle layer allows penetration of air borne substances directly from the atmosphere to the general body surface of gametophytes. Some of these substances are necessary for the function of cells, while others may be toxic and have negative effects on moss growth, development, and reproduction, leading to the formation of ROS. Increased levels of ROS may function as a signal, due to which defense responses are induced to protect the plant from damage. This study demonstrated a distinct perception of the alteration in the production of ROS, photosynthesis physiology and status of antioxidants in *S. orientalis* and *P. eurystomum* under various air pollutants, and promote that mosses can be used as a hyper accumulator to maintain environmental pollution by mitigating oxidative damage. In conclusion, on the basis of the virtue of their structural simplicity and sensitivity to atmospheric pollutants, these miniature plants can be used as day-to-day mirrors of changes in ambient air quality.

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