INTRODUCTION

Earth having an atmosphere is the only planet in the solar systems is responsible for supporting life due to complex and dynamic natural gaseous system. But now the atmosphere of urban areas are under great threat for its air quality as it is mixed up with toxic pollutants which scavenge different ROS and are considered to be a biomarker of oxidative stress [13]. But, plants able to overcome the oxidative damages are occurred in leaves by different toxic pollutants like SO$_2$, NO$_x$ and O$_3$. Leaves are most susceptible parts of a plant to induce acute injury due to their abundance of stomata, which helps to penetrate the pollutants into the sensitive tissues. Road traffic emissions resulted in changes in foliar anatomy and ultimately caused stomatal occlusion, which led reduction of photosynthetic pigments viz., chlorophyll and carotenoids [10]. Moreover, reduction of protein concentration and alteration of peptides is also an indication of ambient air quality which may be achieved due to the inactivation of some enzymes or break down of existing proteins [11, 12]. Pollutants like SO$_2$, nitrogen dioxide and O$_3$ are mainly responsible to produce vast quantities of reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$), nitrite (NO$_2^-$), hydroxyl (OH$^-$), peroxyl (HO$_2^•$) and superoxide (O$_2^-•$) radicals to create oxidative stress [13]. But, plants able to overcome the oxidative stress by using antioxidant enzymes such as CAT, GPX and SOD which scavenges different ROS and are considered to be a biomarker for air pollution stress [14, 15].

T. peruviana formerly known as Cascabela thevetiain is a medicinal and evergreen dicotyledonous shrub that belongs to family Apocynaceae. It is commonly found in the tropics and sub-tropics and it grows about 10-18 feet high. There are two varieties of this
This plant is used in roadsides to develop a green belt in Bhubaneswar. However, the morphology is drastically changed during January to May. Therefore considering the above facts, the present work was designed to assess the shocking effects of air pollution on anatomical and biochemical parameters of *T. peruviana*.

### MATERIALS AND METHODS

#### Chemicals and reagents

Chemicals and reagents including L-methionine, hydroxyamine hydrochloride, nitro blue tetrazolium (NBT), ethylenediamine-tetraacetic acid (EDTA), naphthyl ethylenediamine were obtained from Sisco Research Laboratories (Mumbai, India). Sulphanilamide, tritonX-100, riboflavin, H₂O₂, guaiacol and tetra-methyl-ethylenediamine (TEMED) were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). The protein molecular weight marker was purchased from Bangalore Genei (Bangalore, India). All other chemicals and solvents were of analytical grade.

#### Study area and sampling location

Sampling sites were located in and around the city of Bhubaneswar. Six sampling locations (Rasulgarh, Acharya Vihar, Jayadev Vihar, Baramunda, Pokhariput and Khandagiri) were chosen as the polluted site (named as S1, S2, S3, S4, S5, S6, respectively) while the Khorda was selected as reference site (S7) (fig. 1). The study was carried out from January to May of 2015. The leaf samples were collected and immediately taken to the laboratory for further analysis.

#### Plant materials

*Thevetia peruviana* L. (having yellow flower) was chosen for experimental study as it is widely planted along the divider of roads of Bhubaneswar city (fig. 2). The plant specimen was identified and deposited with voucher number as AMITBTP20150024. The collected leaf samples were washed and kept in a beaker with water for anatomical analysis whereas, for other tests, samples were washed first and then dried by gentle touching with tissue paper.

#### Anatomical studies

With the help of 7 o'clock blade, different transverse sections of leaves were obtained and were kept in petri dish along with water. The fine, selected sections were stained and it would help to distinguish different tissue, cells or inclusions from one another by developing specific colours. The fine thin sections were allowed to immerse in acetone in dark. The homogenate was centrifuged for 3 min at 2800 g. After centrifugation, the supernatant was collected and the procedure was repeated up to 10 ml of the collection with 80 % acetone. The optical density (OD) of the supernatant was determined by spectrophotometrically at different wavelengths like 480 nm, 649 nm and 665 nm for chlorophyll a, chlorophyll b and total chlorophyll, respectively.

But for carotenoid, the absorbance was taken at 480 nm and 510 nm. The chlorophyll content was measured according to Strain et al. [19], whereas the carotenoid contents were calculated as per the method of Duxbury and Yentsch [20]. The results were expressed as mg/g of fresh weight of plant sample.

#### Extraction and estimation of protein

Collected leaf samples (3: 1; buffer volume: fresh weight) were homogenized in pre-chilled mortar and pestle with 50 mmol potassium phosphate buffer (pH 7.8), 50 mmol EDTA, 2 mmol phenyl methyl sulfonyl fluoride (PMSF) and 10 % (w/v) insoluble polyvinylpyrrolidone (PVP) to fine slurry followed by centrifugation at 14000 g for 15 min at 4 °C. The supernatants were taken for protein estimation, SDS-PAGE analysis and antioxidant enzyme (CAT, GPX and SOD) activities. The concentration of protein was done according to the method of Lowry et al. [21] using bovine serum albumin as a standard.

#### Analysis of protein profile by SDS-PAGE

The supernatant containing equal amounts (50 μg) of proteins were separated on 5 % stacking and 55 % resolving polyacrylamide slab gels at a constant current of 35 mA for 4 h [22]. Separated polypeptides on the gel were visualized by the silver staining method [23].

The gels were then scanned and photographed by gel documentation system and analyzed with the quantity one software from Bio-Rad (Bio-Rad, Italy). In order to achieve the precise sizing of the separated polypeptides, a protein molecular weight marker [phosphorylase b (97.4 kDa); bovine serum albumin (66.0 kDa); ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa); lactoglobulin (18.4 kDa)] was used as a standard.

### Table 1: Changes of Chl a, Chl b, total Chl and carotenoid content in leaf samples of *T. peruviana* collected from different parts of the Bhubaneswar and Khorda.

| Collected leaf samples | Chl a (mg/g) | Chl b (mg/g) | Total Chl (mg/g) | Carotenoid (mg/g) |
|------------------------|-------------|-------------|-----------------|------------------|
| S1                     | 16.19±1.74  | 7.74±0.69   | 23.93±2.56      | 7.94±0.92        |
| S2                     | 17.21±1.11  | 8.93±0.72   | 26.14±1.76      | 8.52±0.72        |
| S3                     | 15.52±0.95  | 8.04±0.68   | 23.56±2.18      | 8.96±0.69        |
| S4                     | 12.64±0.83  | 6.82±0.43   | 19.46±1.77      | 7.10±0.54        |
| S5                     | 7.65±0.51   | 3.78±0.56   | 11.43±0.91      | 5.63±0.55        |
| S6                     | 8.36±0.78   | 5.45±0.41   | 13.81±1.06      | 6.39±0.64        |
| S7                     | 19.44±1.08  | 10.12±1.03  | 29.56±2.32      | 10.22±1.21       |

The data represents mean ± SE of replicates (n = 6)
Fig. 1: Location of sample collecting sites from Bhubaneswar and Khurda.
Fig. 2: Collection sites of *T. peruviana* from different parts of the Bhubaneswar and Khurda. A: Rasulgarh; B: Acharya Vihar; C: Jayadev Vihar; D: Baramunda; E: Pokhariput; F: Khandagiri; G: Khorda NHS.
Fig. 3: Transverse section of leaf samples *T. peruviana* collected from different parts of the Bhubaneswar and Khurda. A: Rasulgarh; B: Acharya Vihar; C: Jayadev Vihar; D: Baramunda; E: Pokhariput; F: Khandagiri; G: Khorda NH5.

Fig. 4: Soluble protein estimation from leaves of *T. peruviana*, collected from different parts of the Bhubaneswar and Khurda. S1: Rasulgarh; S2: Acharya Vihar; S3: Jayadev Vihar; S4: Baramunda; S5: Pokhariput; S6: Khandagiri; S7: Khorda NH5. The data represents mean ± SE of replicates (n = 6).
Fig. 5: SDS-PAGE profiling of leaf samples of *Thevetia peruviana* L. collected from different parts of the Bhubaneswar and Khurda. MW: molecular weight (MW) of protein standards are indicated; S1: Rasulgarh; S2: Acharya Vihar; S3: Jayadev Vihar; S4: Baramunda; S5: Pokhariput; S6: Khandagiri; S7: Khorda NH5.

Fig. 6: Changes in the specific activities of CAT (A), GPX (B) and SOD (C) from leaf samples of *Thevetia peruviana* L. collected from different parts of the Bhubaneswar and Khurda. S1: Rasulgarh; S2: Acharya Vihar; S3: Jayadev Vihar; S4: Baramunda; S5: Pokhariput; S6: Khandagiri; S7: Khorda NH5. The data represents mean ± SE of replicates (n = 6)
Antioxidant enzyme assay

CAT activity was determined by measuring the decrease in H$_2$O$_2$ concentration at 240 nm and the activity was calculated by using the molar extinction coefficient of H$_2$O$_2$ (40.0 mmol/cm) [24]. Enzymatic activity was determined at 25 °C using 0.5 ml of enzyme extract (50 µg of protein), 2.0 ml of 100 mmol potassium phosphate buffer (pH 6.8) and 0.5 ml of 10 mmol H$_2$O$_2$. The specific activity was expressed as nKat per mg of protein. GPX activity was assayed as described by Bergmeyer [25]. The assay was done by taking a reaction mixture consisting of 2.8 ml of 100 mmol potassium phosphate buffer (pH 7.0), 0.05 ml of 0.018 M guaiacol and 0.05 ml of 10 mmol H$_2$O$_2$. The reaction was started by the addition of enzyme extract (0.1 ml) equivalent to 50 µg protein. The change in absorbance at 436 nm due to the oxidation of guaiacol to form tetra guaiacol in the presence of H$_2$O$_2$ was measured and expressed in units per mg of protein. SOD activity was assayed by measuring the inhibition of O$_2$-•-driven NO$_2$-formation from hydroxylamine hydrochloride according to Das et al. [26]. The reaction mixture was prepared by mixing 1.110 ml of 50 mmol phosphate buffer (pH 7.8), 0.075 ml of 20 mmol L-methionine, 0.040 ml of 1 % (v/v) triton X-100, 0.075 ml of 10 mmol hydroxylamine hydrochloride and 0.1 ml of 50 µM EDTA. To this mixture, 0.1 ml of enzyme extract (50 µg protein) and 0.08 ml of riboflavin (50 µM) were added. The reaction was started by exposing the mixture to cool white fluorescent light for 10 min. After this period the light was switched off, 1 ml of greiss reagent (prepared freshly by mixing an equal volume of 1 % sulphanilamide in 5 % phosphoric acid and 0.1 % N-1-naphthyl ethylene diamine) was added to each tube and the absorbance was measured at 543 nm. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50 % of NO$_2$-formation. The enzyme activity was calculated from the value of V/V-1, where V is the absorbance of the control (without enzyme) and V is the absorbance of the sample. The activity was expressed as units per mg of protein.

Enzyme activity staining

CAT activity in non-denaturing PAGE was done as described by Woodbury et al. [27]. CAT isoenzymes were visualized by incubating the gels in 0.003 % H$_2$O$_2$ for 10 min at room temperature (25 °C). The gels were then rinsed with distilled water and finally stained in a reaction mixture containing 2 % (w/v) potassium ferricyanide and 2 % (w/v) ferric chloride for 10 min. Activity staining of GPX was performed on 10 % PAGE according to staining procedure of Hamill and Brewbaker [28]. The gels were washed with distilled water and immersed for 30 min at room temperature in 0.018 M guaiacol, rinsed twice with deionized water, and then immersed in a solution of 0.015 % H$_2$O$_2$ in 1 % acetic acid. SOD was localized by using a photochemical method of Beauchamp and Fridovich [29]. The gels incubated in staining buffer [50 mmol potassium phosphate buffer
Anatomical characteristics

The present studies on the *T. peruviana* growing in all parts of Bhubaneswar indicate an adverse effect on the micromorphological structure. In the present investigation, it has been observed that the stomatal and epidermal structures changed which indicates the rate of pollutants is different in important areas of Bhubaneswar. The data was compared with the reference plants grown in NH of Khurda (S7) which is 2.2 KM way from Bhubaneswar. The size of epidermal cells and stomata normally accompanied by an increase in the size of epidermal cells as shown in fig. 3. Almost all plants growing in different parts of the city (Rasulgarh, Achariya Vihar, Jayadev Vihar, Baramunda, Pốrkharītpur and Khandagiri) disturbed its anatomical appearance as compared to the leaves of outside city (Khorda NH5). Our results were in conformity with the findings of Mishra [30] which was studied the effect of environmental pollution on leaf anatomy of *Comelina bengalensis*. Reducing of leaf mesophyll, palisade parenchyma and upper and lower epidermis in *Tannacetum vulgare* was also observed by Stevovi et al. [31]. Stomatal abnormality (clogging and reduction of size) and reduction of the epidermis in *T. peruviana* was also observed by Mishra et al. [12]. The decrease in protein concentration also may be due to the inactivation of some enzymes or break down of existing protein to amino acid through air pollutants such as either through toxic gases or accumulation of heavy metals [2, 12].

**RESULTS AND DISCUSSION**

**Antioxidant enzyme activities**

The results of experiment exhibited that the planted *T. peruviana* when exposed to various polluted areas of Bhubaneswar city showed changes in antioxidative enzymes. According to the results (fig. 6A), the maximum activity (22.12±1.92 Unit/mg of protein) of CAT at leaves was recorded at site S6 whereas, the minimum activity (8.49±1.71 Unit/mg of protein) was observed at site S7. According to the changes in the activity of the CAT enzyme, sites are arranged in the following patterns: S6>S1>S4>5>S2>S5>S7. While analyzing the CAT activity in non-denaturing PAGE, a single isofrom (CAT-I) is appeared in leaf samples of all experimental sites however, the intensity of bands are altered.

The maximum intensities were observed in leaves of S6 and S1 sites plants (Shown in fig. 7A). The changes in GPX activity was also recorded from different sites of the study area, which are illustrated in fig. 6B. The maximum activity was observed in S6 (64.60±1.62 Unit/mg of protein) which was about 6 times higher than reference site S7 (11.23±0.94 Unit/mg of protein). But the maximum decrement was found in S3 (9.12±1.52 Unit/mg of protein). There are two isofroms (GPX-I and GPX-II) appeared in all sites of Bhubaneswar and Khurda.

However, the lowest intensity was observed in leaves of Khurda plants (fig. 7B). No such significant results were noticed in the case of SOD activity (either assay or activity staining) in leaf samples of all collected sites.

The activity was higher in polluted site S5 (34.62±3.41 Unit/mg of protein) as compared to reference one (29.33±1.68 Unit/mg of protein) which indicates as a non-significant (Shown in fig. 6C). The isozyme patterns of SOD was studied through native PAGE and found that only one band (SOD-I) was obtained in leaf samples of all sites but the minimum intensity was observed in S1 and is shown in fig. 7C. Environmental pollutants such as heavy metals, dust and gases are not only responsible to disturb the plant growth and development but also produce more ROS. However, antioxidative enzymes like CAT, GPX and SOD are playing a vital function in the cells to prevent the build-up of ROS [15, 37, 38]. Increased activities of these enzymes are considered as indicators of oxidative stress in plants. Among antioxidative enzymes, peroxidase activity in plants was found to be a sensitive indicator of SOD and NO. These biomarkers are known to evaluate urban air pollution [9].

**CONCLUSION**

On the basis of this study, it is concluded that the air pollutants especially roadside dust and automobile exhaust have a great deterioration effect on the stability level of the leaves of the *T. peruviana* L. in terms of the fluctuating anatomical appearance. The reduction of photosynthetic pigments is also an indication of heavy phytotoxic air pollutant loads in Bhubaneswar city. The study results that there is an alteration of proteins and enhanced activities of antioxidative enzymes to overcome the oxidative damage caused by various pollutants which is an indication of rapid urbanization and threatening towards human health problem.
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AUTHOR CONTRIBUTION

Jyoti Ranjan Rout has interpreted data, wrote the manuscript and acted as the corresponding author. Rout George Kerry and Lidyprava Dash have devoted their time in analyzing the result of the study. Soumya Ranjan Nayak has performed analysis of all samples whereas, Santi Lata Sahoo helped to evaluate and edit the manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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