SCANNING ELECTRON MICROSCOPIC INVESTIGATIONS ON THE LEAF MICRO-MORPHOLOGICAL AND MESOPHYLL PARAMETERS IN ASSOCIATION WITH PHOTOSYNTHETIC ACTIVITY OF THREE MULBERRY (*MORUS* SPP.) GENOTYPES

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ABSTRACT

A scanning electron microscopic study on the micro-morphological and mesophyll parameters of three mulberry genotypes having different yield potentials *viz.* Punjab local, S-30 and China Peking was under taken to find out their association with photosynthetic rate. The results of the present study revealed that the leaf thickness of Punjab local was found maximum (127.42±0.582 µm) and conversely minimum in S-30 genotype (119.17±0.752 µm). Statistically significant differences were found among three genotypes for leaf thickness character against all the other parameters like thickness of palisade and spongy tissues, percentage of palisade parenchyma in mesophyll and the rate of photosynthesis. With regard to the total density of idioblasts per mm^2 leaf surface area, it was observed that sufficient variability exist among the genotypes. The experimental data also indicated the highest photosynthetic rate in S-30 genotype (21.56±0.357 µ mol m^{-2} s^{-1}) with contrastingly low in China Peking (11.06±0.529 µ mol m^{-2} s^{-1}) significantly associating with the respective micro-morphological and mesophyll characteristics. Hence, these structural traits which are found correlated with higher physiological efficiency can be conveniently utilized in the screening and evaluation of mulberry genotypes for further improvement in leaf yield and biomass production by the plant breeding programmes.

Keywords: micro-morphological parameters, mulberry genotypes, photosynthetic rate, PAR, SEM

INTRODUCTION

Mulberry (*Morus* spp.), the only food source of silkworm, *Bombyx mori* Linn., is of great economic importance to the sericulture industry. The quality and quantity of the leaf produced has a direct bearing on the cocoon production and in turn, the economy of the rural farmers. The different factors responsible for a successful cocoon crop are: mulberry leaf (38.2%), climatic conditions (37.0%), rearing techniques (9.3%), silkworm races (4.2%), silkworm eggs (3.1%) and other factors

(8.2%). Therefore, the economics and profitability of sericulture depends primarily on quality of the mulberry leaves produced (Singhal et al. 1999). Genetic and environmental factors influence the growth and quality of mulberry leaves. The effect of water stress on photosynthetic activity in various crops has been well established (Ackerson et al. 1977; Hutmacher & Krieg 1983; Ephrath et al. 1990; Srinivasa Rao & Bhatt 1990; Leidi et al. 1993). Chakrabarti et al. (1996) described the relationship between gas exchange traits and genetic variability in mulberry under irrigated and rainfed conditions. Recently, Babu et al. (2006) have reported the adaptive anatomical characteristics of mulberry leaves genotypes for shade tolerance.

During the evaluation and selection of various mulberry genotypes for the breeding programmes, different morpho-physiological parameters are considered for developing high yielding mulberry varieties. However, it is practically difficult to consider all genotypes for selection and, therefore, they have to be short listed from a large number of progenies based on their easily measurable and useful characters. The literature clearly points out that the prime physiological parameter namely photosynthesis, which is the basis for biological yield, is correlated to stomata frequency for gas exchange and, thus has direct effect on the crop production.

An attempt is, therefore, made to investigate leaf micro-morphology and anatomy by scanning electron microscopy of three mulberry genotypes which may help in identifying certain crucial characters before taking up the mulberry improvement programmes. The survey of literature reveals that such type of studies have not been carried out so far. The ultimate objective of the study is to identify micro-morphological/anatomical and physiological traits which may help in the selection process during early stage of evaluation for short-listing the genotypes during breeding programmes as mulberry is unique with large genetic variability and long gestation period.

MATERIALS AND METHODS

Cultivation of mulberry

For the present study, three mulberry genotypes namely China Peking, Punjab Local and S-30 were selected based on low, medium and high yield potential from the germplasm resource bank of Central Sericultural Research and Training Institute, Mysore, India. These mulberry genotypes were established in random block design (RBD) in plots of size 5.4 m by 3.6 m under irrigated conditions with standard fertilizers (300:120:120 kg NPK/ha/y) and manure application (20 mt compost/ha/y) (Dandin et al. 2000). The spacing between rows and plants was 120 cm x 120 cm. Five replications were maintained per genotype in four different plots. The garden soil used was red loamy with pH of 7.25 to 7.65 and electrical conductivity of 0.11 to 0.13 m mhos/cm. The fully matured mulberry leaves of all the three genotypes

were collected 70 days after bottom pruning was conducted as per standard sericultural practices and used for SEM study.

Procedure for scanning electron microscopy

To study the leaf thickness, palisade and spongy parenchyma and stomata frequency under scanning electron microscope (SEM), the samples of 3 mm x 3 mm, and 3 mm x 1 mm sizes were fixed for 4 hours in glutaraldehyde prepared in 0.2 M sodium cacodylate buffer (pH 7.2). The fixed samples were washed thrice in sodium cacodylate buffer and then dehydrated in an alcohol-acetone series. The dehydrated samples were dried in a critical point drier (EMS - 850) using CO_2 as a transition fluid. The dried samples were mounted on to copper stubs using double side stick cellophane tape. To observe cross sectional area, the 3 mm x 1 mm sized leaf tissues were vertically mounted exposing their cut surfaces. The dried samples were gold coated (20 nm thickness) in a sputter coater (EMS – 550). The coated samples were observed under a transmission electron microscope (JEOL 100 CX II, Tokyo Pvt. Ltd. Japan) attached with scanning device (ASID 4 D) at 20 kV, and photographs were taken at different magnifications. Five samples from each genotype were examined for confirmation of the results. The thickness of leaf and mesophyll tissues was recorded from SEM micrographs and statistically analyzed.

Photosynthetic rate

Photosynthetic rate was measured in all three genotypes *viz.,* Punjab Local, S-30 and China Peking between 10 to 12 hours under natural sun light using a portable photosynthetic system (Model–LI 6200, LI-COR Co. Ltd. Lincoln, NE, USA). The photosynthetically active radiation (PAR) was around 1500 μ mol m^{-2} s^{-1} during the gas exchange measurements. The fully matured and indexed 12^{th} leaf from the top of the shoot was selected, and the gas exchange data was recorded at the time of harvest (70 days).

Statistical analysis

The data were subjected to statistical analysis employing analysis of variance (ANOVA) to ascertain the significance of various characters *viz.* leaf thickness, thickness of palisade and spongy parenchyma, percentage of palisade parenchyma in mesophyll tissue, photosynthetic rate, number of stomata (per mm^2), number of trichomes (per mm^2), and number of idioblasts (per mm^2) among the three mulberry genotypes.

RESULTS AND DISCUSSION

Mesophyll characteristics

Results of the scanning electron microphotographs of transverse section of mulberry leaves of three mulberry genotypes *viz.* Punjab local, S-30 and China Peking

showed variation in leaf thickness, palisade tissue, and spongy parenchyma (Figure 1-6). The leaf thickness of mulberry genotype Punjab local recorded at 127.42±0.582 µm (Figure 1-2) whereas it was 119.17±0.752 µm and 119.61±0.623 µm for genotypes of S-30 (Figure 3-4) and China Peking (Figure 5-6), respectively (Table 1).

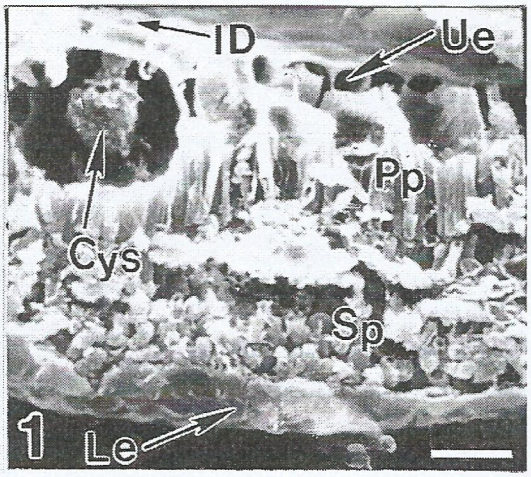
Table 1. Variation in mesophyll characters and the rate of photosynthesis of three different mulberry genotypes.

Genotype	Leaf thickness	Thickness of palisade parenchyma (µm)	Thickness of spongy parenchyma (µm)	Percentage of palisade parenchyma in mesophyll (µm)	Photosynthetic rate ($\mu mol\ m^{-2}s^{-1}$)
Punjab Local	127.42±0.582	62.46 ±0.941	50.77±0.753	50.41±0.660	17.09±0.088
S-30	119.17±0.752	46.72±0.371	36.54±0.649	56.05±0.396	21.56±0.357
China Peking	119.61±0.623	43.22±0.444	59.69±0.703	41.94±0.152	11.06±0.529
Standard Error	0.747	0.635	0.680	0.468	0.332
F Test	***	***	***	***	***
CD at 5% level	2.435	2.072	2.018	1.526	1.083

± = Standard Error
*** Significant at 0.01 level

Babu et al. (2006) while investigating the three different mulberry genotypes under full sunlight and shade conditions have found that the thickness of V1 genotype in sunlight was 104.2 µm and 45.8 µm in the shade, whereas it was 93.8 and 114.6 µm in sunlight and 72.9 and 79.2 µm in shade for K2 and K2 x Kosen genotypes. Further, it was also reported that the leaves of sun-exposed plants tend to be thicker than those of the shade-grown ones (Igboanugo 1993; Mendes et al. 2001), and the light demanding species have the maximum leaf anatomical plasticity (Ashton & Berlyn 1994). When the SEM data of present study for leaf

thickness of three mulberry genotypes were statistically analyzed, there was significant difference among Punjab local, S-30 and China Peking. However, leaf thickness character did not differ between the genotypes of S-30 and China Peking (Table 1). Nonetheless, data for other traits such as thickness of palisade and spongy tissues, percentage of palisade parenchyma in mesophyll, and the rate of photosynthesis, in all the genotypes were found highly significant (Table 1).



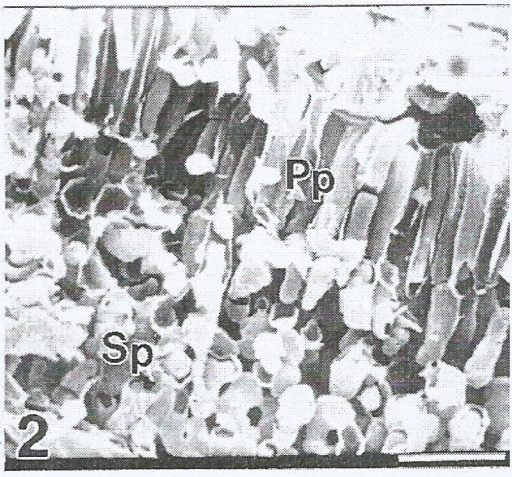
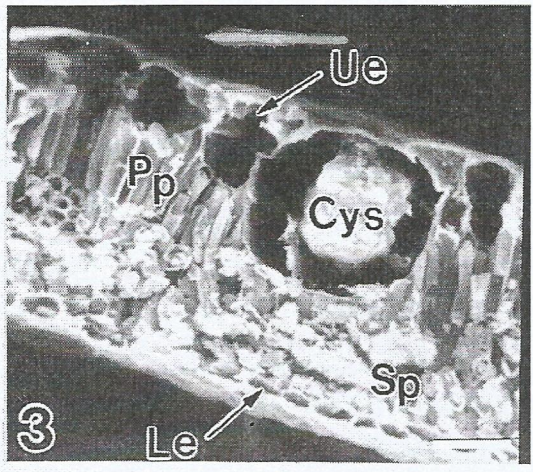


Figure 1-2. Microphotographs of the transverse section of mulberry leaf of Punjab local genotype revealing the upper epidermis (Ue), palisade parenchyma (Pp), spongy parenchyma (Sp) and lower epidermis (Le). Note idioblast (ID) opens in a cystolyth (Cys) just below the upper epidermis. Scale bar = 12 μm for Figure 1, and 10 μm for Figure 2.



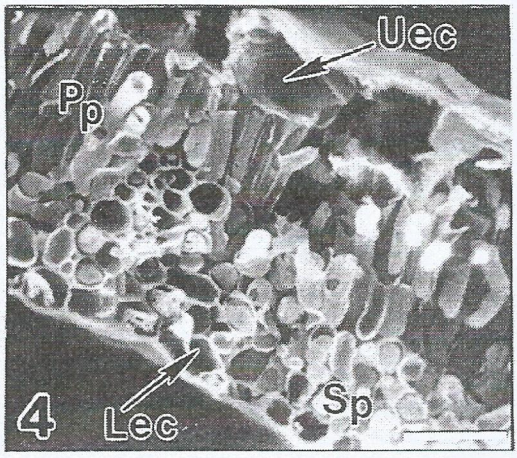


Figure. 3-4. Transverse section of mulberry leaf of S-30 genotype revealing the upper epidermis (Ue), palisade parenchyma (Pp), spongy parenchyma (Sp) and lower epidermis (Le). Scale bar = 12 μm for Figure 3, and 10 μm for Figure 4.

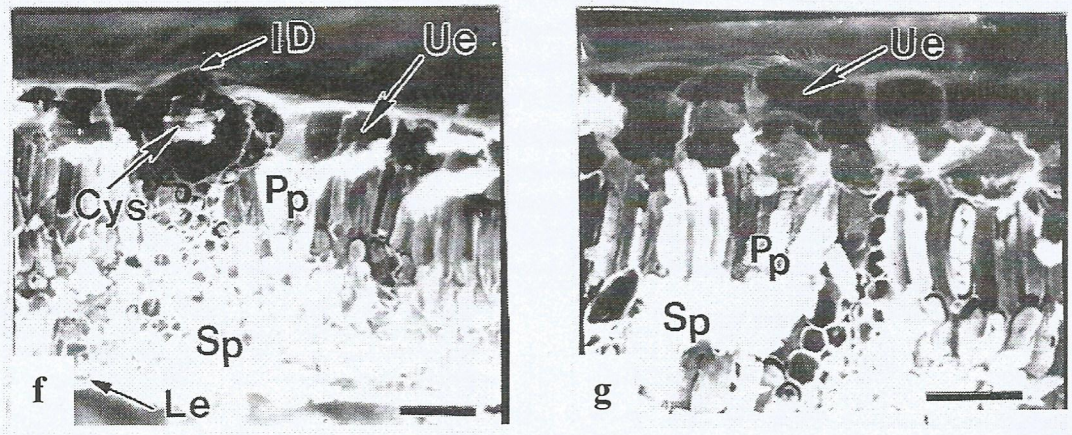


Figure. 5-6. Transverse section of mulberry leaf of China Peking genotype revealing the upper epidermis (Ue), palisade parenchyma (Pp), spongy parenchyma (Sp) and lower epidermis (Le). Note idioblast (ID) opens in a cystolyth (Cys) just below the upper epidermis. Scale bar = 12 µm for Figure 5, and 10 µm for Figure 6.

It is well documented that palisade parenchyma is an important character of the leaves of any plant as it reflects better ability of the plant genotypes to capture solar energy and accounts for photosynthetic efficiency. The thickness of palisade parenchyma in Punjab local genotype (Figure 1-2) was found to be 62.46±0.941 µm whereas it was 46.72±0.371 µm and 43.22±0.444 µm for S-30 (Figure 3-4) and China Peking (Figure 5-6), respectively. There was a significant difference for the character of the thickness of palisade parenchyma among three genotypes studied (Table 1). Further, the values of leaf thickness of three genotypes were highly significant against all the other characters of mesophyll tissue and photosynthetic rate (Table 1). Babu et al. (2006) have earlier studied the percentage of palisade tissue of three mulberry varieties and the data of the leaves grown in sunlight and shade conditions were documented as 48.0% and 40.8% (V1); 37.7% and 28.5% (K2) and 45.4% and 50.0% (K2 x Kosen), respectively.

The healthy leaves of the three genotypes had an almost similar internal structure typical of a dorso-ventral dicot leaf with prominent distinguishable palisade and spongy parenchyma in the mesophyll. However, the thickness of spongy parenchyma distinctly differed among the three genotypes studied. The mean values of the spongy parenchyma of three genotypes were shown in Table 1. It was found maximum in China Peking genotype (59.69±0.703 µm) whereas it was least observed in S-30 genotype (36.54±0.649 µm). The statistical analysis showed a significant difference among the three genotypes for the thickness of spongy parenchyma (Table 1).

Since the mesophyll structure is associated with photosynthetic performance of leaves via the regulation of internal light and CO_2 profiles (Babu et

al. 2006), the percentage of palisade parenchyma is a crucial trait in the mesophyll tissue of mulberry genotypes. During the present study the percentage of palisade parenchyma was found highest i.e. 56.05±0.396 µm in S-30 genotype while the values were recorded minimum for China Peking (41.94±0.152 µm). Further, the data revealed a highly significant difference between the three genotypes for the percentage of palisade tissue in mesophyll (Table 1).

Rate of photosynthesis

Leaf yield of any plant generally depend on the photosynthetic rate, which plays a vital role in biomass productivity. Therefore, it is necessary to consider this prime physiological parameter for mulberry improvement programme. Screening of progeny and short listing by evaluation is the primary step in identification of genotypes in the initial stage for inclusion in primary yield trait in any breeding programme. A rapid screening method is essential to reduce the time and labour required in identification of suitable genotypes for a particular condition (Fotadar et al. 2006). The experimental data revealed that the highest photosynthetic rate recorded was 21.56±0.357 µ mol m^{-2} s^{-1} in S-30 genotype whereas Punjab local stood at second place with the values of 17.09±0.088 µmol m^{-2} s^{-1}. Genotype China Peking was noticed with lowest photosynthetic rate i.e. 11.06±0.529 µ mol m^{-2} s^{-1}. The CD values for the photosynthetic rates measured showed a significant difference among the genotypes (Table 1).

Micro-morphological characteristics of abaxial leaf surface

A scanning electron microscopic study was carried out to find out the micro-morphological characters like density of stomata, trichomes and idioblasts per unit surface area of three mulberry genotypes and the data were subjected to statistical analysis (ANOVA) and presented in Table 2. The average number of stomata was found 895±6.804, 887±6.253 425±3.536 per mm^2 in Punjab local, S-30 and China Peking respectively. Babu et al. (2006) reported a significant reduction in the number of stomata on the leaves of mulberry plants grown under shade condition. Further, a comparatively less stomata frequency in the leaves of shade plants than the sun exposed ones was fairly documented in many plants species (Rocas et al. 1997; Mendes et al. 2001). Since the leaf surface structure is associated with the photosynthetic performance via regulation of internal light and CO_2 profiles, the genotype China Peking seems not to be much useful for mulberry productivity improvement programmes as it has minimum number of stomata per mm^2. On the other hand, Punjab local and S-30 genotypes can be useful for breeding purposes as both the genotypes have more stomata frequency. Though the data is not showing significant difference between the genotypes of Punjab local and S-30, yet the genotype China Peking was found significant when compared to others.

Table 2. Variation in number of stomata, trichomes and idioblasts in three mulberry genotypes.

Genotypes	Number of stomata (Per mm^2)	Number of Trichomes (Per mm^2)	Number of idioblasts (Per mm^2)
Punjab Local	895±6.804	112.5±5.853	67.0±3.364
S-30	887±6.253	140.4±3.855	29.0±2.702
China Peking	425±3.536	168.6±5.212	34.0±2.270
Standard Error	6.379	5.474	1.522
F Test	***	***	***
CD at 5% level	20.805	17.851	4.964

± = Standard Error
*** Significant at 0.01 level

Plant species exhibit wide variation in types and densities of trichomes within the families. Trichomes are the epidermal appendages that consist of one or more cells derived from a single proto-dermal cell (Kesavacharyulu et al. 2004). In many plants, trichomes have been used to classify genera and species (Metcalf & Chalk 1979; Mehta et al. 1979) including mulberry (Katsumata 1971; Fujita & Uchikawa 1986). The role of trichomes in plant defense against herbivore is known since long (Callahan 1957; Beck 1965; Levin 1973; Norris & Kogan 1980; Jermy 1984). The foliar trichomes when present in high density are reported to cause physical hindrance and discourage phytophagous insects, and affects acceptability of foliage (Singh et al. 1971; Levin 1973).

Trichomes play a major role in acceptability as feed by insects (Kesavacharyulu et al. 2004) and in some cases were reported to synthesize, metabolize, accumulate, and secrete a variety of substances (Norris & Kogan 1980; William et al. 1984; Purushothaman & Vasanth 1988; Pedro et al. 1991; Werker 1993). The secretions from glandular trichomes probably play a role to attract the pollinators (Ascensao et al. 1995). In the present study, the glandular trichomes were present on the abaxial surface of three mulberry genotypes were recorded as 112.5±5.853 per mm^2 in Punjab local (Figure 7-8), 140.4±3.855 per mm^2 in S-30 (Figure 9-10), and 168.6 5.212 per mm^2 in China Peking (Figure 11-12).

Significant differences were observed among these three genotypes (Table 2; Figures 7-12).

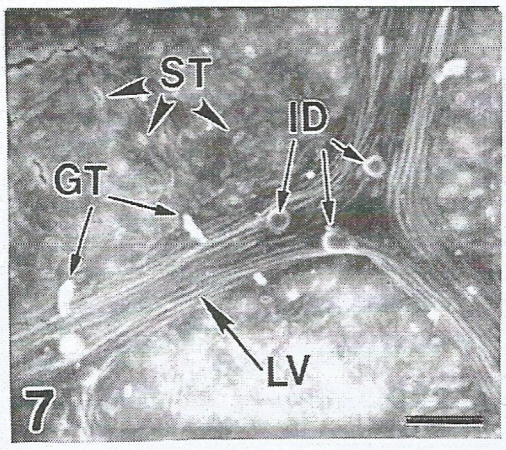


Figure 7. Abaxial leaf surface of mulberry genotype of Punjab local showing the leaf vain (LV), glandular trichomes (GT), idioblast (ID) and stomata (ST). Scale bar = 30 μm.

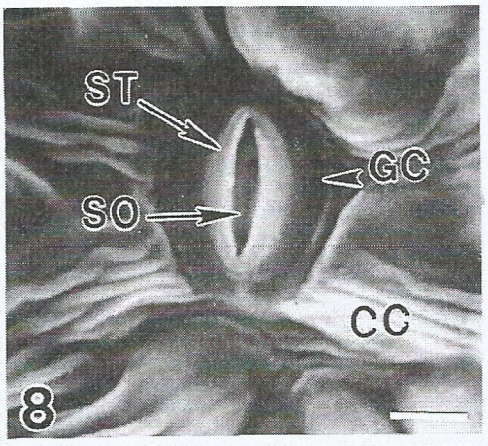


Figure 8. A magnified view of stomata of Punjab local reveals the stomata opening (SO), guard cell (GC) and companion cell (CC). Scale bar = 2 μm.

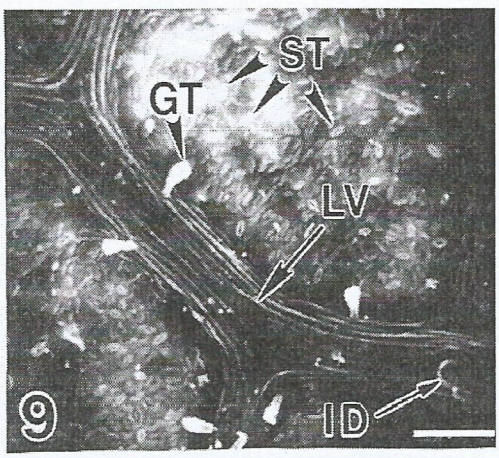


Figure 9. Abaxial leaf surface of mulberry genotype of S-30 showing the leaf vain (LV), glandular trichomes (GT), idioblast (ID) and stomata (ST). Scale bar = 30 μm.

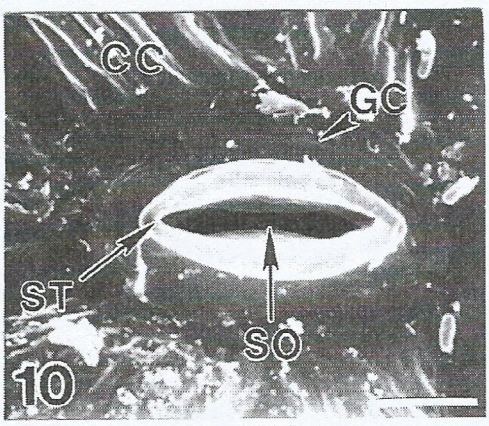


Figure 10. Microphotograph reveals the stomata (ST), stomata opening (SO), guard cell (GC) and companion cell (CC). Scale bar = 2 μm.

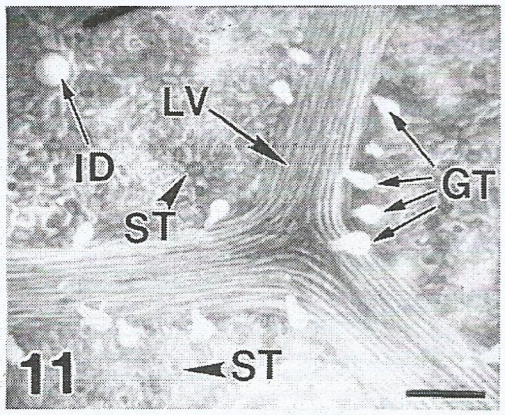


Figure 11. Abaxial leaf surface of mulberry genotype of China Peking showing the leaf vain (LV), glandular trichomes (GT), idioblast (ID) and stomata (ST). Scale bar = 30 μm.

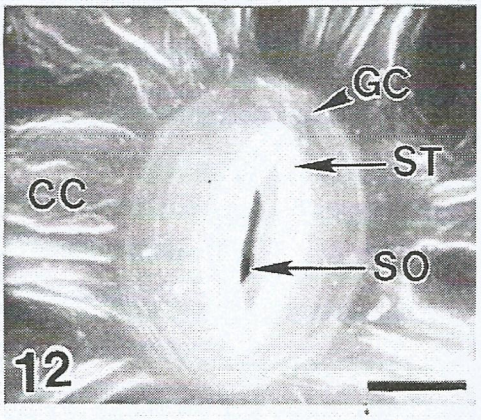


Figure 12. A magnified view of stomata of the genotype of China Peking reveals the stomata opening (SO), guard cell (GC) and companion cell (CC). Scale bar = 2 μm.

While considering the total density of idioblasts per mm^2 leaf surface area, it was observed that sufficient variability exists among these genotypes. The density of idioblasts in genotype Punjab local recorded maximum values (67.0±3.364) and lowest in S-30 (29.0±0.702), whereas the number was medium in China Peking (34.0±2.270; Figures 7-12). There was a significant difference in number of idioblasts among three mulberry genotypes studied.

CONCLUSION

Overall, the results of the present SEM investigation revealed that the differences in micro-morphological and mesophyll characteristics are directly associated with variability in light interception and photosynthetic efficiency of mulberry genotypes. These micro-morphological and photosynthetic traits can prove their potential in the selection and screening of improved mulberry varieties in a relatively shorter span of time.

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