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LEAF ARCHITECTURE, ORNAMENTATION AND LEAF AREA ESTIMATION IN EXOTIC *MEDICAGO SATIVA* L. (PAPILIONACEAE) - GROWN IN PAKISTAN

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ABSTRACT

Some 90 trifoliate leaves of various sizes were randomly selected from six 70-day old seedlings of late vegetative stage of *Medicago sativa* L. (fifteen leaves from each seedling) raised in Karachi (Pakistan) from seeds obtained from New Zealand. The linear measurements from the leaflets of these leaves were recorded i.e. length (LM) and width of leaflets (LB) at the broadest points on the margins. The leaves were studied for their architecture, ornamentation and leaf area estimation. The seedling shoot was pubescent. The exotic taxon was distinct from the local taxon in leaf architecture (shape and larger size of the leaflets and larger petiolar length. Leaf exhibited size-related variability in leaflet shape. Terminal leaflet of a trifoliate leaf was longer and broader than left or right lateral leaflets. Length /Width ratio averaged to 1.82 to 1.84 and didn't vary amongst the three leaflets significantly. It appeared that the overall leaf shape as given by the aspect ratio (L /W) is generally maintained at least in mature leaves. The average area of the terminal leaflet (4.57 cm^2) was significantly higher than that of the left or right lateral leaflets (3.98 and 3.87 cm^2, respectively). Apical angle (AA) and basal angle (BA) averaged to 82^o to 84^o indicating on an average acute angular placement. However, since AA and BA ranged from 68^o to 100^o, there were some cases of obtuse AA and BA. The value of the multiplication factors (k = leaf area $_{measured}$ / (LM x LB) for terminal, left and right lateral leaflets (k1, k2 and k3) in each case were non-normal, highly leptokurtic in distribution with low lateral spread. The ks values concentrated around the mean value and averaged to k1= 0.626408, k2 = 0.659049 and k3 = 0.651420. The terminal leaflet varied in area from 0.25 to 10.37 cm^2, left leaflet from 0.45 to 9.45 cm^2 and right lateral from 0.22 to 9.68 cm^2. The area of a mean leaf of late vegetative stage seedlings was measured to be 12.4203 ± 0.6939 and estimated to be 12.3832 ± 0.6600 cm^2. The leaf area $_{measured}$ and leaf area $_{estimated}$ correlated highly positively with each other (r = 0.9834) and relationship between them was isometric. The average leaf area $_{measured}$ didn't differ significantly with average leaf area $_{estimated}$ (t = 0.0389, NS). There were several types of stomata in *M. sativa*- anisocytic, anomocytic, and tetracytic stomata on stipule, tetracytic stomata on stem, anisocytic, staurocytic, tetracytic, and contiguous stomata on dorsal surface of leaf, and anisocytic, staurocytic, tetracytic, contiguous stomata and several stomata with common subsidiaries on ventral surface of leaf. Stomatal density on dorsal and ventral surfaces of leaves was moderate in magnitude, 100.86 ± 2.80 and 113.03 ± 5.58 per mm^2, respectively. The mean estimated total leaf area per seedling amounted to 646.33 ± 60.92 cm^2 varying by 29.80 % (432.72 to 854.44 cm^2). The estimated total leaf area per seedling (Z) related significantly with shoot length (X) and the number of leaves per seedling (Y) as given by the following equation (r = 0.9989):

$$Z = -1.391 + 0.04182 X + 12.377 Y$$

Key-words: Leaf architecture, ornamentation, leaf area, *Medicago sativa* (an exotic from New Zealand), Pakistan.

INTRODUCTION

Leaf architecture and surface ornamentation of plants are important in morphological studies - important as the emerging domain of plant science (Paria, 2014). The works of Metcalfe and Chalk (1950), Shah and Gopal (1969), Willis (1972), Hickey (1973), Kothari and Shah (1975), Gupta and Murty (1977), Bara and Baruah (1979), Nenggan (1983-84), Smith and Scott (1985), Das and Paria (1999), Croxidale (2000), Dasti *et al.* (2003), Freire *et al.* (2005), Wang *et al.(*2006), Gan *et al.* (2010), Miller and Miller (2011),Tripathi and Mondial (2012), Lu *et al.* (2012), Khan *et al.* (2015) etc. are few of the important references in this domain – many of which dealt with Family Leguminosae.

Medicago sativa L. (lucerne) is the World's oldest named forage crop, known in written from the Hittites (between 1400 BC-1200 BC) (Russelle, 2011) and widely spread around the World between 500 BC and 400 BC

(Michaud *et al.,* 1988) in Europe, N. Africa, Asia and Pacific countries. It is rated as high yielding plant of high quality forage. In this paper, leaf architecture and surface ornamentation of *Medicago sativa* L. (an exotic from New Zealand and cultivated in Karachi under normal cultural conditions) is described. Leaf area is the single determinant of plant productivity (Linder, 1985). Leaf area estimation in this species is also undertaken through determination of multiplication factors Ks for each leaflet separately and also allometrically. There are numerous such studies (to cite a few, Kemp, 1960; Jain and Misra, 1966; Williams *et al*., 1973; Aase *et .al.*, 1978; Hatfield *et. al.*, 1976; Elasner and Jubb, 1988; Chinamuthu *et. al*., 1989; O'Neal *et al*., 2002; Williams III and Martinson, 2003; Kathirvelan and Kalaiselvan, 2007, Cristofori *et al.*, 2007; Khan, 2008, 2009, Ahmed and Khan, 2011, Khan *et al*, 2015 a and c) conducted with very many different species. Such simple and accurate methods eliminate the need of expensive leaf area meters (Gamiely *et al.*, 1991). These studies shall hopefully facilitate us in our agronomic studies with this species.

MATERIALS AND METHODS

The plants of *Medicago sativa* L. were grown in the Botanical field Federal Urdu University of Science and Technology, Karachi, under normal cultural practices, from seeds supplied by department of plant protection, Ag Research, Hamilton, New Zealand. Some 90 trifoliate leaves of various sizes were randomly selected from six 70-day old seedlings of late vegetative stage (fifteen leaves from each) (Fig. 1A) and the linear measurements from the leaflets of these leaves were recorded i.e. length of leaflet (LM) and width of leaflets (LB) at the broadest points on the margins. To measure the leaf area the outlines of the leaves were drawn on graph paper and the area was measured with possible accuracy. The multiplication factor, k, for a leaflet was calculated by employing the formula, k = leaf area $_{\text{measured}}$ / (LM x LB) for each leaflet of the leaf. The average ratio or the factor k was employed to estimate three leaflet areas of a leaf as leaf let area $_{\text{estimated}}$ = k_i (LM x LB) where k_i is the k for the i^{th} leaflet of the leaf (Lu *et al.,* 2012). Hickey (1973) and LAWG (1999) were followed for description of leaf architecture. Leaf epidermal impressions were made with clear nail polish (Wang *et al.*, 2006). Stomatal nomenclature suggested by Prabhakar (2004) being simple and based upon structure of stomata and not their ontogenetic pathways was adopted to ascertain stomatal types. He recognized eleven types of stomata. This nomenclature does not recognize actinocytic and stephanocytic stomata and categorize them as anomocytic type. As a basic criterion, all the cells abutting the guard cells are considered distinct by Prabhakar (2004) from the other epidermal cells by virtue of their position (i.e. abutting nature to the guard cells) hence he prefers to call them subsidiaries. The data was analyzed statistically (Zar, 2010). The skewness and kurtosis and their errors were calculated following Shaukat and Khan (1979).

Fig. 1A. A late vegetative seedling of 70-day old *M. sativa* grown from New Zealand seeds in Karachi (Pakistan) under field conditions.

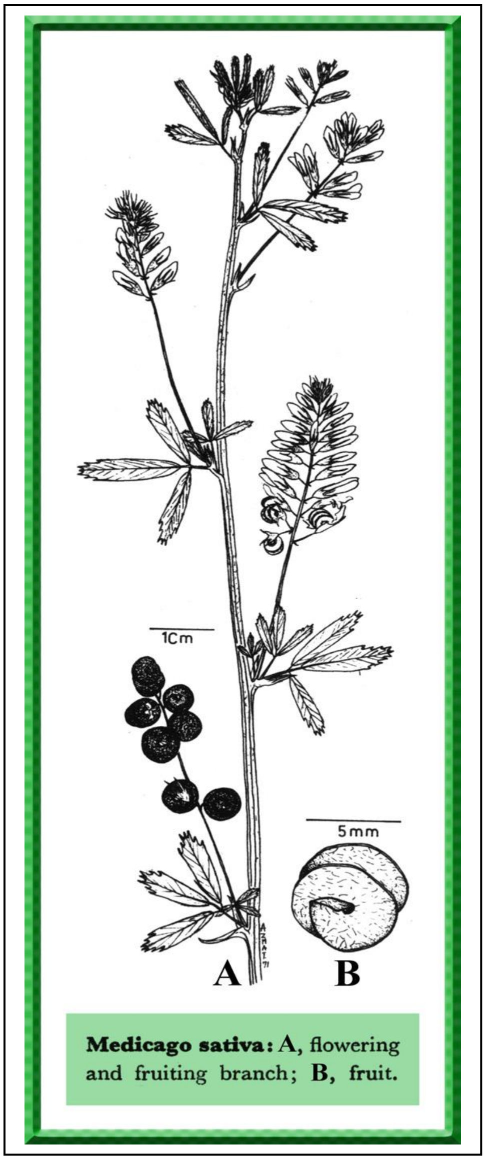

Fig. 1B. *M. sativa* L. habit from (Ali, 1977).

The abundance of stomata was determined by counting them in 42 frames or fields of vision at 45 x 10 X magnification. Each field of vision at this magnification was around 0.10174mm^2 in area. The density of stomata was expressed per mm^2. The sampling for the stomatal abundance was random in the laminar region of the leaflet.

RESULTS AND DISCUSSION

Some eight species of genus *Medicago* including *M. sativa* are reported from Pakistan (Ali, 1977). *M. sativa* (Lucerne, Alfalfa) is an erect to sub erect perennial herb fodder, widely cultivated in Pakistan and distributed throughout world. Its habit as appeared in Ali (1977) is given in Fig. 1B). According to the taxonomic description by Ali (1977), *M. sativa* is *mostly erect to sub erect perennial herb, 30-60 cm, pubescent to subglabrous, leaflets 5-20 mm long, 3 x10 mm broad, obovate to sub linear, dentate at apex, appressed pubescent, entire or dentate at base. Inflorescence a peduncled receme, peduncle much longer than petiole. Calyx teeth as long as the tube. Corolla 6-12 mm long, violet to pale lavender. Fruit falcate or in a loose spiral of* $1\frac{1}{2}$ *- 4 turns, glabrous to appressed pilose, 10-20 seeded.*

Like local species, exotic counterpart from New Zealand, appeared to have pinnately trifoliate leaflets which are apically toothed. The stipules are large and adnate (Fig. 2A). Stem is hairy (Fig. 2B). Stipules are pale green in the mid part and dark green on the wing or marginal part. The petiole of the exotic species appeared to be much larger [mean = 2.394 ± 0.1705 cm, CV= 45.05%, 1.10 - 5.0cm, N =40) and rachis admeasured to be 0.675 ± 0.1145 cm, CV = 107.68%, 0.3-1.0, N = 40). In local species, these parameters were shorter. Leaflets are narrower in local taxon as compared to the Kiwi counterpart. Leaflet lamina in in-hand sample is symmetrical, elliptic, average Length /width ratio 1.82 to 1.84, margin entire, apical region of the leaflet dentate, texture membranous, petiole normal. Leaflet apex generally acute (apex angle < 90^o) and leaflet base also acute (base angle < 90^o) but often both were obtuse also. Leaflet apex extension zero and leaflet base extension zero.

Leaf exhibited size-related variability in leaflet shape (Fig. 3). Leaflets appeared to exhibit differential rate of growth in length and breadth. Initially, the growth appeared to be more in linear direction. Table 1 presents the linear dimension of the leaflets. Terminal leaflet of a trifoliate leaf was longer (3.46 cm) than left (3.14cm) and right (3.13cm) leaflets (t = 2.29and 2.30, respectively, p < 0.001). The lateral leaflets of a leaf didn't vary in length from each other (t = 0.0184, NS).

The terminal leaflet was broader (1.95 cm) than the lateral left and right leaflets (t = 2.0 and 2.25, respectively, p < 0.001). The two lateral leaflets didn't vary in width (t = 0.27, NS) (Table 1).

The length of the three leaflets distributed normally and varied from 27.76 to 29.37%. Leaflets broadness, however, distributed normally only in case of one leaflet. The two lateral leaflets didn't vary in width (t = 0.27, NS). It is clear from the above description that in hand taxon was quite distinct from the local species at least in leaflet morphology and petiolar size (Fig. 1A and 3). *M. sativa* is a complex taxon composed of eight subspecies – *sativa, falcata, glutinosa, coerulea, varia, hemicycla, polychroa, and tunetana* with both diploid and tetraploid occurring naturally (Quiros and Bauchan, 1988; Pembleton, 2010) and great attention has been paid in breeding program of this species all over the world. (Radović *et al.*, 2001, 2009; Michand *et al.*, 1988; Kalu and Fick, 1981). The subspecies *sativa* and *falcata* have been the focus of cultivar development and commercial production (Irwin, *et al.*, 2001). Annicechiarico (2006) has described diversity in genetic structure, distinctiveness and agronomic value of Italian lucerne and Benabderrahim *et al.* (2005) compared local alfalfa ecotypes for their performance under Tunisian conditions. The assessment of *M. sativa* at sub specific level has not been made as yet in Pakistan and we do not know much about the Kiwi counterpart.

Venation of leaflets is pinnate simple (craspedodromous (Gr. *kraspedon* = edge = border and *dromos* = course) i.e. secondary veins terminating at the margin (Fig.3). Dorsally, the mature leaflets are devoid of pubescence but ventrally, apex, margins, and blade are pubescent – denser on midrib and basal part of the leaflet (Fig. 4).

Leaf shape consistency

Verwijst and Wen (1996) Opined that L/W ratio (aspect ratio) may give some indication to the consistency of leaf shape. Aspect ratio (Table 5) in present analysis followed the symmetrical distribution in all three leaflets of a leaf. It showed significant correlation with Leaf $area_{measuresd}$ (r = 0.301, 0.333, or 0.334 in case of the three leaflets of a trifoliate leaf. Although the leaf size dependence on L/W ratio was too meager in the three leaflets (9.1%, 11.2% and 10.1%). L/W ratio averaged to 1.82 to 1.84 and didn't vary amongst the three leaflets significantly (t = 0.087 – 0.453, NS) in pairs of comparison. L/W varied from 1.5 to 2.0 in 82.3% of the cases in terminal leaflets, 70.30% cases in left lateral leaflets and 72.4 % cases in right lateral leaflets. L/ W ratio in the three leaflets varied by 16.15 to 17.93% 0nly. It appears that the overall leaf shape as given by the aspect ratio is maintained at least in mature leaves.

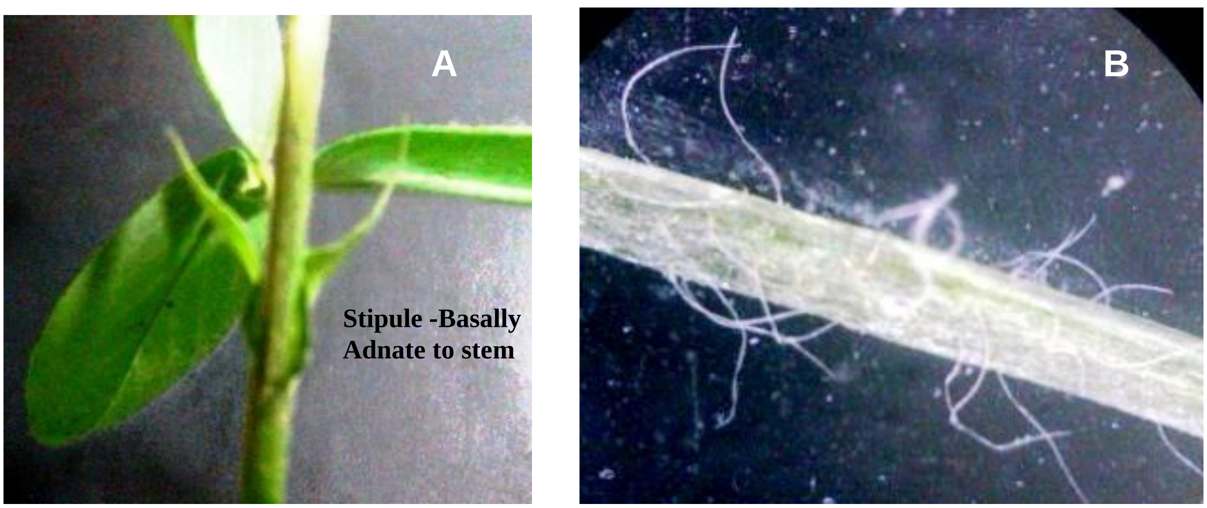

Fig. 2. Adnate type of large foliaceous stipule (1-2 cm in length) (A) and stem bearing trichomes (B).

Multiplication ratios or factors

Multiplication factors – k1, k2 and k3 for terminal, left lateral and right lateral leaflets, respectively are presented in Table 4. The value of the ks in each case were non-normal, highly leptokurtic in distribution with low lateral spread. The ks values concentrated around the mean value and averaged to k1= 0.626408, k2 = 0.659049 and k3 = 0.651420.

Leaflets area measured

The average area of the terminal leaflet (4.57 cm^2) was significantly higher than that of the left or right lateral leaflet (3.98 and 3.87 cm^2, respectively). The size of the leaflets in all cases distributed normally and varied by 50-54% (Table 2). The terminal leaflet varied in area from 0.25 to 10.37 cm^2, left leaflet from 0.45 to 9.45 cm^2 and right lateral from 0.22 to 9.68 cm^2.

Leaflets areas estimated

The area of the leaflets and the leaf was determined by determining multiplication factors or ratio for each leaflet separately, k1, k2, and k3 for terminal, left lateral and right lateral leaflets, respectively. The estimated area of the leaflets didn't differ significantly from the measured areas of the respective leaflets (terminal-terminal leaflets: t = 0.066, NS; left lateral -left lateral leaflets: t = 0.055, NS and right lateral- right lateral leaflets: t = 0.04, NS). The estimated size of the leaflets and leaf distributed normally. The estimated areas of the three leaflets varied by 48.65, 51.26 and 52.10 % in the terminal, left lateral and the right lateral leaflets, respectively (Table 2).

Apical and basal angles

Apical (AA) and basal angles (BA) of leaflets as determined following Hickey (1973) and LAWG (1999) are presented in Table 3. The variation in terms of CV (%) in magnitude of AA and BA for each leaflet type was substantially low (around or below 10% generally) except BA for terminal leaflet varied somewhat larger (14.23%). AA and BA averaged to 82^o to 84^o indicating on an average acute angular placement. Since AA and BA ranged from 68^o to 100^o, there were a number of cases of obtuse AA and BA (Fig. 7). The leaflets in general, however, coincided with the elliptic geometry in shape i.e. the line of the widest breadth was situated near 0.5 LM of the leaflets.

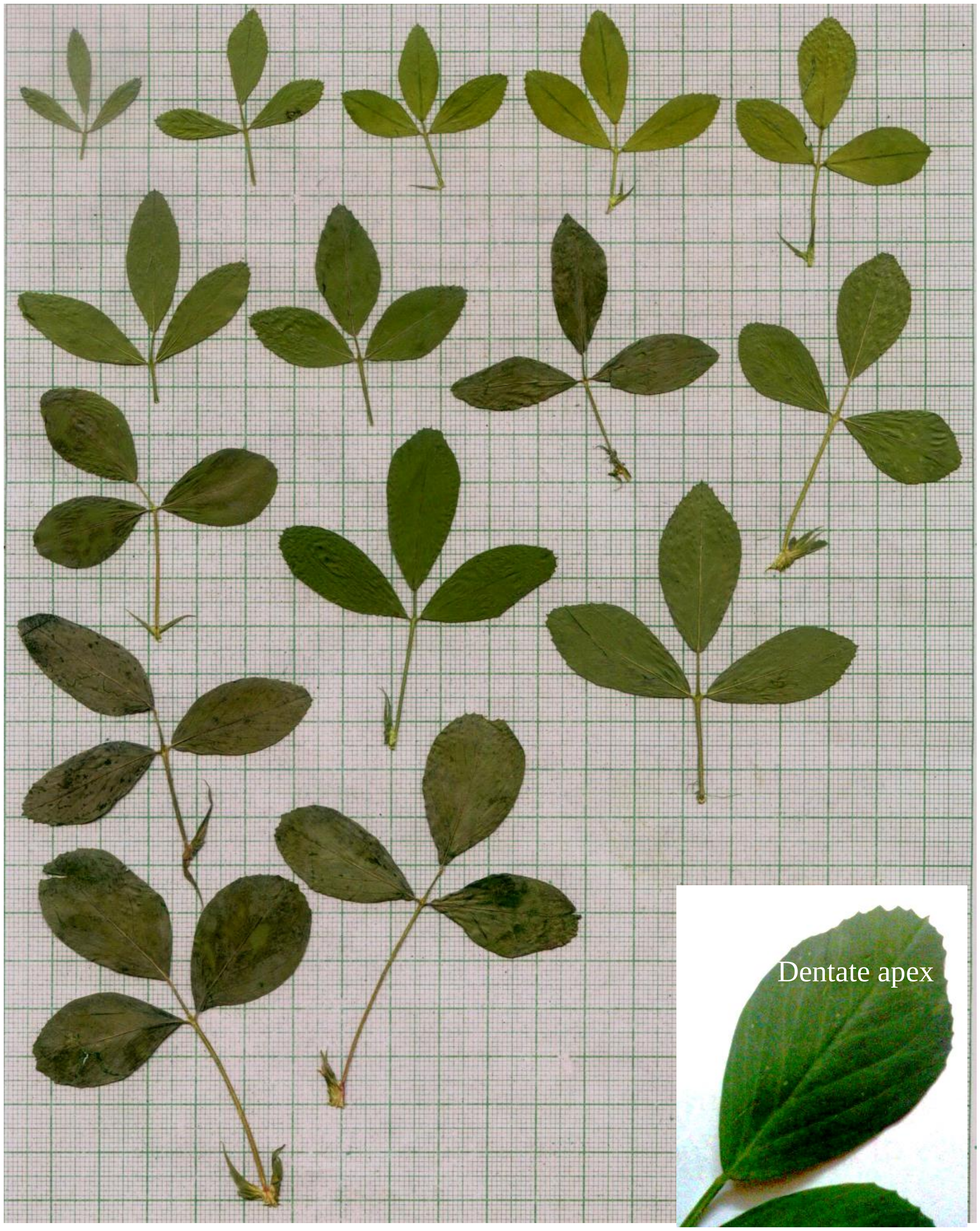

Fig. 3. Size – related leaf shape variability in *Medicago sativa*. The shape variability appears to be age-related - differential rates of growth in length and breadth. The inset shows the dentate apex of the leaflet.

Trichomes

The plant is hairy. The trichomes on stem and leaves are non-glandular long, thin, tender, and two-celled. The lower cell is small and upper cell straight or curved and much longer (Fig. 4). Thirty nine species of trifolieae were studied for their trichomes by Gupta and Murty (1977). *M. sativa* L. was reported to bear non-glandular, uniseriate, two-celled, stalk cell short and long apical cell straight or curved trichomes on leaflets (590.9 x 8.51 µm), pedicels (299.86 x 7.98 µm), and calyx (252.7 x 7.86 µm) and glandular trichomes with head biseriate or multiseriate on pedicel (39.90 x 18.62 µm and calyx (67.82 x a8.62 µm).

Stomatal types

Stomata on stem, stipule, and leaves were studied with clear nail polish imprint under optical microscope. Following types of stomata were observed. *M. sativa* is amphistomatic species. Amphistomatosis is common in many leguminous species. Of 45 species of order Leguminales, 31 species were reported to be amphistomatic and 14 hypostomatic (Tripathi and Mondial, 2012). Stomatal types on stem and leaf were as follows:

1) Stipule - Anisocytic, anomocytic, and tetracytic (Fig. 5).
2) Stem - Tetracytic (four subsidiaries – two polar and two lateral subsidiaries) - Fig. 6.
3) Leaf - Dorsal surface: Anisocytic, staurocytic, tetracytic, and contiguous stomata (Fig, 8).
   Leaf – Ventral surface: Anisocytic, staurocytic, tetracytic, contiguous stomata and several stomata with common subsidiaries. Three such stomata in close association are much apparent on ventral leaf surface (Fig. 9).

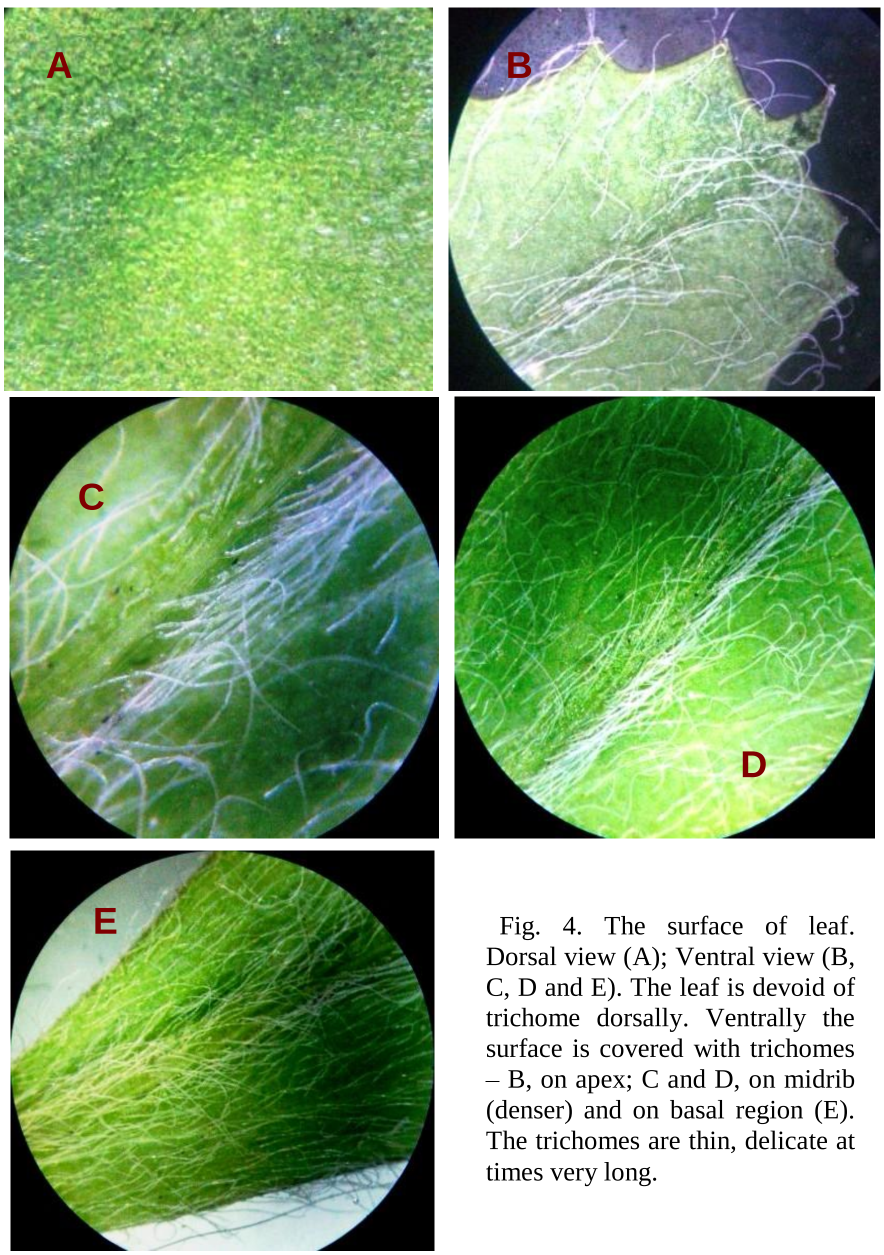

Fig. 4. The surface of leaf. Dorsal view (A); Ventral view (B, C, D and E). The leaf is devoid of trichome dorsally. Ventrally the surface is covered with trichomes – B, on apex; C and D, on midrib (denser) and on basal region (E). The trichomes are thin, delicate at times very long.

Paracytic stoma which is quite wide spread in Papilionaceae (Metcalfe and Chalk, 1950; Tripathi and Mondal, 2012; Khan *et al.*, 2014, 2015a and b) but this type of stomata was not found in *M. sativa*. Other types of stomata found in *M. sativa* were, however, quite common in several members of Papilionaceae such as *Erythrina suberosa* (Khan *et al..*, 2014)), *Bauhinia racemosa* (Khan *et al.*, 2015a), *Pongamia pinnata* (Khan *et al.*, 2015b). There are anisocytic stomata in *Glycine soja, Pisum sativum, Sesbania grandiflora* and *Trigonella foenum-graceum*. The stomata on leaf of *Alhagi maurorum* are paracytic and anisocytic and on stem anomocytic type (Bokhari and Dasti, 1991). Contiguous stomata have been reported in *Datura stramonium*, *Hyoscymus niger*, *Lycopersicon pimpinelli* and *Petunia* etc. of the family Solanaceae (Inamdar and Patil, 1976).

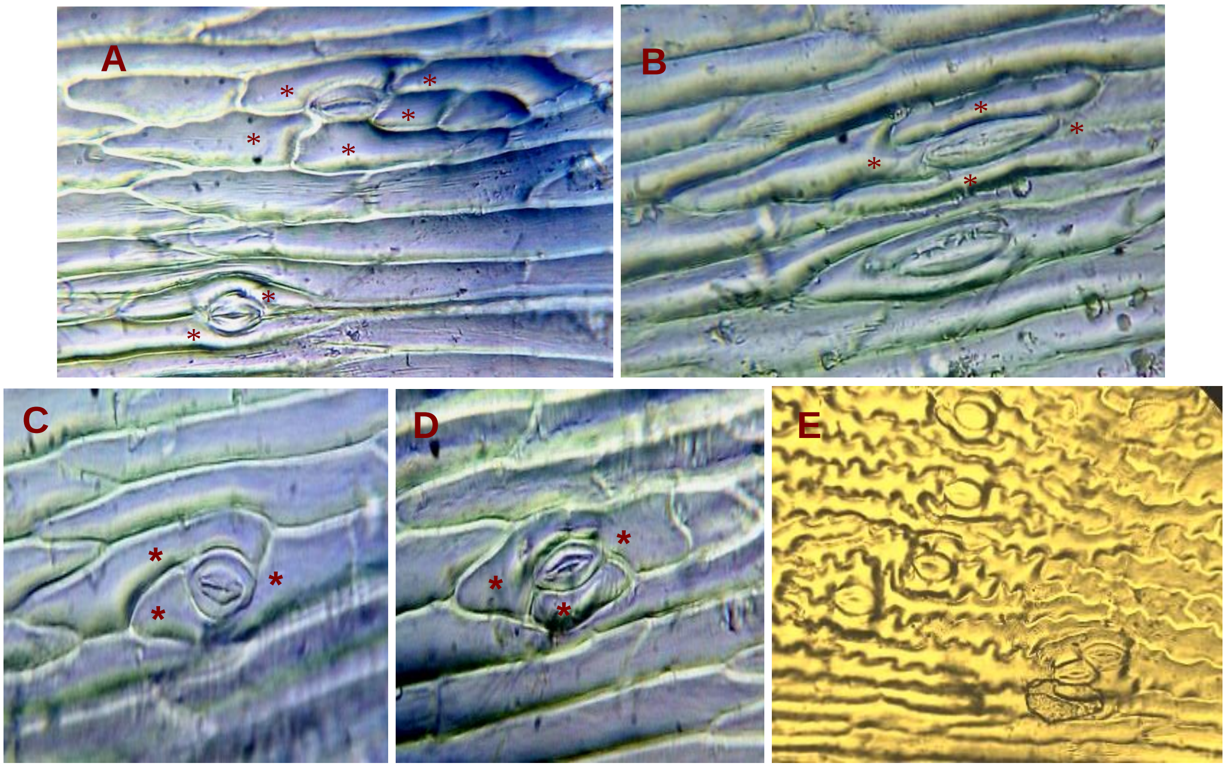

Fig. 5. Ornamentation of stipules with stomata A) Anomocytic and other paracytic, B) Tetracytic, C and D) Anisocytic. All Subsidiaries in A – D are elongated and smooth walled. E, General surface showing smooth walled elongated cells and subsidiary cells with wavy anticlinal contour also.

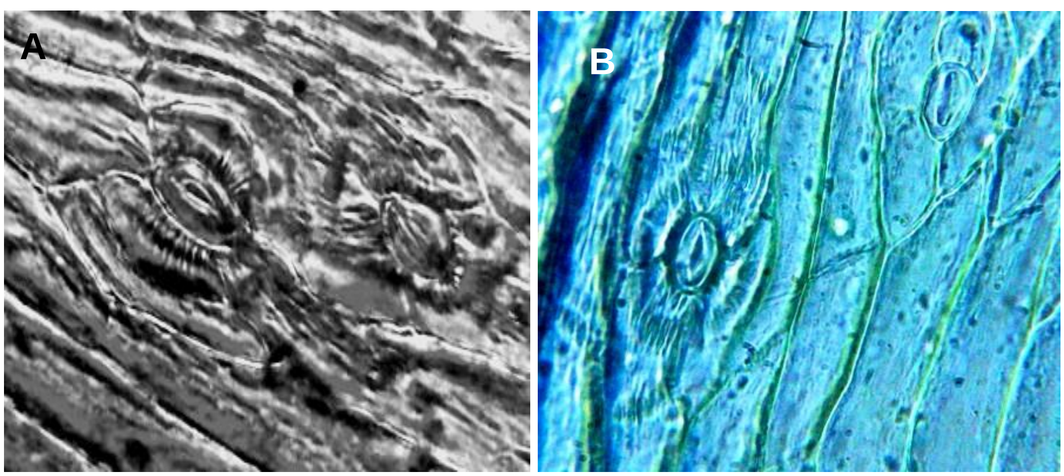

Fig. 6. Stem stomata A) Tetracytic –two subsidiaries are polar and two lateral, B) tetracytic and anisocytic. Note the peculiar cuticular striation in the lateral subsidiaries running radially over the periclinal surface. Striations run longitudinally over the periclinal surface of the two polar subsidiaries (A).

Table 1. Length and width of the leaflets.

Parameter	Length (cm)			Width (cm)		
	Terminal leaflet	Left lateral	Right lateral	Terminal leaflet	Left lateral	Right lateral
N	87	87	87	87	87	87
Mean	3.4621	3.1391	3.1333	1.9529	1.7759	1.7529
SE	0.10303	0.09493	0.09868	0.06434	0.06065	0.06154
Q_2	3.600	3.300	3.300	2.00	1.800	1.800
CV	27.76	28.21	29.37	30.73	31.85	32.75
g_1	-0.424	-0.383	-0.536	-0.503	-0.437	-0.400
Sg_1	0.258	0.258	0.258	0.258	0.258	0.258
g_2	-0.181	0-0.466	-0.101	0.171	-0.243	-0.183
Sg_2	0.511	0.511	0.511	0.511	0.511	0.511
Min	1.2	1.2	1.00	0.40	0.50	0.50
Max	5.6	5.0	5.0	3.20	2.90	3.00
K s-z	0.993	1.274	1.205	1.475	1.177	1.427
p	0.278	0.078	0.109	0.026	0.125	0.034

Table 2. Leaflet and leaf area estimated and measured (cm^{2}).

Parameter	AREA ESTIMATED				AREA MEASURED			
	Terminal leaflet	Left Lateral	Right Lateral	Leaf Area	Terminal leaflet	Left Lateral	Right Lateral	Leaf Area
N	87	87	87	87	87	87	87	87
Mean	4.5435	3.9666	3.8731	12.3832	4.5661	3.9841	3.8701	12.4203
SE	0.23696	0.218015	0.21635	0.66001	0.24739	0.23226	0.22388	0.69391
Q_2	4.5227	4.0070	3.8694	12.4493	4.710	4.060	3.7700	12.74
CV	48.65%	51.26%	52.10	49.71	50.64%	54.38%	53.96%	52.11%
g_1	0.114	0.254	0.296	0.171	0.077	0.430	0.338	0.211
Sg_1	0.258	0.258	0.258	0.258	0.258	0.258	0.258	0.258
g_2	-0.139	-0.017	0.334	0.058	-0.335	0.244	-0.036	-0.0152
Sg_2	0.511	0.511	0.511	0.511	0.511	0.511	0.511	0.511
Min	0.30	0.40	0.39	1.31	0.25	0.45	0.22	0.92
Max	10.22	9.56	9.77	29.55	10.37	9.95	9.68	29.77
K s-z	0.701	0.654	0.701	0.765	0.687	0.683	0.688	0.730
P	0.709	0.785	0.710	0.602	0.732	0.739	0.731	0.661

Table 3. Apical and basal angles of the leaflets.

Parameter	Apex angle			Base angle		
	Terminal leaflet	Left lateral	Right lateral	Terminal leaflet	Left lateral	Right lateral
N	76	76	76	70	70	70
Mean	84.65	83.63	84.07	81.952	83.80	82.14
SE	0.8625	0.8661	0.9873	0.9263	0.8362	0.8854
Q_2	86	82.5	85.0	81	85	80
CV	8.88	9.028	10.24	14.23	8.35	9.02
g_1	-0.597	-0.228	-0.241	-0.709	-0.439	0.562
Sg_1	0.276	0.276	0.276	0.287	0.287	0.287
g_2	0.516	0.206	-0.169	0.965	0.530	1.978
Sg_2	0.545	0.545	0.545	0.566	0.566	0.556
Min	62	62	61	60	64	68
Max	100	100	100	100	100	100
KS-z	1.388	1.028	1.106	1.320	0.854	1.072
P	0.042	0.241	0.173	0.061	0.460	0.201

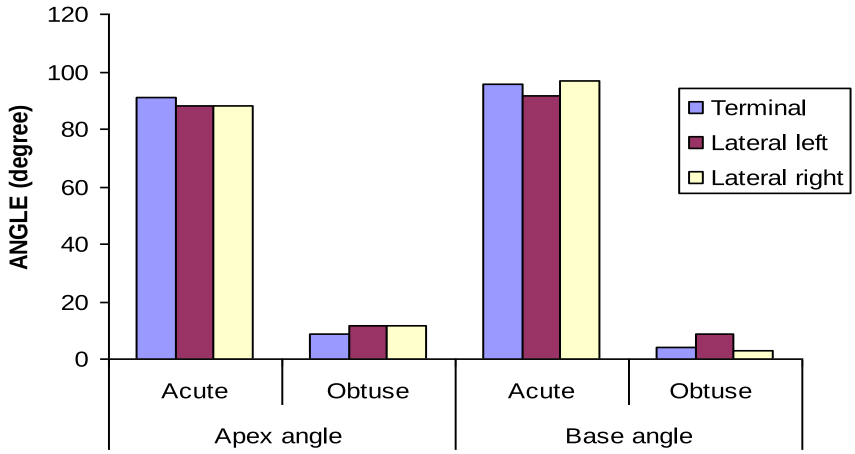

Fig. 7. Percent proportion of acute and obtuse angle types of apex and base of terminal, lateral left and lateral right leaflets of *M. sativa* leaves. Acute angle: ≤ 90 º and obtuse angle: > 90º.

Table 4. Magnitude of 'k' factors for three leaflets.

Statistical Parameters	Multiplicative factor		
	k1	k2	k3
N	90	90	90
Mean	0.626408	0.659049	0.651420
SE of mean	0.009098	0.011185	0.0111346
Median	0.645916	0.658434	0.656406
CV (%)	13.78	16.09	16.22
Skewness	-1.994	0.599	0.294
SE of Skewness	0.254	0.254	0.254
Kurtosis	5.623	8.394	5.490
SE of Kurtosis	0.503	0.503	0.503
Minimum	0.23755	0.24008	0.23992
Maximum	0.76389	1.17669	1.05714
KS-z	1.644	1.672	1.262
p	0.009	0.067	0.083

Table 5. Magnitude of length /width ratio for three leaflets of leaves.

Statistical Parameters	Terminal leaflet	Left lateral	Right lateral
N	87	87	87
Mean	1.826846	1.822903	1.844405
SE of mean	0.03271	0.031577	0.035463
Median	1.83333	1.77775	1.857143
CV (%)	16.70	16.15	17.93
Skewness	0.903	0.874	-0.098
SE of Skewness	0.258	0.258	0.258
Kurtosis	2.007	1.227	-0.470
SE of Kurtosis	0.511	0.511	0.511
Minimum	1.100	1.2083	1.050
Maximum	3.300	2.800	2.444
KS-z	1.052	0.887	0.422
p	0.219	0.411	0.994

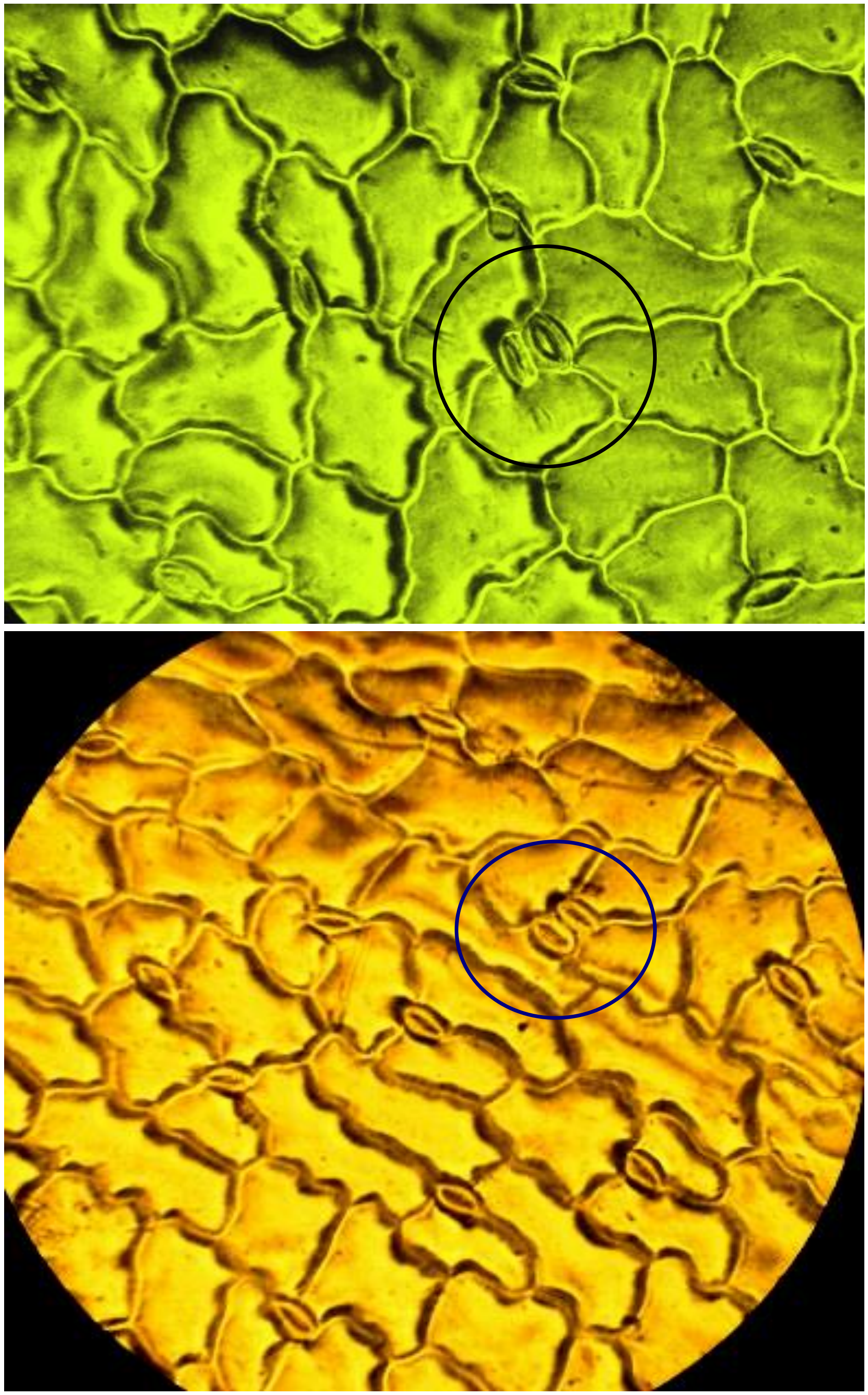

Fig.8. Stomata on dorsal leaf surface.

Contiguous stomata as observed in *M. sativa* were also found in *Erythrina indica* but rarely. They were, however, frequent in *Lathyrus sativus* (Shah and Gopal, 1969), *Melilotus albus*, *Alysicarpus vaginallis, Aeschymonene indica* and *Desmodium* spp (Kothari and Shah, 1975; Bora and Baruah, 1979). Anieusa and Silas (2012) have reported contiguous stomata in *Acalypha*. There were more than one type of stomata on leaf surface of *M. sativa* are also reported by Shah and Gopal (1969), Ahmad *et al.* (2009), Saheed and Illoh (2010) and Anieusa and Silas (2012). Metcalfe and Chalk (1979) have also reported several types of stomata in Papilionaceae. Drought and salinity increase the occurrence of contiguous stomata (Gan *et al.*, 2010). Croxidale (2000) has suggested that structure, development and patterning of stomata on the leaf surface is the function of complex processes. They should be viewed on organ, ecological, physiological and evolutionary perspective.

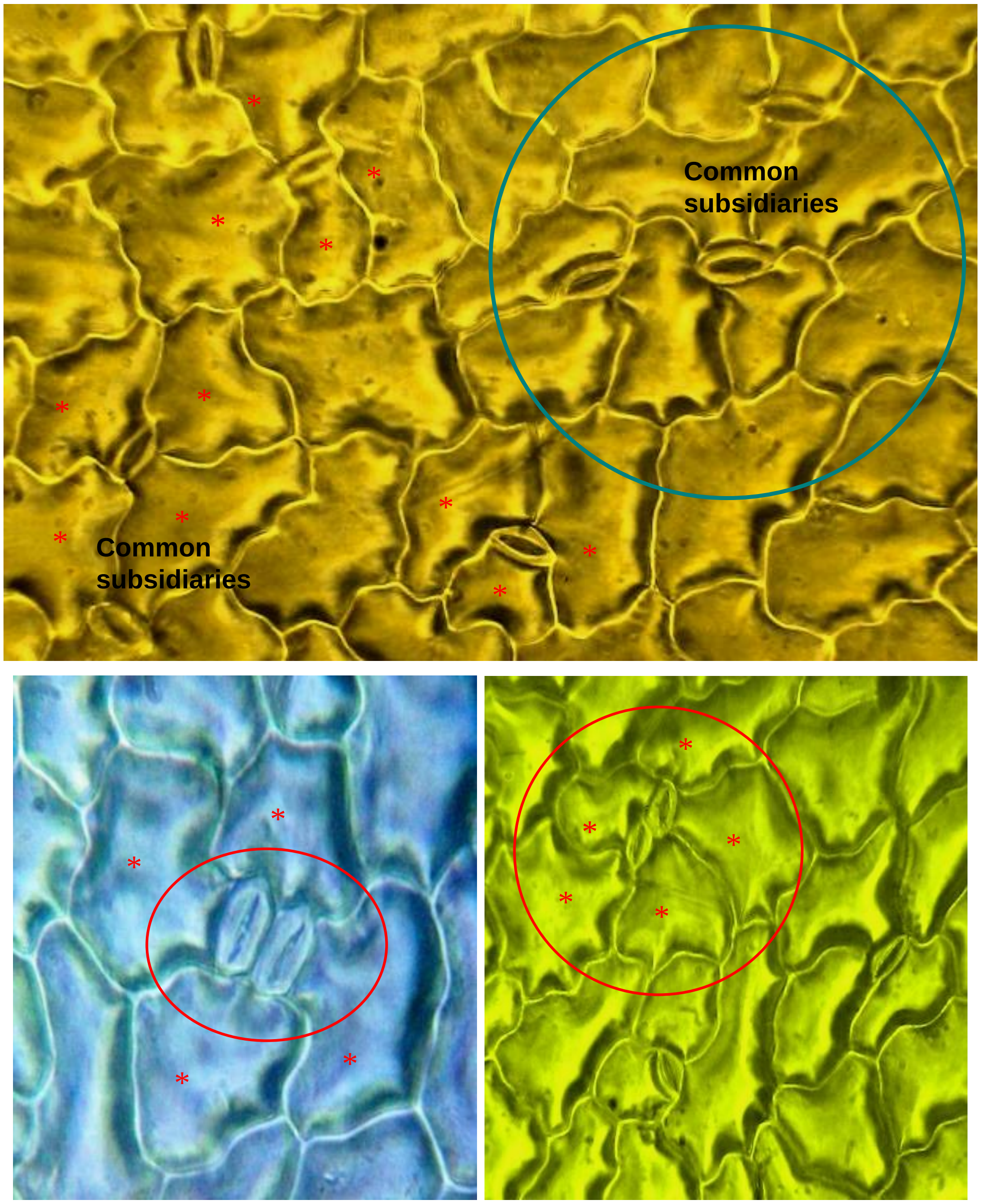

Fig. 9. Stomata on ventral surface of leaf.

Foliar stomatal density

Stomatal density on dorsal and ventral surfaces of leaves was moderate in magnitude, 100.86 ± 2.80 and 113.03 ± 5.58 per mm^2, respectively (Table 7). The stomatal density distributed normally over the two surfaces. There was more variation in stomatal number per frame on ventral surface (31.98%) than that on the dorsal surface (18.01%). There were significantly higher number of stomata on ventral surface ($t = 1.95$, $p < 0.0001$). Stomatal frequency is known to vary on upper and lower surfaces of leaf (Ekenayake *et al.*, 1998) and greater density of stomata on ventral surface of leaf is common in species occurring in xeromorphic environments (Esau, 1974; Cutter, 1986). Stomatal density in perennial papilionaceous tree, *Pongamia pinnata*, has been reported to be 211.59 ± 2.6 per mm^2 (Khan *et al.*, 2015b) and *Erythrina suberosa* 110.28 ± 2.07 per mm2 (Khan *et al.*, 2014). Stomata are variable even in the members of a tribe or even within a genus (Metcalfe and Chalk, 1950) or in a species as well. Environmental warming is reported to decrease inter-stomatal distance in maize (Zheng *et al.*, 2013).

Leaf area Estimation

Linear simple and multiple correlation and regression models obtained by regression of leaflet area of three leaflets with their respective lengths or breadths separately or in combination or as power law predictive equations yielded significant results (Table 7 and 8). It was apparent that LL was somewhat better related to leaflet area than breadth. The comparison of the three models on the basis of r, r^2, and F ratio values suggested that the power law equation were relatively better to define leaflet areas on the basis of LL X LB – explanatory value of these equations being 94.4 to 94.7% (Table 8).

Table 6. Foliar stomatal density per $mm.^2$

Statistical Parameters	Leaf Surface	
	Dorsal	Ventral
N	42	42
Mean	100.86	113.033
SE of mean	2.804	5.57754
Median	98.2898	113.033
CV (%)	18.01	31.98
Skewness	- 0.915	- 0.148
SE of Skewness	0.365	0.365
Kurtosis	2.506	-0.436
SE of Kurtosis	0.717	0.717
Minimum	39.32	39.32
Maximum	137.61	176.92
KS-z	1.178	0.575
p	0.125	0.895

Table 7. Linear correlation and regression between leaflet areas measured (Y) and leaflet length and breadth.

Leaflet	Leaflet length (LL)				Leaflet breadth (LB)			
	r	a	b	F	r	a	b	F
Terminal	0.922	-3.114	2.218	481.17	0.915	-2.323	3.527	440.02
Lateral left	0.918	-3.068	2.247	457.13	0.903	-2.156	3.457	374.50
Lateral right	0.900	-2.520	2.039	357.73	0.929	-2.055	3.380	537.60

Table 8. Predictive equation of multiple regression of leaflet area with LL and LB, linear regression with LL x LB and power law regression for leaflet area with LL X LB.

Multiple linear correlation and regression:

Area terminal leaflet = -3.270 + 1.243 LL + 1.809 LB ± 0.71736, R = 0.952; R^2 = 0.906; F = 404.9 ($p < 0.001$)

Area left lateral leaflet = -3.002 + 1.358 LL + 1.550 LB ± 0.77436; R = 0.936; R^2 =0.875; F = 294.54 ($p < 0.001$)

Area right lateral leaflet = -2.645 + 0.850 LL + 2.198 LB ± 0.67462, R = 0.948; R^2 = 0.898, F = 369.9 ($p < 0.001$)

Regression with multiplicative parameter (LL x LB):

Area terminal leaflet = -0.036 + 0.636 (LL x LB) ± 0.58322, R = 0.968; R^2 = 0.937; F = 1267.1 ($p < 0.001$)

Area left lateral leaflet = -0.041 + 0.669 (LL X LB) ± 0.66341; R = 0.953; R^2 =0.907; F = 832.05 ($p < 0.001$)

Area right lateral leaflet = -0.008 + 0.652 (LL x LB) ± 0.52977, R = 0.968; R^2 = 0.936, F = 1251.17 ($p < 0.001$)

Power law equations:

Area terminal leaflet = 0.56715. LL x $LB^{1.019778}$ ± 0.17210, R = 0.973; R^2 = 0.947; F = 1524.14 ($p < 0.001$)

Area left lateral leaflet = 0.634336. LL X $LB^{1.014955}$ ± 0.17704; R = 0.972; R^2 = 0.944; F = 1429.34 ($p < 0.001$)

Area right lateral leaflet = 0.646414. LL x $LB^{0.997658}$ ± 0.17844, R = 0.973; R^2 = 0.947, F = 1518.4334 ($p < 0.001$)

The leaflets areas were, in present studies, also determined mathematically by calculating multiplication ratio or factor (ks) for various leaflets as described earlier. The three ks (k1, k2 and k3) for terminal and two lateral left and right leaflets, respectively, are described in Table 4. k1, k2 and k3 averaged to, k1= 0.626408, k2 = 0.659049 and k3 = 0.651420.

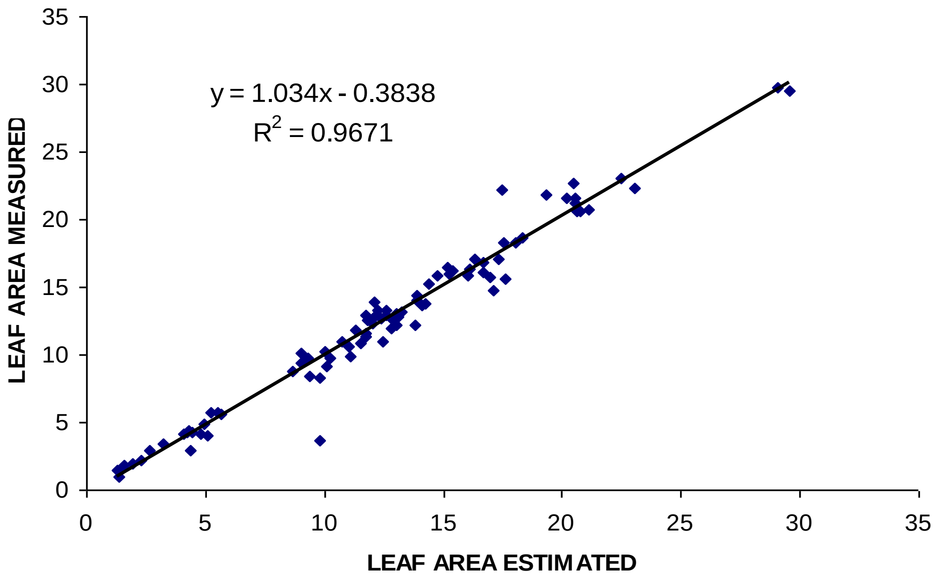

Fig. 10. Relationship between leaf area measured and leaf area estimated on the basis of k factor values (k1, k2, and k3 for terminal and two lateral leaflets, respectively. The relationship between the two variable is typically isometric (b = 1.0). Leaf area estimated = Sum of areas of three leaflets estimated via k values.

The location and dispersion parameters of measured and estimated leaflet areas for terminal, left lateral and right lateral leaflets are presented in Table 2. The measured and estimated average areas were not significantly different from each other in any of the leaflets (terminal leaflet: t = 0.087, NS; left lateral: t = 0.032, NS and right lateral: t = 0.0096, NS).

The area of the leaf was estimated through simple summation of the estimated areas of the three leaflets of a leaf calculated on the basis of numerical values of k1, k2 and k3 for the three leaflets (Table 2). The area of a mean leaf of late vegetative stage seedlings was measured to be 12.4203 ± 0.6939 and estimated to be 12.3832 ± 0.6600 cm^2. The leaf area $_{measured}$ and leaf area $_{estimated}$ correlated highly positively with each other (r = 0.9834) and relationship between them was isometric (Fig. 10). The average leaf area $_{measured}$ didn't differ significantly from the average leaf area $_{estimated}$ (t = 0.0389, NS).

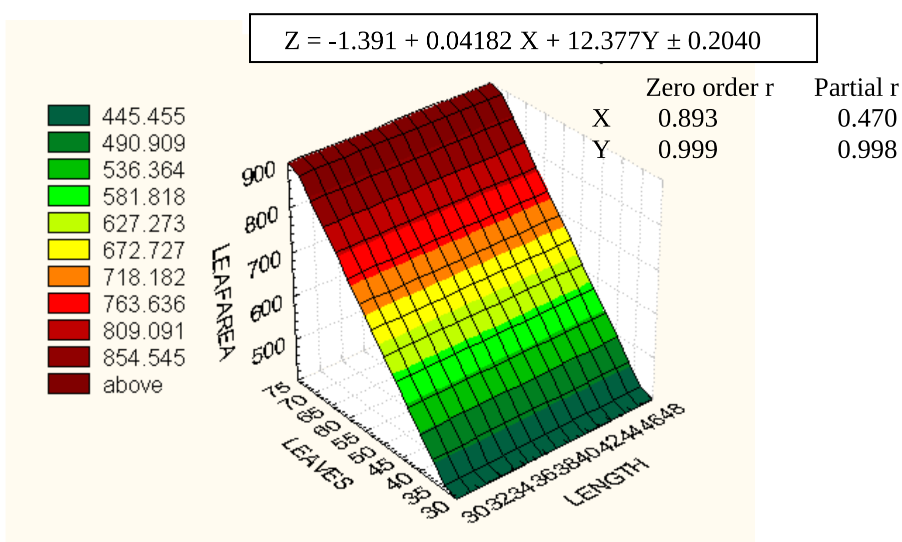

Fig. 11. Relationship of shoot length (cm; x-axis), number of leaves (y-axis) and estimated leaf area per seedling (cm^2; z-axis) of late vegetative stage seedlings (N = 10) of *M. sativa*.

Above-given results on leaf area estimation of *M. sativa* are highly significant and could be useful in experimental agronomic studies with this taxon. Since Huxley (1924) who was the first to undertake such studies, many workers have undertaken leaf area estimation allometrically as well as mathematically and have obtained useful results with many plant species e.g., *Fragaria* spp. (Demirsoy *et al.* (2005); *Xanthosoma* spp. (Goenaga and Chew (1991); *Arachis hypogaea* (Kathirvelan and Kalaiselvan, 2007); hazel nut (Cristofori *et al*. (2007); millet (Persaud *et al.* (1993); *Prunus avium* (Citadani and Peri, 2006); in 15 fruit spp. (Uzun and Celik, 1999); sunflower (Bange *et al.* (2000), cotton (Akram-Ghaderi and Sultani, 2007), *Nicotiana plumbaginifolia (*Khan, 2008*), improved genotypes of Coffea arabica* and *C. canephora* (Brinate *et al.*, 2015), *Ficus religiosa* (Khan, 2009), *Vicia faba* (Erdoğan, (2012), *Simmondsia chinensis* (Khan *et al.*, 2015c), *Capparis cartilaginea* (Khan *et al.*, 2015d), etc.

In view of the simplicity and convenience and the accuracy of estimation, using mean k coefficients (k1= 0.626408, k2 = 0.659049 and k3 = 0.651420 for terminal, left lateral and right lateral leaflet, respectively) while measuring length and breadth of the leaflets may be recommended for the estimation of leaf area in *Medicago sativa*. It may, however, be mentioned that environmental interactions may influence any such model in plants (Robbins and Pharr, 1987).

Mean total leaf area per late vegetative seedling

The estimated area of the mean-sized leaf (MLA) of the seedlings of late vegetative stage (70-day old) studied was 12.3832 cm^2 ranging from 1.31 to 29.55 cm^2. From this statistics the total leaf area per seedling was calculated as multiple product of number of leaves per seedling with mean area per leaf (MLA) for a sample of ten seedlings. The mean estimated total leaf area per seedling amounted to 646.33 ± 60.92 cm^2 varying by 29.80 % (432.72 to 854.44 cm^2). The estimated total leaf area per seedling (Z) related significantly (Fig. 11) with shoot length (X, 33-46 cm) and the number of leaves per seedling (Y, 36-69) as given by the following equation (r = 0.9989):

$$Z = -1.391 + 0.04182 X + 12.377 Y$$

It was evident from the zero order and partial correlations for this relationship (Fig. 11) that the total leaf area of a seedling was relatively more closely correlated with number of leaves than the length of the shoot.

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