

## Antioxidant, photosynthesis and growth characteristics of plants grown in high sulphur coalmine overburden

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North East region of India suffers from high sulphur coal mine overburden (OB) and plants those survive under such condition have adopted to such harsh environment. Here, we have investigated the effect of coal mine OB substrate on biochemical, physiological and growth of two shrubs Cassia (*Cassia sofera* L.) and Dhaincha (*Sesbania rostrata* L.); two tree species Gomari (*Gmelina arborea* L.) and Sisso (*Dalbergia sisso* L.); and two monocots Citronella Grass (*Cymbopogon winterianus* Jowitt) and Lemon Grass (*Cymbopogon citratus* L). The mine OB was found to be acidic (pH 2.0) with no true soil behavior, 12.5% sulphur and more than double the trace and heavy metals compared to normal control soil. Overall, high lipid peroxidation, membrane damage, peroxidase, glutathione reductase, ascorbate peroxidase, superoxide dismutase enzymes activities along with osmolyte proline and total soluble sugar was found in mine OB plants compared to control plants. While antioxidant activities were higher, nitrate reductase activity was significantly low but reduced leaf area, total shoot and root biomass. However, the responses of antioxidant activities in mine OB plants and control plants did not follow a standard pattern of higher activities in roots followed by old>young leaves or *vice versa*. Further, in mine OB plants, lipid peroxidation was found significantly higher for young citronella leaves. The levels of higher glutathione reductase was found in roots than the leaves except for old leaves in Gomari while ascorbate peroxidase was found in the leaves than the roots. Overall, all the species showed higher antioxidant enzyme activities, osmolyte accumulation and membrane damage possibly due to acidity and significantly higher limit of trace and heavy metals in mine OB substrate.

**Keywords:** Adaptation, Bioremediation, *Cassia sofera*, Citronella grass, Coal mine, *Cymbopogon citrates*, *Cymbopogon winterianus*, *Dalbergia sisso*, Dhaincha, *Gmelina arborea*, Gomari, Lemon grass, Mining, Oxidative stress, Reclamation, ROS, *Sesbania rostrata*, Sisso

Abandoned coal mine and overburden (OB) dumping ground of coal mine reject materials, mine tailings of Northeast India are major environmental concern due to its no true soil behavior, acidity, excess amount of trace and heavy metal<sup>1,2</sup>. Mine OB dumping grounds in general remain barren for long time but can be restored by introducing tolerant plants and mimicking the environment<sup>3,4</sup>. Studies revealed that plants grown in mine OB and other abiotic stress environment changed its morphological, physiological and biochemical behaviour for its survival<sup>5,6</sup>. Apart from morphological and physiological changes, plants undergo biochemical changes as well to prevent cellular damage caused by reactive oxygen species (ROS). To overcome such oxidative damage, plants have evolved complex antioxidant system composed

of enzymatic and non-enzymatic endogenous mechanisms<sup>7-9</sup>. The enzymatic antioxidant includes peroxidase (POD), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX) and monodehydro ascorbate reductase (MDAR)<sup>10,11</sup>. Other endogenous antioxidants are ascorbic acid, glutathione,  $\alpha$ -tocopherol and carotenoids; osmolyte proline, glycine betaine and sucrose<sup>12-15</sup>. However, the enzymatic and other endogenous antioxidant activities are dependent on the substrate characters and amelioration can be done through amendment<sup>16,17</sup>.

Documentation of stress response characters of high sulphur coal mine OB tolerant plants is important as it may help in enhancing plant survival, and thereby restoration of the environment. Details of screening of high sulphur coal mine tolerant plant species to rejuvenate mine OB plants were discussed earlier<sup>2</sup>. In this study, we have made an attempt to document the stress related characters of six native plants of northeast India Cassia, Dhaincha, Sisso, Gomari, Lemon grass and

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**Abbreviations:** APX, Ascorbate peroxidase; GR, Glutathione reductase; MDA, Malondialdehyde; NR, Nitrate reductase; POD, Peroxidase; SOD, Superoxide dismutase.

Citronella including its physiological tolerance to high sulphur coal mine OB. Stress was given to characterization of morphological, nitrogenase and antioxidant enzyme activities, cell damage, osmolyte accumulation and net photosynthesis. Comparison of roots and leaf antioxidant enzyme activities; between grass, shrubs and tree species was also done.

## Materials and Methods

### Characteristics of control soil and mine substrate

Mine waste materials were collected from mine overburden dumping ground of Tirap collieries, Assam, India. The pH of soil and coalmine OB materials was determined in 50% (w/v) soil/mine OB suspension. Total carbon content was determined by potassium dichromate oxidation while microbial biomass carbon by chloroform fumigation. Trace and heavy metal content (Mg, Ca, Mn, Fe, Cd Cr, Cu, and Pb) in coalmine OB and in control soil was analyzed by atomic absorption spectrometer (AAS) Analyst-100 Perkin Elmer, Inc., USA). For this, wet digestion methods described in AOAC<sup>18</sup> (1990) were used for sample preparation to determine essential, trace and heavy metals in mine OB and control soil.

### Preparation of planting pots

The collected mine OB and control soil were filled in to pots having the bottom diameter 20 cm, top diameter 26 cm and height 30 cm. It was then kept in green house under controlled condition.

### Plant material

Shrubs Cassia (*Cassia sofera* L.) and Dhaincha (*Sesbania rostrata* L.) and tree species Sisso (*Dalbergia sisso* L.) of Leguminosae and Gomari (*Gmelina arborea* L.) of Verbanaceae, and Lemon grass (*Cymbopogon citratus* L.) and Citronella grass (*C. winterainus* Jowitt.) of Poaceae were selected. Details of screening of plant species have been reported in our earlier studies<sup>2</sup>.

### Generation of plant seedling

Seeds of Cassia, Dhaincha, Sisso and Gomari were sowed in nursery beds and the seedlings were allowed to grow for a period of 30 days. The temperature of the green house was maintained at 30°C with a photoperiod of 12 h day and night.

### Transplantation

The pots filled with coal mine OB and control soil were placed in the greenhouse. Six pots were arranged

in a randomized block for each species and replicated six times and 30 days old healthy seedlings were transplanted to already prepared pots. Plants were observed regularly for water and nutrients for three months. The root, young leaf and lower fully expanded old leaf from the main shoot were taken for biochemical comparison. In all the analysis minimum three plants were taken.

### Antioxidant and enzyme activity

#### Crude enzyme extraction

Crude enzymes for assay of POD, SOD, APX and GR were extracted by grinding 150 mg of fresh plant sample (leaf or root) in 4 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 3% PVP and 1 mM L-ascorbic acid at 4°C. The homogenates were centrifuged at 4°C for 20 min at 10000 rpm; supernatant was collected and used immediately for assays.

POD (EC.1.11.1.7) activity was determined with pyrogallol as substrate and the activity was calculated using the molar extinction coefficient 2.47 mM<sup>-1</sup> cm<sup>-1</sup>. SOD (EC. 1.15.1.1) activity was determined by the method of Beauchamp & Fridovich<sup>16</sup> by following the photo-reduction of nitro blue tetrazolium. Non-illuminated and illuminated reactions without supernatant served as calibration standards. APX (EC. 1.11.1.11) activity was done according to Dalton *et al.*<sup>17</sup>, while GR (EC. 1.6.4.2) activity was determined following rate of NADPH oxidation by measuring the decrease of absorbance at 340 nm<sup>19</sup>. Nitrate reductase (NR) (EC. 1.6.6.1) activity was assayed according to the Jawoski<sup>20</sup>.

#### Total protein

Total protein content in the plant extract (root and leaf) was determined according to Lowry *et al.*<sup>21</sup> using bovine serum albumin (BSA) as a standard.

#### Accumulation osmolyte

Proline accumulation was determined according to Bates *et al.*<sup>22</sup>. Total soluble sugar content was measured spectrophotometrically using glucose as standard according to Sadasivam & Manickam<sup>23</sup>.

#### Membrane stability

The effect of stress on membrane stability (leaf and root) was assessed by measuring the UV-absorbing substances (UVAS) in water<sup>24</sup>. The final absorbance (A<sub>280</sub>) was measured and relative leakage ratio (RLR) was calculated using following formula:

$$\text{RLR (\%)} = (A_{280} / A'_{280}) \times 100$$

*Oxidative damage due to lipid*

The oxidative damage of lipid was determined by measuring the malondialdehyde (nM malondialdehyde mL<sup>-1</sup>) equivalents<sup>19</sup>.

**Photosynthesis**

Net photosynthesis and stomatal conductance of the plants grown in mine substrate were determined using infra red gas analyzer system CIRAS 3, NUTECH, USA as per the instruction. These measurements were carried out between 10:00 to 14:00 h. Minimum three fully expanded leaves in each plant were recorded.

**Growth measurement**

For comparison of growth, plants were uprooted from the pot, loosely adhered mine OB's and soil was removed. The growth data such as root length, shoot length and leaf size were recorded. The shoot and root dry biomass were measured after drying the sample in hot air oven at 65°C till constant weight was obtained after repeated measurement at 2 h intervals.

**Statistical analysis**

All the data were subjected to paired comparison between mine substrate and control plants. Analysis of variance (ANOVA) was done to compare the significant difference plant species grown in mine OB substrate and compared by Tukey's test at  $P < 0.05$  level. The analysis was performed by SPSS programming.

**Results****Substrate characteristics**

Characteristic of mine OB and control soil is described in Table 1. The mine OB was acidic in nature (pH 2.0-3.5) with low moisture holding capacity and low macro nutrients, high sulphur (12.5%) compared to the control soil. In mine OB, the trace and heavy metals were also significantly higher in content than that of control soil.

**Effect of mine OB on osmolyte, antioxidant, photosynthesis and growth**

Comparison of effects of coal mine OB on membrane stability, osmolyte accumulation and antioxidant enzyme activities to that of control soil plants in old and young leaves and root are described in Tables 2 and 3. Compared to control plants, a significantly highest relative leakage rate (RLR) ( $P < 0.05$ ) was found for all the test plants. Overall increase of higher RLR compared to control plants was 25.9-69.3% in young leaves; and 43.6-88.8% in

Table 1—Comparison of chemical characteristics of coal mine overburden (OB) of Tirap colliery with control soil

| Parameters   | Type of substrate |             |
|--|-------------------|-------------|
|  | Control soil      | Mine OB     |
| pH   | 6.1-6.4**         | 2.0-3.5     |
| Cation exchange (Eh mV)  | 28-137**          | 198 – 138   |
| Organic Carbon (%)   | 1.24±0.11**       | 0.05±0.001  |
| Phosphorus (%)   | 0.02±0.01*        | 0.009±0.002 |
| S (%)  | 0.25±0.01         | 12.57±2.6   |
| Moisture (%)   | 20.69±1.0*        | 15.39±1.    |
| Water holding capacity (%)                                     | 32.22±1.8**       | 10.46± 2.2  |
| Microbial biomass C (%)  | 1.52±0.11**       | 0.005±0.01  |
| Trace and heavy metal content (µg <sup>-1</sup> dry substrate) |                   |             |
| Aluminium  | 30±0.92***        | 315.3±11.2  |
| Chromium   | 8.1±0.03          | 15.6±1.09   |
| Iron   | 10.2±0.2*         | 32.6±2.6    |
| Cobalt   | 1.43±0.1          | 3.53±0.1    |
| Nickel   | 0.76±0.0**        | 8.93±0.9    |
| Copper   | 0.7±0.02          | 1.5±0.01    |
| Zinc   | 0.5±0.30          | 1.2±0.1     |
| Cadmium  | 1.73±0.1          | 4.76±0.76   |
| Lead   | 0.4±0.01**        | 7.7±0.21    |

[The values are mean of three replications; ± = standard deviations of observed values; asterisks represent significant difference between mine OB and control soil; \* = significant at  $P < 0.05$ ; \*\* = significant at  $P < 0.01$  by paired comparison]

old leaves. Though there was appreciably higher lipid peroxidation (LP) and sugar accumulation in all the mine OB plants, it was mostly insignificant compared to control plants. In comparison to sugar, proline accumulation in mine OB plants was significant to that of control soil plants. Proline accumulation in mine OB plants was more compared to that of control plants which was 33.8 to 121.6 µMol g<sup>-1</sup> for different plant parts.

The POD activities of mine OB plants were significantly higher in Cassia, Dhaincha and Citronella leaves and also in roots of Dhaincha and Gomari. Overall increment in POD activity was 68.5-1448%. The APX activity was significantly ( $P < 0.01$  and  $P < 0.05$ ) higher in all the mine OB plants to that of control plants except Sisso in old leaf. Interestingly, the APX activities were appreciably higher for all the plant parts. Like GR activities the SOD activities were also significantly higher 1.0-319.9% except Sisso in old leaves. Overall, the response of antioxidant activities of mine OB plants to that of control plants were not similar ad uniform. Instead, lower NR activities were found for all the mine OB plants than the control plants. Similarly, mine OB plants showed an overall

Table 2—Comparison of membrane stability and osmolyte accumulation in old-, young- leaves and roots of plant species grown in coal mine overburden (OB) and in control soil

| Plant parts | Plant      | Substrate    | RLR (%)     | LP ( $\mu\text{mol g}^{-1}$ fresh weight) | Proline ( $\mu\text{g}$ of tyrosine $\text{g}^{-1}$ cell mass) | Sugar (%) |
|-------------|------------|--------------|-------------|---|--|-----------|
| Old leaf    | Cassia     | Mine OB      | 88.9±0.9*** | 1.9±0.5*                                  | 36.1±1.2**   | 4.1±0.1*  |
|             |            | Control soil | 20.8±0.2    | 0.6±0.1                                   | 24.3±1.1   | 2.9±0.1   |
|             | Dhaincha   | Mine OB      | 43.6±0.3*** | 1.7±0.09*                                 | 45.9±2.1**   | 3.8±0.2*  |
|             |            | Control soil | 5.6±0.1     | 0.5±0.01                                  | 17.1±0.2   | 2.3±0.3   |
|             | Sisso      | Mine OB      | 25.1±1.2**  | 1.4±0.1*                                  | 34.1±2.1**   | 5.4±0.8*  |
|             |            | Control soil | 14.9±1.1    | 0.9±0.01                                  | 26.0±2.3   | 4.0±0.9   |
|             | Gomari     | Mine OB      | 26.4±0.6*   | 1.4±0.6                                   | 30.0±2.3*  | 6.5±0.2*  |
|             |            | Control soil | 22.0±1.1    | 1.0±0.1                                   | 26.5±1.2   | 4.9±0.3   |
|             | Citronella | Mine OB      | 69.1±1.6**  | 1.1±0.2                                   | 32.1±2.2**   | 4.4±0.2*  |
|             |            | Control soil | 49.5±1.0    | 0.8±0.01                                  | 18.9±1.8   | 3.6±0.3   |
|             | Lemongrass | Mine OB      | 56.7±0.5**  | 2.8±0.1*                                  | 29.0±2.1**   | 3.5±0.7   |
|             |            | Control soil | 40.9±0.1    | 0.8±0.1                                   | 14.6±1.1   | 4.3±0.5   |
| Young leaf  | Cassia     | Mine OB      | 52.7±1.6*** | 3.6±0.8**                                 | 48.9±1.8**   | 4.1±0.1*  |
|             |            | Control soil | 8.2±0.2     | 1.2±0.2                                   | 30.3±0.8   | 2.6±0.2   |
|             | Dhaincha   | Mine OB      | 26.0±1.8*** | 2.3±0.1                                   | 29.0±0.6**   | 3.3±0.3   |
|             |            | Control soil | 8.4±0.7     | 1.2±0.02                                  | 15.8±0.7   | 2.9±0.1   |
|             | Sisso      | Mine OB      | 69.4±1.2*** | 1.9±0.7                                   | 44.3±2.7**   | 3.0±0.3   |
|             |            | Control soil | 41.9±0.1    | 1.7±0.2                                   | 24.1±1.1   | 2.4±0.5   |
|             | Gomari     | Mine OB      | 61.7±1.2*** | 2.2±0.3                                   | 44.3±2.3**   | 8.2±0.2   |
|             |            | Control soil | 4.3±0.2     | 1.7±0.7                                   | 25.0±1.7   | 8.2±0.2   |
|             | Citronella | Mine OB      | 30.4±1.1*** | 4.3±0.6*                                  | 30.1±1.1**   | 4.8±0.7   |
|             |            | Control soil | 6.3±0.8     | 1.5±0.1                                   | 18.8±0.8   | 4.0±0.6   |
|             | Lemongrass | Mine OB      | 32.8±0.8**  | 3.2±0.8*                                  | 33.8±1.2**   | 4.5±0.2   |
|             |            | Control soil | 8.2±0.2     | 1.2±0.2                                   | 8.1±0.3  | 4.1±0.3   |
| Root        | Cassia     | Mine OB      | 61.8±1.1**  | 5.2±0.7*                                  | 70.5±1.8***  | 1.6±0.1   |
|             |            | Control soil | 40.0±1.2    | 2.3±0.2                                   | 41.6±0.9   | 3.6±0.7   |
|             | Dhaincha   | Mine OB      | 88.0±0.4**  | 1.4±0.2                                   | 121.6±1.2***   | 3.0±0.3   |
|             |            | Control soil | 39.4±0.2    | 1.4±0.1                                   | 60.9±0.7**   | 3.9±0.4   |
|             | Sisso      | Mine OB      | 63.7±1.7**  | 2.5±0.2**                                 | 30.2±0.3*  | 2.3±0.3   |
|             |            | Control soil | 35.1±0.9    | 0.2±0.01                                  | 20.9±0.8   | 3.3±0.1   |
|             | Gomari     | Mine OB      | 58.5±1.6**  | 1.4±0.1**                                 | 52.5±2.1**   | 3.6±0.1   |
|             |            | Control soil | 27.2±0.9    | 0.2±0.02                                  | 15.7±0.9   | 4.6±0.1   |
|             | Citronella | Mine OB      | 86.3±1.2*** | 1.4±0.4*                                  | 21.1±1.1*  | 2.0±0.2   |
|             |            | Control soil | 25.5±0.1    | 0.2±0.01                                  | 14.6±0.2   | 3.8±0.3   |
|             | Lemongrass | Mine OB      | 60.6±1.8*** | 1.7±0.02*                                 | 47.1±1.6**   | 2.5±0.7   |
|             |            | Control soil | 25.1±0.2    | 0.2±0.01                                  | 14.7±1.1   | 6.0±0.7   |

All the data's were mean of six observations with three replication each;  $\pm 1.0$  = standard error means of observed values; \* asterisk is th significant difference of paired comparison between mine OB and control soil plant species by 't' test; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$  \*\*\* =  $P < 0.001$

low net photosynthesis, reduced stomatal conductance (Table 4). The root growth, shoot length, leaf area and total biomass in mine plants were also low compared to control plants (Table 5).

#### Effect of mine OB on osmolyte, antioxidant, photosynthesis of different plant plants

Comparison of RLR, LP, proline and sugar accumulation in roots, young- and old- leaves of the mine OB plants is described in Fig. 1. Compared to leaves, the roots showed significantly decreased total soluble sugar content. In Cassia, the highest RLR

(68%) and sugar (15%) was found in old leaf followed by root > young leaf. The lipid peroxidation (LP) (408%) and proline (95%) were found the highest in roots followed by young > old leaf. In Dhaincha, roots had significantly higher RLR (238%) and proline (316%) and young and old leaves for LP (230%) and sugar (28.4%), respectively. In Gomari, significantly higher RLR (134%), LP (623%) and sugar (128%) were found in old leaves, while 75% higher accumulation of proline was found in root. In case of Sisso, higher RLR was found in root (143%), LP and sugar in young leaves and proline (81.8%) in

Table 3—Comparison of membrane antioxidant, nitrate reductase activity in old-, young leaves and roots of plant species grown in coal mine OB and control soil

| Plant parts | Plant      | Substrate    | POD<br>( $\mu\text{M mg}^{-1}$<br>protein) | AXP<br>( $\mu\text{M mg}^{-1}$<br>protein) | GR<br>( $\mu\text{M mg}^{-1}$<br>protein) | SOD<br>( $\text{U mg}^{-1}$<br>protein) | NR<br>( $\mu\text{MNH}_3$ release<br>$\text{mg}^{-1}$ protein) |           |
|-------------|------------|--------------|--|--|---|---|--|-----------|
| Old leaf    | Cassia     | Mine OB      | 3.24±0.7**                                 | 177.53±1.0**                               | 48.73±1.2**                               | 59.18±2.2*                              | 1.25±0.1   |           |
|             |            | Control soil | 0.21±0.01                                  | 135.89±1.8                                 | 24.90±1.6                                 | 33.14±1.8                               | 6.75±0.2   |           |
|             | Dhaincha   | Mine OB      | 1.53±0.02*                                 | 264.4±2.1**                                | 61.43±1.1**                               | 62.24±1.5**                             | 1.30±0.3   |           |
|             |            | Control soil | 0.42±0.01                                  | 193.8±2.2                                  | 35.53±1.8                                 | 56.86±2.7                               | 3.40±0.5   |           |
|             | Sisso      | Mine OB      | 1.51±0.01*                                 | 176.91±1.3                                 | 93.26±1.9**                               | 6.87±1.1                                | 2.17±0.1   |           |
|             |            | Control soil | 0.45±0.01                                  | 176.23±1.8                                 | 52.13±2.1                                 | 13.63±1.2                               | 7.22±0.9   |           |
|             | Gomari     | Mine OB      | 0.78±0.02*                                 | 238.3±1.9**                                | 52.59±1.3**                               | 12.64±1.8*                              | 2.36±0.3   |           |
|             |            | Control soil | 0.20±0.01                                  | 183.16±1.7                                 | 44.70±2.6                                 | 8.83±1.6                                | 4.36±0.7   |           |
|             | Citronella | Mine OB      | 0.39±0.03*                                 | 307.33±1.3*                                | 48.26±2.2**                               | 63.86±2.1*                              | 1.38±0.3   |           |
|             |            | Control soil | 0.16±0.01                                  | 303.56±2.3                                 | 29.40±1.8                                 | 54.32±1.2                               | 5.02±0.1   |           |
|             | Lemongrass | Mine OB      | 0.16±0.01**                                | 301.00±2.7**                               | 56.30±1.3*                                | 79.06±1.9*                              | 1.04±0.5   |           |
|             |            | Control soil | 0.08±0.01                                  | 157.97±3.1                                 | 43.23±2.1                                 | 65.52±1.8                               | 3.86±0.3   |           |
|             | Young leaf | Cassia       | Mine OB                                    | 0.90±0.01*                                 | 566.40±3.2***                             | 30.82±2.2*                              | 8.04±1.1*  | 0.26±0.01 |
|             |            |              | Control soil                               | 0.33±0.02                                  | 66.26±2.3                                 | 26.30±2.6                               | 5.86±1.1   | 1.24±0.2  |
| Dhaincha    |            | Mine OB      | 1.02±0.02**                                | 66.06±2.7*                                 | 33.53±2.5*                                | 183.45±2.2*                             | 0.53±0.09  |           |
|             |            | Control soil | 0.42±0.01                                  | 54.32±2.1                                  | 25.26±1.8                                 | 172.04±2.3                              | 0.80±0.02  |           |
| Sisso       |            | Mine OB      | 1.02±0.02**                                | 154.66±3.1**                               | 53.33±1.3**                               | 126.67±2.7***                           | 0.34±0.07  |           |
|             |            | Control soil | 0.18±0.03                                  | 124.80±2.2                                 | 36.10±1.7                                 | 28.39±1.9                               | 2.41±0.2   |           |
| Gomari      |            | Mine OB      | 0.59±0.01*                                 | 133.26±2.7**                               | 44.57±2.1*                                | 21.36±1.3                               | 0.41±0.01  |           |
|             |            | Control soil | 0.35±0.05                                  | 93.78±2.3                                  | 32.86±2.3                                 | 14.59±1.2*                              | 0.48±0.03  |           |
| Citronella  |            | Mine OB      | 1.06±0.02**                                | 432.93±2.1**                               | 39.53±2.2*                                | 182.04±2.3**                            | 0.33±0.01  |           |
|             |            | Control soil | 0.75±0.02                                  | 135.20±2.4                                 | 31.33±2.1                                 | 83.83±2.3                               | 0.84±0.02  |           |
| Lemongrass  |            | Mine OB      | 1.98±0.06*                                 | 150.11±2.3**                               | 54.63±1.8                                 | 193.32±2.1***                           | 0.17±0.01  |           |
|             |            | Control soil | 1.01±0.03                                  | 134.90±2.8                                 | 50.40±1.9                                 | 88.24±2.3                               | 0.87±0.01  |           |
| Roots       |            | Cassia       | Mine OB                                    | 1.02±0.02                                  | 183.10±3.1***                             | 308.1±2.3***                            | 149.57±2.5**   | 0.39±0.02 |
|             |            |              | Control soil                               | 1.05±0.02                                  | 31.21±2.1                                 | 23.7±2.1                                | 131.81±2.6   | 1.57±0.01 |
|             | Dhaincha   | Mine OB      | 1.85±0.07                                  | 169.11±2.2**                               | 242.2±3.2***                              | 168.59±2.1**                            | 0.24±0.01  |           |
|             |            | Control soil | 2.09±0.06**                                | 156.00±2.1                                 | 68.1±3.1                                  | 65.47±2.3                               | 1.58±0.05  |           |
|             | Sisso      | Mine OB      | 1.16±0.05                                  | 67.10±2.7*                                 | 176.1±3.3**                               | 111.24±2.7**                            | 0.47±0.05  |           |
|             |            | Control soil | 0.76±0.02                                  | 59.15±1.8                                  | 93.1±2.3                                  | 26.49±2.5                               | 1.69±0.05  |           |
|             | Gomari     | Mine OB      | 0.61±0.02*                                 | 87.10±1.3**                                | 43.9±2.7**                                | 75.81±2.1**                             | 0.05±0.01  |           |
|             |            | Control soil | 0.32±0.02                                  | 18.24±1.2                                  | 18.2±2.1                                  | 16.06±2.3                               | 1.34±0.02  |           |
|             | Citronella | Mine OB      | 1.71±0.03                                  | 199.10±1.9***                              | 184.3±1.8***                              | 147.23±1.8***                           | 0.15±0.01  |           |
|             |            | Control soil | 2.19±0.06                                  | 38.11±2.1                                  | 24.11±1.1                                 | 53.53±1.9                               | 3.41±0.03  |           |
|             | Lemongrass | Mine OB      | 1.89±0.07                                  | 180.00±2.1***                              | 235.01±2.6**                              | 148.33±2.7***                           | 0.33±0.03  |           |
|             |            | Control soil | 2.13±0.09                                  | 18.40±2.7                                  | 21.9±1.1                                  | 40.03±1.8                               | 1.59±0.01  |           |

[All the data's were mean of six observations with three replication each; ±1.0= standard error means of observed values; \* asterisk is the significant difference of paired comparison between mine OB and control soil plant species by 't' test; \*= $P < 0.05$ ; \*\*= $P < 0.01$ ; \*\*\*= $P < 0.001$ ]

Table 4—Comparison of net photosynthesis rate and stomatal conductance of the plants grown in mine OB and control soil

| Plant      | Substrate    | Net photosynthesis ( $\mu\text{mol cm}^{-2} \text{S}^{-1}$ ) | Stomatal conductance<br>( $\mu\text{mol cm}^{-2} \text{S}^{-1}$ ) |
|------------|--------------|--|---|
| Cassia     | Mine OB      | 0.5 ±0.07  | 18.3±1.2  |
|            | Control soil | 10.0±0.2**   | 40.4±0.7**  |
| Dhaincha   | Mine OB      | 0.3±0.02   | 13.8±0.5  |
|            | Control soil | 9.3±0.5**  | 46.9±0.5**  |
| Gomari     | Mine OB      | 0.2±0.01   | 8.7±0.6   |
|            | Control soil | 15.5±0.3***  | 45.4±1.2**  |
| Sisso      | Mine OB      | 0.4±0.01   | 19.7±1.0  |
|            | Control soil | 14.6±0.4***  | 36.6±1.1**  |
| Citronella | Mine OB      | 0.3±0.01   | 22.3±1.6  |
|            | Control soil | 5.5±0.1***   | 32.4±0.5**  |
| Lemongrass | Mine OB      | 0.2±0.01   | 18.3±1.1  |
|            | Control soil | 6.1±0.3***   | 35.0±1.2**  |

[All the data's were mean of three individual observations; ±1.0= standard error means of observed values; \* asterisk is the significant difference of paired comparison between mine OB and Control soil plant species by 't' test; \*\*= $P < 0.01$ ; \*\*\*= $P < 0.001$ ]

Table 5—Comparison of root length, shoot length and biomass of the plant species grown in mine OB and in control soil

| Plant      | Substrate    | Root length (cm) | Shoot length (cm) | Leaf area (cm <sup>2</sup> ) | Biomass (g) |
|------------|--------------|------------------|-------------------|------------------------------|-------------|
| Cassia     | Mine OB      | 5.2±0.7ns        | 21.0±0.5**        | 24.5±1.5**                   | 4.1±2.1     |
|            | Control soil | 6.2±0.6          | 26.0±1.1          | 45.5±1.2                     | 5.2±1.5ns   |
| Dhaincha   | Mine OB      | 2.0±0.2ns        | 31.3±0.8**        | 5.4±0.4*                     | 5.1±2.1     |
|            | Control soil | 3.3±0.2          | 45.6±0.6          | 9.3±0.4                      | 6.9±2.2*    |
| Gomari     | Mine OB      | 32.0±1.6ns       | 23.0±1.2**        | 5.7±1.8*                     | 17.3±1.8**  |
|            | Control soil | 31.9±2.7         | 46.6±1.2          | 14.8±3.1                     | 21.7±2.6    |
| Sisso      | Mine OB      | 23.0±2.7***      | 62.6±0.3*         | 70.9±7.9*                    | 7.1±2.2*    |
|            | Control soil | 47.6±2.6         | 65.8±0.9          | 146.1±1.2                    | 10.7±2.5    |
| Citronella | Mine OB      | 5.8±1.2**        | 23.8±2.5*         | 13.7±1.8*                    | 4.6±1.2*    |
|            | Control soil | 12.2±1.2         | 33.8±3.3          | 22.1±2.5                     | 8.2±2.2     |
| Lemongrass | Mine OB      | 7.9±2.1**        | 25.7±2.6**        | 19.3±2.1*                    | 2.1±1.2*    |
|            | Control soil | 18.3±2.3         | 41.0±2.5          | 23.0±1.2                     | 4.7±2.2     |

[All the data's were mean of three individual observations; ±1.0= standard error means of observed values; \* asterisk is the significant difference of paired comparison between mine OB and control soil plant species by 't' test; \*=*P* <0.05; \*\*=*P* <0.01; \*\*\*=*P* <0.001]

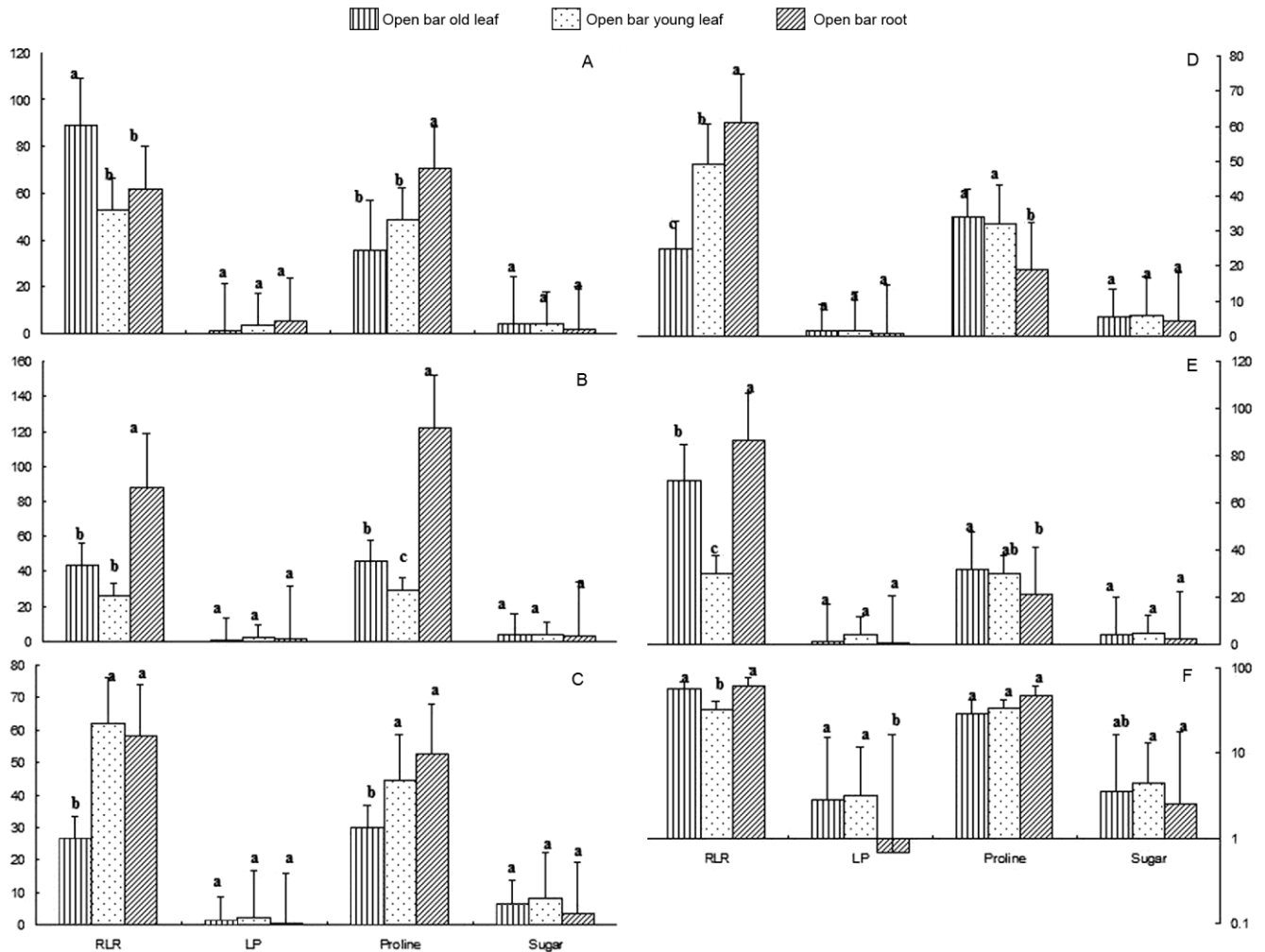


Fig. 1—Comparison of membrane stability and osmolyte accumulation in old/young leaves and roots in the plant species tolerant to coal mine OB. [A-F denotes Cassia, Dhaincha, Gomari, Sisso, Citronella and Lemon grass, respectively. All the data's were mean of three individual observations; Error bars= standard error means of observed values; error bars followed by similar letters are not significantly different from each other between leaves and root according to student's 't' test (*P* <0.01); RLR= relative leakage rate (%); LP= lipid peroxidation (nM malonaldehyde released mL<sup>-1</sup>); Proline= (μMol tyrosine released mg<sup>-1</sup> cell mass); sugar (%)]

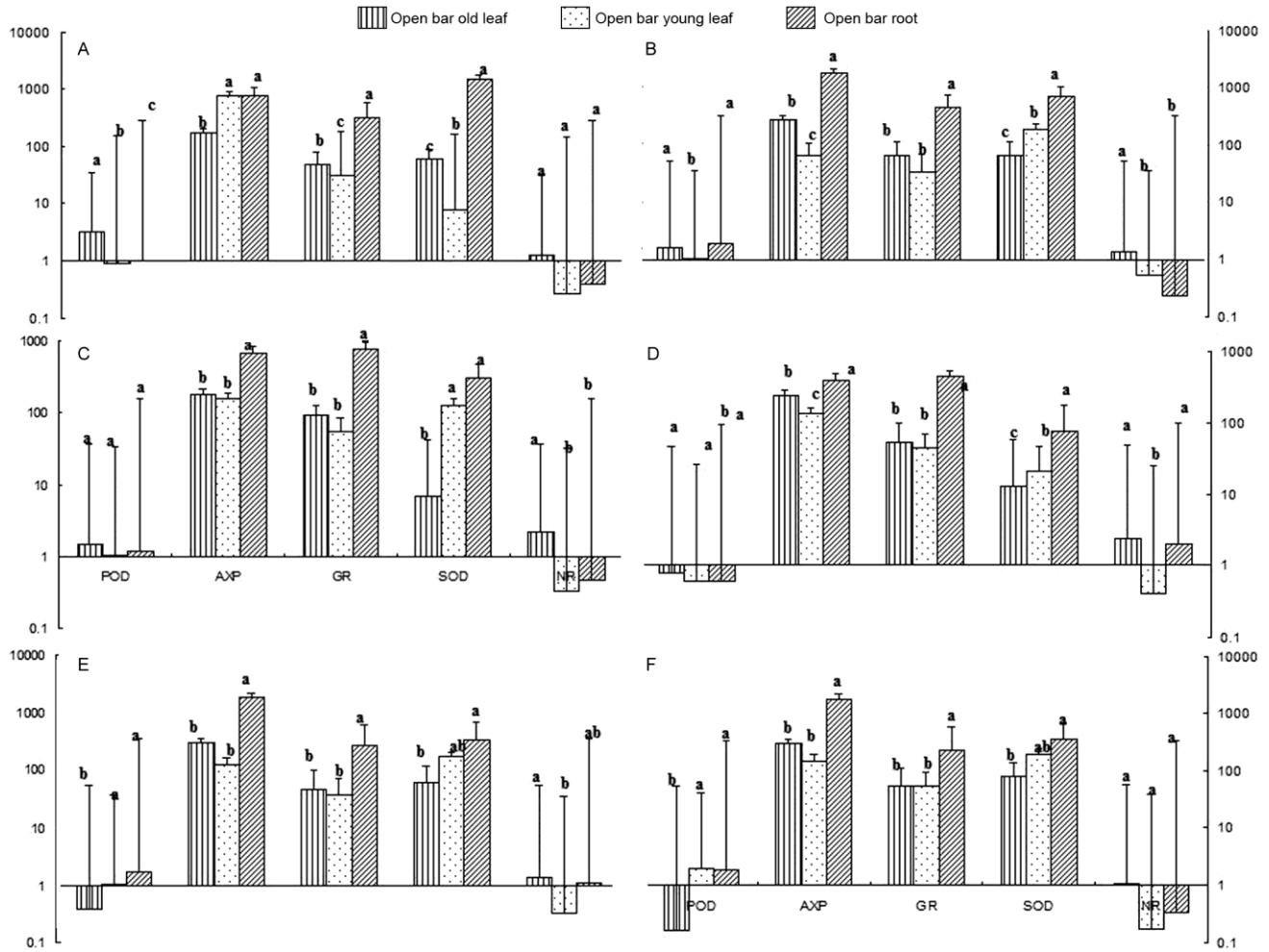


Fig. 2—Comparison of antioxidant and nitrate reductase enzyme activity in old/young leaves and roots in the plant species tolerant to coal mine overburden (OB). [A-F denotes Cassia, Dhaincha, Gomari, Sisso, Citronella and Lemon grass, respectively. All the data's were mean of three individual observations; Error bars= standard error means of observed values; Error bars= standard error means of observed values; error bars followed by similar letters are not significantly different from each other between leaves and root according to student's 't' test ( $P < 0.01$ ); POD= Peroxidase activity ( $\mu\text{M mg}^{-1}$  protein); AXP= GR= Gltathione reductase activity ( $\mu\text{M mg}^{-1}$  protein); SOD= superoxide dismutase activity ( $\text{U mg}^{-1}$  protein); NR= Nitrate reductase activity ( $\mu\text{M NH}_3$  released  $\text{mg}^{-1}$  protein)]

old leaves. Both Citronella (183%) and Lemon grass (85%) showed highest RLR in root; LP and sugar in young leaves while proline in old leaves (52.4%) and root 62.9%), respectively. In antioxidant enzyme activities, all the tested plants showed significantly higher APX (78-2458%), GR (115-1326.7%) and SOD (340-4340%), respectively (Fig. 2). Except Citronella and Lemon grass, the other species showed highest POD activities in old leaves. No similar trend of highest antioxidant in roots followed by leaves or *vice versa* was observed.

**Comparison of osmolyte, antioxidant and photosynthesis in different mine OB plants**

Significantly highest and lowest RLR activities were found in Cassia (88.8%) and Gomari (25.1) in old leaves. In young and old leaves, highest RLR

activity was found in Gomari (61.7%) followed by Dhaincha (25.9%) while in root it was Dhaincha (88%) and lowest in Gomari (58.5%). The highest LP activities in old as well as young leaves and roots were found for Lemon grass (2.7), Citronella (4.3) and Cassia (5.2) while lowest was found in Dhaincha (0.7), Sisso (1.3). Significantly highest proline accumulation in old leaves and roots was found in Dhaincha (45.8 and 121.6) and lowest in Lemon grass (28.9) and Sisso (18.8). In young leaves, it was highest in Cassia and lowest in Dhaincha. Significantly higher sugar in old and young leaves (6.5 and 8.1%) was found in Gomari followed by Sisso root (4.2%), Citronella (3.4%), Dhaincha (3.3%) and Cassia (1.6%), respectively. In old leaves, significantly highest POD, APX, GR, SOD were found in Cassia (3.2), Citronella (307.3),

Gomari (93.2) and Lemon grass (79.0), while it was lowest in Lemon grass (0.1), Gomari both for APX (176.9) and SOD (6.8) and Citronella (48.2), respectively. In young leaves, overall highest antioxidant enzymes POD, GR and SOD were found in Lemon grass (1.9, and 54.6, 193.3), and AXP in Cassia (766.4), respectively.

## Discussion

Highly acidic nature (pH 2.0-3.0) with low water holding capacity, higher cation exchange rate, >12% S with above permissible limits of trace and heavy metals content are the harsh environmental condition of high sulphur coal mine OB<sup>2,3</sup>. Except few tolerant plant species, other plants do not grow. Even among the tolerant ones, few specific plants require more than 2-3 decades to establish. In our previous study, we have reported some of the plants tolerant to high sulphur coalmine OB<sup>2</sup>. Therefore, to enhance the survivability of plants and to mimic the environment of mine OB, the documentation of stress related characters of plants is essential. In the present study, an appreciably higher GR, POD, AXP, SOD activities were found in young leaf, old leaf and root of mine OB plants compared to control plants. The disturbances in equilibrium due to heavy and trace metals, temperature variations, nutrient deficiency and no true soil behavior of mine OB along with acidity and sulphur may escalate antioxidant activities. It has been reported that plants grown under adverse environmental condition showed higher antioxidant activities to prevent cellular and other damage<sup>25-27</sup>. The findings of the present study are in agreement with several reported works<sup>28-30</sup>. Compared to antioxidant enzyme activities the contrasting decrease of NR activities under mine OB stress condition is due to low uptake capability of nitrogen which is in general deficient in coal mine OB. Sharma *et al.*<sup>31</sup>, have reported decreased NR activities under stress in root and leaves of rice seedlings.

Higher accumulation of osmoprotectant proline under drought stress is also well documented<sup>32,33</sup>. Like antioxidant enzyme activities, the three to four fold increases in proline and sugar accumulation in young and old leaves of mine OB plants to that of control plants were also supports the effect of mine OB. The water deficit, salinity, low and high temperature in mine OB condition also leads to accumulation of solute in mine OB is in agreement with earlier reported works<sup>34,35</sup>. The plants grown in mine OB also had higher basal roots, shortened

nodal length and smaller leaves with an overall low photosynthesis and stomatal conductance.

Compared to leaves, roots of all the mine substrate plants have shown almost six times higher GR, APX and SOD activity. However, some workers reported decreased GR activity of root compared to leaf under stress conditions<sup>35,36</sup>. Overall, higher POD activities were found in leaf than root and old leaves. The increased POD activity in all plant parts, particularly young leaves, indicates that the plants try to overcome oxidative stress by raising POD activity to a higher level. In case of SOD, compared to young and old leaves root exhibited much higher level of SOD activities. In young leaf, there was considerable amount of MDA but compared to young leaves, in old leaves MDA generation was much more. Higher MDA highlights the oxidative membrane damage. The present finding of increased electrolyte leakage in leaf and root of mine OB plants is supported by earlier reported works<sup>36,37</sup>.

Species to species variations of stress activities of mine OB plants occurred as a general trend but no specific pattern of membrane stability and osmolyte accumulation. The variation in the present study is due to the nature of the plant i.e., shrubs, trees and grass. The variation in change of osmolyte and membrane stability of grass species, shrubs and trees were reported earlier<sup>37,38</sup>. There was also variation in net photosynthesis (NP) in the mine substrate plants (Table 4). Higher NP was found in Cassia followed by Sisso > Dhaincha = Citronella > Gomari = Lemon grass, respectively.

## Conclusion

Overall, higher osmolyte accumulation, membrane damage and antioxidant enzyme activities with stunted growth parameters were found in mine OB plants. Citronella and Lemon grass showed higher antioxidant characters. The higher antioxidant activities in present findings were due to high sulphur, above threshold limit of trace and heavy metals, temperature variation, nutrient deficiency and no true soil behavior of mine OB. Higher antioxidant enzyme activities activity with stunted growth supports the adaptability of the plants to high sulphur coalmine OB.

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