INT. J. BIOL. BIOTECH., 17 (3): 607-638, 2020.

MORPHOLOGICAL CHARACTERISTICS OF SEEDLINGS OF SUNFLOWER (*HELIANTHUS ANNUUS* VAR. US 666)

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ABSTRACT

Seedling morphology of *Helianthus annuus* var. US 666 is described. Seedlings were phanerocotylar-epigeal-reserve type. Hypocotyl grew rapidly bringing cotyledons aboveground while enclosed in pericarpic shell. The primary leaves showed brochidodromous venation. Leaves were wide elliptical with apex acute and often mucronate, dorsiventral and yellow green ventrally. The photosynthetic area (PA) of seedlings increased with age in quadratic manner as follows: PA (mm^2) = - 9.2066 (Age in days) ^{2} + 348.88 (Age in days) – 983.55; R^2 = 0.8974. The leaves were simple, opposite, petiolate. Cotyledons were atrichomatous and leaves exhibited three types of trichomes, 1) Glandular moniliform multicellular uniseriate trichomes (GTs), 2) Glandular short-stalked capitate trichomes (CGTs) and 3) Non-glandular apically pointed trichomes (NGTs) of variable sizes. NGTs from mature leaf had reticulate epicuticular ornamentation with spike like projections, particularly on the lower cell. Cotyledonary epidermal cells were straight to slightly curve in anticlinal contour but wavy in leaves. The foliar pavement epidermal cells were quite intricate in shape with U-shaped undulations fitting like the pieces of the Jigsaw puzzle. The waviness varied substantially, (1-) 2-12 (-13) crests per cell and averaged around six and varied on dorsal and ventral surfaces of primary and secondary leaves by 30.15 to 39.0%, respectively. Waviness was found not to vary significantly between dorsal and ventral surfaces of primary leaf. In the pooled samples, dorsal surface showed waviness 6.01 ± 1.02 crests per cell and ventral surface had waviness averaged to 6.05 ± 0.130 crests per cell. If viewed according to Prabhakar (2004), four types of stomata were observed in the laminar islands of leaf – tetracytic, anisocytic anomocytic and staurocytic on dorsal and ventral surfaces of primary, secondary, or tertiary leaves. Cotyledons were more diverse in stomata - Anomocytic, tetracytic, anisocytic, staurocytic, isotricytic, paracytic, and diacytic stomata were recorded. Often paracytic stomata changing to isotricytic or anisocytic were also observed. Clustering of stomata was often seen, and contiguousness of stomata was common. Stomata have been described for their density and relative abundance on the surface of various organs. According to the classical schemes of stomatal classification, stomata of in-hand sunflower variety were just of one kind, anomocytic stomata, being abutted with indistinct neighbouring cells. Classical schemes of stomatal classification appear to be more suitable from ontogenic viewpoint. Stomatal size in terms of length (L) and breadth (B) have been described. In 72.8% cases of stomata the L / B ratio fell within a class of 1.26 to 1.75.

Key Words: *Helianthus annuus* var. US 666, Seedling Morphology, Micromorphology, Trichomes and Stomatal types and their relative occurrence.

INTRODUCTION

Asteraceae is an exceptionally large family consisting of 1620 genera and 23600 species (http://www.britannica.com/plant/Asteraceae). A host of the studies have been conducted on foliar epidermal structure of the taxa of this family, however a few important ones may be cited as (Tuberosa *et al.*, 1985; Adedeji and Jewoola, 2008; Qureshi *et al.*, 2002; Ahmad, 2005; Milan *et al.*, 2006; Zarinkumar, 2007; Badmus and Afolayan, 2010; Hayat *et al.*, 2009, 2010; Inceer and Ozean, 2011; Kumekawa *et al.*, 2013; Paniagua-Ibáñez *et al.*, 2015; Bano *et al.*, 2015; Tahir *et al.*, 2016). Only a few studies as regards to the epidermal characteristics in Asteraceae have been conducted in Pakistan. Qureshi *et al.* (2002) carried out taxonomic studies of six species of the genus *Sonchus* (Asteraceae). Ahmad (2005) conducted morphological and anatomical studies of 23 species of the genus *Saussurea* (Asteraceae) from Pakistan. Hayat *et al*. (2009) reported that foliar trichomes of genus *Artemisia* are good taxonomic markers. They studied the stomatal variation in 24 taxa of the genus *Artemisia* which can be utilized to resolve taxonomic issues at infra-generic level. Bano *et al.* (2015) described micromorphological characters of 12 asteraceous species of alpine zone of Deosai plateau. However, the available information on the leaf epidermal-anatomical characteristics of this family still appears to be quite limited. *Helianthus annuus* is an annual and economically valuable species of Family Asteraceae. Information on epidermal micromorphology of *H. annuus* is rather limited with reference to stomatal frequency (Lovett and Cambell, 1973, Rawson and Craven, 1975, Dhopte and Aher, 1976) particularly in various seedling components. The study of leaf surface of plants is taxonomically important in general (Stace, 1965) and for the family Asteraceae in particular (Adedeji and Jewoola, 2008). Vidhu *et al.* (1985) presented stomatal description of *Tagetes erecta* as influenced by the growth regulators and Inamdar *et al* (1980) investigated the stomatal behaviour in sunflower cotyledons under various treatments of growth regulators in sunflower cultivar EC 68414. To our knowledge, the data available on seedling morphology of sunflower is still meager and whatever data are available appear to be related with the *H. annuus* as a species and only a few have mentioned the sub-specific taxonomic specifications of the plant under studies. This paper describes the micromorphology of *H. annuus* var. US 666 in detail at the seedling stage.

MATERIALS AND METHODS

The seeds of *H. annuus* Var. US 666 were obtained from Seed Certification Department, Karachi. Sunflower seeds are technically fruits. The fruits of *H. annuus* var. US 666 (containing seed enclosed within the shell of

pericarp), were sown in sandy loam soil in pots (irrigated to 75% MWHC) in March 2020. Seedling started emergence on third day of incubation. The seedlings were studied for their morphological characters including stomatal types. Seedling type was described according to Garwood (1996). Hickey (1973) was followed for description of leaf. Leaf epidermal impressions were made with clear nail polish (Wang *et al.*, 2006). All imprints were obtained with freshly collected samples. Stomatal nomenclature suggested by Prabhakar (2004) was followed to describe stomata. This classification is based upon structure of stomata and not their ontogenetic pathways. And so, no distinction of distinct and indistinct neighbouring cells (NCs) was maintained by him. Indistinct NCs were considered equally important as distinct NCs due to their abutting nature to stoma. Stomata were also viewed according to the classical schemes of stomatal classification. Length and width of stomata were measured in μm with calibrated micrometer. The data was analyzed statistically (Zar, 2010).

Wherever needed, similarity between samples was determined by Czekanowski (1913) coefficient of similarity (SI). SI = [2 min (X_i, Y_i) / $\sum$ (X_i + Y_i)] * 100, where X_i equals to a measure belonging to one sample and Y_i is this measure for the other sample to be compared. Characters of waviness and the distribution of stomatal types on cotyledon and leaf were the measures employed in this study. Dormant cotyledons were not observed. Observations on cotyledons, hypocotyl, epicotylar stem and leaves were made on 3- to 20-day old seedlings.

Seedling emergence

Seedlings began emerging on third day of incubation. Maximum emergence (90%) was achieved within 5 days of incubation. Seedlings were fast growing with rapid hypocotylar growth. Radicle came out of the diaspore first and the hypocotylar growth resulted in emergence of cotyledons aboveground while still enclosed in pericarpic shell. The cotyledons underwent expansion and growth. The seed coat was found associated with pericarp shell. Cotyledons became green soon and started expansion. The seedlings when emerged were curved which straighten later on. The cotyledons remained with seedlings for c 20 days. The shriveled cotyledons were abscised after some time.

Table 1. Morphometric characteristics of variously aged seedlings of *H. annuus* var. US 666 grown in sandy loam soil in pot.

Parameters	Seedlings					
	3-day (3) *	7-day (9)	10-day (2)	15-day (5)	20-day (3)	25-day (1)
Hypocotyl length (cm)	6.5	12.43 ± 1.25	14.55 ± 0.95	13.56 ± 1.03	12.33 ±2.68	13.5 ± 2.1
Area per cotyledon (mm^2)	136 ±15.0	296.25± 13.07	346.75 ± 46.6	332.8± 18.01	295.0 ± 25.7	Shriveled
Cotyledon area per seedling	272.0 ± 5.06	598.75 ± 34.23	679.0 ± 149	665.6 ± 43.8	590.0 ± 72.0	-
Epicotyl length (cm)	-	-	2.9 ±0.29	6.76 ± 0.31	6.50 ± 0.29	14.0**
Number of Epicot. internodes	-	-	1	1	2	3
Number of Primary leaves	-	-	2	2	2	2
Number of Sec. leaves	-	-	-	2	2	2
Number of Tert. leaves	-	-	-	2	2	2
Number of Quaternary leaves	-	-	-	-	-	2
Leaf area / Prim. Leaf (mm^2)	-	-	389.5 ± 66.51	544.0 ± 53.4	595.7 ± 94.7	545.5
Leaf area / Sec. leaf (mm^2)	-	-	-	171.6 ± 37.7	357.7± 133.1	409
Leaf area / Tert. Leaf (mm^2)	-	-	-	95.25 ± 40.0	81.3 ± 29.2	125.5
Leaf area of a Quat. leaf	-	-	-	-	-	5.0
Total Photosyn. Area (mm^2)	272	599	1458	2287	2660	1770***

*, Number of seedlings studied. **, Three internodes in 25-day old seedling measured individually in length from base to apex: 8.5, 5.0 and 0.5 cm, respectively in pots. Petiolar length measured around 2 cm in case of primary leaf; 1.3cm in case of secondary leaf and 1.3 mm in tertiary leaf. ***, Both cotyledons consumed.

Seedling

Seedling morphometry is described in Table 1. Hypocotyl is the most rapidly growing organ of the seedling that brings the cotyledons aboveground. Hypocotyl is green c. 2.0-2.5 mm in diameter but somewhat weak being long – reaching around 14.5 ± 0.95 cm on 10^{th} day of emergence. It is somewhat swollen at the point of cotyledons insertion. Cotyledons are fleshy, thick food-laden and fragile. They are concave on upper side and convex on the lower side. Cotyledons are petiolate and opposite. They are smooth shining and greener on upper side. They are obtuse at the apex. At the time of release from the pericarp the cotyledons are on an average 136 ± 15 mm^2 in size. Cotyledons undergo continuous expansion after emergence reaching to an average size of 346.75 ± 46.6 mm^2 on 10^{th} day. Cotyledons are not only food-laden, but they are also the primary photosynthetic organ. Their substantial expansion in sunflower has also been reported by Lovell and Moore (1970). According to them such expansion of sunflower cotyledons is due to the increase in cell size and not the cell number. Venation in cotyledons is not apparent (Fig. 1). Cotyledons are sometimes abnormal in shape – discreetly cup like in shape (Fig. 2). On protrusion

of cotyledons from the pericarp, the membranous seed coat may often be seen with cotyledon or attached to the pericarp. This is more or less transparent structure, the surface cells of which are polygonal – generally tetrangular but also triangular in outline, parenchymatous, closely-fitting with little intercellular spaces (Fig. 3A). Epicotylar stem is densely studded with hairs (Fig. 3 B). Seedlings, as per Garwood (1996) classification, are phanerocotylar-epigeal- reserve type (cotyledons thick fleshy). The cotyledons are raised aboveground on emergence while enclosed in pericarp due to rapid elongation of hypocotyl. There appears a good degree of regenerative potential in sunflower seedlings as removal of epicotylar stem from mid-point induced the leaf initiation from the cotyledonary axil (Fig. 14B). This feature appears to be economically important. Pilson and Decker (2002) have reported that in wild sunflower (*H. annuus*) removal of the primary capitulum resulted in the production of more inflorescences, which compensated fully for the initial loss.

The pair of primary leaves was produced in plane opposite to that of the cotyledons and the subsequent leaves were produced in opposite decussate manner. The leaves were simple, petiolate, and opposite. The primary leaves showed brochidodromous venation. Leaves were wide elliptical with apex acute and often mucronate, dorsiventral and yellow green ventrally. Leaves were trichomatous on surfaces, margins, and petiole (Fig. 4). Leaf size increased progressively with age (Table 1). The total photosynthetic area per seedling was the highest in 20-day old seedlings. It varied with age of seedling through a second-degree polynomial model:

$$\text{Photosynthetic area } (mm^2) = -9.2066\,(\text{Age in days})^2 + 348.88\,(\text{Age in days}) - 983.55;\ R^2 = 0.8974$$

The photosynthetic area of the seedling decreased in 25-day old seedlings owing to the fact that cotyledons shriveled after around 20 days of growth after emergence. Yellowing of cotyledon was seen to initiate in some seedlings at an age of 15 days after emergence. They shriveled while they were around 20-day old and abscised later on.

Fig. 1. Sunflower (*H. annuus* var. US 666) seedlings – initially seedlings are curved but they soon straighten. Hypocotyl grows rapidly bringing cotyledons (enclosed in pericarp) aboveground.

Fig. 2. Abnormal cotyledons (cup-like) in a four-day old seedling.

A few leaves of some seedlings were abnormal in shape. They were broader and notched at the apex (Fig. 5). Such leaves were trichomatous on both sides and had stomata similar to normal leaves. Foliar variations are quite common in sunflower. Abnormal leaf of *H. annuus* observed in a field-grown control plant (c 2m in height) in a pathological experiment conducted by Dr. M.J. Zaki in Dept. of Botany, Univ. Karachi is depicted in Fig. 6. Generally, in this species one petiole bears one leaf. In the abnormal leaf an additional leaf was borne on the petiole of the original leaf. It was apically notched as also found here (Fig.5).

Surface micromorphology

Micromorphologically, the epidermis may be distinguished in three components – Trichomes, epidermal pavement cells and the stomata (Esau.1965) which in sunflower seedlings may be described as follow:

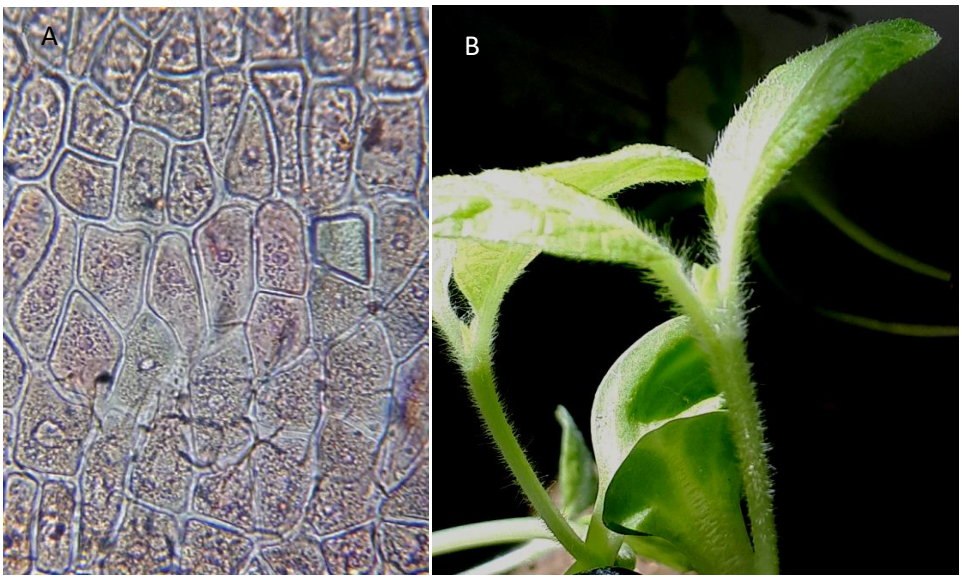


Fig. 3. A) Surface structure of membranous seed coat (45 x 10 X, zoom 4X); B) Hairy epicotyl (7-day old seedling).

Fig. 4. Dorsal (A) and ventral (B) sides of primay leaf of 10 - day old seedlings of sunflower shown with eglandular trichomes which are denser and longer on veins. Glandular trichomes are not visible here. They are shown in a separate figure. The venation is of brochidodromous type. Petiole is densely hairy.

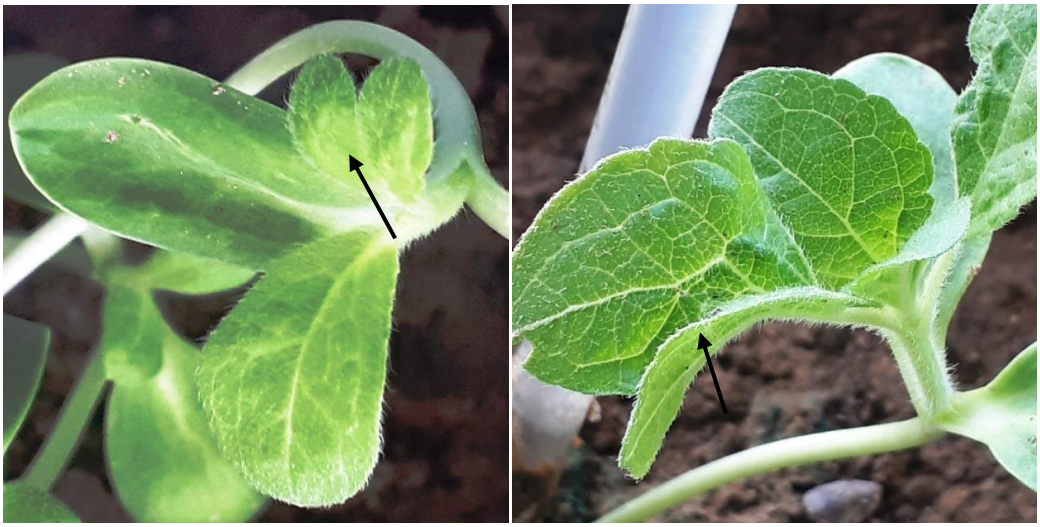


Fig. 5. Abnormal leaves (arrows) in sunflower seedlings.

Fig. 6. Abnormal leaf of *H. annuus* observed in a field-grown control plant (c 2m in height) in a pathological experiment conducted by Dr. M. J. Zaki in Dept. of Botany, Univ. Karachi. Generally, a petiole bears single leaf. In the image the additional leaf was not only borne on the petiole of the original leaf, but it was deformed in shape also as noticed above (Fig.5).

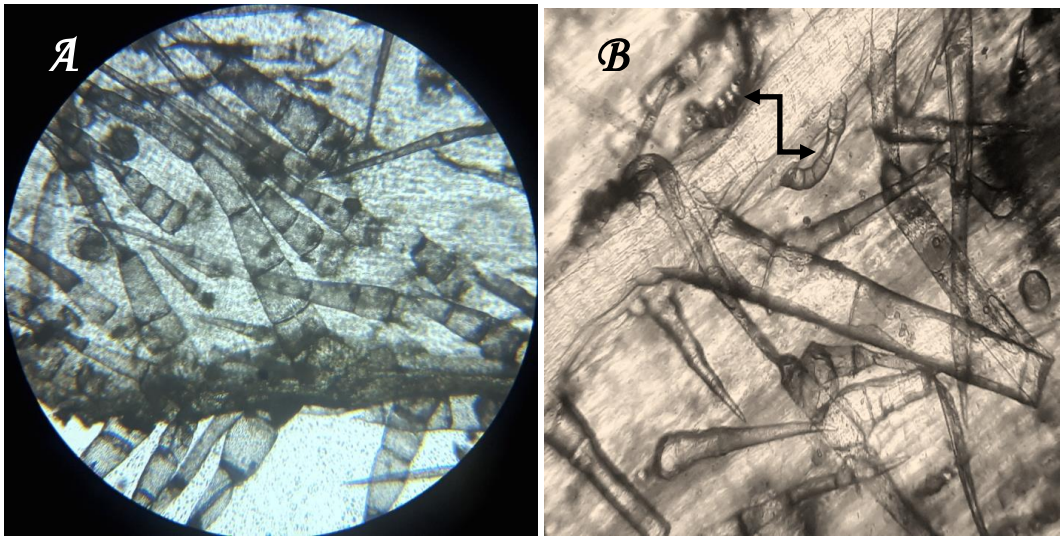


Fig. 7. Surface view of epicotyl (A) and the petiole (B) showing trichomes. Glandular trichomes are present on both petiole (indicated by an arrow) and the epicotyl.

Trichomes

In sunflower seedlings, all epicotylar structures – stem, leaf petiole, leaf blade and the margins of the leaves were trichomatous. Both glandular trichomes (GTs) and non-glandular trichomes (NGTs) of variable sizes were recorded in sunflower Var. US 666 seedlings. GTs were of two types: 1) Glandular moniliform multicellular uniseriate trichomes and 2) shortly stalked capitate trichomes (CGTs). NGTs were conical with pointed apical cell. Cotyledons including cotyledonary petiole were devoid of trichomes.

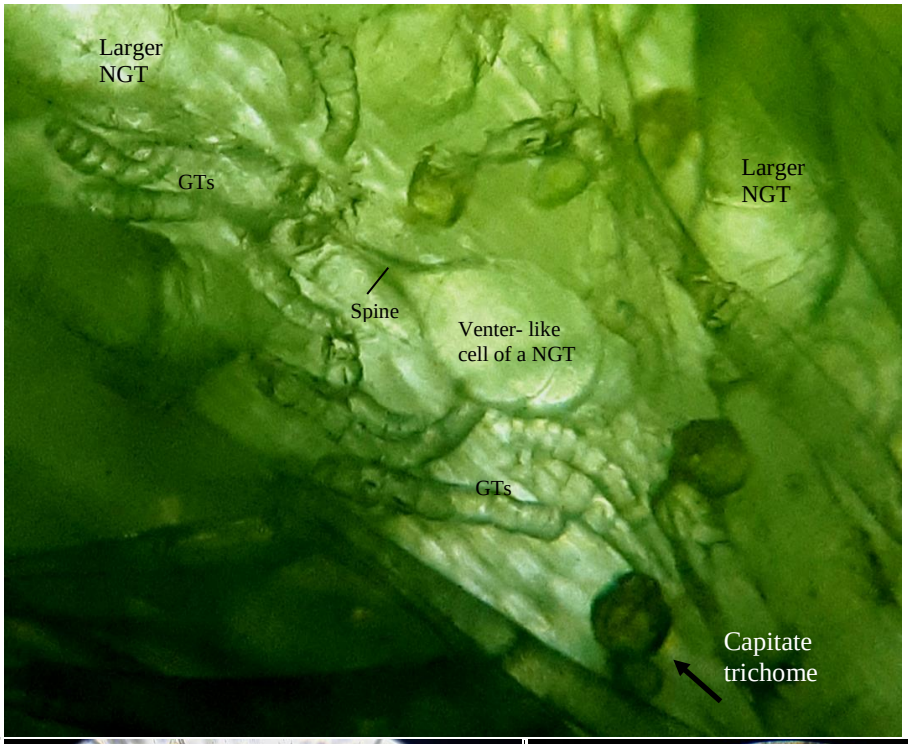


Fig. 8. The surface view of a young and fresh leaf showing the multicellular glandular (GTs) and non-glandular multicellular trichomes (NGTs) in the midrib region. The NGTs are longer and sometimes with large venter -like cell above the basal cell. Trichomes have varying number of constituent cells. The apical cell of NGTs is conical, pointed at the apex. GT is generally curved, moniliform and larva-like in appearance. Capitate trichome (arrow) with a small stalk may be seen in the lower right corner of the image. (Magnification: 45 x

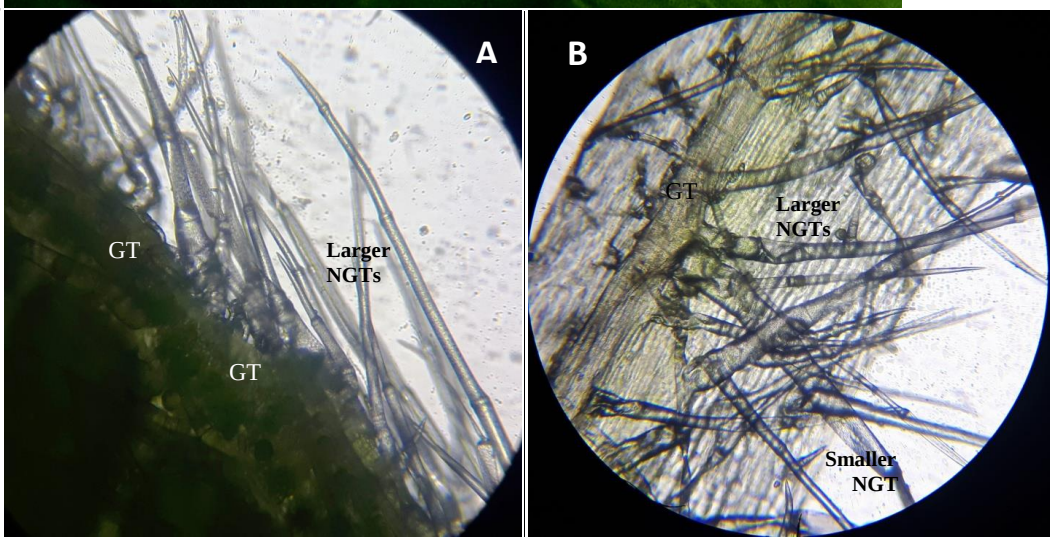


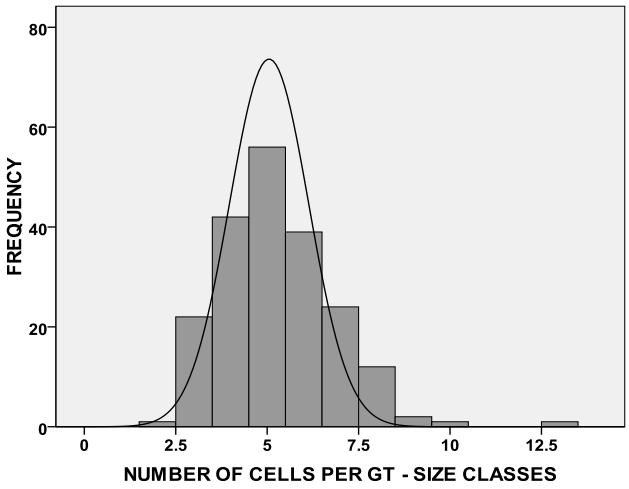
Fig. 9. Young leaf of *H. annuus* var. US 666 showing glandular larva-like trichomes (GTs) and non-glandular trichomes (NGTs) on the leaf margin (A) and midrib (B).

Basal cells of NGTs are modified in shape and are radially arranged (generally 7 in number) as also reported in *Tridax procumbens* (Suseela *et al.,* 2002). Trichomes are first cells to differentiate and develop in a basipetal manner in *Arabidopsis* (Larkin *et al.,* 1996).

GTs: The GTs as studied in a young fresh leaf were moniliform, larva-like, multicellular, and uniseriate with spherical to disk- shaped cells (Fig. 8). They were also observed in mature leaf blade, its petiole, and margins and the epicotylar stem (13 D, E, F, 14 A). The number of constituent cells per GT of younger leaves of the seedlings varied from 2 to 13 (mean = 5.28 ± 0.111) i.e. the variation amounted to 29.65%. Their distribution tended to be asymmetrical (positively skewed and leptokurtic) (Fig. 10). In around 60% of the observations, the trichomes were composed of 5 - 7 cells. In some GTs, the basal cell was quite elongated. Fig. 11 A is adopted here from Louisa Howard, Dartmouth College, UK (public domain) is referred to here for comparison and clarity – A six-celled GT is clearly visible besides an NGT and a roundish stoma on the vascular nerve. Moniliform GTs of sunflower were referred to as linear glandular trichomes (LGT) by Aschenbrenner *et al.,* 2013). These trichomes were reported by

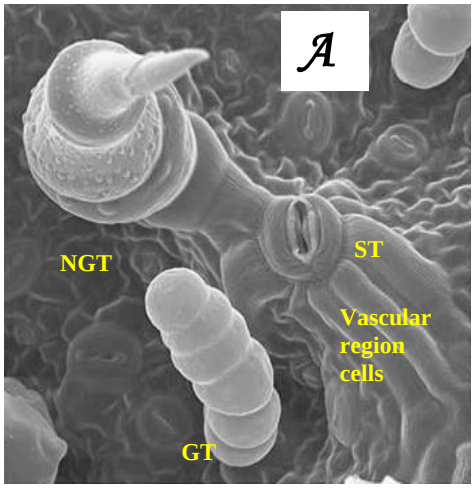
them from phyllary, petiole, stem, leaves, chaffy bracts, ray, and disc florets distributed amongst the linear pointed NGTs. LGTs have been reported by them from several species of *Helianthus* and other genera like *Aldoma, Heliomeris, Lagascea, Rudbeckia* etc. LGTs development is hypothesized to be completed at a stage where leaf primordia are still embedded in plumula. This appears to be similar to the CGTs development in sunflower leaf (Spring and Bienert, 1987) and anther appendages of disc florets (Göpfert *et al.*, 2005). They are reported to be influenced by light. Earlier, Monteiro *et al.* (2011) have reported early development of capitate glandular trichomes (CGTs) on leaf primordia in *Stevia rebaudiana* (Asteraceae). This appears to be the reason of well-formed crop of GTs and CGTs in young leaf of in hand *H. annuus* var. US 666 seedlings. Capitate glandular trichomes (CGTs), in our studies, were present amongst LGTs and GTs on young leaf. Aschenbrenner *at al.* (2013) reported them from the anther tip of sunflower and both surfaces of leaf as well. CGTs are reported to be metabolically active (Aschenbrenner *et al.*, 2013).

NGTs: These trichomes were linear, long, multicellular, and uniseriate with apical cell acicular - much variable in number of constituent cells and size (Fig. 9, 11A). The number of cells per NGT on the surface of epicotylar stem of 10-day old seedling was observed to be 4.45 ± 0.2291 ranging from 2 to 10 (CV: 32.56%) (Fig. 11 B). The most conspicuous class of 5 cells per NGT occupied 50% of the total observations and a class of 3 cells per NGT occupied c 30% of the whole set of observation. Around 92.5 % of the data set was represented by a class of 3 - 5 cells per NGT (Fig. 11B).



Number of cells per GT
N = 200
Mean = 5.28 ± 0.111
Median = 5.00
CV = 29.65%
Skewness = 0.884
SE of skewness = 0.172
Kurtosis = 2.249
SE of kurtosis = 0.342
Minimum = 02
Maximum = 13
K-S Test* = 0.175
P < 0.0001
Shapiro-Wilk = 0.925
P < 0.0001

Fig. 10. Frequency distribution of number of constituent cells per glandular trichome (GT) on leaves of sunflower seedlings. K-S Test*, Kolmogorov-Smirnoff test for normalcy with Lilliefors significance correction.



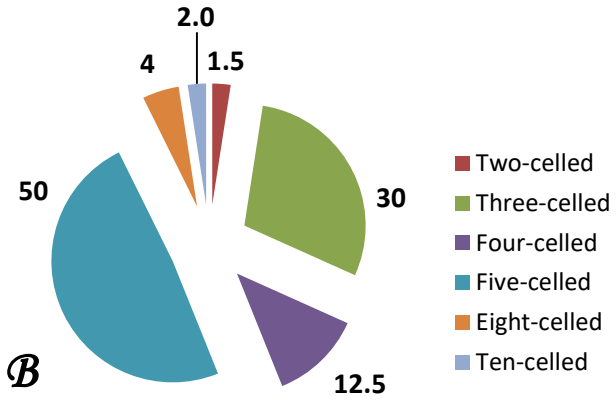


Fig. 11. A) SEM of *H. annuus* trichomes adopted from Louisa Howard, Dartmouth College, UK. (Public Domain) – Acronyms: GT, glandular trichome; NGT, non-glandular trichomes and ST, Stomata. B) The % frequency distribution of variously-celled NGTs on epicotylar stem of 10-day old seedling. N = 40; Mean number of cells per NGT = 4.45 ± 0.2291 varying from 2 to 10 (CV: 32.56%). Fifty percent of the trichomes had five cells per trichome and 30% had three cells.

Density of GTs and NGTs: The trichomal density per mm^2 as studied on a leaf blade of a mature leaf of 21 cm^2 in size from a 5-month old sunflower Var. US 666 is presented in Table 2. Density of GTs and NGTs on dorsal surface varied from that on ventral surface. GTs were significantly higher in number on dorsal surface (54.06 ± 1.865 per mm^2) as compared to that on ventral surface (35.93 ± 2.014 per mm^2) where as NGTs were slightly larger in number on ventral surface (38.99 ± 1.393 per mm^2) against 30.58 ± 1.347 per mm^2 on dorsal surface. Distribution of GTs and NGTs on both leaf surfaces was asymmetrical.

Table 2. Density per mm^2 of glandular (GT) and Non-glandular (NGT) trichomes on a leaf (21.0 cm^2 in size) of a 5–month old sapling, bearing inflorescence (stem height c 53 cm and stem diameter c 0.8 cm)) of *H. annuus* var. US 666 grown in pot. GTs and NGTs were observed on the laminar islands of the leaf.

Statistics	GT (Dorsal)	GT Ventral	NGT Dorsal	NGT Ventral
N	90	90	90	90
Mean	54.06	35.93	30.579	38.99
SE	1.865	2.014	1.3468	1.3931
Median	19.15	29.49	29.490	39.316
CV (%)	32.73	53.78	38.68	33.90
Skewness	0.526	0.520	0.217	0.204
SE skewness	0.254	0.254	0.254	0.254
Kurtosis	-0.003	0.098	-0.365	-0.509
SE kurtosis	0.503	0.503	0.503	0.503
Minimum	19.66	Zero	9.83	9.83
Maximum	108.12	88.46	59.97	68.80
K-S Test*	0.165	0.154	0.170	0.153
p	0.0001	0.0001	0.0001	0.0001
Shapiro-Wilk	0.948	0.953	0.924	0.943
p	0.001	0.002	0.0001	0.001
Distribution	Asymmetrical	Asymmetrical	Asymmetrical	Asymmetrical

GTs (dorsal) - Some 86.7% of the observations belonged to the class: 21-75 GTs per mm^2.

GTs (Ventral) – Some 62.2 % of the observations belonged to a class: 21-60 GTs per mm^2.

NGTs (dorsal) - Some 82.2 % of the total observation belonged to a class: 11-40 NGTs per mm^2.

NGTs (ventral) – Some 82.3% of the total observations belonged to class: 21-60 NGTs per mm^2.

K-S Test*, Kolmogorov-Smirnoff test with Lilliefors significance correction.

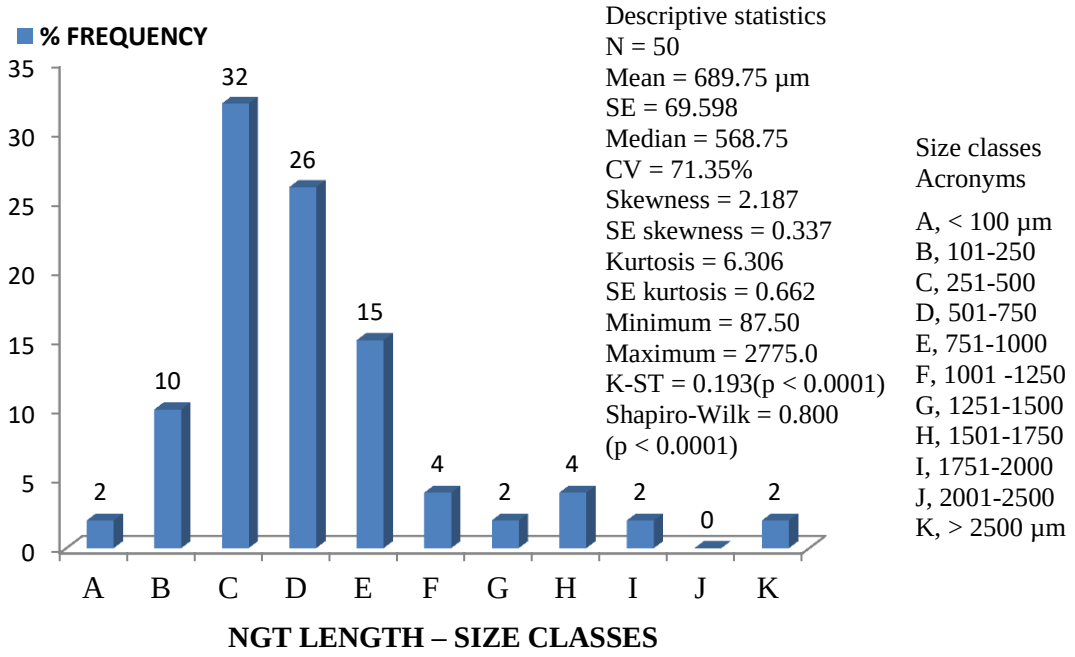


Fig. 12. Distribution of NGT lengths (µm) on the surface of epicotylar stem of 10-day old seedling. The distribution was asymmetrical (positively skewed and highly leptokurtic).

Size variation of NGTs: NGTs varied substantially in size. On epicotylar stem of 10-day old seedling, NGTs were found to vary in size from 87.5 µm to 2775 µm (CV = 71.35%) and averaged to 689.75 ± 69.60 µm, following asymmetrical distribution – positively skewed and leptokurtosis (Fig. 12). Larger trichomes generally had 5 cells per trichome (uniseriate) and smaller trichomes were three-celled structures. Modal class of NGTs length varied from 251 to 500 µm.

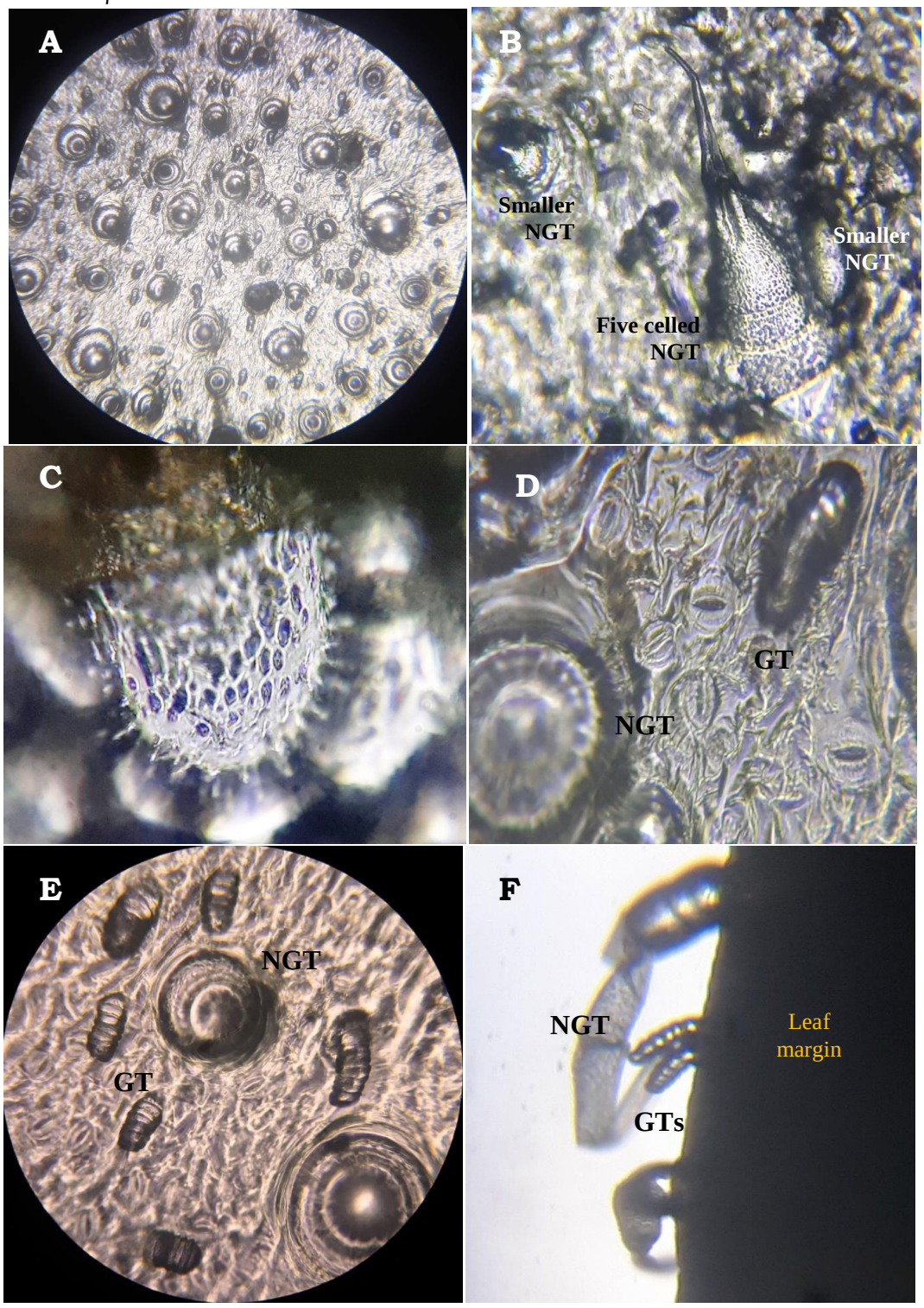


Fig. 13. Mature trichomes of a leaf from 6th node of five-month old sunflower plant grown in pot. A) General distribution of GTs and NGTs on dorsal surface of leaf (Mag.: 10 x 10 X). B) Trichomes of different sizes (Mag.: 45 x 10 X; zoom 2X) – A five-celled NGT with epicuticular ornamentation. C) Close up view of reticulate epicuticular ornamentation with spike like projections on lower cell of an NGT. D and E) surface showing GT, NGT and stomata. F) GTs and NGTs on margin of the leaf.

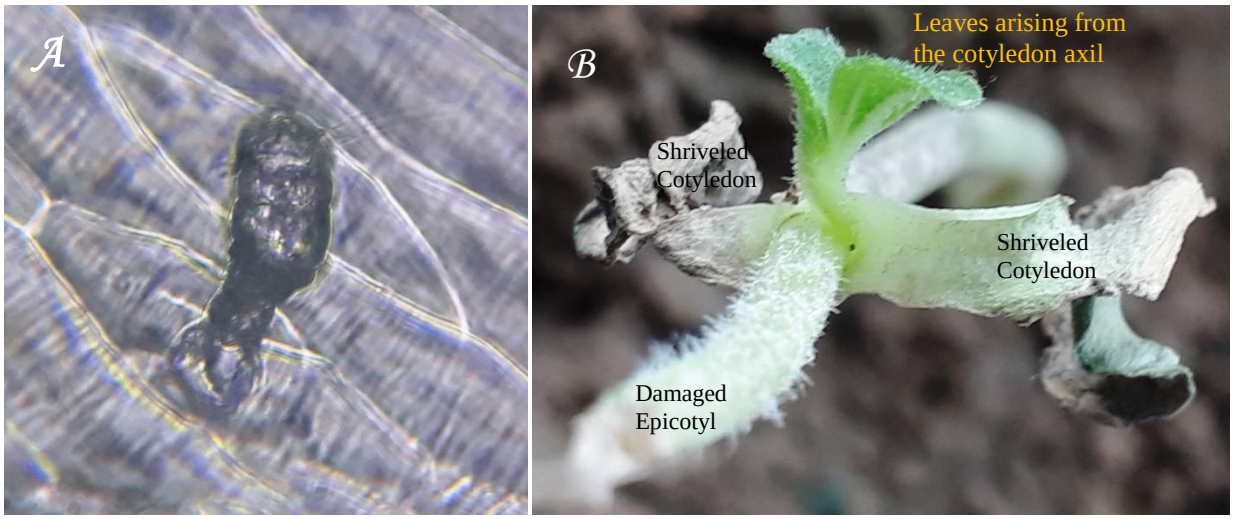


Fig. 14. A) GT on petiole of a primary leaf. B) The leaf initiation from the cotyledonary axil on damage to the epicotyl in a 15-day old seedling. It took five days to produce leaves on the total expense of the cotyledons.

Ornamentation of NGTs: Trichomes from a leaf measuring c 21 cm^2 of a 5-month old sunflower plant grown in pot are presented in Fig. 13. Two types of trichomes were found on blade and margins of this leaf which were almost regularly distributed on the surface. Trichomes present on leaf blade islands were generally smaller than those present on the vascular nerve. The apical cell of NGT is apically pointed whereas lower cells bear reticulate epicuticular ornamentation with outwardly oriented spikes (Fig. 13B, C and D).

Epidermis

The epidermal cells of cotyledons are straight in anticlinal contour sometimes arched. The foliar epidermal cells are, however, wavy in the anticlinal walls. Epidermal cells of cotyledons (Figs. 18-23) and leaves (primary, secondary, or tertiary) were convex papillose on dorsal as well as ventral periclinal surfaces irrespective of leaf age (Fig. 25 - 27). In genus *Senecio*, irregular, polygonal to rectangular epidermal cells have been reported by Joshi and Bajracharya (2015). Sussela *et al*. (2002) have reported wavy epidermal cells in *Tridax procumbens*.

Epidermal pavement cells: The pavement epidermal cells of leaf in *H. annuus* were quite intricate in shape with U-shaped undulations. They fit like the pieces of the Jigsaw puzzle. The protrusions or lobes of one cell fitting in the indentations or concavities of the adjacent neighbouring cell i.e. the lobes were perfectly interlocking. Waviness of the epidermal cells on primary and secondary leaves of 15-day old seedlings and a 20-day old seedling is presented in Table 3. The waviness varied substantially, (1-) 2-12 (-13) crests per cell and averaged around six and varied on dorsal and ventral surfaces of primary and secondary leaf, 30.15 to 39.0% (Table 3). Waviness was found to be statistically insignificant between dorsal and ventral surfaces of primary leaf (t = 0.71101, NS) and secondary leaf (t = 01.1348, NS). Dorsal and ventral epidermises in pooled samples also exhibited no significant difference in waviness (t = 0.0485, NS). In the pooled samples, dorsal surface showed waviness 6.01 ± 1.02 crests per cell and ventral surface had waviness averaged to 6.05 ± 0.130 crests per cell (Table 3). The distribution was more or less similar on dorsal surface leaf (8.02 cm^2 in size) from a 20-day old seedling. It may be deduced from the frequency distribution that the predominating size class of waviness was 5-7 crests per cell occupying 50 or slightly higher percentages of frequency. This class occupied 51.6 to 57.3 of the observations in the pooled samples of dorsal (N = 340) and ventral (N = 275) surfaces. In all cases of observation, the waviness tended to distribute asymmetrically as indicated by Kolmogorov-Smirnoff test with Lilliefors significance correction and Shapiro-Wilk test of normality. Dorsal surfaces of primary and secondary leaves had 85.02% similarity in waviness and ventral surfaces of primary and secondary leaves showed 75.4% similarity on the basis of waviness frequency distribution following Czekanowski (1913) coefficient of similarity.

The waviness of epidermal anticlinal contour varied with the size of the epidermal cells. The smaller cells had lesser number of lobes and larger cells had larger number of lobes. Jigsaw puzzle type waviness in foliar epidermal cells is also reported in *Artemisia tangutica* (Hayat *et al.*, 2010). Wavy anticlinal walls may occur in several Families Amaranthaceae, Boraginaceae, Labiatae, Scrophluriaceae, Polygonaceae, Portulacaceae, etc. (Ormrod and Renney (1968) and *Sesbania bispinosa* (Papilionaceae) (Khan and Zaki, 2019). The wavy contours in epidermal pavement are considered to be of biomechanical benefits (Jacques *et al.*, 2014; Sapala *et al.*, 2018).

Waviness is known since Areschoug (1897) and Anheisser (1900) to vary in sun and shade leaves. Watson (1942) proposed that waviness was determined by the cells outer cuticle with cell expansion being limited at regions of the cell wall that have a hardened cuticle, but not at regions where the cuticle is still hardening. The depth of undulation increases with shade (Watson, 1942) and waviness decreases from base of *Sinapis alba* to the tip (Rippel, 1919). With few exception waviness appears to be higher on lower surface (Watson, 1942; Misra (2009). This waviness appears to be affected by the environmental conditions prevailing during leaf development and its formation is considered to be regulated by sub-cellular self-organizing components – subcellular cytoskeleton organization of microtubules, cellulose microfibrils and actin (Panteris *et al.,* 1994; Jacques *et al.,* 2014; Sapala *et al.,* 2018). In sunflower, the degree of waviness of the anticlinal walls of the epidermal cells appears to be influenced by the size (age) of the leaf. In very mature large leaf, the waviness is not discretely apparent due to cuticular deposition and striation (Fig. 35).

Table 3. Waviness in terms of the number of crests per cell in dorsal and ventral epidermis of leaves of *H. annuus* seedlings. In each case, the distribution tended to be asymmetrical (AS). Modal class in bold italics.

Number of Crests per cell	Frequency (%)						
	15-day Primary Leaf		15-day Sec. Leaf		Pooled 15-day data		20-day leaf
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal (Primary)
	N = 210	100	130	175	340	275	210
% Frequency distribution							
1	-	-	0.8	2.3	0.3	1.5	-
2	2.4	3.0	2.3	5.7	2.4	4.7	2.9
3	4.3	2.0	8.4	16.6	5.9	11.3	3.8
4	12.8	16.0	12.3	15.4	12.6	15.6	10.4
5	15.7	17.0	***23.9***	***20.6***	18.8	***19.3***	16.2
6	***24.8***	***18.0***	19.2	18.8	***22.6***	18.5	***19.1***
7	15.2	***18.0***	16.3	11.5	15.9	13.8	15.7
8	12.9	12.0	8.3	4.5	10.9	5.8	14.8
9	7.6	7.0	6.2	3.5	7.1	4.8	11.9
10	4.3	7.0	13	0.5	3.2	2.9	2.6
11	-	1.0	-	-	-	0.3	0.5
12	-	3.0	0.2	-	0.3	1.1	1.9
13	-	-	-	0.6	-	0.4	-
Descriptive statistics							
Mean	6.17	6.39	5.95	5.92	6.01	6.05	6.46
SE	0.128	0.222	0.166	0.148	1.02	0.130	0.143
CV%	30.15	34.8	36.0	39.0	31.29	39.1	31.98
G1	0.053	0.489	0.282	0.473	0.134	0.535	0.143
Sg1	0.168	0.241	0.212	0.184	0.132	0.147	0.168
G2	-0.432	-0.043	0.289	0.810	-0.210	0.474	-0.116
Sg2	0.334	0.478	0.422	0.365	0.264	0.293	0.334
Minimum	2	2	1	1	1	1	2
Maximum	10	12	12	13	12	13	12
K-S T*	0.137	0.132	0.130	0.109	0.128	0.120	0.112
p	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Sh-W	0.966	0.957	0.971	0.961	0.973	0.964	0.972
p	0.0001	0.002	0.006	0.0001	0.0001	0.0001	0.0001
Distribution	AS	AS	AS	AS	AS	AS	AS

K-S T*, Kolmogorov-Smirnoff test with Lilliefors significance correction. Sh-W, Shapiro-Wilk test.

Stomata

Stomata of sunflower seedlings were studied on hypocotyl, cotyledons, epicotyl stem and leaves of various sizes (ages) – primary, secondary, and tertiary. The neighbouring cells of stomata were indistinct. They, however, may briefly be described as follows following Prabhakar (2004) who considers indistinct neighbouring cells to be subsidiaries due to their abutting nature.

Hypocotylar stomata: Anisocytic, anomocytic and contiguous stomata (Fig. 15).

Stomata of Cotyledonary petiole: Anisocytic, tetracytic, staurocytic and paracytic (Fig. 16). Stomata at this surface were quite widely placed. Stomatal density averaged to 33.81 ± 1.735 per mm^2 varying from 9.83 to 58.97 stomata per mm^2: CV= 37.40%. (Fig. 17). A cluster of three stomata (triplet) was recorded on this surface (Fig. 30A).

Stomata of petiolar area merging into cotyledonary blade: Tetracytic, staurocytic, anomocytic, anisocytic and contiguous.

Stomata of cotyledonary blade: The stomata on cotyledonary surface were studied on dorsal surface of cotyledons of various ages i.e. 3-, 7- , 10- 15- and 20-day old. Differential degree of stomatal maturity was obvious in 3-day old cotyledons which varied spatially. At this age, the epidermal cells were periclinally convex (papillose). Stomatal were variously shaped – squarish, triangular to Pentagular, wide elliptical and oval. Pentagular stomata were generally large and surrounded by five or more cells (neighbouring cells). Epidermal cells were straight in anticlinal contour, rarely curve. Anomocytic, tetracytic, anisocytic, staurocytic, isotricytic, paracytic stomata were recorded. Often paracytic changing to isotricytic was observed (Fig. 18, 20). Three Contiguous stomatal initials were also seen (Fig. 20).

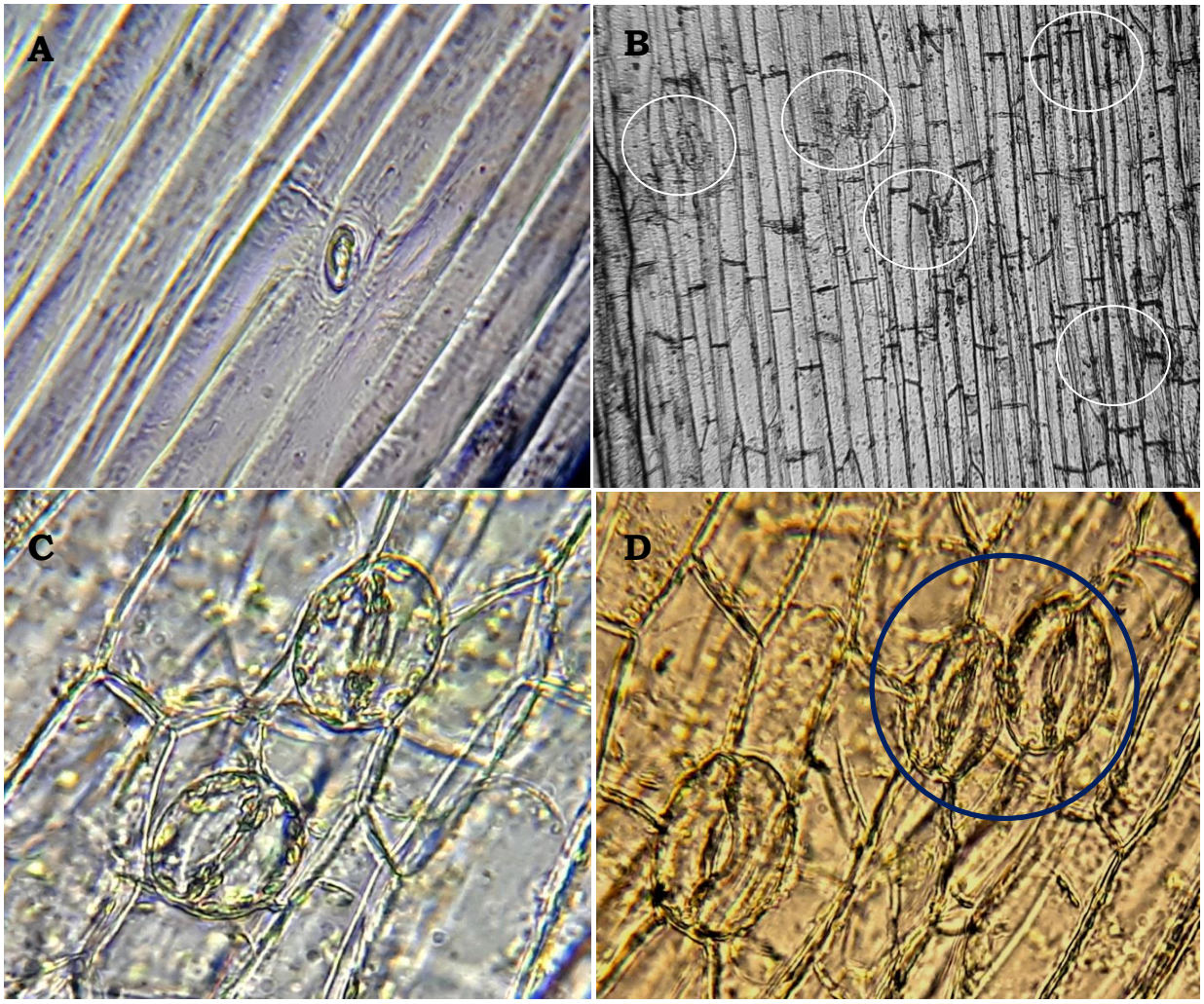


Fig. 15. Surface of hypocotyl. A) Early hypocotyl showing elongated epidermal cells; B) Maturing hypocotyl showing profuse divisions of the epidermal cells and stomata) shown inside the circles. C) One anisocytic and other anomocytic stoma with common subsidiaries. D) Contiguous stomata.

In seven-day old cotyledons, anomocytic, tetracytic and anisocytic stomata were observed. Epidermal cell walls were generally curved, often straight. Epidermal cells were papillose particularly on ventral surface in 10-day old cotyledon (Fig. 19). With increase of age, the epidermal surface became somewhat more papillose. At 16-day of age there was greater deposition of cuticle and surface structure became more and more unclear (Fig. 21, 22). Surface of twenty–day old cotyledons was more unclear due to cuticular striae and profuse development of cell walls (Fig. 23). At this age, they were senescent, thinner, and shriveled. They remained with the seedling for further some time and ultimately abscised. In brief, anisocytic, tetracytic, staurocytic, anomocytic, isotricytic, paracytic, diacytic and contiguous stomata and those in formative stages (in young cotyledons) were recorded. The contiguous stomata of

various types, juxtaposed, superimposed, or oriented at around right angle on cotyledons are shown in Fig. 28, 29, 30A.

Stomata of Leaf petiole: On this surface two types of stomata were observed – anisocytic and tetracytic (Fig. 24).

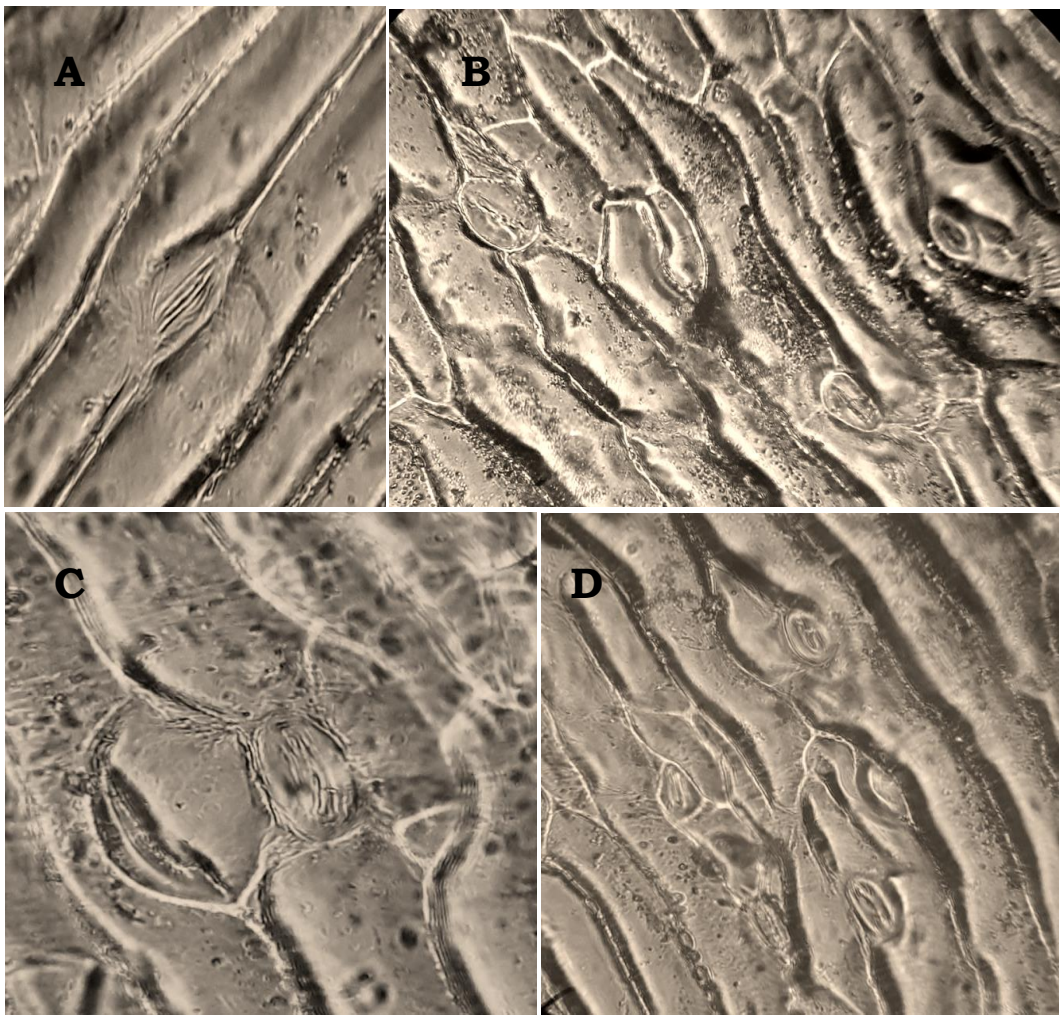
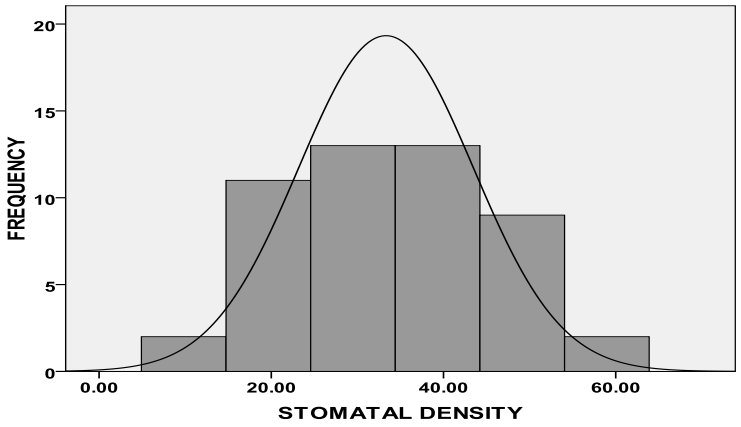


Fig. 16. Various stomatal types of the dorsal surface of the cotyledonary petiole. A) Paracytic stoma in young petiole of 3-day old seedling. B, C and D) Cotyledonary petiole of 10-day old seedling – Anisocytic (B and D), anomocytic (B), tetracytic (C), staurocytic (D) and paracytic (D) stomata.



N = 50
Mean = 33.81
SE = 1.7348
Median = 29.487
CV= 37.46%
Skewness = 0.077
SE skewness = 0.337
Kurtosis = - 0.729
SE kurtosis = 0.662
Minimum = 9.83
Maximum = 58.97
K-ST = 0.158 ($p < 0.003$)
Shapiro-Wilk = 0.933 (0.007)

Fig. 17. Stomatal density per mm^2 on dorsal surface of cotyledonary petiole (flat, 5 mm long; 2-2.5 mm broad) of 7-day old seedling. The distribution was asymmetrical due to platykurtosis.

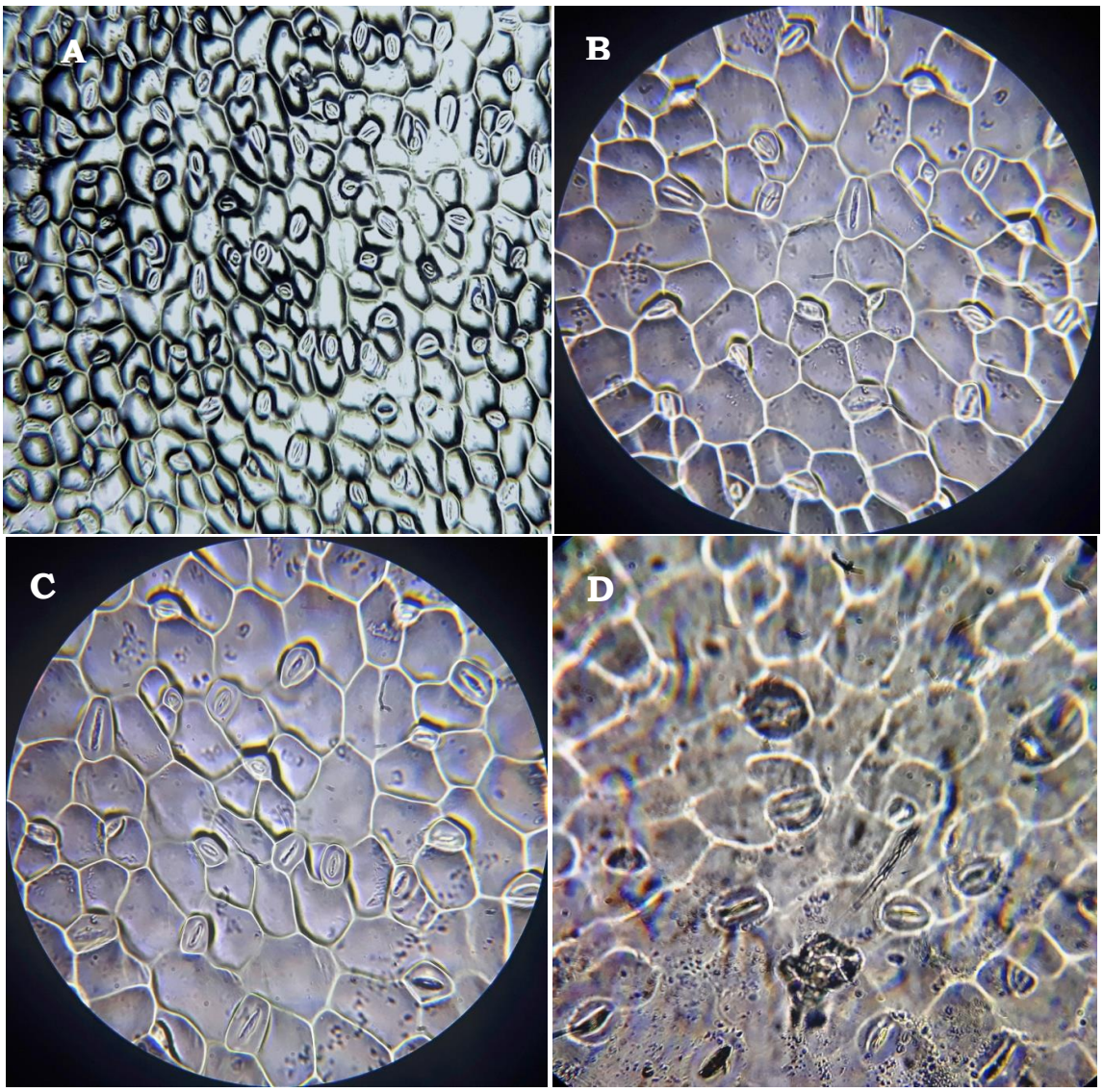


Fig. 18. Stomata of a three-day old cotyledon (A, B and C dorsal surface and D ventral surface). Anticlinal surface of epidermal cells is generally smooth on dorsal side and smooth to curvy on ventral side.

Foliar stomata: Leaves of sunflower seedling were amphistomtatous. Abaxial surface of leaves of sunflower had larger number of stomata which may probably be attributed to the fact that it is less exposed to sun (Martin and Glover, 2007). Amphistomaticity is considered to be light related in plants. Leaves of *Ambrosia cordifolia*, a composite, produced at high light intensities were amphistomtatous but those produced at low light intensities were hypostomatous. Although stomatal density at upper surface increased with increasing light intensity, the total stomatal density (upper + lower) was not substantially affected by high light intensity because density of stomata on the lower surface was reduced at high light intensity (Mott and Michaelson (1991). Stomata were generally absent on vascular nerves which may help prevent water loss.

Four types of stomata were observed in the laminar islands – tetracytic, anisocytic anomocytic and staurocytic on dorsal and ventral surface of primary, secondary or tertiary leaves (Fig. 25, 26, 27), doublet contiguous stomata (Fig. 30B) on dorsal surface of leaf of 15-day old seedling, a cluster of five contiguous stomata was observed on the dorsal surface of this 15-day old seedling (Fig. 31). The stomata were oriented in various directions. They were oval, wide-elliptical, elliptical, or round in shape. Often stomata were arranged in concentric manner around a trichome (Fig. 32). The outer ledges of guard cells were quite developed and stomatal pore lying somewhat deep. Stomata were oriented in various directions and had common neighbouring cells between them. There were some stomata without pore. Stomata were rare on veins. They may be seen on edges of the veins. In that case they are small, round

and generally anisocytic and few tetracytic. They are round 11.0 x 9.6 um in length and breadth. Cuticle appeared to increase gradually with size (age) of the leaf as is obvious from Fig. 35. The ventral epidermis in this mature leaf (21 cm^2), from sixth node of 160-170 days old sapling in pot, became complex and had cuticular striations not only on surface of epidermal cells but also present on the guard cells. Sheet cuticularization obscured waviness. Contiguous stomata occurred quite often.

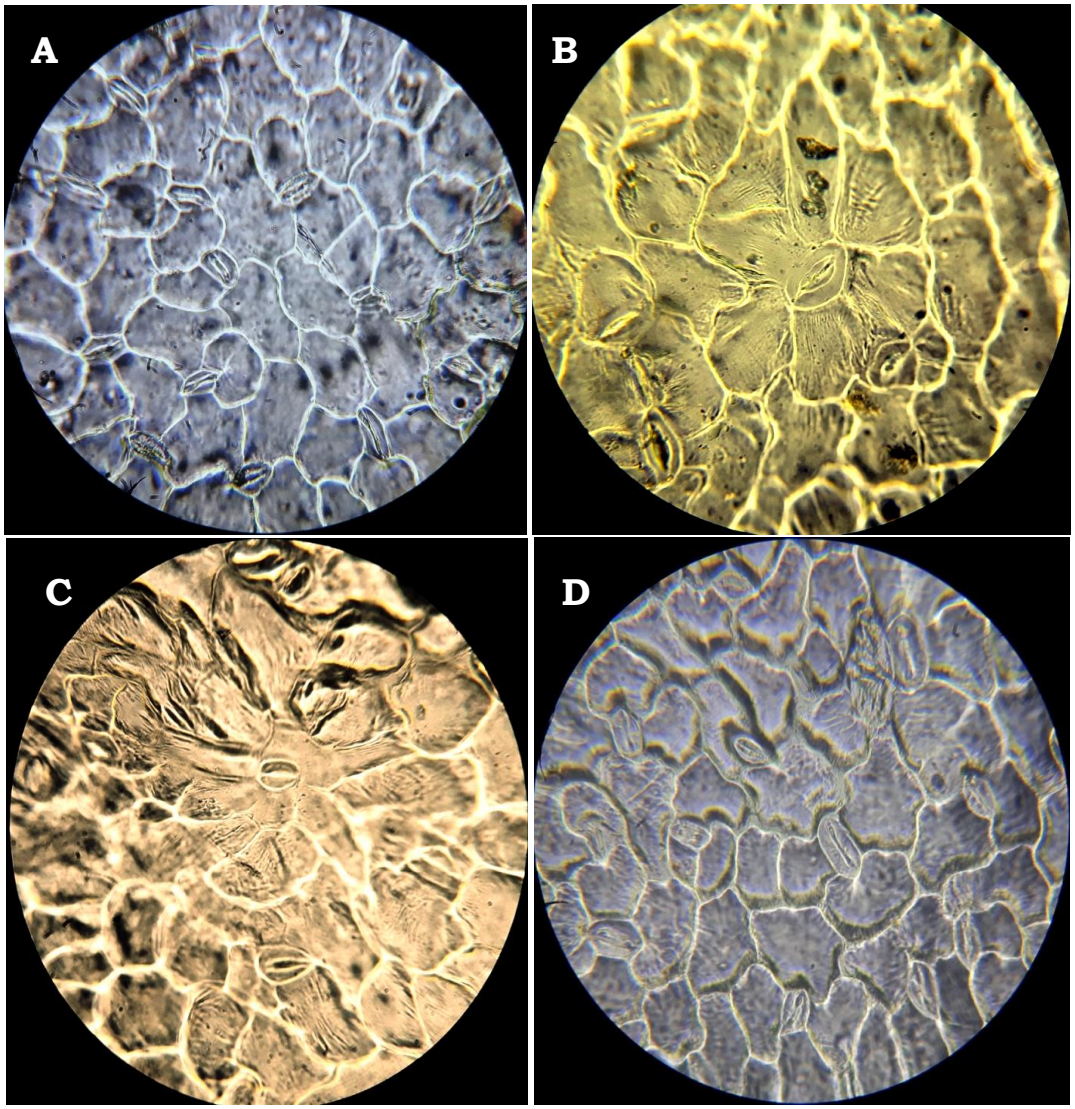


Fig. 19. Dorsal surface of 7- and 10-day old cotyledon - A, C and D from dorsal side of 7-day old cotyledon and B from ventral side of 10-day old cotyledon showing various types of stomata (tetracytic, anomocytic and staurocytic). Papillose nature of epidermal cell is apparent in 7-day old cotyledon (dorsal surface) – periclinal plateau and anticlinal precipice is well obvious.

Stomata of *H. annuus* have been studied by a number of workers - Mahbub Ur Rahman (2013) in Bangla Desh, Kaur and Nagpal (2016) in India, Tahir *et al.,* (2016) in AJ & K, Pakistan , Tuberosa *et al.* (1985) in Italy, etc. Most of the workers have reported anomocytic stomata from this species. Mahbub Ur Rahman (2013) haS also reported anisocytic stomata in sunflower. In general, Dilcher (1974) and Metcalf and Chalk (1979) have been followed to classify stomata in Asteraceae. Only few have followed other scheme (s) of stomatal classification. Tahir *et al.* (2016) and Khan *et al*. (2016) have adopted the scheme proposed by Prabhakar (2004) for delimitation of mature stomata in Asteraceae species. Tahir *et al*. (2016) studied thirteen species of Asteraceae for variations in stomatal attributes and reported tricytic, tetracytic, anisocytic, and anomocytic stomata from the species studied. Anomocytic stoma was reported to be the solo type in *H. annuus* leaves. The same four types of stomata were also recorded in genus *Senecio* – anomocytic, tricytic, anisocytic and tetracytic (Joshi and Bajracharya (2015) while using stomatal terminology of Metcalfe and Chalk (1950; Metcalfe, 1961 and Pant and Kidwai, 1966). Besides, previously recorded

anomocytic and anisocytic stomata, Hayat *et al.* (2010) following the stomatal nomenclature scheme of Dilcher (1974) and Metcalfe and Chalk (1979), reported Anomotetracytic (tetracytic with indistinct subsidiaries, paratetracytic (four subsidiaries – two polar cells and two lateral cells, may be of unequal sizes), diacytic (two subsidiaries not parallel to pore) and paracytic (two subsidiaries parallel to pore) in *Artemisia tangutica.* Gole *et al.* (2013) have reported diacytic and paracytic stomata in *Chrysanthemum indicum*. It may be emphasized here that as per Dilcher (1974) or similar classificatory schemes, only one type of stomata are found in *H. annuus* Var. US 666 and that is anomocytic (indistinct neighbouring cells). Inamdar *et al.* (1980) have also described only anomocytic stomata in cotyledons of *H. annuus* Var. EC 68414. Suseela *et al. (*2002) described anomocytic stomata in *Tridax procumbens* on the basis of indistinct neighbouring cells.

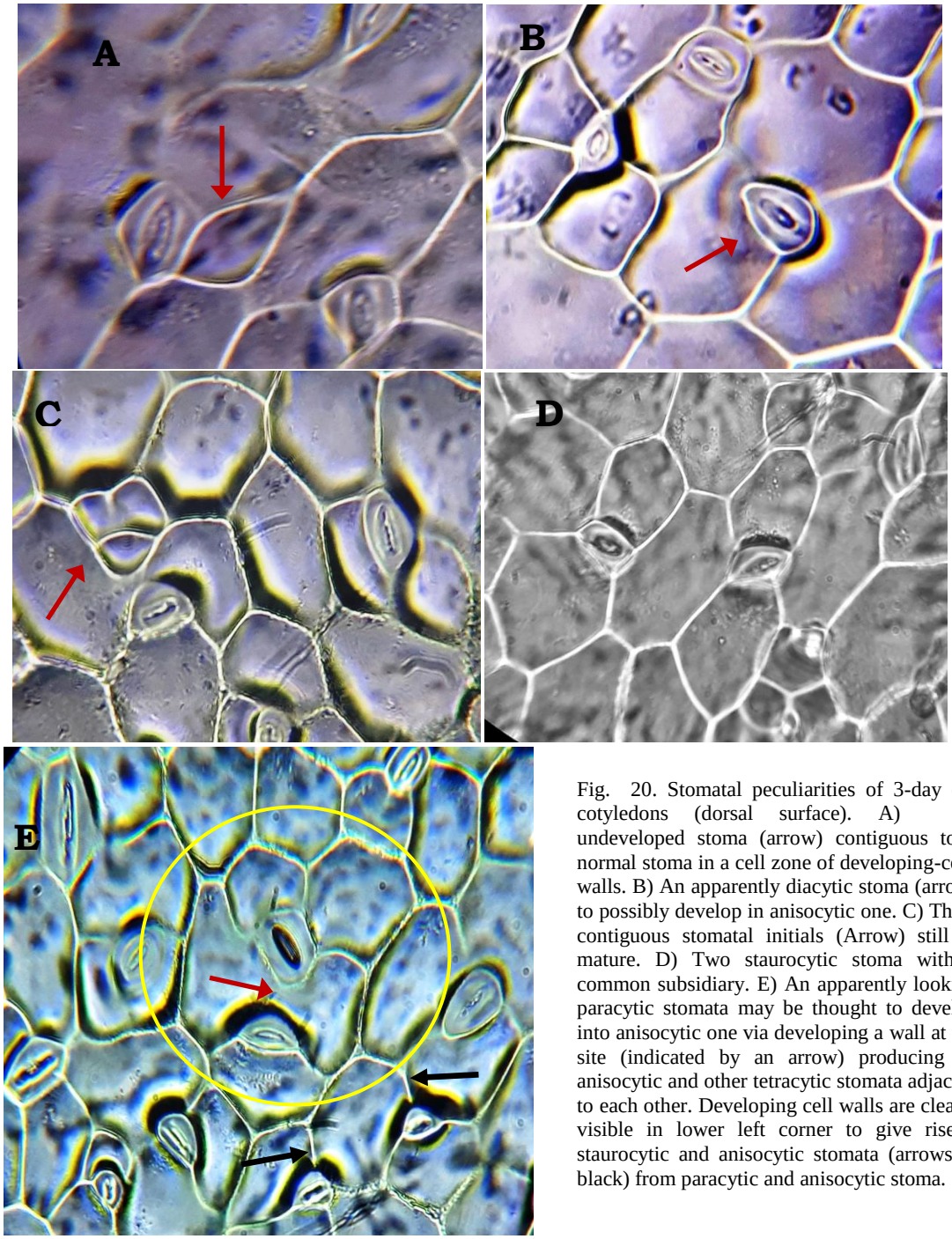


Fig. 20. Stomatal peculiarities of 3-day old cotyledons (dorsal surface). A) An undeveloped stoma (arrow) contiguous to a normal stoma in a cell zone of developing-cell-walls. B) An apparently diacytic stoma (arrow) to possibly develop in anisocytic one. C) Three contiguous stomatal initials (Arrow) still to mature. D) Two staurocytic stoma with a common subsidiary. E) An apparently looking paracytic stomata may be thought to develop into anisocytic one via developing a wall at the site (indicated by an arrow) producing an anisocytic and other tetracytic stomata adjacent to each other. Developing cell walls are clearly visible in lower left corner to give rise a staurocytic and anisocytic stomata (arrows in black) from paracytic and anisocytic stoma.

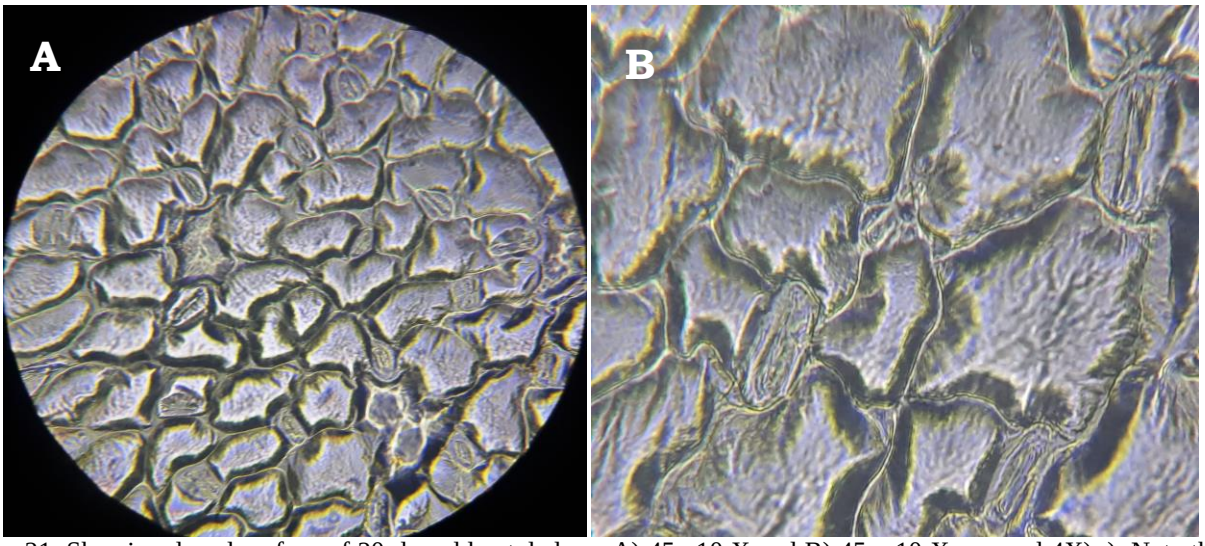


Fig. 21. Showing dorsal surface of 20-day old cotyledons. A) 45 x10 X and B) 45 x 10 X zoomed 4X).). Note the papillose nature of epidermal cells.

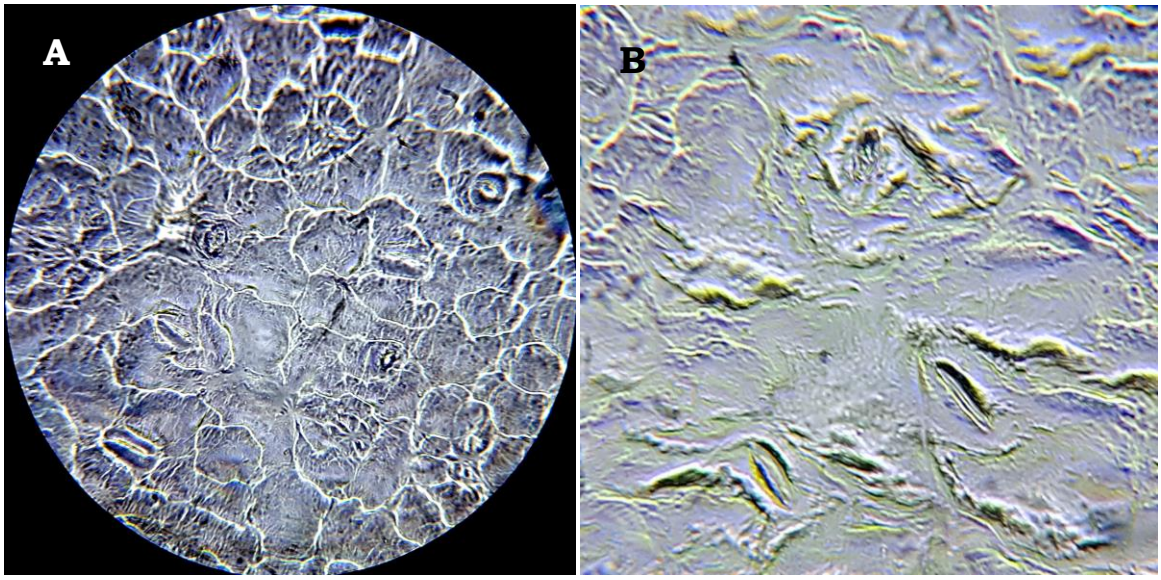


Fig. 22. Ventral surface of 16-day old cotyledons. A, Cuticular deposition. (45 x 10 X); B, 45 x 10X, zoomed 4X.

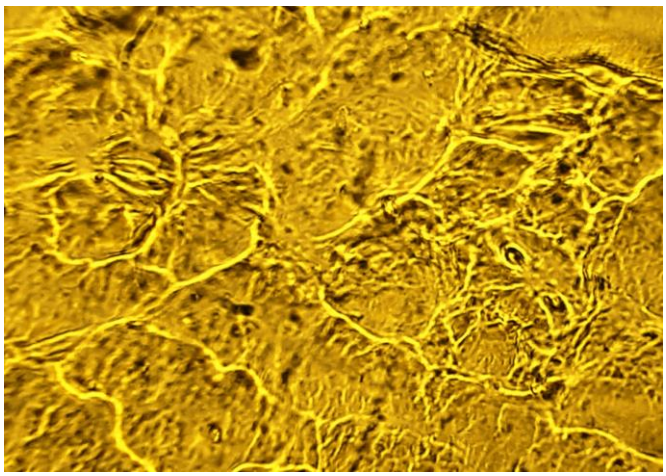


Fig. 23. Ventral surface of nearly 20-day old cotyledon. Cuticular deposition and profuse division in epidermal cells is clearly seen. It may be mentioned that at this age cotyledons are senescent and get exhausted very soon and shriveled. The shriveled cotyledons remained with the seedling for some time and ultimately abscised.

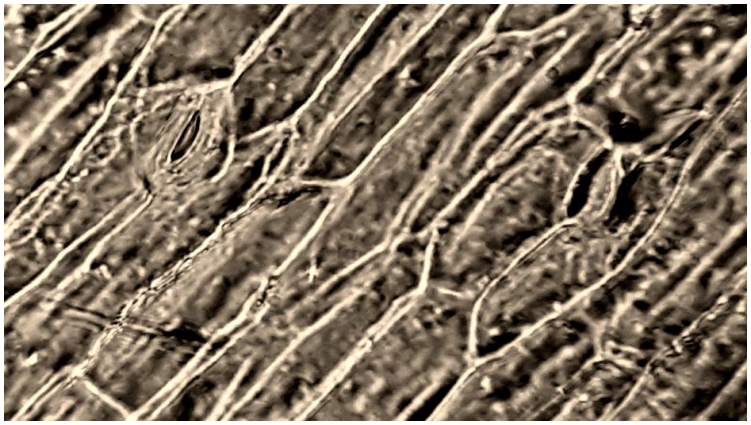


Fig. 24. Tetracytic and anisocytic stomata on the surface of leaf petiole.

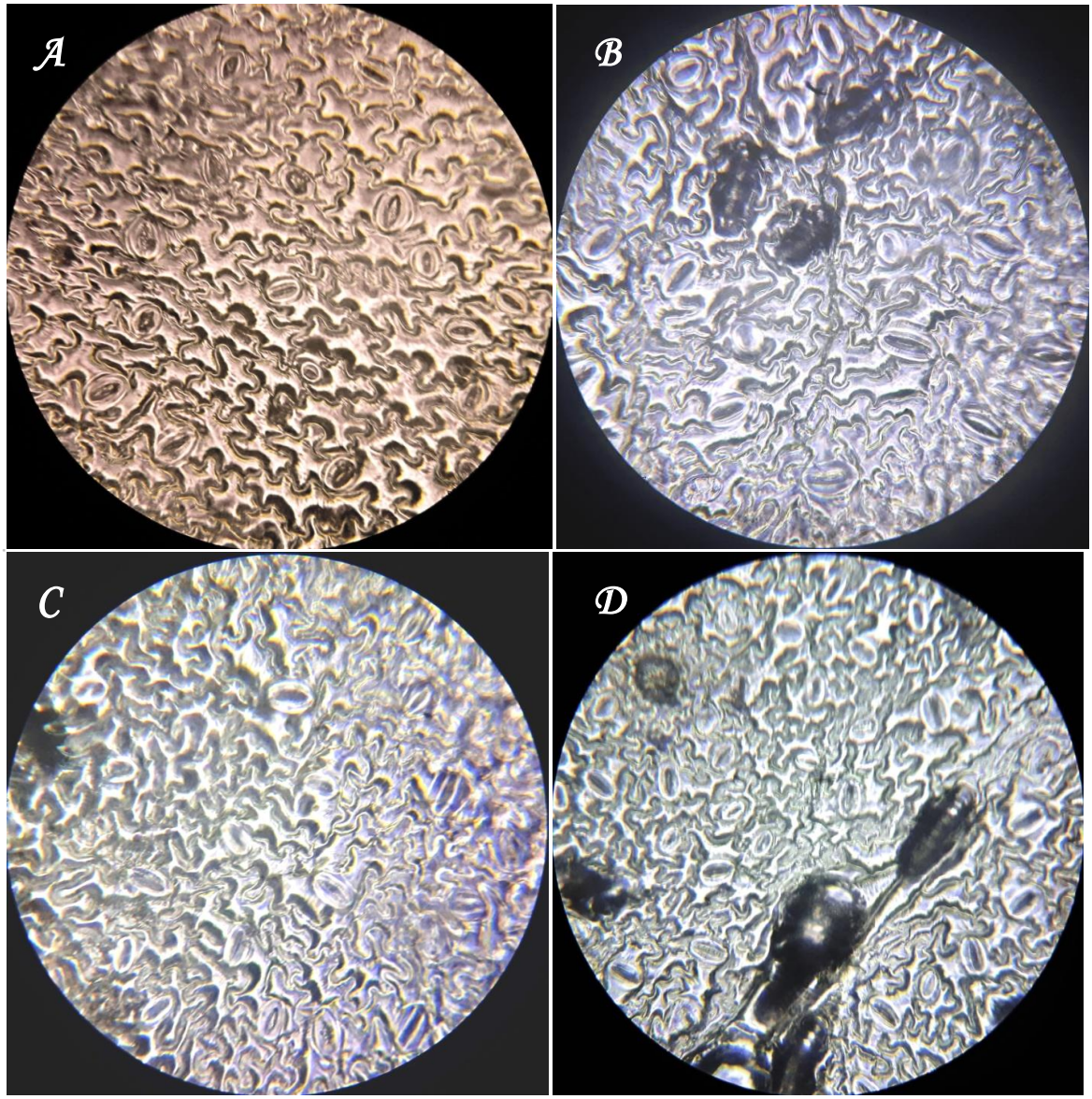


Fig. 25. Surface of leaves of 15-day old sunflower seedlings. A, Primary leaf (dorsal); B, Primary leaf (Ventral); C, Secondary leaf (dorsal) and D, Secondary leaf (Ventral). Magnification: 45 x 10 X. Conspicuous waviness of the epidermal cells is obvious all over.

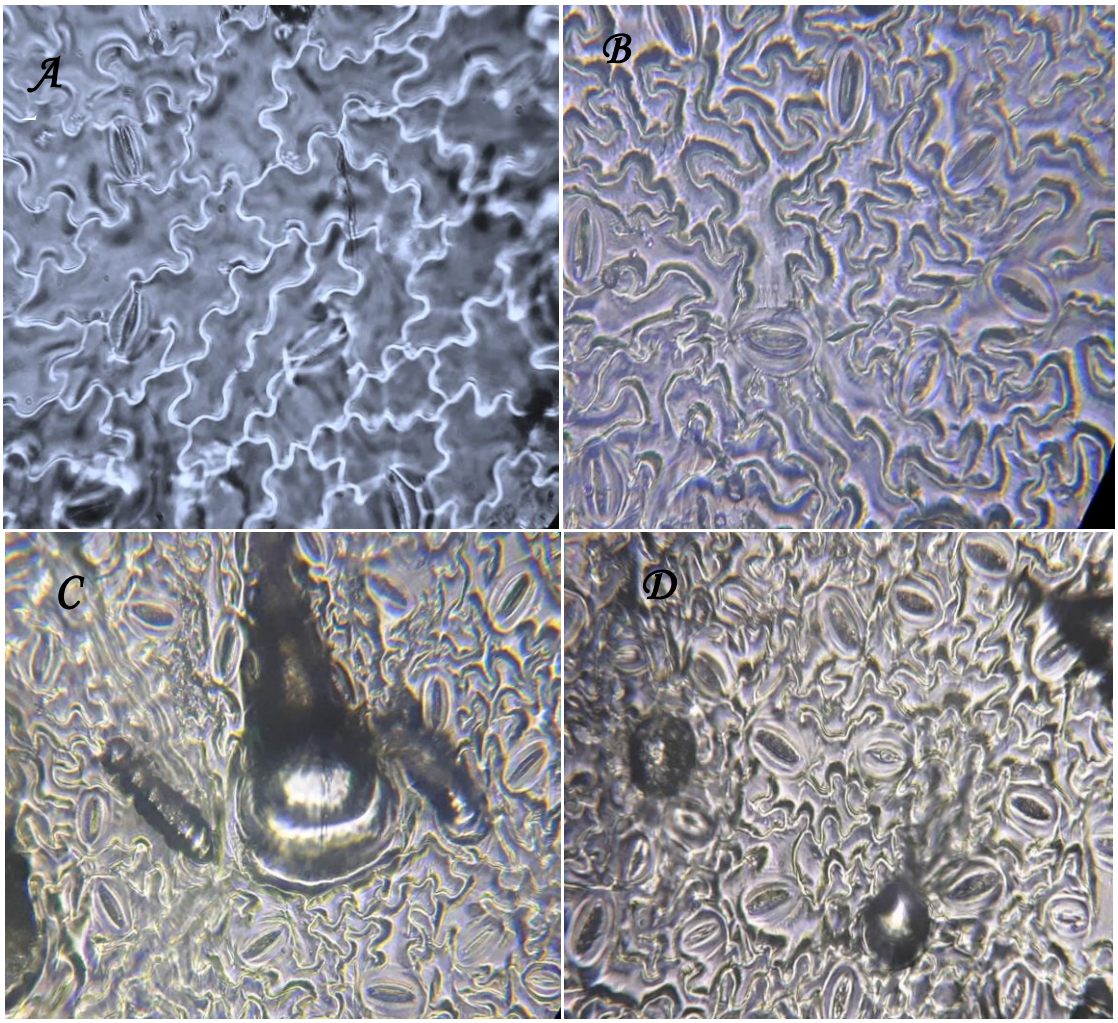


Fig. 26. Surface of leaves of 20-day old sunflower seedlings. A, Primary leaf (dorsal); B, Primary leaf (Ventral); C, Secondary leaf (dorsal) and D, Secondary leaf (Ventral). Magnification: 45 x 10 X. Conspicuous waviness of the epidermal cells is obvious all over.

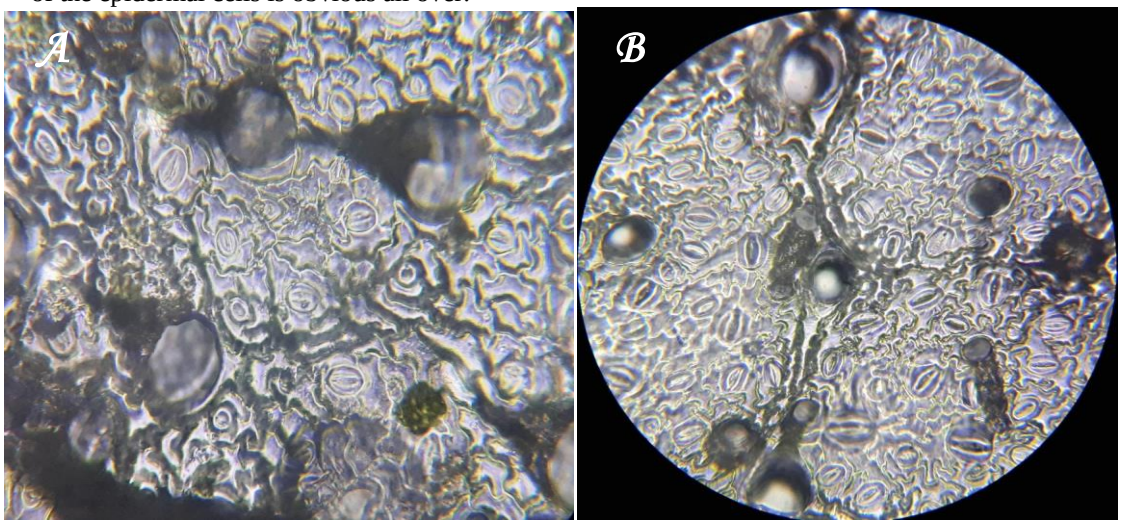


Fig. 27. Fig. Surface of tertiary leaves of 20-day old sunflower seedlings. A, Tertiary leaf (dorsal); B, Tertiary leaf (Ventral); C, Magnification: 45 x 10 X. Conspicuous waviness of the epidermal cells is obvious all over.

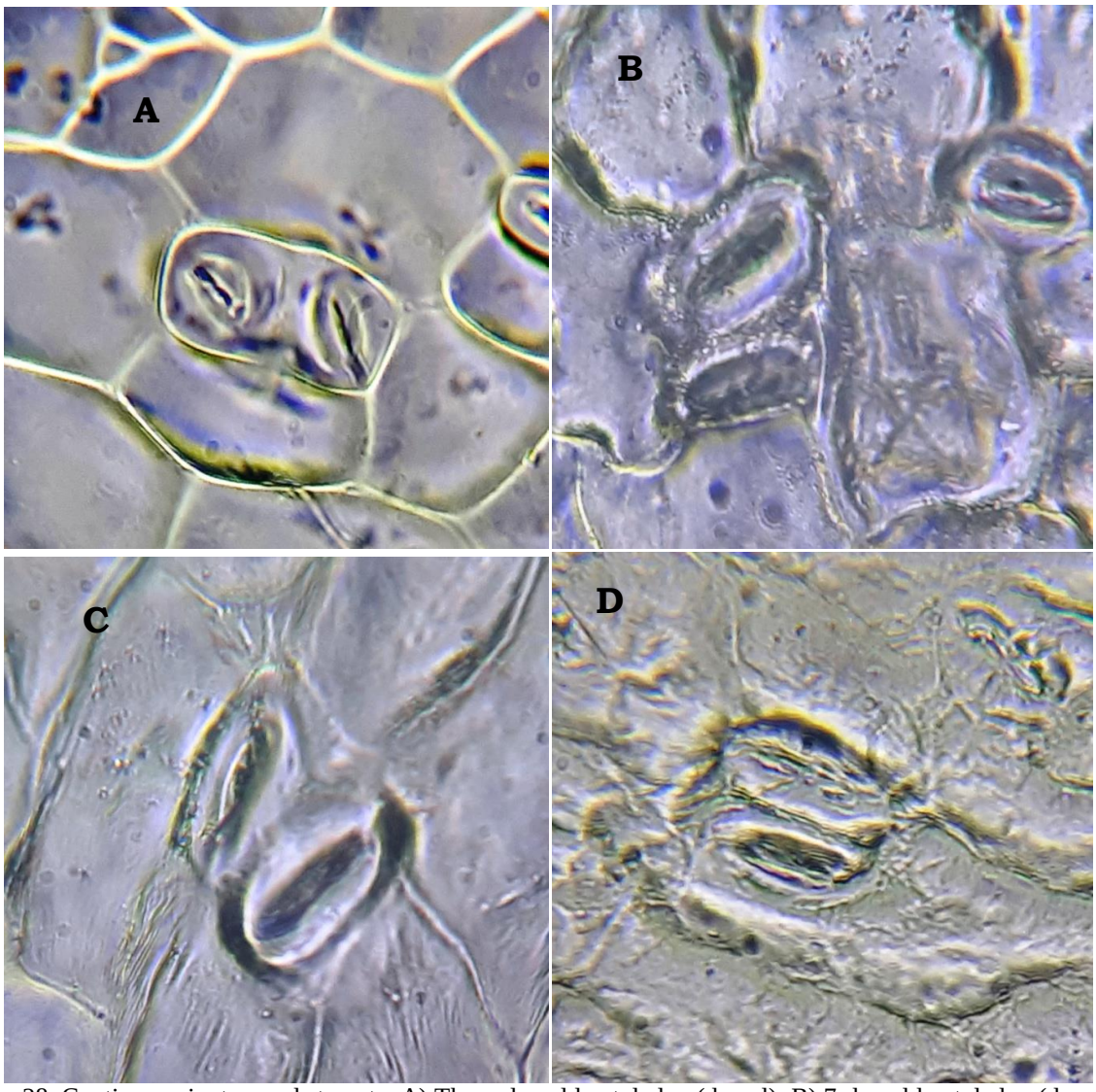


Fig. 28. Contiguous juxtaposed stomata. A) Three-day old cotyledon (dorsal); B) 7-day old cotyledon (dorsal); C) 7-day old cotyledon (ventral); D) 20-day old cotyledon with thick cuticular encrustation (dorsal). Mag.: 45 x 10 X, zoomed 1.4X.

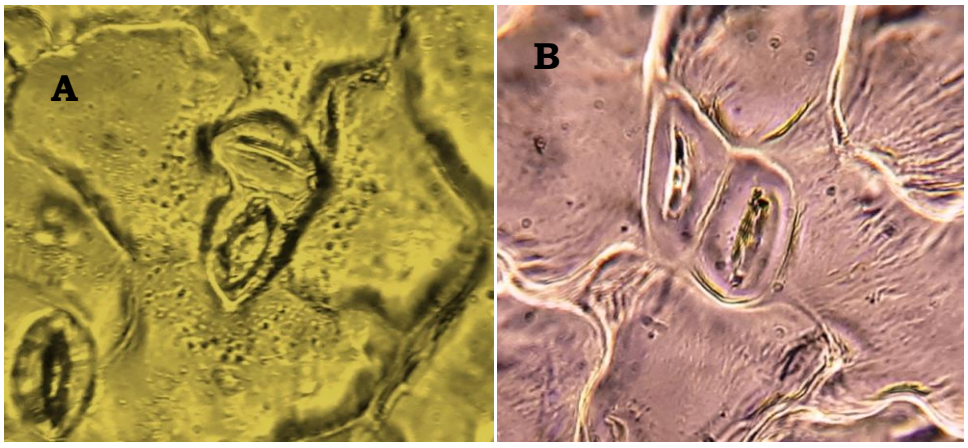


Fig. 29. A) Contiguous stomata (at right angle) in 5-day old cotyledon (dorsal) - Magnification 45 x 10X, zoomed 1.2 X. B) 7-day old cotyledon (ventral) – juxtaposed contiguous stomata; one stoma wide elliptical and other triangular – magnification: 45 x 10 X, zoomed 1.2X)

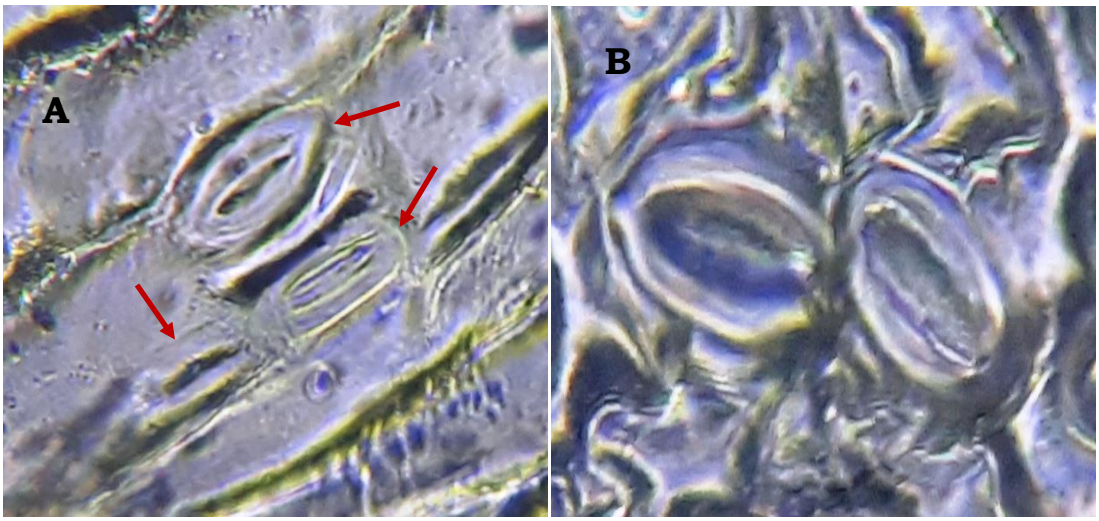


Fig. 30. A group of three closely placed stomatal apparatus on cotyledonary petiole Magnification: A - 45 x 10X – zoomed 2X. B) Contiguous stomata, more or less at right angle, on 15-day old leaf (dorsal). Magnification – 45 x 10 X, zoomed 4X.

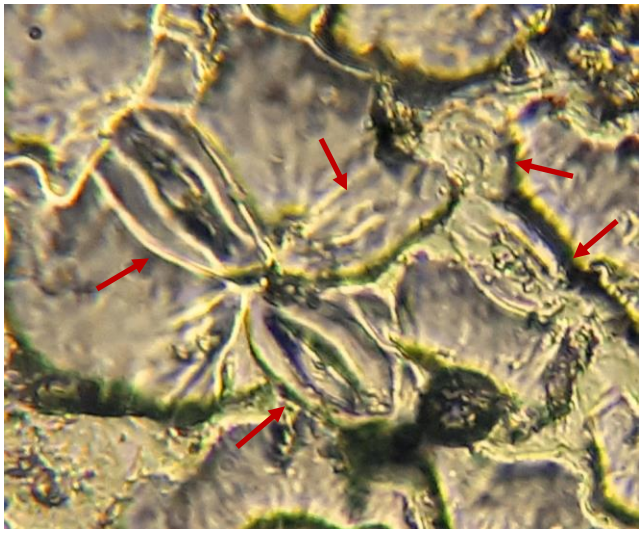


Fig. 31. A discrete group of five contiguous stomata (shown by arrows) observed on the dorsal surface of leaf of 15-day seedling of *S. annuus* var. US 666. Leaf size: 7.33 cm^2.
It is a unique feature of the taxon. Contiguous stomata are often seen in plants, but such developmental abnormality has not probably been observed previously.
Magnification: 45 x 10 X; zoomed 4 X)

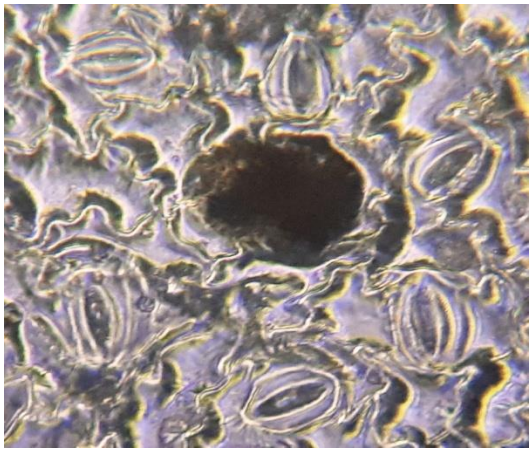


Fig. 32. Nail polish imprint of the dorsal surface of leaf of 20-day old seedling showing six stomata arranged concentrically around a trichome. This was quite frequently seen. Magnification: 45 x 10 X.

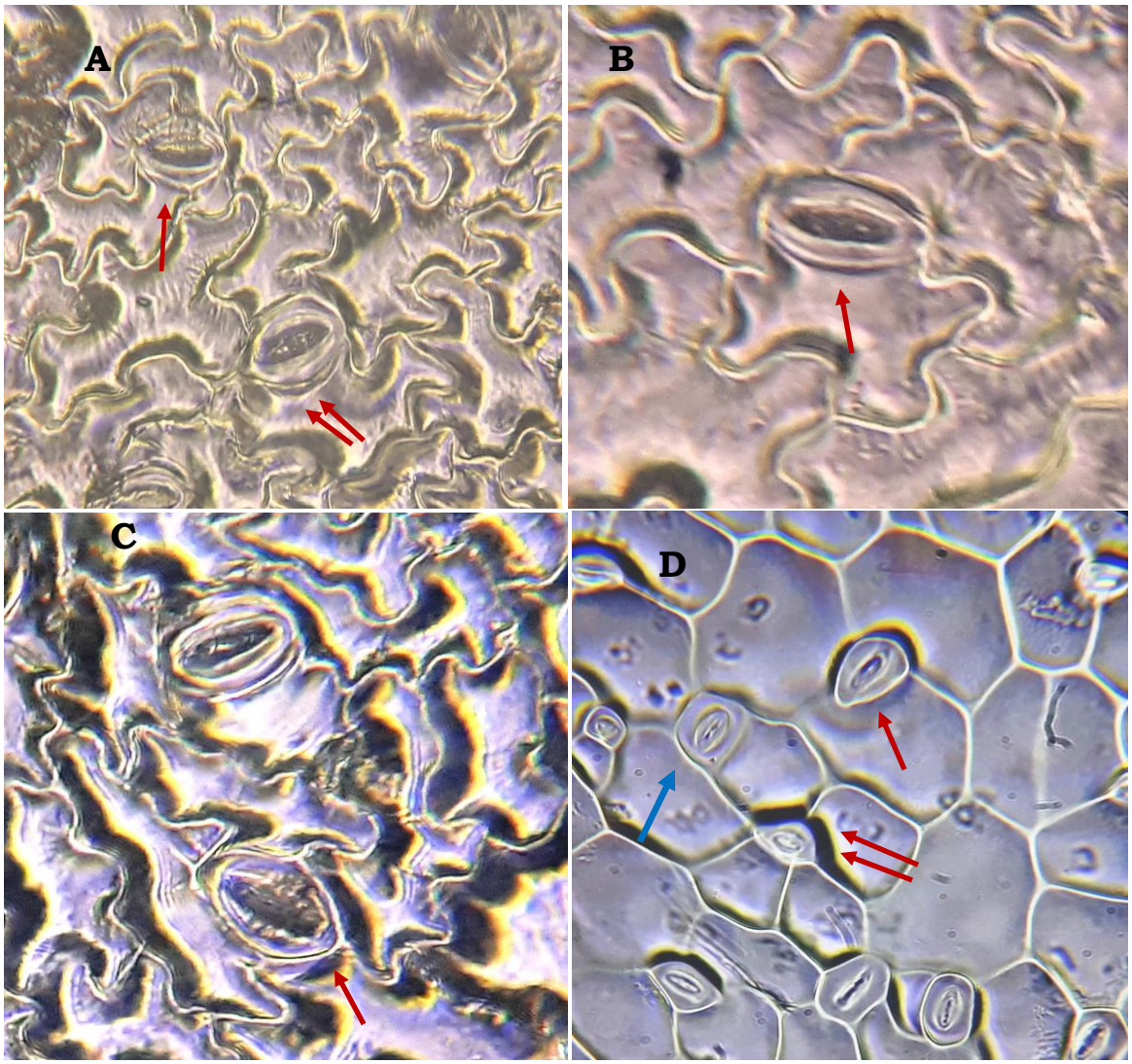


Fig.33. Various types of stomata in sunflower leaves (A, B C) and Cotyledon (D) - A) Anomocytic (arrow in red) and tetracytic (double red arrows); B) Anisocytic; C) Staurocytic and D) Possible isotricytic (red arrow), Tetracytic (green arrow) and Anomocytic (double red arrows). Magnification: A, B and C -45 x 10 X, zoomed 4X and D, 45 x 10 X, zoomed 1.4 X).

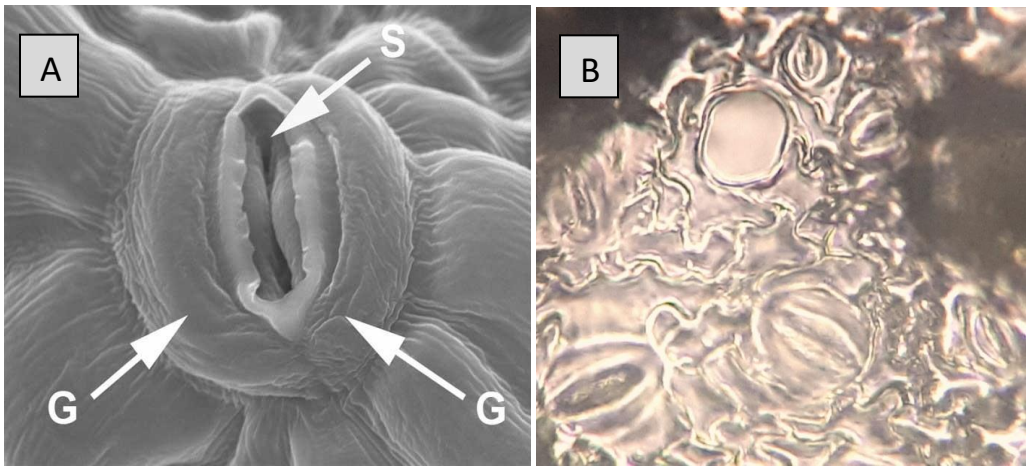


Fig. 34. Sunflower: A) Anomocytic stoma on the surface of a sunflower leaf – S, stoma; G, Guard cell. (Adopted from Louisa Howard, Dartmouth College, UK; Public Domain). B) Scar of a fallen trichome on the leaf surface.

Table 4. Stomatal density per mm^2 on dorsal (upper) and ventral (lower) surfaces of cotyledons of various ages.

Parameters	3- day old Cotyledon		5-day old cotyledon		7-day old cotyledon		10-day old cotyledon	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Cotyledon (mm^2)	212	231	214	243	263	271	368	386
N (frames of vision)	60	50	75	70	60	50	50	50
Mean	188.55	207.59	188.85	165.04	113.03	108.12	91.99	96.91
SE	2.828	4.446	3.258	3.458	2.1348	3.991	2.412	2.679
Median	186.75	216.24	196.58	167.09	108.12	108.119	88.46	98.29
CV (%)	11.62	15.14	14.98	17.53	14.63	26.10	18.53	19.53
Skewness	0.864	-0.386	-0.513	-0.055	0.221	0.749	-0.145	0.543
SE of skewness	0.309	0.337	0.277	0.287	0.309	0.337	0.337	0.337
Kurtosis	0.789	-0.029	-0.431	0.101	0.2 13	0.328	-0.339	0.705
SE of kurtosis	0.608	0.662	0.548	0.565	0.608	0.662	0.662	0.662
Minimum	147.43	127.78	117.90	98.29	78.83	68.80	58.97	58.97
Maximum	245.72	265.38	235.90	249.72	157.26	186.75	127.78	147.83
K-S T*	0.174	0.128	0.141	0.109	0.202	0.120	0.138	0.171
p	0.0001	0.038	0.001	0.039	0.0001	0.069	0.019	0.001
Shapiro-Wilk	0.925	0.974	0.952	0.982	0.944	0.944	0.957	0.743
p	0.001	0.330	0.007	0.430	0.019	0.022	0.065	0.018
Distribution	AS	AS	AS	AS/S	AS	AS/S	AS	AS

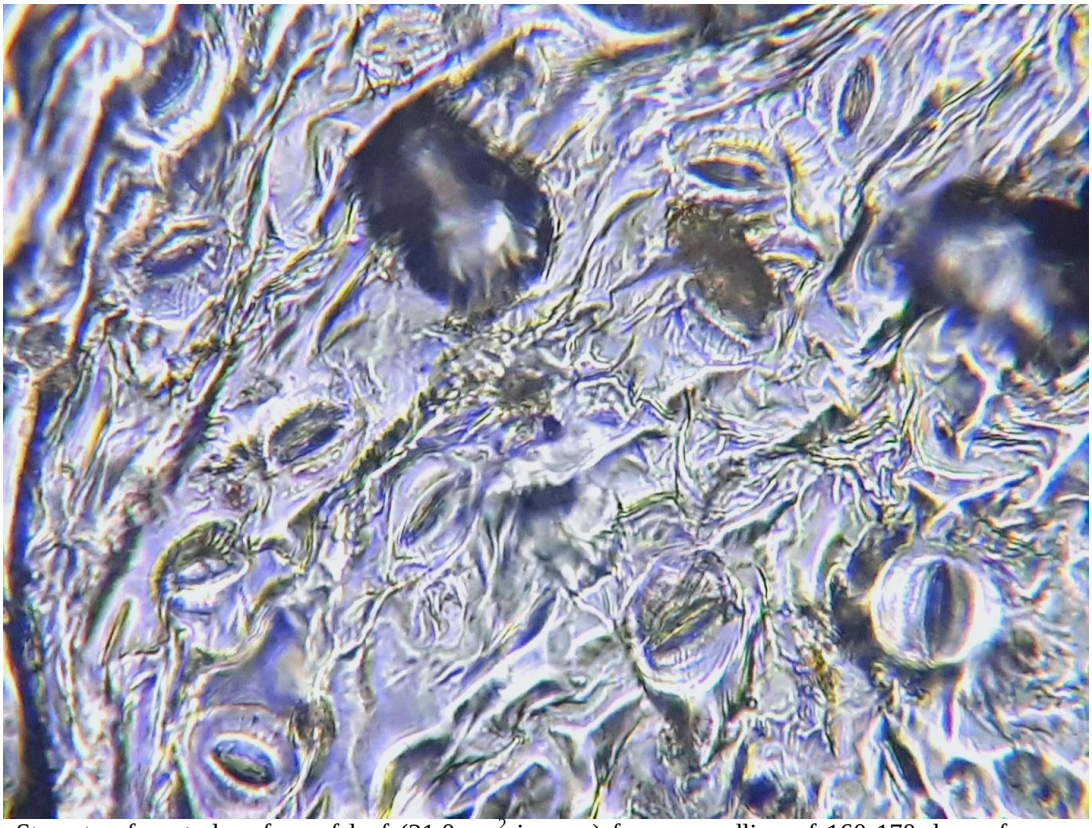


Fig. 35. Stomata of ventral surface of leaf (21.0 cm^2 in area) from a seedling of 160-170 days of age – profuse cuticularization of epidermal cells and cuticular striations extending to guard cells are apparent.

Stomatal density per mm^2 (SD): SD on cotyledonary and foliar surfaces is described in Table 4 and 5, respectively.

Cotyledonary stomatal density: Distribution of stomatal density on upper and lower surfaces of variously aged (size) cotyledons tended to be generally asymmetrical. The mean SD varied from 91.99 ± 4.45 to 188.5 ± 3.26 per mm^2 on upper and from 96.91 ± 2.68 to 207.59 ± 4.45 per mm^2 on lower surface. The sizes of cotyledons for dorsal surface SD estimate ranged 212 to 368 mm^2 and ventral estimate 231 to 386 mm^2) (Table 4). In the sample cotyledons SD varied from 11.62 to 26.1% as determined by coefficient of variability (CV %).

Table 5. Stomatal density per mm^2 on dorsal and ventral surfaces of variously sized leaves.

Parameters	15-day prim leaf		15-day sec. leaf		20-day Prim. Leaf		20-day Sec. leaf		20-day Tert. leaf	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
Leaf area cm^2	4.6	6.0	3.0	3.12	8.8	9.4	7.8	7.75	2.6	2.3
N	50	70	50	50	50	50	50	50	50	50
Mean	149.6	148.7	193.6	299.9	101.8	129.3	242.8	287.4	406.1	450.4
SE	4.798	3.137	7.254	4.994	2.861	3.557	5.374	6.296	6.136	8.870
Median	147.4	147.4	186.7	294.9	98.3	127.7	245.7	280.1	402.99	442.3
CV (%)	22.42	17.68	26.49	11.77	19.86	19.44	15.65	15.48	10.68	13.92
skewness	0.430	0.056	0.340	-0.405	0.092	-0.048	-0.612	0.507	-0.054	0.267
SE skewness	0.337	0.287	0.346	0.337	0.337	0.337	0.337	0.337	0.337	0.337
Kurtosis	-0.429	-0.170	-0.924	1.116	-0.649	-1.033	-0.199	0.717	-0.796	-0.618
SE Kurtosis	0.662	0.566	0.662	0.662	0.662	0.662	0.662	0.662	0.662	0.662
Minimum	88.46	88.46	117.9	196.6	58.97	78.63	147.4	186.7	314.53	314.5
Maximum	226.1	206.4	314.5	373.5	147.4	176.92	304.7	412.8	481.62	589.7
K-S T*	0.171	0.105	0.121	0.104	0.147	0.124	0.151	0.133	0.098	0.115
p	0.001	0.054	0.066	0.020	0.008	0.053	0.006	0.095	0.200	0.095
Shapiro-Wilk	0.958	0.978	0.948	0.961	0.965	0.960	952	0.960	0.965	0.975
p	0.080	0.268	0.029	0.093	0.138	0.087	0.043	0.095	0.146	0.368
Distribution	S	S	AS	AS	AS	AS	AS	S	S	S

Table 6. Foliar stomatal density per /mm^2 in *H. annuus* and *Aster hispidus* as reported in the literature.

S. No.	Dorsal surface	Ventral surface	Locality of study	Reference
Helianthus annuus				
1.	178.82 ± 14.17	208.23 ± 8.92	AJ & K, Pakistan	Tahir *et al*. (2016)
2.	-	276.16	India	Kaur and Nagpal (2016)
3.	100.33 ± 7.31	125.67 ± 6.07	Gandzolo, Bologna, Italy	Tuberosa *et al.* (1985)
4.	114.87 ± 5.04	144.23 ± 4.98	Monselice Pedoro, Italy	Tuberosa and Paradisi (1985)
5.	120.0	175.0	-	Mouseth (2011)
6.	218.78 ± 52.33*	263.14 ± 58.32*	(Pakistan) Variety US 666	Present study (seedling data)
Aster hispidus (Foliar stomatal density per mm^2)				
A. hispidus var. *hispidus*		70.66 ± 13.29		Kumekawa *et al.* (2013)
A. hispidus var. *leptocladus*		28.52 ± 6.78		Kumekawa *et al.* (2013)
A. hispidus var. *insularis*		33.42 ± 4.01		Kumekawa *et al.* (2013)

*, Average for all types of leaves (See Table 5).

Table 7. Percentages of various stomatal types on dorsal surface of variously aged cotyledon.

Stomatal Types	3-day old Cotyledon	5-day old Cotyledon	7-day old Cotyledon	10-day old Cotyledon	15-day old Cotyledon	Cotyledonary petiole (10-day old)	Cotyl-petiole merging Area (10 day old) **
	N = 362*	N = 185	N = 138	N = 140	264	N = 50	N =56
Anisocytic	17.13	23.78	18.12	18.57	15.15	52.0	14.29
Tetracytic	40.68	60.54	39.85	51.43	39.77	32.0	53.57
Staurocytic	15.20	4.32	16.67	13.57	6.81	8.0	17.86
Anomocytic	23.28	11.35	24.64	16.43	38.25	-	14.29
Isotricytic	0.55	-	-	-	-	-	-
Paracytic	-	-	-	-	-	8.0	-
Diacytic	-	-	0.72	-	-	-	-
Developing	2.49	+	-	-	-	-	-
Contiguous	+ +	+	+	+	+	+	+

*, Number of stomata studied; **Cotyledon – petiole merging area.

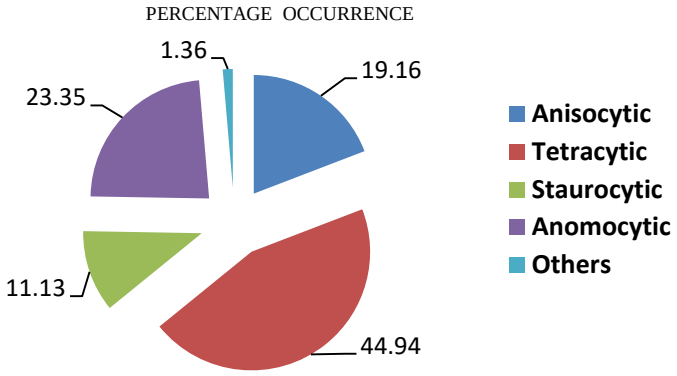


Fig. 36. Percentage occurrence of various stomatal types on dorsal surface of cotyledons of variously-aged seedlings (data pooled; N = 1195). The category of other stomata included paracytic, diacytic, isotricytic and developing ones.

Table 8. *Per cent* stomatal types on dorsal and ventral surfaces of various leaves.

Parameters	15-day prim leaf		15-day sec. leaf		20-day Prim. Leaf		20-day Sec. leaf		20-day Tert. leaf	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
N	300	100	70	80	65	120	160	100	260	180
Tetracytic	53.0	42.0	45.71	40.0	69.23	55.83	35.0	43.33	56.54	36.11
Anisocytic	25.3	18.0	20.00	11.25	18.26	9.17	35.63	15.0	24.23	30.56
Anomocytic	20.0	40.0	32.86	48.75	9.03	35.0	27.5	40.03	19.23	33.33
Staurocytic	1.7	-	1.44	-	3.8	-	1.88	1.67	-	-

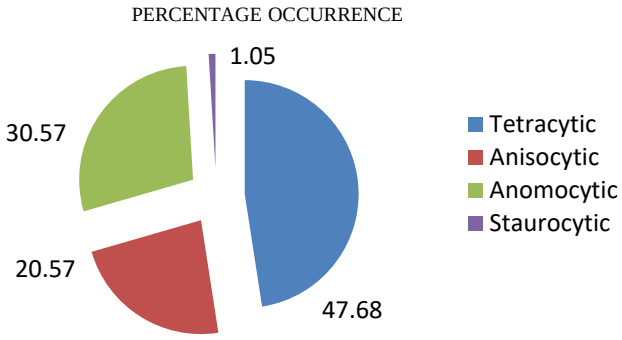
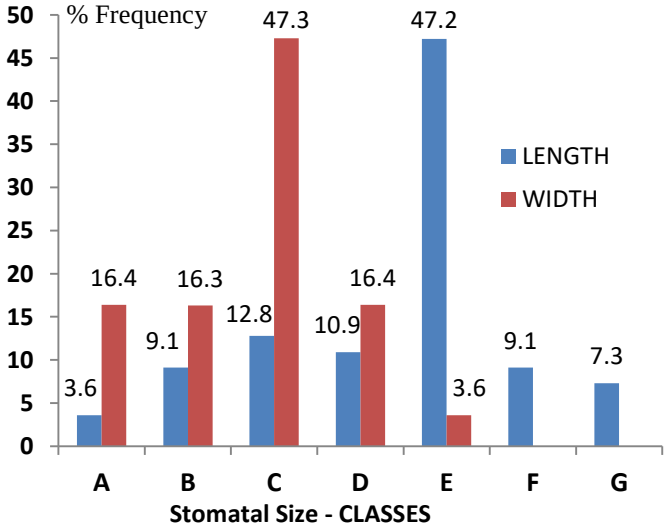


Fig.37. Percentage occurrence of various stomatal types on dorsal and ventral surfaces (collectively) of leaves of variously aged seedlings (data pooled; N = 1435). The data on contiguous stomata not included. The major proportion was occupied by tetracytic stomata followed by anomocytic and anisocytic stomatal types.



Length (µm)
Mean = 24.50
SE = 0.9355
Median = 25.60
CV = 28.30%
Skewness = -0.558
SE Skewness = 0.322
Kurtosis = -0.175
SE kurtosis =0.634
Minimum = 9.6
Maximum = 38.4
KST* = 0.200
P < 0.0001
Shapiro–Wilk= 0.938
P < 0.007

Width (µm)
Mean = 16.39
SE = 0.6157
Median = 16.00
CV = 27.86%
Skewness = 0.250
SE skewness = 0.322
Kurtosis = -0.183
SE kurtosis = 0.634
Minimum = 9.60
Maximum = 28.80
K-ST* = 0.139
P < 0.010
Shapiro-Wilk = 0.946
P < 0.016

Fig. 38. Distribution of stomatal length and width on ventral surface of secondary leaf of 15-day old seedling. Acronyms: A, < 10 µm; B, 11-15µm; C, 16-20 µm; D, 21-25 µm; E, 26-30 µm; F, 31-35 µm; G, > 35 µm. Both parameters (length and width) were asymmetrical in distribution.

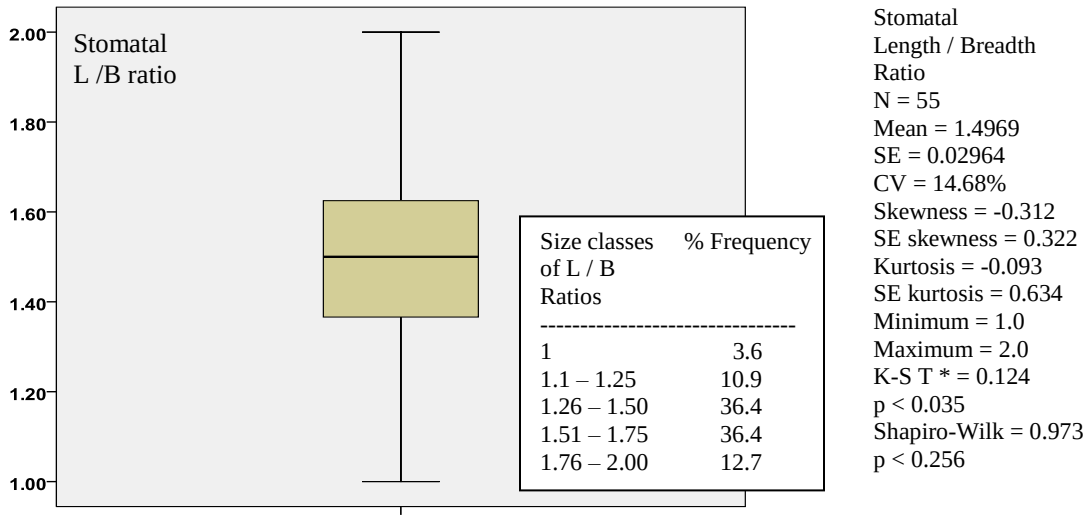


Fig. 39. Frequency distribution of stomatal length / Breadth ratio. *, Kolmogorov- Smirnoff test with Lilliefors significance correction. In 72.8% cases the ratio fell within a class of 1.26 to 1.75.

SD on cotyledonary surfaces decreased significantly with the increase of cotyledonary area (expansion).

SD per mm^2 = 243.53 – 15.67 Cotyledonary area (cm^2); r^2 = 0.8584 ---- Dorsal surface
SD per mm^2 = 245.50 – 16.12 Cotyledonary area (cm^2); r^2 = 0.8846 ---- Ventral surface

Cotyledons in *H. annuus* var. US 666 underwent continuous expansion after emergence reaching to an average size of 346.75 ± 46.6 mm^2 on 10^{th} day. Substantial expansion in sunflower cotyledons has been reported by Lovell and Moore (1970). They reported that such expansion is due to increase in cell size and not the cell number and may be considered to attribute in reduction of stomatal density in larger cotyledons. In *Cassia fistula* seedlings, young cotyledons were reported to bear higher stomatal density on both upper and lower surfaces than larger (older) ones (Khan and Zaki, 2019).

Foliar stomatal density per mm^2: SD was estimated on both surfaces of primary, secondary, and tertiary leaves of *H. annuus* var. US 666 seedlings of 15 and 20days of age (Table 5). Following trends were observed.

1) The stomatal density tended to be higher on ventral surface of leaf as compared to the dorsal.
2) Foliar SD significantly declined with size of the leaf. Smaller (young) leaves had higher SD and larger leaves had lower SD values. SD did not vary with the nature of the leaf (primary, secondary, or tertiary) but with the foliar area.
3) Foliar SD related to the leaf area in quadratic manner (second degree polynomial) as given below (leaf area in cm^2):

Foliar SD per mm^2 = 591.45 + 9.469 (leaf $area^2$) – 131.45 (leaf area); R^2 = 0.4100 ... Dorsal surface
Foliar SD per mm^2 = 611.85 + 6.161 (leaf $area^2$) – 104.06 (leaf area); R^2 = 0.6635 ... Ventral surface

Data on stomatal density of *H. annuus* leaves given by some workers is presented in Table 6. Significant variation in SD in a number of species of Family Asteraceae, as studied by Tahir *et al.* (2016), have been reported ranging from 60.00 ± 7.67 (*Galinsoga parviflora*) to 328.23 ± 21.77 stomata per mm^2 (*Tagetes erecta*) on dorsal surface and from 152.94 ± 11.76 (*Conyza canadensis)* to 414.11 ± 15.34 stomata per mm^2 (*Tagetes erecta*) on ventral surface. They have also recorded higher SD on ventral surface of leaf. Our data on both surfaces was higher than their records. This may probably be attributed to the fact that our data came from seedling of this species (*H. annuus* var. US 666) which appears not to be the case with data of the earlier workers presented in Table 6. Greater frequency of stomata on ventral surface is common in species occurring in xeromorphic environments (Cutter, 1986).

It is known since Salisbury (1928) that stomatal density is related to leaf size inversely. Stomatal density on young and small terminal leaflets of *C. fistula* was reported to be quite higher than that on relatively larger leaflets (Khan and Zaki, 2019). The decrease in SD in larger leaves, as compared to the smaller ones, may be attributed to the foliar epidermal cells' expansion. Young leaves have large number of stomata but as leaf expands the density declines (Gay and Hurd, 1975). Also, lower radiation is known to affect stomatal densities (Rawson and Craven (1975).

Relative abundance of cotyledonary stomatal types: The occurrence of various stomatal types on cotyledonary blade, petiole, and the cotyledonary petiole to cotyledonary blade transition area, of *H. annuus* var. US 666 seedling, is presented in Table 7 and Fig. 36. Stomatal types were more diverse in young (3-day old) cotyledon (Table 7) but with the maturity of cotyledons usual four types (tetracytic, anisocytic, staurocytic and anomocytic) of stomata were present. Stomatal types such as isotricytic, paracytic and diacytic or developing ones were only seen in young cotyledon, probably due to the transformation of diacytic or paracytic types in anisocytic stomata and anisocytic ones into tetracytic or anomocytic stomata as indicated by the transformation visible in Fig. 20. However, the stomatal types on various organs of *H. annuus* var. US 666 may be arranged in following order of relative abundance on the basis of their occurrence percentages (Table 7).

Cotyledonary petiole

Anisocytic (52%) > Tetracytic (32%) > Staurocytic (8%) = Paracytic (8%)

Cotyledonary petiole area merging with cotyledonary blade

Tetracytic (53.57%) > Staurocytic (17.86%) > Anomocytic (14.29%) = Anisocytic (14.29%)

Cotyledonary surface (Dorsal)

Tetracytic (44.54%) > Anomocytic (23.35%) > Anisocytic (19.16%) > Staurocytic (11.13%) > other (1.36%)

It is obvious that tetracytic stomata were predominant on the cotyledonary blade but anisocytic stomata were more frequent on the cotyledonary petiole (52%). On cotyledonary blade, tetracytic stomata were the most frequent (44.54%) and other stomatal types (anomocytic, anisocytic and staurocytic) were of lesser frequency of occurrence. On the basis of relative abundance of various stomatal types on three areas of cotyledon were 54.29 to 59.16% similar as determined by following Czarnowski's (1913) coefficient of similarity. It follows that there was significant variation of stomatal types on the cotyledonary surfaces.

Relative abundance of foliar stomatal types: The percent occurrence of various stomatal types on dorsal and ventral foliar surfaces of *H. annuus* var. US 666 seedling is presented in Table 8 and Fig. 37. The order of percent occurrence was observed to be as follows:

Leaf surface (Dorsal)

Tetracytic (51.90%) > Anisocytic (24.28%) > Anomocytic (21.72%) > Staurocytic (1.76%)

Leaf surface (Ventral)

Tetracytic (43.45%) > Anomocytic (30.573%) > Anisocytic (16.796) > Staurocytic (0.33%)

Leaf surface Pooled (Dorsal + Ventral surfaces)

Tetracytic (47.68%) > Anomocytic (30.57%) > Anisocytic (20.54%) > Staurocytic 1.05%)

It is clear that tetracytic stomata were of substantially higher occurrence followed by anisocytic stomata on dorsal leaf surface. Ventrally, tetracytic stomata maintained their higher frequency but anomocytic type significantly surpassed the anisocytic stomata. It follows from the data that stomatal occurrence on various organs of the sunflower seedling is quite complex and dynamic phenomenon which should depend on a number of extrinsic and intrinsic factors. As regards to the frequency of various stomatal types, dorsal and ventral surface were 85.54% similar to each other as determined by Czekanowski (1913) coefficient of similarity on the basis of percent frequency distribution of various stomatal types on the two surfaces. The occurrence of various stomata types on one and the same vital surfaces is frequently occurring in plants (Shah and Gopal, 1969; Ahmad *et al.*, 2009). In such cases stomata may be of lesser taxonomic significance (Pant and Kidwai, 1964). However, Shah and Gopal (1970) opined that frequent type of stomata may be used as taxonomic character. Epidermal surface structure proved to be a significant diagnostic feature in separation of genera *Senna* and *Chamaecrista* from their initial genus *Cassia* (Saheed and Illoh, 2010).

Stomatal size: The size of stomata was measured in terms of stomatal length (L) and breadth (B) on ventral surface of secondary leaf of 15-day old seedling (Fig. 38). Stomata varied in size considerably. Stomatal length in sunflower averaged to 24.50 ± 0.936 µm and breadth averaged to 16.39 ± 0.6157µm. The both parameters varied more or less equally (length: 9.6 – 38.4 µm; CV: 28.30% and width: 9.6-28.80µm; CV: 27.86%) and both distributed asymmetrically.

Solereder (1908) suggested that length / breadth ratios may give the actual shape of the stomata. The range of possible variation may, however, be limited since the shape can only be 1) broader than longer, 2) roundish, 3) broadly elliptical, 4) narrowly elliptical and 5) angular. Stomatal length / breadth (L /B) ratios in our data for sunflower seedling averaged to 1.4969 ± 0.0296 varying from 1.0 to 2.0 (CV = 14.68%) and tended to distribute normally as suggested by Shapiro-Wilk test of normality (Fig. 39). Only 3.6% of the stomata had L / B ratio = 1 that

is to say that they were round in shape. Some 10.9% of the stomata had L / B ratio of 1.1 – 1.25 that is they were slightly elongated in shape (oval). Around 72.8 % of stomatal L/B ratios fell in the L /B size class of 1.26 – 1.75 that they were moderately elongated (elliptical to wide elliptical). Some 12.9% of the stomata were quite larger and much elongated that is twice or nearly twice in length than their breadth. There was, therefore, considerable diversity of stomatal size and shape in the seedlings of *H. annuus* var. US 666 seedlings. Tahir *et al.* (2016) reported stomatal size (guard cell size; L x B) in sunflower as 22.72 ± 3.39 x 5.43 ± 4.79 µm (Leaf dorsal surface) and 27.93 ± 3.30 x 5.59 ± 3.46 µm (Leaf ventral surface). The stomatal length of this estimate was although comparable to our data for length but magnitude of breadth in their case was substantially lesser. Mean L / mean breadth ratio from their data was 4.996. Vidhu *et al.* (1985) reported that Length / Breadth ratio of stomata in control leaves of *T. erecta* to be 1.33 on abaxial side and 1.6 on adaxial side. They reported that in this species, Solereder' s (1908) third and fourth categories were reported to be common, second category was in more restricted occurrence and first and fifth categories were not observed at all. There were angular stomata in sunflower but there occurred no first category of stomata (broader than longer).

Contiguous and clustered stomata

Contiguousness of stomata in *H. annuus* var. US 666 seedlings was frequently seen on cotyledons. Three types of contiguous stomata (CS) were observed – juxtaposed, superimposed and those oriented at right angle. Juxtaposed CS were found on dorsal surface 3-day, (Fig. 28A), 7-day, and 20-day old cotyledon (Fig. 28B; 28D). The ventral surface of 7-day old cotyledon also had juxtaposed CS (Fig. 28C; 29 B). CS oriented at right angle were present on dorsal surface of 5-day old cotyledon (Fig. 29 A) and leaf of 15-day old seedling (Fig.30 B). Contiguousness of 5 stomata in a peculiar fashion was observed on dorsal surface of large leaf (7.33 cm^2) of a 15-day old seedling (Fig. 31). A discrete cluster of three stomata was evident on the cotyledonary petiole (Fig. 30A). Vidhu *et al.* (1985) have reported three linearly arranged contiguous stomata in *Tagetes erecta* adaxial and abaxial leaf surfaces under influence of 500 ppm IAA. Four contiguous stomata were also reported by them in this species under 100 ppm IAA on adaxial surface of leaf.

Contiguous stomata are found in many species of diverse families of angiosperms (Shah and Gopal, 1969; Inamdar and Patel, 1969; Gopal and Shah, 1970; Patel and Shah, 1971; Kothari and Shah, 1975; Inamdar and Gangadhara, 1976; Inamdar and Patel, 1976; Inamdar *et al.*, 1983; Vidhu *et al.*, 1985; Hashemloian and Azimi, 2014; Khan *et al.*, 2014; 2016) and clustering of stomata is reported from more than sixty species (Gan *et al.*, 2010). Drought and salinity increase the occurrence of CS (Gan et al., 2010). Pollution due to heavy traffic density has also been considered to increase frequency of abnormal stomata in *Albizia lebbeck* (Khan, 2020). The structure, development, and patterning of stomata on leaf are quite complex processes (Croxdale, 2000) and may be due to many extrinsic and intrinsic factors (Inamdar and Patel, 1976). A number of aberrant stomatal types (including contiguous stomata – juxtaposed, superimposed, obliquely oriented or oriented at right angle have been reported in several Solanaceae even under normal conditions of seedling growth (Inamdar and Patel, 1976).

In epidermis, mature stomata are usually separated from each other by a minimum of one cell (Sachs, 1978) which appears to ensure that there is minimum overlap between diffusion shells (Sachs, 1978; Casson and Gray, 2008). It is, however, known quite earlier that stomatal pattern is altered in response of environment (Salisbury, 1928) and several aberrations may be seen in stomatal structure. In cotyledons of *Helianthus annuus var.* EC 68414 control plants, Inamdar *et al.* (1980) reported anomocytic stomata (sensu Dilcher, 1974) and anomocytic stomata with a single subsidiary cell, paracytic and cyclocytic in various treatments of growth regulators. In addition to normal types, abnormalities such as contiguous stomata, division of guard cells, single guard cells, cytoplasmic connections, degeneration of guard cells, persistent stomatal cells and giant stomata are noticed in different concentrations of various growth regulators treatments. Giant stomata with abnormally big pores are commonly met with in kinetin treatments. Exogenously applied growth substances are also reported to bring several variations in the morphology of stomata in *Tagetes erecta* (Vidhu *et al.*, 1985) e.g. production of diacytic stomata, anomocytic stomata having three cells surrounding the stomata making them anisocytic, persistent stomatal initials, Variously oriented contiguous stomata (CS) juxtaposed, superimposed, at right angles) in control and treated leaves, Equal or unequal contiguous stomata, cytoplasmic connections guard cells of adjacent stomata, One and a half CS, single guard cell, degenerate guard cells, transversely divided guard cells, reduction in stomatal size etc. Gopal (1992) considered abnormalities such as contiguous stomata, degenerate guard cells, abortive and single guard cells recorded in six succulent species of genus *Senecio* (Asteraceae) as natural phenomena. Ontogeny of stomata, if not the stomatal density, stomatal index, and sizes of epidermal and guard cells, is viewed to be genetically controlled (Sharma and Dunn, 1968).

REMARKS

1. The structure and development of foliar stomata and stomatal density are age - related phenomena. Stomatal density is known to reduce with mature leaves as compared to young leaves and stomatal types undergo

transformation with age due to development of new cell walls in subsidiaries or neighbouring cells (Stace, 1965; Khan and Zaki, 2019b). Stomatal studies have generally ignored the age or size of leaf of a plant and that has never been mentioned in most of the works published on the subject. Stomatal parameters should be studied in relation to the age of the leaf.

2. The published schemes on stomatal classification are many. In the present studies, simple scheme of classification of mature stomata proposed by Prabhakar (2004) was followed which gave no consideration to the ontogenetic pathway of stomatal development and considered neighbouring cells (distinct or indistinct) equally important in view of their position (abutting stoma). This way a number of stomatal types were recognized in seedlings of *H. annuus* Var. US 666. The Prabhakar's (2004) classification, however, is bound to ignore a great deal of technical information on ontogeny of stomata in plants. The importance of ontogenetic information regarding stomata is well-emphasized by many workers (Vesque, 1889; Metcalfe and Chalk, 1950; Tomlinson, 1969; Van Cotthem, 1970; Fryns-Claessens and Van Cotthem, 1973; Dilcher, 1974; Wilkinson, 1979; Rasmussen, 1981, Willmar and Fricker, 1996). If the concept of the above classical schemes of classification is followed, only one type of the stomata that is "anomocytic" characterizes *H. annuus* var. US 666 owing to indistinct nature of neighbouing cells. In cotyledons of *H. annuus var.* EC 68414 under normal conditions of growth, Inamdar *et al.* (1980) also reported anomocytic stomata (sensu Dilcher, 1974). Under influence of growth regulators some other types of stomata (paracytic, cyclocytic, anomocytic with one subsidiary or three unequal subsidiaries (simulating anisocytic type) were also found by them but ontogeny of all stomata was reported to be of perigenous type. Suseela *et al. (*2002) described anomocytic stomata in *Tridax procumbens* on the basis of the indistinct neighbouring cells surrounding stomata. Anomocytic stomata in *H. annuus* Var. US 666 appear to be produced agenously (see Willmer and Fricker, 1996). Idu *et al.* (2006) reported agenously produced anomocytic stomata in some species of Fabaceae (*Albizia zygia, Amphimas pterocarpoides, Baphia nitida, Bauhinia rufescens, Pilostigma thiinghii, tetrapleura tetraptera,* etc.). The anomocytic stomata bear no distinct subsidiary cells and the immediate ground cells abutting stomata are referred to as neighbouring cells (NCs) (Van Cotthem, 1970; Fryns-Claessens and Van Cotthem, 1973).

The number of NCs in *H. annuus* var. US 666 varied relatively with the organ types (Table 9).

- On cotyledonary petiole, the number of NCs was generally three in 52% and four in 40% stomata. There were 2% stomata with two NCs each.
- On cotyledonary dorsal surface, c 58% stomata had four NCs. In 23% stomata NCs were ≥ 5.
- The cotyledon-petiole merging area had predominantly four NCs in 71% of the stomata.
- Foliar stomata were largely abutted with four NCs. Stomata with two or five NCs were present in substantial number but no foliar stomata had two NCs. Stomata with two NCs were found to be restricted to the cotyledon.

The scheme of Prabhakar (2004) makes it easy to identify stomata but renders the results not comparable to the vast amount of data accumulated on the subject in the past on the basis of classical schemes. Moreover, Prabhakar's scheme appears to be an artificial scheme ignoring vital morphogenetic information on ontogeny of the stomata, the valid base for taxonomic conclusions (Kondo, 1962; Pant, 1965; Stace, 1965; Maroti, 1966). In our opinion, study of stomata should not ignore their ontogenetic peculiarities. The study at seedling stage should obviously provide requisite information on stomatal ontogeny. Schemes of Van Cotthem (1970) and Dilcher (1974) appear to be more suitable for this purpose.

3. It appears imperative that taxonomists should reach a consensus on use of certain comprehensive scheme of stomatal classification that should universally be employed in stomatal studies to facilitate comparison.

Table 9. Percent proportion of various number of Neighbouring cells (NCs) abutting stomata on cotyledonary and foliar surfaces.

Number of NCs	Cotyledon* Dorsal	Cotyledonary Petiole	Petiole – Cotyl. merging area	Leaf (dorsal)**	Leaf (Ventral)**	Leaf (pooled)**
2	0.14	8.0	Zero	Zero	Zero	Zero
3	18.66	52.0	14.29	24.68	16.80	20.57
4	57.66	40.0	71.43	53.66	43.78	49.25
≥ 5	23.19	-	14.29	21.72	39.42	30.57

*, Data of 3, 5, 7, 10 and 15-day old cotyledons pooled. **, Data of 10- and 20-day old primary, secondary and Tertiary leaves pooled.

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(Accepted for publication June 2020)