# Non-uniform, patchy stomatal closure of a plant is a strong determinant of plant growth under stressful situation

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**The stomatal response of cassia (*Cassia streata* L.) and dhaincha (*Sesbania rostrata* L.) to a coalmine overburden (OB) substrate was studied with a view to rehabilitate such areas. Plants raised in unmined soil were used as controls. The mine OB induced significant increase in stomatal index (SI) with diminished stomatal size and a stomatal closure in the test plants. The leaf water status measured as water content was significantly enhanced, while the relative growth rate was markedly reduced. This is indicative of slow but sustainable growth of the species in mine OB adverse conditions.**

**Keywords:** *Cassia streata*, coalmine overburden, leaf water content, *Sesbania rostrata*, stomata.

NEARLY hundred years ago Francis Darwin showed that stomata on leaves respond to environmental stimuli. Now much information on the mechanisms of stomatal opening and closing and on stomatal response to varying environmental conditions has been generated[1]. Yet there are still unanswered questions surrounding stomatal behaviour in response to the environment. The basic function of stomata is to regulate $CO_2$, $O_2$ and water vapour exchange between the plant and the environment[2]. Stomata are sufficiently sensitive to respond to changes in the environment, and either open or close in an effort to acclimatize[2–5]. Changes in the degree of stomatal opening reflect the cumulative effect of many physiological responses by a leaf to its environment[6,7]. Measurements of the degree of stomatal opening on a leaf surface provide a convenient visual indication of stomatal responses to environmental conditions[8,9]. The dimensions of stomatal pores have a great effect on the rate of gas exchange for the entire leaf, determined by the response of all stomatal pores on a leaf to ambient environmental conditions[2]. The ecological implications of stomatal response are the focus of current research. Information on stomatal response in many species to typical opencast mining areas is not easily accessible. The present investigation was carried out to investigate the stomatal behaviour of cassia (*Cassia streata*) and dhaincha (*Sesbania rostrata*) species grown on a coalmine overburden (OB) substrate. *C. streata* and *S. rostrata* were selected on the basis of their performance under high sulphur containing coalmine OB as observed in a prescreening test. Therefore, understanding of this behaviour should help attempts to rehabilitate colliery-devastated areas in Assam, India.

Mine tailings/OB samples, together with unmined soil taken from areas growing natural vegetation, were collected from the Tirap colliery area in Assam. The mine tailings and unmined soil were manually broken to a fine tilth and used as separate substrates in earthen pots (bottom diameter 10 cm, height 18.5 cm) for plant-growth experiments. The characteristics of the substrates are given in Table 1. Nitrogen and total organic carbon of the substrates were estimated according Kjeldhal digestion and potassium dichromate oxidation[10,11], and phosphorus spectrophotometrically[12]. The particle size distribution of the substrates was determined using a laser diffraction particle size analyser (model CILAS 1180) using sodium carbonate and hexametaphosphate as the dispersing agents.

Seeds of two drought-tolerant plant species, *C. streata* and *S. rostrata*, were sown in a nursery bed (40 cm × 60 cm) under polyhouse conditions. The seedlings were allowed to grow in the nursery bed for a period of 21 days. Uniform-sized seedlings from the bed were transferred to experimental pots prepared with mine OB and unmined soil. The seedlings were raised under standard polyhouse conditions for a period of 90 days. Intermittent irrigation was used to simulate a moderate moisture stress situation.

The relative water content (RWC) was determined taking the fully expanded topmost leaf of the main shoot at 30, 60 and 90 days of plant growth[13]. The fresh weight of the sample leaves was recorded and the leaves were immersed in distilled water in a petri dish. After 2 h, the leaves were removed, the surface water was blotted-off and the turgid weight recorded. Samples were then dried in an oven at 80°C to constant weight. RWC was calculated using the formula:

$$\text{RWC (\%)} = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Turgid weight} - \text{dry weight}} \times 100.$$

To compute the relative growth rate (RGR), the plant dry weight (shoots) was obtained at weekly intervals[14]. Samples were dried at 70°C to constant weight. RGR was calculated using the equation:

$$\text{RGR} = \log W_2 - \log W_1/(t_2 - t_1),$$

where $W_1$ and $W_2$ are the plant dry weight (g $g^{-1}$ $week^{-1}$) at times $t_1$ and $t_2$ respectively.

Light intensity during the experiments was recorded using a Guarda FF-101 lux meter (Figure 1).

The topmost fully expanded leaf from the main shoot was considered for stomatal studies. Peelings of leaf epi-

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**Table 1.** Characteristics of coalmine overburden (OB) and unmined soil substrates

| Substrate | Component (%) | | | | | | | | Microbial biomass (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | pH | C | N | P | K | Silt | Sand | Clay | |
| Unmined soil | 6.5 | 9.29 ± 2.3 | 1.47 ± 0.21 | 0.56 ± 0.23 | 0.21 ± 0.02 | 31.27 ± 3.1 | 32.25 ± 6.0 | 28.63 ± 2.1 | 305 ± 4.5 |
| Mine OB | 2.5 | 1.32 ± 1.1 | 0.002 ± 0.001 | 0.025 ± 0.001 | 0.005 ± 0.001 | 39.99 ± 2.8 | 39.94 ± 2.6 | 21.93 ± 3.5 | 64.33 ± 2.2 |

Data are mean ± SD ($n$ = 5).

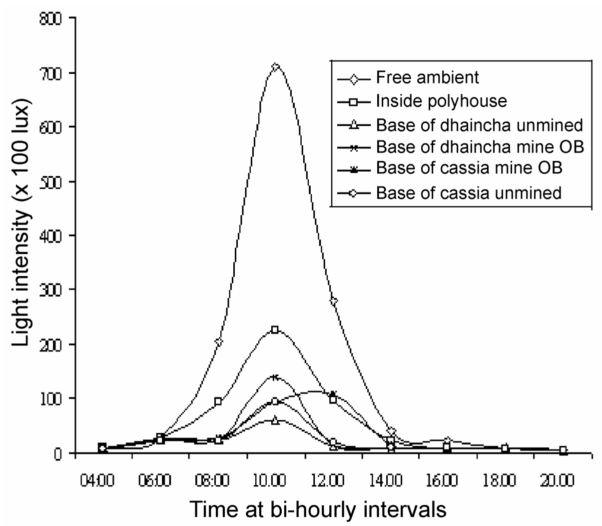


**Figure 1.** Light intensity: free ambient, inside the polyhouse and at the base of dhaincha (*Sesbania rostrata*) and cassia (*Cassia streata*) plants grown in coalmine overburden (OB) and in unmined soil.

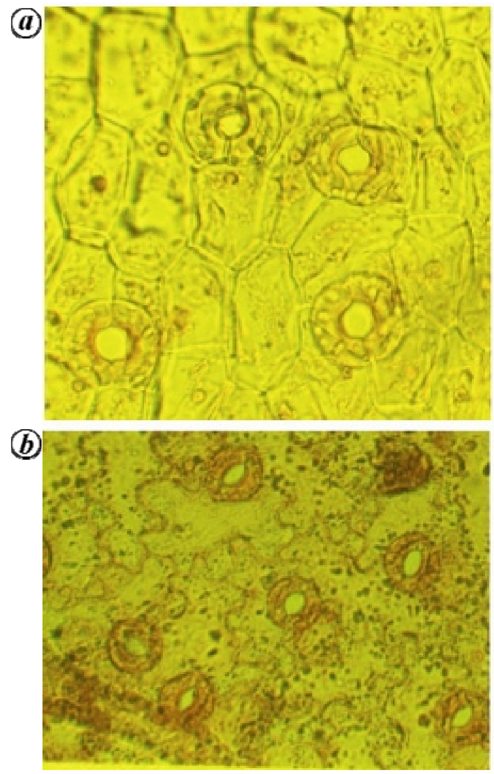


**Figure 2.** Typical photographs of stomata of *C. streata* (***a***) and *S. rostrata* (***b***). The photographs were taken using the Leitz Orthomate E digital camera at 40× exposure, connected to a microscope.

dermal layers were taken at 2 h intervals between 04:00 and 16:00 h. The number of stomata and epidermal cells was counted in microscopic fields using a Leitz microscope at 40× magnification. Measurements of stomatal components such as guard cells, stomatal pores and stomata were carried out using ocular and stage micrometers. Photographs were taken using a Leitz Orthomate E digital camera attached to the microscope (Figure 2). The stomatal index (SI) was calculated by dividing the number of stomata in a microscopic field by the combined number of epidermal cells and stomata in the same field, expressed as a percentage[15].

Data generated in the experiments were analysed using the Analysis Tool Pack in Microsoft® Excel 97 SR-1. Analysis of variance (ANOVA) was carried out to test the null hypothesis[16] that the sample means were significantly different from each other at a significance level of $P > 0.01$. The standard errors of simple means were computed at a 95% level of confidence.

Plants grown on mine OB substrate showed higher stomatal frequency, as indicated by greater SI values (Table 2). The increase in SI under mine OB was greater in cassia (17.04%) than in dhaincha (6.78%) plants. However, dhaincha recorded greater SI values than cassia for mine OB and unmined substrates. Stomata are the gateway of plants to obtain resources for the vital processes of photosynthesis and respiration, as the epidermis is almost impervious to gases and water vapour. Stomatal behaviour is directly related to plant–water relations and is also closely associated with plant growth[17]. Stomatal frequency and size of the stomatal pores are significance in this regard[18]. However, stomatal frequency as well as the geometry of substomatal cavities and the length of the stomatal pore, can be regarded as more or less fixed and invariant for the leaves of a given plant species[19]. In the present study, the mine OB caused a variation in SI, with a greater response in cassia than in dhaincha plants. The potential for water loss increased in proportion to increase in the number of stomata on a leaf.

The mine OB also influenced the dimensions of stomatal components such as guard cells, pores and stomata in both cassia and dhaincha (Table 3). The greatest effect of the mine OB was on pore size, with a 22.46% reduction for cassia and a 19.68% reduction for dhaincha. The guard cell diameter was 18.84 and 19.68% lower and stomatal diameter was 17.77 and 9.45% lower in cassia and dhaincha respectively, compared to plants grown in the unmined soil. The mine OB induced closure of stomata per unit leaf area in both cassia and dhaincha plants (Figure 3). In cassia, stomatal closure was 14.29% higher for mine OB plants than the unmined control plants. In dhaincha, this increase was 11.80%. The internal resistance to outward movement of water vapour for a leaf primarily depends on stomatal pores, with a smaller aperture resulting in greater resistance. The mine OB induced significant changes in pore size in cassia and dhaincha plants. This must have reduced transpirational water vapour loss from the leaves. A concomitant shrinkage of guard cells was observed in the stomata, contributing to the diminished stomatal size in both cassia and dhaincha plants grown on mine OB substrate.

RWC was higher in plants raised on mine OB substrate (Figure 4). A 4.52% increase was recorded in cassia, while the increase in dhaincha was 0.65%. The percentage of closed stomata per unit leaf area is a significant feature of plant adaptation. Higher percentage of stomatal closure was observed for cassia and dhaincha plants raised on mine OB compared to unmined soil. Plant RGR was considerably lower when grown on mine OB substrate, as assessed on a per-week basis (Figure 5). A 65.51% reduction was recorded for cassia. For dhaincha, the reduction was 62.21%. When the water status of these leaves was assessed, higher RWC was recorded. Despite better water status, the biomass production rates of cassia and dhaincha plants were considerably reduced by the mine OB substrate.

Guard cells are continuously in motion and thus regulate stomatal pores. By varying the stomatal pores, the plant controls resource exchange; at any point in time, not all

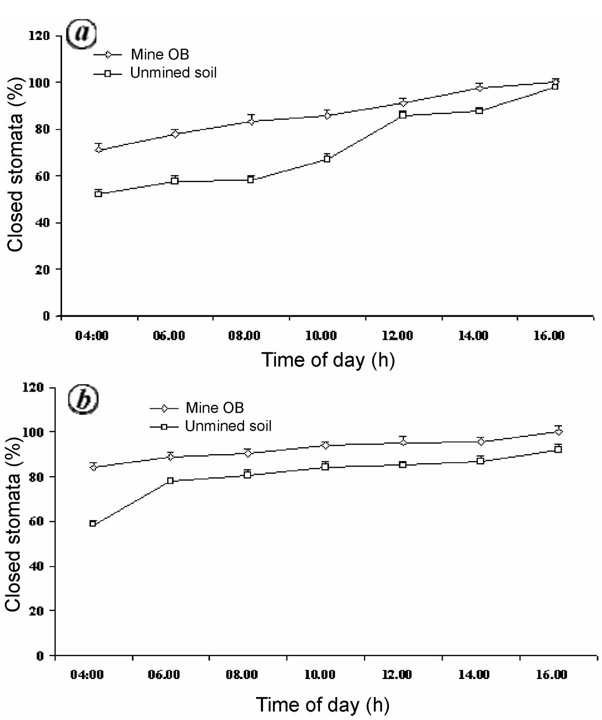


**Figure 3.** Percentage of closed stomata in leaves of plants grown in coalmine OB and in unmined soil. ***a***, *C. streata*; ***b***, *S. rostrata*. Data are mean of three observations at 30, 60 and 90 days of plant growth. Error bars represent standard deviation of observed values.

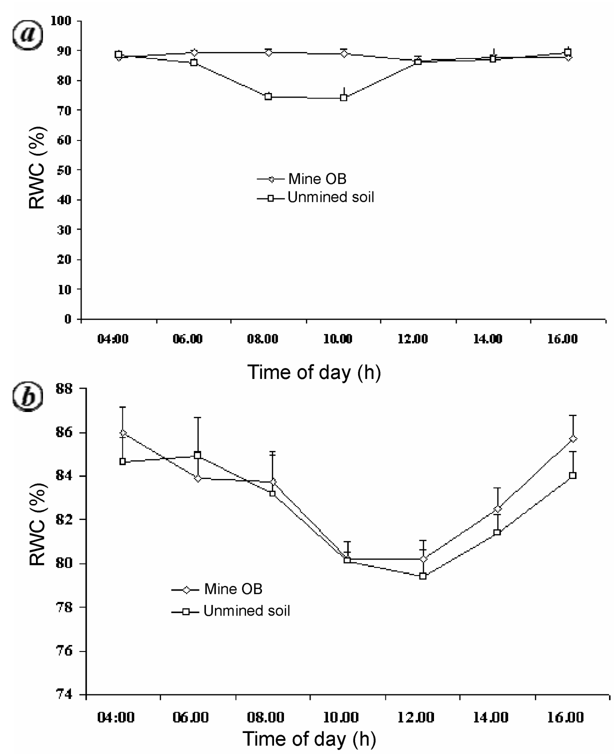


**Figure 4.** Relative water content (RWC) of plants grown in coalmine OB and unmined soil. ***a***, *C. streata*; ***b***, *S. rostrata*. Data are mean of three observations at 30, 60 and 90 days of plant growth. Error bars represent standard deviation of observed values.

**Table 2.** Stomatal index of *Cassia streata* and *Sesbania rostrata* grown in coalmine OB substrate and unmined soil respectively

| Plant species | Substrate | Stomatal index (%) |
|---|---|---|
| *C. streata* | Unmined soil | 9.33 ± 2.5 |
| | Mine OB | 10.92 ± 3.1 |
| *S. rostrata* | Unmined soil | 14.90 ± 2.8 |
| | Mine OB | 15.91 ± 4.1 |

Data are mean ± SD ($n$ = 5).

**Table 3.** Dimensions of stomatal components of *C. streata* and *S. rostrata* leaves grown in coalmine OB and unmined soil substrates

| | | *C. streata* | | | *S. rostrata* | | |
|---|---|---|---|---|---|---|---|
| Time (h) | Substrate | Guard cell diameter (μm) | Pore diameter (μm) | Stomatal diameter (μm) | Guard cell diameter (μm) | Pore diameter (μm) | Stomatal diameter (μm) |
| 04:00 | Unmined soil | 2.45 ± 0.27 | 2.22 ± 2.36 | 6.55 ± 1.58 | 3.30 ± 0.12 | 3.37 ± 0.002 | 7.10 ± 0.68 |
| | Mine OB | 2.45 ± 0.03 | 1.37 ± 2.04 | 4.80 ± 8.10 | 2.19 ± 0.12 | 2.46 ± 0.01 | 6.10 ± 4.73 |
| 06:00 | Unmined soil | 3.81 ± 0.11 | 2.20 ± 1.58 | 5.85 ± 4.83 | 3.42 ± 0.56 | 3.82 ± 0.01 | 7.60 ± 1.26 |
| | Mine OB | 3.00 ± 0.16 | 1.22 ± 0.03 | 5.20 ± 1.173 | 2.02 ± 0.16 | 2.62 ± 0.06 | 6.65 ± 0.70 |
| 08:00 | Unmined soil | 3.62 ± 0.72 | 3.56 ± 0.27 | 7.75 ± 2.25 | 2.72 ± 0.37 | 2.22 ± 3.44 | 7.42 ± 0.5 |
| | Mine OB | 2.45 ± 0.55 | 2.75 ± 0.91 | 5.00 ± 2.74 | 2.37 ± 0.32 | 2.45 ± 0.00 | 6.20 ± 2.0 |
| 10.00 | Unmined soil | 3.67 ± 0.155 | 3.4 ± 0.033 | 5.95 ± 11.6 | 3.05 ± 0.13 | 2.45 ± 0.01 | 6.52 ± 1.80 |
| | Mine OB | 3.15 ± 0.33 | 2.46 ± 0.01 | 4.57 ± 0.08 | 2.77 ± 0.28 | 2.40 ± 0.33 | 6.27 ± 0.2 |
| 12.00 | Unmined soil | 4.05 ± 0.59 | 3.46 ± 0.07 | 4.10 ± 0.60 | 1.95 ± 0.15 | 2.55 ± 0.03 | 4.7 ± 0.92 |
| | Mine OB | 3.15 ± 0.33 | 3.05 ± 0.02 | 3.70 ± 0.92 | 1.95 ± 0.15 | 2.40 ± 0.02 | 4.75 ± 0.8 |
| 14:00 | Unmined soil | 3.97 ± 0.52 | 2.4 ± 0.23 | 5.10 ± 1.57 | 2.40 ± 0.44 | 3.60 ± 0.00 | 6.52 ± 0.9 |
| | Mine OB | 3.11 ± 1.79 | 2.36 ± 1.32 | 5.25 ± 0.51 | 2.00 ± 0.06 | 2.60 ± 0.01 | 6.8 ± 2.5 |
| 16:00 | Unmined soil | 2.95 ± 1.25 | 2.17 ± 8.74 | 6.40 ± 6.40 | 2.42 ± 0.20 | 2.39 ± 0.01 | 5.77 ± 0.56 |
| | Mine OB | 2.59 ± 0.41 | 1.84 ± 0.04 | 5.77 ± 5.77 | 2.19 ± 0.12 | 1.46 ± 0.01 | 4.55 ± 2.78 |

Data are mean ± SD ($n$ = 5).
Data shown are the average of three observations at 30, 60 and 90 days of plant growth.

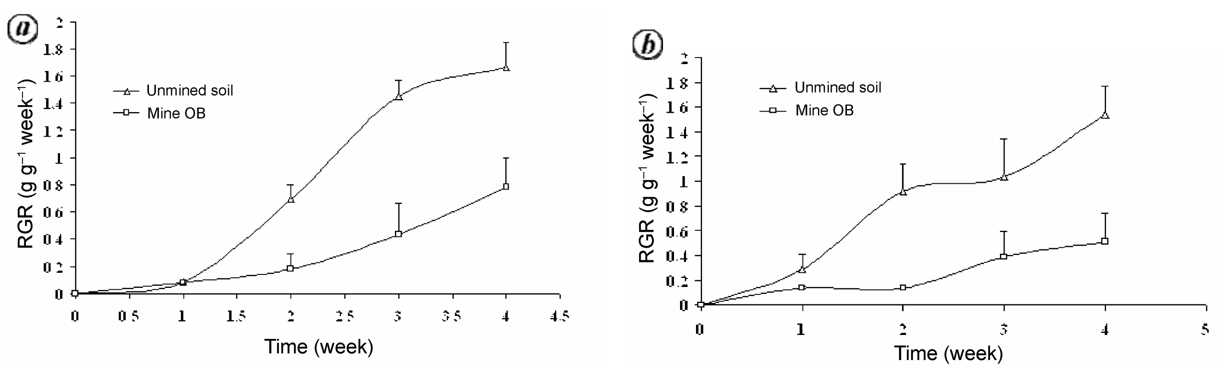


**Figure 5.** Relative growth rate (RGR) of plants grown in coalmine OB and unmined soil. ***a***, *C. streata*; ***b***, *S. rostrata*. Error bars represent standard deviation. RGR was determined on a weekly basis by comparing the total biomass of the plant up to four weeks.

stomata are open to the same extent. This non-uniform, patchy stomatal closure, which is more pronounced under stress situations, indicates that the mine OB substrate represents a stressful growth condition.

Studies have shown that leaves which grow in drier environment and higher light intensity tend to have smaller and numerous stomata than those grown in wet and shady conditions[19]. Under nutrient-deficient conditions the stomata respond sluggishly[3]. The role of moisture stress and acidity in stomatal closure is now well established. The light intensity and temperature fluctuation (minimum 28°C and maximum 34°C) during the experimental period were found to be optimal for plant growth and were not stressful for the cassia and dhaincha plants. It was the edaphic environment created by the mine OB substrate that evoked a strong response in stomatal closure of the test plants. As the silt and sand fractions were high, with only 20.07% clay, the mine OB was inefficient at holding water, with intermittent irrigation used to simulate moderate stress. The low pH and poor nutrient status of the mine OB might have compounded the stress, leading to effective stomatal closure. Plants regulate their diurnal water status at a favourable level by controlling the stomatal aperture[3,17,20]. With better relative water content the test plants, especially cassia, exhibited strong adaptation potential on mine OB. Although leaf photosynthesis decreases, stomatal closure contributes towards maintaining high water content and potential in the leaves[21]. Cassia and dhaincha plants also showed sustainable growth, but at a much reduced rate.

Stomatal response is undoubtedly a sensitive indicator of plant growth under stressful situations. The greater number of stomata per leaf with stomatal components of reduced size clearly indicate that cassia and dhaincha are potential candidates for rehabilitation of areas under typical coalmine tailings.


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ACKNOWLEDGEMENTS. We are grateful to Dr P. G. Rao, Director, RRL, Jorhat for support. We thank Dr B. K. Gogoi, Head, Biotechnology Division, RRL, Jorhat for support and guidance. CSIR, New Delhi provided financial assistance. We acknowledge the National Environmental Engineering Research Institute, Nagpur for coordination of the Network Mode Project IWMC.

Received 4 June 2007; revised accepted 4 April 2008

# Simultaneous detection of one RNA and one DNA virus from naturally infected citrus plants using duplex PCR technique

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**Citrus tristeza clostero virus (CTV), an aphid-transmitted RNA virus having a genome size of about 19.3 kb, singly or as a mixed infection with Citrus mosaic badna virus (CMBV), a non-enveloped bacilliform DNA virus having genome of 7.5 kb, plays a significant role in causing citrus decline, particularly in sweet orange in India. Rapid detection techniques are important in the prevention of spread of these two diseases in field conditions. Since CMBV is weakly immunogenic, sero-diagnosis is not the preferred diagnostic method. Similarly, for detection of CTV though serological techniques like ELISA are being widely used, production of polyclonal antibodies to various isolates of CTV is often limited by various factors, namely low yields of the virus in plant tissues, uneven distribution, difficulties in production of sufficient quantities of infected tissue, contamination by host proteins in purified preparation, international quarantine, etc. As an alternative, a rapid and reliable PCR based detection protocol has been standardized. Sets of primers were designed based on the respective virus isolate sequence data available in GenBank, to obtain anticipated products of calculated size.**

**Keywords:** Citrus plants, detection protocol, duplex PCR technique, RNA and DNA virus.

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