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SURFACE MICROMORPHOLOGY AND THE STOMATAL COMPLEXES OF FENUGREEK (*TRIGONELLA FOENUM-GRAECUM* L., FAMILY PAPILIONACEAE) SEEDLINGS

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ABSTRACT

Surface micromorphology, stomata and trichomes of 20-day old Phanerocotylar-epigeal seedlings of fenugreek (*Trigonella foenum-graecum* L.) were studied from its Karachi accession. Hypocotyl was relatively thick, short, and light green in colour. In some seedlings it was pink due to the accumulation of anthocyanin. Cotyledons were thicker and shiny. Epicotyl was trichomatous. Eglandular trichomes, variable in length, were simple, unicellular, pustulate (covered by small blisters-like pimples, pusticulate) and, present on cotyledons, petiole, rachis and petiolule and leaflets (densely trichomatous ventrally). Lower part of hypocotyl showed capitate glandular trichomes (possibly glandular). Primary leaves were simple petiolate, orbicular to oval but subsequent leaves were trifoliate, alternate, adenately stipulate. The terminal leaflet was always larger than the lateral leaflet. Epidermal cells were irregular in shape. Epidermal cells in young leaf were straight to curvy in anticlinal contour. At adult stage epidermal cells, in cotyledons and the leaves both, were large in size and sinuous in anticlinal contour. In pooled sample of both lateral and the terminal leaflets, waviness was larger on ventral surface (8.04 ± 0.106, varying from 4-14) as compared to dorsal surface (7.54 ± 0.12, varying from 4 to 11 wave crests per cell ($t = 3.079$, $p < 0.001$). Cotyledons and leaves were amphistomatous and isostomatous as indicated by Kirkham's (2014) stomatal ratio on adaxial / abaxial surface. Stomata were elliptical, wide elliptical, to round. Some stomata were giant with relatively larger surrounding cells. In *T. foenum-graecum*, cytoplasmic connections between adjacent stomata were rare. Contiguous stomata were, however, common. Stomatal density was lower on cotyledons (averaging to 51.93 ± 1.72 stomata per mm^2 on dorsal surface and 50.43 ± 2.17 stomata per mm^2 on ventral surface). Stomatal density per mm^2 was higher on leaflets as under:

Lateral leaflet: 97.81 ± 2.19 (dorsal) and 113.6 ± 3.170 (Ventral).
Terminal leaflet: 106.65 ± 1.78 (dorsal) and 113.44 ± 2.42 (Ventral).

Stomtal length was significantly ($t = 6.195$, $p < 0.001$) larger (35.18 ± 0.697 µm) on sun-exposed dorsal surface of cotyledon than that on the ventral surface (29.22 ± 0.643 µm). Similarly, stomatal width was significantly ($t = 6.77$, $p < 0.001$) larger on dorsal surface (18.67 ± 0.452 µm) than that on the ventral surface (14.30 ± 0.462 µm) of cotyledons. Stomatal size (L x W) of lateral and terminal leaflets averaged to as follows.

Lateral leaflet (Dorsal): 22.88 ± 0.525 x10.53 ± 0.377 µm; Lateral leaflet (Ventral): 23.40 ± 0.403 x 10.97 ± 0.322 µm.
Terminal leaflet (Dorsal): 22.57 ± 0.349 x 10.68 ± 0.374 µm and Terminal leaflet (Ventral): 21.43 ± 0.529 x 10.58 ± 0.286µ.

The neighbouring epidermal cells (NCs) were indistinct in cotyledons as well as leaflets and thus stomata were of anomocytic type according to classical structural-morphological schemes (subsidiaries cells (SCs) equal to zero). The number of NCs varied from 2 to 6 but generally 3or 4 or less frequently 5. Three- or four NCs per stoma were abundant - 55.14 and 41.69%, respectively on ventral and dorsal surface. In pooled sample of leaflets, according to the classification of Prabhakar (2004), the stomata with two NCs (so called paracytic) were rare, stomata with 3NCs ranked as anisocytic were abundant (around 55%), stomata with 4NCs (41.69%) were either with tetracytic (37.92) or staurocytic (3.77%) arrangements and stomata with 5 or 6NCs were anomocytic (c 3% in all). The differences arising due to Prabhakar's system were discussed in detail and Prabhakar's system was found to suffer from irrationality and artificiality. His contention, NCs = SCs, is highly questionable in view of recent studies on molecular signatures of SCs.

Key-words: *Trigonella foenum-graecum* L., Surface micromorphology, trichomes, stomatal complexes.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L., family Papilionaceae) is a tap-rooted and erect annual with pinnate trifoliate leaves reaching to a height of 40-80 cm. It is called "Methi" in Urdu, "Fenugrec" in French, "hu-lu-pa" or "kü-ton" in Chinese, "Hulabana" in Arabic, "Fieno Greco" in Italian, etc. – to mention few vernacular names. Its nomenclature is based on Trigonella = Latin, small triangular flowers and foenum-graecum = "Greek hay". It is also called "bull horn" or "goat horn" because of projection of horns (Rosengarten, 1969; Tucker and De Baggio, 2009). Its 2N set of chromosomal number is 16. It is a flavouring agent in cuisines of Indo-Pakistan subcontinent. Fenugreek was used for different purposes in ancient times especially in Egypt and Greece (Beyzi *et al.,* 2010; Tucker and De Baggio, 2009). It is coumarin-odouring (Huber-Morath, 1970). Besides being a medicinal plant especially in Indian Ayurvedic and Unani systems (Srinivasan, 2006), it has a potential of good quality forage (Mir *et al.,* 1997). Its seeds are used as spices and tender shoot and leaves as vegetable. There are various folkloric uses of fenugreek. Acharya *et al.* (2007) and Aher *et al.* (2016) have reviewed evidence-based research on medicinal uses of this plant in humans. Sheikhlar (2013) reviewed it in animal growth and health. Fenugreek seeds improve insulin sensitivity and the mitochondrial function (Li *et al.,* 2018). Some people are, however, allergic to fenugreek, including those with peanut allergies or chickpea allergies (Ouzir *et al*. (2016; NCCIH, 2020). Fenugreek seeds can cause diarrhea, dyspepsia, abdominal distention, flatulence, perspiration, and a maple-like smell to sweat, urine or breast milk (Ouzir *et al.,* 2016; fenugreek Drug.com.Dec.2020; NCCIH, 2020). There is a risk of hypoglycemia particularly in people with diabetes, and it may interfere with the activity of antidiabetic drugs (Ouzir *et al*., 2016; NCCIH, 2020). Because of the high content of coumarin-like compounds in fenugreek, it may interfere with the activity and dosing of anticoagulants and antiplatelet drugs (Ouzir *et al*. (2016; NCCIH, 2020). Al-Ashaban *et al.*

(2010) have reported that chronic treatment with fenugreek may cause highly significant spermatotoxic effects in male mice.

Fenugreek exhibits considerable morphological, physiological and genetic diversity (Marzougui *et al.,* 2009; Gangopadhayay *et al.,* 2009; Soori and Najd, 2012; Al-Maamari *et al.,* 2020). Mukhtar and Riaz (2021) reported on micromorphology of *T. foenum-graecum* from Pakistan but there appears certain reasons to reinvestigate micromorphology of this species. In the present paper, surface micromorphology, stomata and trichomes of seedlings of this species are studied from its Karachi accession.

MATERIAS AND METHODS

Seeds and seedling collection: The seeds of *T. foenum-graecum* were purchased from the Karachi market. They were small, yellow, and rectangular-rhomboidal in shape. The seeds were sown in garden loam type of soil without any treatment except that healthy and average-sized seeds were sterilized with 1% sodium hypochlorite for two minutes and rinsed with distilled water. The soil moisture was kept around 70% of MWHC in spring season of 2022 and maximum temperature around 28^{o}C. The emergence of seedling began after around two days of incubation (as also reported by Kadam, 2019) and completed near 60% within five days. Cotyledons were closely appressed on emergence but soon started unfolding and underwent some expansion.

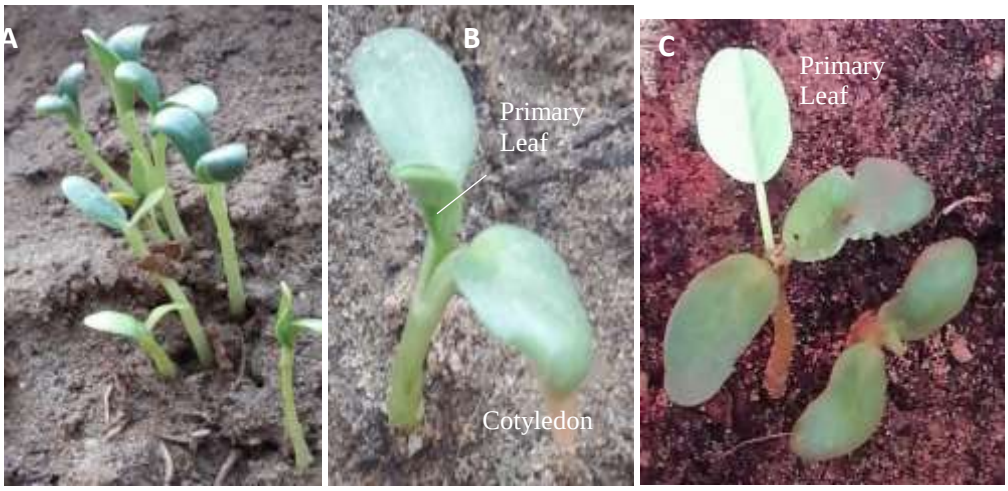

Fig. 1. Newly-emerged Seedlings of *T. foenum-graecum from soil* (A), Emergence of folded primary leaf (B) and unfolded simple primary leaf (C).

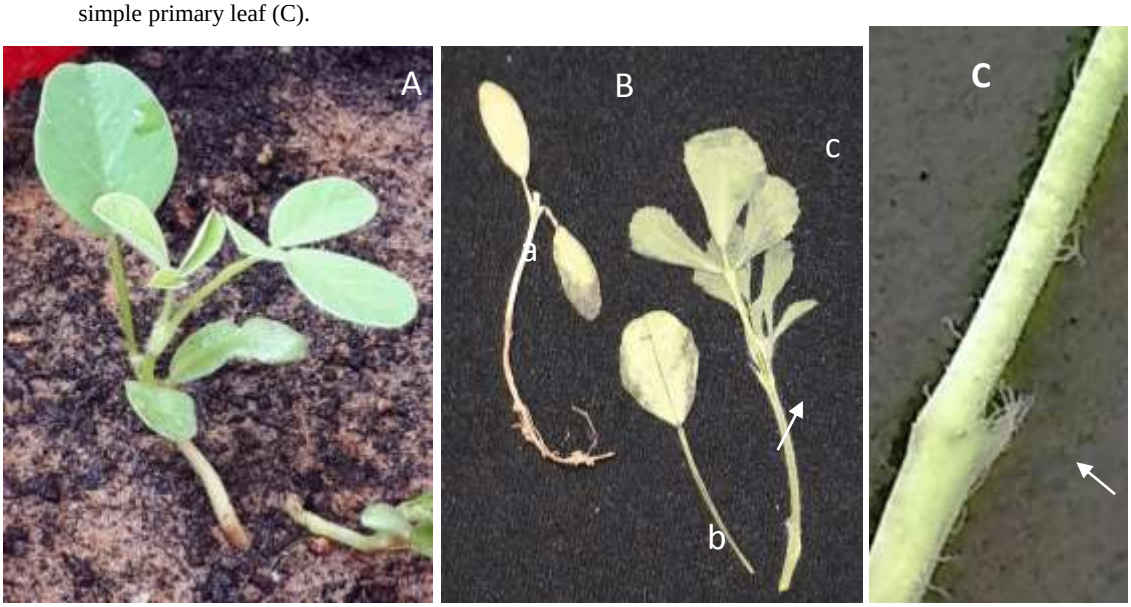

Fig. 2. Seedling of *T. foenum-graecum*. A) A 15-day old seedling grown in pot in April, 2022, bearing wide elliptical opposite cotyledons, primary simple leaf and trifoliate secondary and tertiary leaves. B) Parts from a near mature plant – grown in Karachi in spring season from the same stock of seeds in Karachi. C) The adenate triangular hairy stipule shown by an arrow.

Twenty-day old seedlings were employed for micromorphological investigations. Seedlings type was described according to Garwood (1996). Hickey (1973) and Ash *et al*. (1999) were followed for description of leaf. The impressions of surfaces of cotyledons, lateral and terminal leaflets were made with clear nail polish (Wang *et al.*, 2006) and studied under compound optical microscope for ornamentation and micromorphological structure. There are several schemes available which are important in identifying the stomata in plants (to cite a few- Metcalfe and Chalk (1950), Van Cotthem (1970), Dilcher (1974, Wilkinson (1979), Baranova (1987, 1992), Prabhakar (2004), Carpenter (2005), etc. The present study was undertaken to elaborate and elucidate stomatal characteristics of *T. foenum-graecum* on the basis of classical structural-morphological schemes of stomatal classification of Metcalfe and Chalk (1950), Van Cotthem (1970) and Dilcher (1974) and relatively recent artificial approach of Prabhakar (2004). The earlier classifications basically undertook considerations regarding distinctiveness of stoma-surrounding cells by their size, shape, in possession of papillae or in their contents - no matter how they are derived ontogenetically (Wilkinson, 1979).

It may be mentioned that Prabhakar's (2004) classification may give quite varying results (Khan *et al.,* 2020) as compared to Dilcher's scheme. As a basic criterion, all the cells abutting the guard cells were considered distinct by Prabhakar (2004), by virtue of their position, whether they differentiate from the other epidermal cells or not. Only on the basis of their abutting nature to the guard cells, he prefers them to call subsidiaries. This nomenclature does not recognize actinocytic and stephanocytic stomata and categorize them as anomocytic type.

Stomatal size was measured microscopically at 45 x 10 X magnification for cotyledons, lateral leaflets (1.0 to 1.2 cm^2 in size) and terminal leaflets (1.6 to 2.0 cm^2 in size) from 15-20 days old seedlings. Stomatal ratio, sensu Kirkham (2014), was calculated as ratio of stomatal density (frequency) on adaxial surface to that on abaxial surface.

RESULTS AND DISCUSSION

Seedling: The micromorphology of *T. foenum-graecum* is presented in Figs. 1-16 and briefly described in Table 1-7.The seed germination was epigeal as cotyledons emerged above ground (Fig. 1A & B). According to Garwood's (1996) classification of seedlings, the seedling was Phanerocotylar-epigeal type. Hypocotyl was relatively thick, short, and light green in colour. In some seedlings hypocotyl was pink due to the accumulation of anthocyanin (Fig. 1C). At 15 days of age seedling was c10-12 cm in height.

Hypocotyl: Short, thick, light green, sometimes pink due to anthocyanin accumulation.

Epicotyl: Trichomatous, light green.

Cotyledons: Long cotyledonary petiole (1.5 cm) and blade wide elliptical in shape – around 1.6 cm in length and c. 05 cm wide. They were dark green and shiny whereas cotyledonary petiole was light green in colour (Fig. 1). Cotyledons had trichomes on both surfaces. Cotyledons were thicker than the leaves.

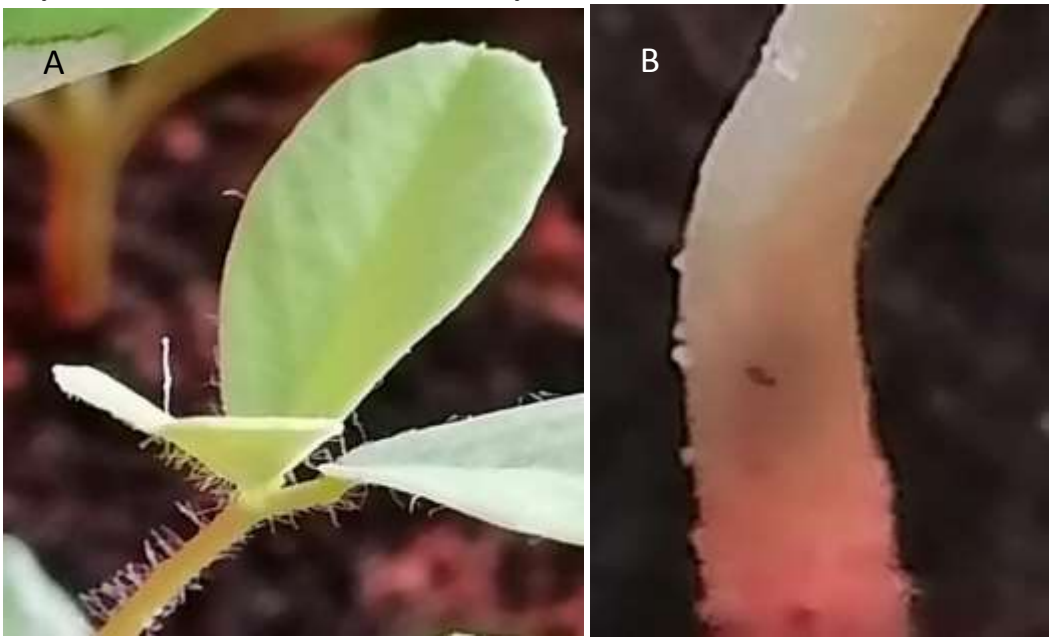

Fig. 3. Pusticulate (Pustulate) trichomes on leaves (A). The basal part of the hypocotyle (B) showing protuberances, possibly capitate glandular trichomes. See Fig. 5A also.

Leaves: There are two types of leaves in fenugreek seedling. The primary leaf of the seedling is simple, orbicular to oval in shape, entire-margined, dorsiventral. It emerged on fourth day and was initially folded by the midrib (Fig.

1B) but soon unfolded. The unfolded primary leaf was obtuse at the apex provided with small notch. The subsequent leaves were trifoliate (Fig. 2). The leaflets of trifoliate leaves were also folded by midrib when young but soon they unfolded. The leaves were arranged alternately. Stipules were adnate to petiole, triangular-lanceolate, green and hairy (Fig. 2C) and the terminal leaflet was always larger than the lateral leaflet. The lateral leaflets were opposite. The leaflets were orbicular or oblong in shape with upwardly pointing dentation at the obtuse apex and up to around midway of the lamina (dentation = 8.58 ± 2.75 teeth per leaflet, N = 12 varying from 6 to 14, CV= 46.2%).

Cotyledonary and Foliar epidermis: Epidermal cells are irregular in shape. Epidermal cells in young leaf were straight to curvy in anticlinal contour. At adult stage epidermal cells in case of both cotyledons and the leaves both were large in size and sinuous in anticlinal contour. Periclinally, as per Barthlott *et al.* (2017) classification, the epidermal cells were convex and at margins the cell walls sloped down to meet the low-lying walls. Striations of cuticle was observed on ventral surface in form of bands parallel to each other. The epidermal cells in vascular region were quite elongated and at times had prominent cuticular striations. The epidermal cells abutting stomata were indistinct from rest of the ground epidermal cells. In both, control diploid and mixoploid plants of *T. foenum-graecum,* epidermis was reported to be sinuous by Omezzine *et al*. (2012). Anitha and Priyadarshini (2012) although didn't describe epidermal characteristics but their figure clearly indicated epidermal cells to be sinuous. Wall formation in neighbouring cell was also observed sometimes as also reported earlier in tribe Trifolieae by Shah and Kothari (1975).

Table 1. Waviness in terms of wave crests per cell of epidermis of lateral and terminal leaflets of *T. foenum-graecum* seedling.

Parameters	Lateral leaflet		Terminal Leaflet		Pooled Dorsal	Pooled Ventral
	Dorsal	Ventral	Dorsal	Ventral		
N	90	90	90	125	180	215
Mean	7.54	7.94	7.53	8.10	7.54	8.04
SE	0.173	0.129	0.176	0.157	0.123	0.106
Median	8.0	8.0	8.0	8.0	8.0	8.0
CV (%)	21.79	15.78	22.28	21.60	21.91	19.33
G1	0.052	0.184	0.081	0.224	0.066	0.272
Sg1	0.254	0.254	0.254	0.217	0.181	0.166
G2	-0.387	-0.288	0.381	0.834	-0.406	1.037
Sg2	0.503	0.503	0.503	0.4530	0.360	0.330
Minimum	4	4	4	4	4	4
Maximum	11	11	11	14	11	14
KS-T	0.143	0.185	0.132	0.128	0.138	0.151
p	0.0001	0.0001	0.001	0.0001	0.0001	0.0001
Shapiro-Wilk	0.959	0.925	0.958	0.963	0.958	0.951
p	0.007	0.001	0.005	0.001	0.001	0.001

Waviness
N = Number of observations
SE = St. Error of mean
CV = Coefficient of variability (%)
G1 = skewness
Sg1 = St. Error of skewness
G2 = Kurtosis
Sg2 = St. Error of kurtosis
KS-T = Kolmogorov-Smirnoff test with Lilliefors significance correction
p = probability
Shapiro-Wilk = Shapiro-Wilk Test

Waviness of Epidermal pavement cells: The leaves of *T. foenum-graecum* exhibited sinuous anticlinal contour (Fig. 6 – 12). Agbagwa and Okoli (2012) have reported the anticlinal walls of foliar epidermis of some *Abrus* spp. to be irregular, wavy or arcuate. Khan and Zaki (2019b) also reported waviness in epidermal anticlinal contour of *Sesbania bispinosa* seedling. The pavement cells of epidermis in leaf in *T. foenum-graecum* were quite intricate in shape with U-shaped undulations and 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. The waviness of contour varied with the size of the cells. The smaller cells had lesser number of lobes and larger cells had larger number of lobes. The epidermal waviness was not significantly different on dorsal surfaces of lateral leaflet (7.54 ± 0.173 crests per cell) from dorsal surface of terminal leaflet (7.53 ± 0.176) as evident from t- test (t = 0.041, NS). Similarly, waviness of epidermis of ventral surface of lateral leaflet (7.94 ± 0.129 crests per cell was not significantly different (t = 0.785, NS) from waviness of ventral surface of terminal leaflet (8.10 ± 0.157 crests per cell). In pooled sample for both lateral and the terminal leaflets, waviness was larger on ventral surface (8.04 ± 0.106, varying from 4-14) as compared to dorsal surface (7.54 ± 0.12, varying from 4 to 11)) (t = 3.079, p < 0.001) (Table 1).

Watson (1942) reported the greater tendency toward waviness on the lower side of leaves with few exceptions. Misra (2009) also reiterated that undulations are more pronounced on the lower side of leaf than upper surface. The waviness appears to be affected by the environmental conditions prevailing during leaf development. 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 top (Rippel, 1919). It also appears to be the function of the age of the organ – as the undulations of the anticlinal contour of epidermal pavement cells were only observed in 7-day old mature cotyledons and three-day old cotyledons exhibited no undulation in the pavement epidermal cells in *Sesbania bispinosa* (Khan and Zaki, 2019b). The formation of undulations in the epidermal cells 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). The wavy contours in epidermal pavement are considered to be of biomechanical benefits (Jacques *et al.,* 2014; Sapala *et al.,* 2018).

Trichomes: The trichomes are of two types. 1) Simple unicellular, pustulate (covered by small blister-like pimples, pulsaculate), non-glandular present on cotyledon petiole, rachis and petiolule of leaflets in variable length (Fig. 3 and 4) and 2) there were capitate glandular trichomes on the lower part of hypocotyl (Fig. 3 and 5). Eglandular trichomes were denser on the ventral surface of leaflets (Fig. 5). Few non-glandular trichomes were quite long – as large as 544 µm on the leaf margin and rachis. The trichomes gradually weathered with leaf maturity - declining their density. The lower part of stem had some protuberances, possibly capitate glandular trichomes (Fig. 3B and 5).

The length of trichome of first type may reach to 1205 – 2451 µm (Anitha and Priyadarshini, 2012). Patil *et al.* (2015) reported only simple type of trichomes *in T. foenum-graecum.* Rajbar and Hajmoradi (2016) reported two types of trichomes (papillose-spinulate and glandular trichomes in *Trigonella spruneriana* from Iran.

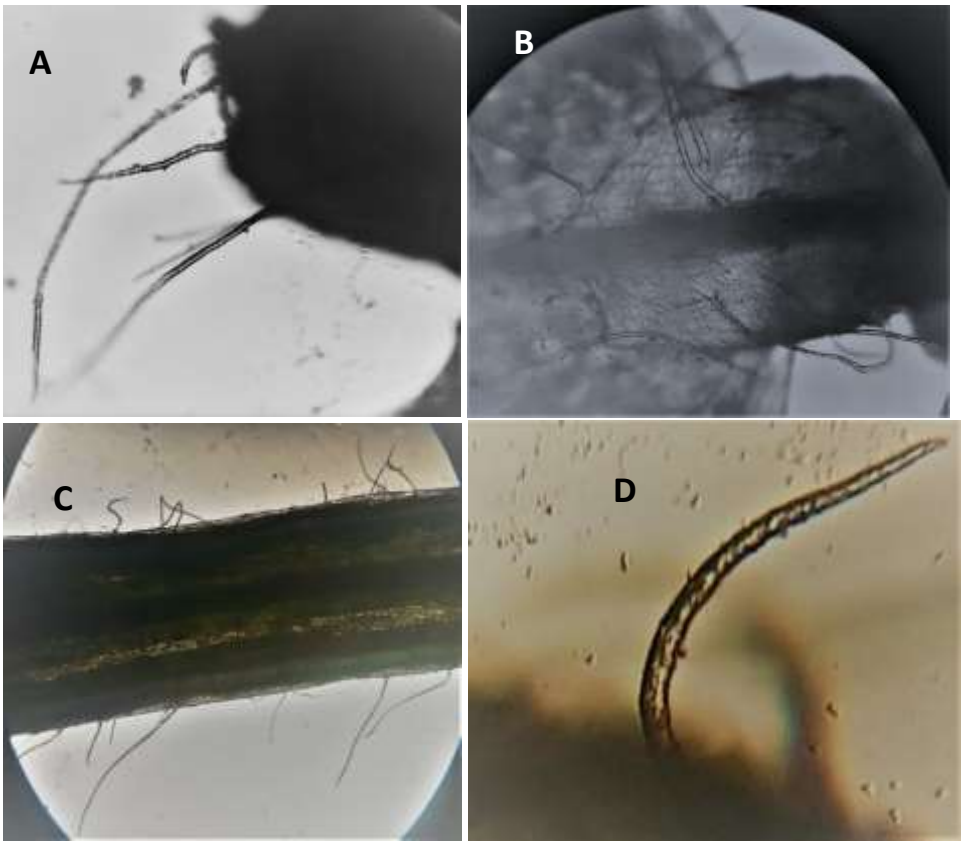

Fig. 4. Leaf of *T. foenum-graecum* seedling. Trichomes on the leaf apex and petiolule (A, and B) and petiole (C). An enlarged view of eglandular trichome (D) on the leaf base - trichomes are long, unicellular, and delicate, pustulate, and pointed at the apex. Magnification: A, B and C) 4 x 10X, zoom 1.4 X; C) 4 x, 10X; zoom 1.6 X. D) 10 x 10 X, zoom 4.0 X.

Stomata complexes: The leaves of *T. foenum-graecum* were amphistomatous. Epidermal micromorphology of various structural components of seedling is given in Table 2. Stomata were elliptical, wide elliptical, to round. Cotyledons and leaves were amphistomatous. Some stomata were relatively larger with larger surrounding cells (giant). In *T. foenum-graecum* there was cytoplasmic connection between adjacent stomata sometimes (Fig. 14D). Inamdar and Patel (1976) have reported cytoplasmic connection between stomata in family Solanaceae. Stomata on all parts of *T. foenum-graecum* were generally oriented in various directions.

Neighbouring cells of stomatal complexes (NCs): The cells abutting stomata were indistinguishable (indistinct) from rest of the epidermal cells and therefore they cannot be ranked as subsidiary cells). Such stoma-abutting cells are referred to as neighbouring cells (NCs) by Van Cotthem (1970) and this state is called a zero-subsidiary cell (SCs) state. The number of NCs in *T. foenum-graecum*, however, varied from 2 to 6 but generally 3, 4 or less frequently 5 with varying arrangement (Table 3 & 4). Three- or four NCs per stoma was predominant - 55.14 and 41.69%, respectively on ventral and dorsal surface.

Table 2. Epidermal micromorphology of various components of seedlings.

Organ	Epidermis	Trichome	Stomata
Hypocotyl base	Straight to curvy	Capitate, Glandular	-
Rachis & Petiole	Straight to curvy	Eglandular, pulsalute, pointe at apex	Elliptical, large, Stomata with three NCs (Fig. 5B). Stomata oriented in various direction.
Cotyledon dorsal	Large, sinuous, periclinally convex Striation present, Trichomal present.	Eglandular, pulsalute, pointed at apex.	Elliptical, NCs varying in number (Fig. 6). Stomata oriented in various directions.
Cotyledon Ventral	Large, sinuous, periclinally convex Striations present, Trichomal scars present.	Eglandular, pulsalute, pointed at apex	Elliptical, NCs varying in number (Fig. 7C). Stomata oriented in various directions.
Lateral Leaflet	Large, sinuous, periclinally convex Striations present, Trichomal scars present. Thick cuticular sheet.	Eglandular, pulsalute, pointed at apex	Elliptical, NCs varying in number- 2 to 5 (Fig. 8, 9). Common NSs between stomata. Stomata oriented in various directions. Contiguous stomata present (Fig. 13).
Terminal Leaflet	Large, sinuous, periclinally convex Striations present, Trichomal scars present. (Fig. 10). Thick cuticular sheet and striations (Fig.15) in mature leaves mask the epidermal structure (Fig. 15A).	Eglandular, pulsalute, pointed at apex. Varying number of basal pavement cells of trichome may be as high as ten (Fig. 15B).	Elliptical, NCs varying in number- 3 to 6 (Fig. 11, 12). Common NSs between stomata. Stomata oriented in various directions. Sometimes giant stomata present (Fig. 14C). There appear cytoplasmic connection between adjacent stomata present (Fig. 14D). Contiguous stomata present (Fig. 16).

Table 3. *Per cent* abundance of stoma types on cotyledons and leaflets of *T. foenum-graecum*.

Number of Neighbouring cells	Cotyledon (Adaxial) (N =115) *	Cotyledon (Abaxial) (N = 70)	Lateral Leaflet (Adaxial) (N = 300)	Lateral Leaflet (Abaxial) (N = 300)	Terminal Leaflet (Adaxial) (N = 350)	Terminal Leaflet (Abaxial) (N = 350)
1 Cell	Zero	Zero	Zero	Zero	Zero	Zero
2 Cells	Zero	Zero	Zero	Zero	0.30	0.30
3 Cells	**56.50**	**38.6**	**58.60**	**54.67**	**56.90**	**50.90**
4 Cells	**37.4**	**42.9**	**40.0**	**43.0**	**41.10**	**42.60**
5 Cells	6.10	18.6	1.3	2.33	1.70	6.0
6 Cells	-	-	-	-	-	0.30

*, Number of stomata observed).

Table 4. Stomatal types on leaflets (pooled for dorsal and ventral surfaces of lateral and terminal leaflets) on the basis of number of NCs.

Number of Neighbouring cells	Dorsal (650) *	Ventral (650) *	Pooled (1300) *
1 Cell	Zero	Zero	Zero
2 Cells	0.154	0.154	0.154
3 Cells	**57.612**	**52.615**	**55.154**
4 Cells	**40.615**	**42.769**	**41.692**
5 Cells	1.538	4.308	2.923
6 Cells	Zero	0.154	0.077

*, Number of stomata observed.

Stomatal complexes on the basis of structural-morphogenetic classificatory schemes: According to any of the structural-morphogenetic schemes given by Metcalfe and Chalk (1950) or Van Cotthem (1970) or Dilcher (1974) or Wilkinson (1979) or Carpenter (2005) the indistinct neighbouring cells in *T. foenum-graecum* indicated to the anomocytic type of stomatal complex prevalent in this species and no other kind of stomatal complex can be assigned in this species. Rashid *et al.* (2019) investigated 17 species of genus *Medicago* L., *Melilotus* Mill., *Trifolium* and *Trigonella* L. (Trifolieae) from various localities of Pakistan and reported anomocytic stomata (following Wilkinson's key given in Metcalfe and Chalk, 1979) in these species. Anomocytic stomata arise perigenously directly from the meristemoids by straight division and without cutting off any subsidiary cells (Pant, 1965; Fryns-Claessens and Van Cotthem, 1973). Willmer and Fricker (1996) called them agenous. 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.). They may, however, arise mesogenously (Pant and Verma, 1963) or mesoperigenously (Pant and Mehra, 1964).

Stomatal complexes based on Prabhakar's artificial classification

Following the artificial classification for structural delimitation of mature stomata (Prabhakar, 2004), a number of stomatal complexes were identifiable in *T. foenum-graecum*. In cotyledons, the order of abundance of stomatal types varied with the dorsal and ventral surfaces (unequally exposed surfaces to sun). Stomata with 3NCs (accepted as subsidiaries by Prabhakar (2004) – anisocytic complex) were relatively more abundant on dorsal surface (56.5%) than that on the ventral surface (Table 3). Stomata with 4NCs were much higher in abundance on ventral surface (42.9%) than that on the dorsal surface (37.4%). No 4NCs staurocytic arrangement of stomatal complex was observed on ventral surface of cotyledon. Stomata with 5NCs were 18.6% on ventral and 7.61% on dorsal cotyledonary surface.

In pooled sample of leaflets (Table 4), the stomata with two NCs parallel to the guard cells (accepted as subsidiaries by Prabhakar, 2004 - paracytic) were rare, stomata with 3NCs were ranked anisocytic were predominant (around 55%), stomata with 4NCs (41.69%) were either with tetracytic (37.92) or staurocytic (3.77%) arrangements and stomata with 5 or 6NCs were anomocytic (c 3% in all) as per Prabhakar's opinion.

Abnormal stomata

In *T. foenum-graecum*, some abnormal stomata were also observed e.g., Stomata with common NCs, contiguous stomata (placed juxtaposed or two stomata placed more or less at right angle or laterally) on dorsal as well as ventral surfaces of leaflets (Fig. 9, 10, 13, 16). In rare cases, guard cells development was arrested. Abnormal stomata have been reported in papilionaceae earlier (Shah and Kothari, 1975; Dave and Bennet, 1989; Mukherji *et al.*, 2000). Recently, Agbagwa and Okoli (2012) reported contiguous stomata from some species of *Abrus* (Papilionaceae). Contiguous stomata are reported to be formed of two or more adjacent meristemoids or readjustments during epidermal maturation or as a result of budding from guard cell (e.g., in *Lathyrus sativus*, Shah and Gopal, 1969). Concluding their studies in the view of the results of the earlier studies by several workers, Inamdar and Patel (1976) suggested the development of abnormal stomata may be due to several extrinsic and intrinsic factors during stomatal development. The occurrence of abnormal development of stomata in Solanaceae (Inamdar and Patel, 1969, 1976) and Cruciferae (Rao and Inamdar (1981) have been reported – in form of persistent stomatal initials, arrested stomatal type, single guard cells, divided guard cells, degenerate guard cells and cytoplasmic connection between two nearby stomata and contiguous stomata (juxtaposed, superimposed, obliquely placed and oriented at right angle types).

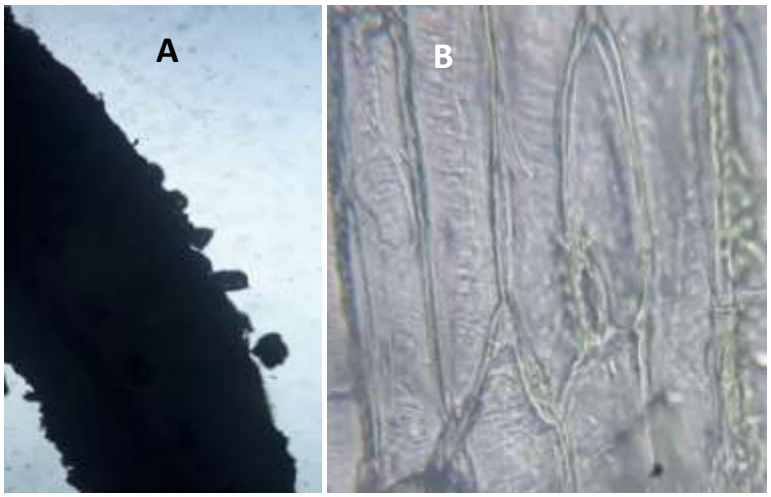

Fig. 5. Capitate trichomes on basal part of hypocotyl (A) and stoma surrounded by three indistinct neighbouring cells on rachis (B). Magnification: A, 5 x 10X and B) 45 x 10X.

Table 5. Stomatal types reported in various species of *Trigonella* after Taia (2004) and Mukhtar and Riaz (2021).

S. No.	Species	Stomatal type	Epidermal anticlinal contour	Wax encrustation
After Taia (2004)* (Egyptian Flora). Dilcher (1974) terminology followed				
1.	*Trigonella arabica*	Paracytic	Straight	-
2.	*T. anguina*	Anomocytic	Straight	-
3.	*T. occulta*	Paracytic	Straight	-
4.	*T. monspelica*	Paracytic	Straight	+
5.	*T. stellata*	Paracytic	Straight	+
6.	*T. laciniata*	Paracytic	Straight	-
7.	*T. maritima*	Diacytic	Straight	-
8.	*T. hamosa*	Diacytic	Undulate	-
9.	*T. media*	Paracytic	Straight	-
After Zarinkamar (2007)* – Combination of Dilcher (1974) & Wilkinson (1979) -Iran				
1.	*Trigonella brachycarpa*	Anomocytic & anisocytic	-	-
2.	*T. Calliceras*	Anomocytic	-	-
3.	*T. gladiata*	Anomocytic & anisocytic	-	-
4.	*T. monospeliana*	Anomocytic & anisocytic	-	-
5.	*T. spicata*	Anomocytic & anisocytic	-	-
After Rajbar and Hajmoradi (2016)*- Iran				
1.	*Trigonella spruneriana*	Anisocytic (19.6-39.7% in its population)**		-
After Patil *et al.* (2015) - India – key of stomatal identification not mentioned.				
1	*Trigonella foenum-graecum*	Anomocytic	-	-
After Mukhtar and Riaz (2021) (Pakistan Flora) – reportedly key of Metcalfe and Chalk (1950) followed.				
1.	*T. anguina*	Anomocytic	NOTE: Present study, on the basis of key of, Dilcher (1974) indicated anomocytic stomata in *T. foenum-graecum*. However, on the basis of Prabhakar's (2004) artificial key, anisocytic, tetracytic and anomocytic stomata were pre-dominant and paracytic and staurocytic stomata were rare.	
2.	*T. corniculata*	Anomocytic		
3.	*T. cachemiriana*	Anomocytic		
4.	*T. emodi*	Anomocytic		
5.	*T. fimbriata*	Anomocytic (Sunken)		
6.	*T. foenum-graecum*	Anisocytic (Sunken)		
7.	*T. gharuensis*	Anisocytic		
8.	*T. gracilis*	Anisocytic		
9.	*T. hamosa*	Anomocytic		
10.	*T. monantha spp. monantha*	Anomocytic		
11.	*T. monantha ssp. Incisa*	Anomocytic		
12.	*T. monospelica*	Anomocytic (Sunken)		
13.	*T. occulta*	Tetracytic		
14.	*T. podperae*	Anomocytic		
15	*T. pubescens*	Paracytic		

*, *T. foenum-graecum* not studied. **, only abstract seen.

Stomatal density per mm^2 (STD)

Stomatal density on Cotyledon: STD was low on cotyledon as compared to that on leaves. It averaged to 51 93 ± 1.72 (N = 60, varying from 19.66 to 78.64, CV= 78.64%) on dorsal surface of cotyledon and averaged to 50.43 ± 2.17 (N = 50, varying from 9.83 to 78.84, CV= 30.4%) on ventral surface (Table 5). The density size class of 31-60 stomata/ mm^2 occupied 73.4% of total observations (N = 60) on dorsal surface and 64% of the observation (N = 50) on ventral surface. The STD distribution was asymmetric on both surfaces. Stomatal ratio *sensu* Kirkham (2014) was 1.029 in case of cotyledons, obviously owing to somewhat higher density on dorsal surface.

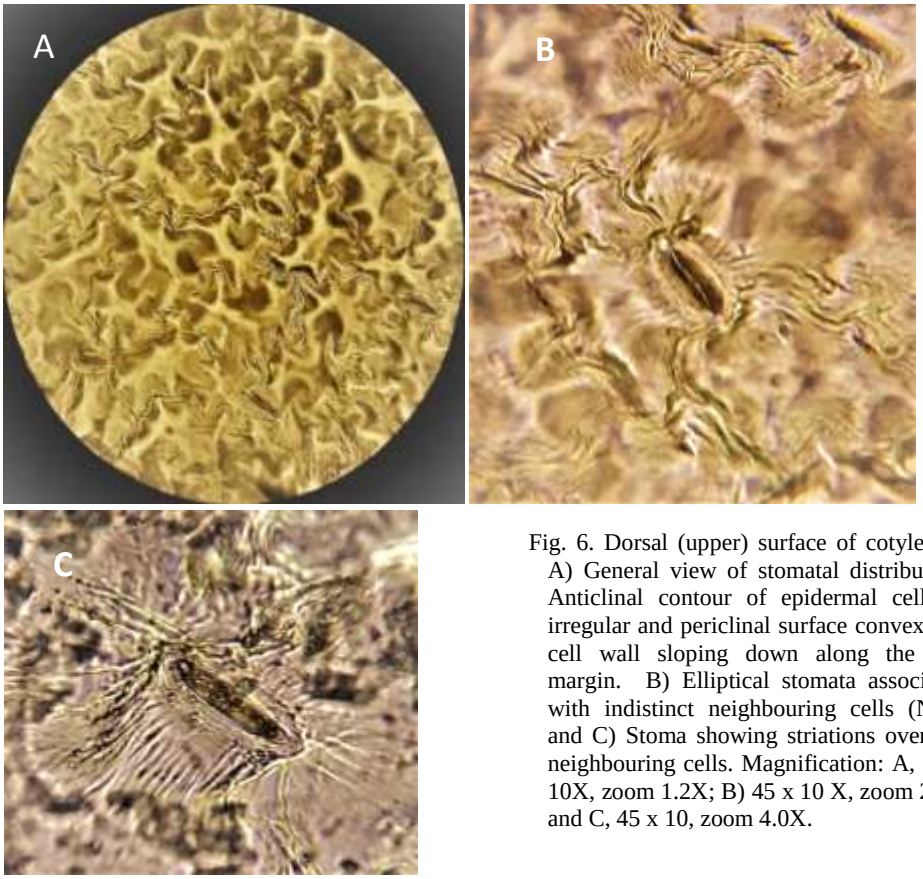

Fig. 6. Dorsal (upper) surface of cotyledon. A) General view of stomatal distribution. Anticlinal contour of epidermal cells is irregular and periclinal surface convex and cell wall sloping down along the cell margin. B) Elliptical stomata associated with indistinct neighbouring cells (NCs) and C) Stoma showing striations over the neighbouring cells. Magnification: A, 45 x 10X, zoom 1.2X; B) 45 x 10 X, zoom 2.6X and C, 45 x 10, zoom 4.0X.

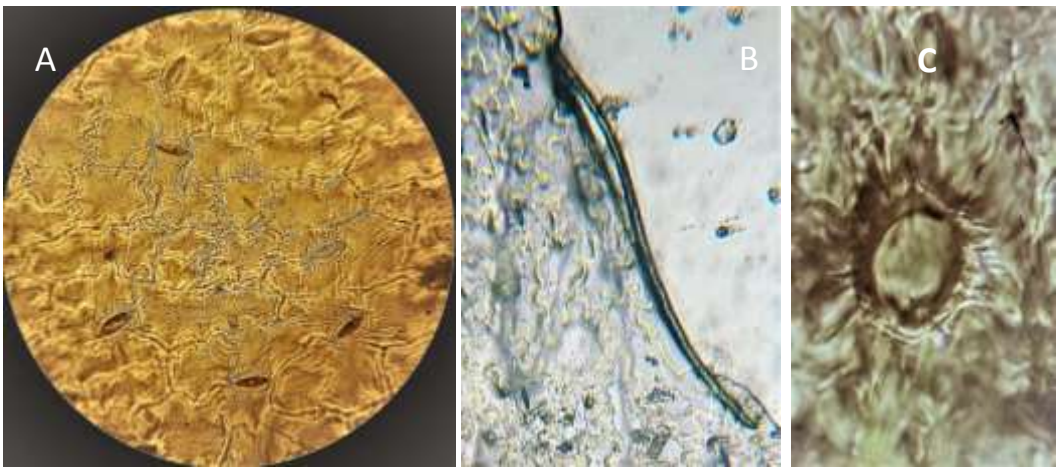

Fig. 7. Ventral (lower) surface of cotyledon showing stomata associated with varying number of neighbouring cells (NCs, indistict) and epidermal cells of of irregular shape (A), a long non-glandular trichome with pointed apex (B) – anticlinal walls are sinuous. Trichomal scar (C). Magnification: A, 45 x 10X, zoom 1.2X; B, 45 x 10 X and C, 45 x 10 X, zoom 2.0X).

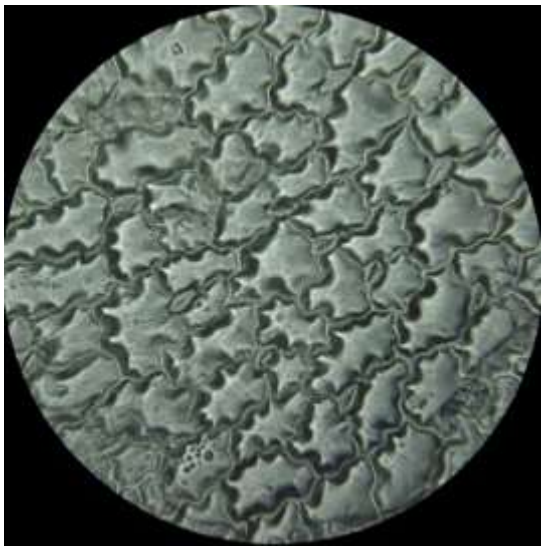

Fig. 8. Dorsal surface of a lateral leaflet showing stomatal distribution. Anticlinal contour of epidermal cells is irregular and periclinal surface convex and cell wall sloping down along the cell margin. Stomata associated with varying number of NCs (indistinct). Magnification: 45 x 10 X, zoom 1.3X.

Stomatal density on Lateral Leaflets: STD on dorsal surface of lateral leaflet averaged to 97.81 ± 2.19 (N = 60, varying from 24.49 to 137.62, CV = 17.30). The modal class occupied a proportion of 56.7 % of the total observations. STD on the ventral surface of lateral leaflet averaged to 113.6 ± 3.17, somewhat denser than that on dorsal side. It varied from 49.15 to 176.94 (N 80, CV = 24.93%). Around 76.7% of the total observations (N = 60) had 76-150 stomata per mm^2 on dorsal side and c 56 % of observations had STD in a size class of 76-100 stomata per mm^2 on ventral surface (Table 6). Stomatal ratio (adaxial / abaxial) *sensu* Kirkham (2014) was 0.861 on lateral leaflet.

Stomatal density on Terminal Leaflets: On dorsal surface of terminal leaflet, in our studies, STD averaged to 106.65 ± 1.78 (varying from 68.81 to 157.28, CV= 16.7%). The modal class (76-100 stomata per mm^2) occupied a proportion of 42.0 % (N = 100). On ventral surface, STD averaged to 113.44 ± 2.42 varying from 58.9 to 176.9 (CV = 21.3%). The class occupying 76-150 STD had 88% of the total observations (N = 100). STD on both surfaces was symmetric in distribution (Table 6). Stomatal density in *T. foenum-graecum* has been reported to be 92 stomata per mm^2 by Anitha and Priyadarshini (2012) from India. In our case stomata were not only larger in number on ventral surface but somewhat larger than the the estimate of Anitha and Priyadarshini (2012). This appears to be related to the stomatal index somewhat higher on ventral surface (12.69%) than on the dorsal surface ((Kadam, 2019). Rashid *et al.* (2019) reported stomatal index in *T. foenum-graecum* to be 83%. In present studies, the stomatal ratio *sensu* Kirkham (2014) was found to be 0.940.

The lateral as well terminal leaflets were, therefore, isostomatous in *T. foenum-graecum*. The structure and development of foliar stomata and stomatal density are age - related phenomena. Stomatal density is known to decline in 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, 2019a). 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.

Environmental factors are known to cause changes in stomatal distribution. Warming causes stomatal distribution in maize to be more regular. High temperature significantly decreases the average nearest neighbour distance between stomata on both surfaces of leaves (Zheng *et al.*, 2013).

Cotyledonary stomatal size

Stomtal length was significantly ($t = 6.195$, $p < 0.001$) larger (35.18 ± 0.697 µm) on sun-exposed dorsal surface of cotyledon than that on the ventral surface (29.22 ± 0.643 µm). Similarly, stomatal width was significantly ($t = 6.77$, $p < 0.001$) larger on dorsal surface (18.67 ± 0.452 µm) than that on the ventral surface (14.30 ± 0.462 µm) (Table 7).

Foliar stomatal size:

Stomatal size (L x W) of lateral and terminal leaflets averaged to as follows (Table 8):

Lateral leaflet (Dorsal): 22.88 ± 0.525 x10.53 ± 0.377 µm.
Lateral leaflet (Ventral): 23.40 ± 0.403 x 10.97 ± 0.322 µm.
Terminal leaflet (Dorsal): 22.57 ± 0.349 x 10.68 ± 0.374 µm.
Terminal leaflet (Ventral): 21.43 ± 0.529 x 10.58 ± 0.286 um.

Stomatal length on dorsal and ventral surfaces of lateral leaflet didn't vary significantly from each other (t = 0.7225, NS). Similarly, stomatal width didn't vary significantly from that of ventral surface (t = 0.8558, NS).

The parameters of length and width of stomata were insignificantly different between dorsal and the ventral surface of the terminal leaflet (t = 0.1758, NS and t = 0.6309, NS), respectively. The stomatal size in colchicine treated plants of *T. foenum-graecum* was reported to be larger than control diploid plants i.e. stomata measured 13.20 ± 1.01 x 10.21 ± 0.70 µm in diploid plants and 14.83 ± 0.94 x 12.25 ± 0.73 um (Omezzine *et al.,* 2012). The stomata of our accession was obviouly larger than that reported by Omezzine *et al.,* 2012). The stomatal pore in *T. foenum-graecum* is reported to be 9.5 (9.71) 10.1 µm in length and 1.59 (2.28) 2.41 µm in width (Mukhtar and Riaz, 2021).

Table 6. Stomatal density per mm^2 on cotyledon and leaf of *T. foenum-graecum*.

Statistical Parameters	Cotyledon		Lateral leaflet		Terminal leaflet	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
N	60	50	60	80	100	100
Mean	51.93	50.43	97.81	113.55	106.65	113.44
SE	1.718	2.1701	2.1846	3.165	1.780	2.418
Median	49.149	49.149	98.298	117.957	108.127	108.127
CV (%)	25.63	30.47	17.30	24.93	16.67	21.31
G1	- 0.031	-0.327	-0.931	0.265	0.249	0.223
Sg1	0.309	0.337	0.309	0.269	0.241	0.241
G2	-0.383	0.002	3.272	-0.134	-0.116	0.058
Sg2	0.608	0.662	0.608	0.532	0.478	0.478
Minimum	19.66	9.83	29.49	49.15	68.81	58.98
Maximum	78.64	78.84	137.62	176.94	157.28	176.94
K-ST	0.152	0.187	0.129	0.108	0.121	0.117
P	0.002	0.0001	0.014	0.023	0.001	0.002
Sh-Wilk	0.950	0.951	0.910	0.970	0.968	0.978
P	0.016	0.038	0.0001	0.059	0.017	0.094
Distribution	AS	AS	AS	AS	AS	AS

Acronyms: SE, Standard error of mean; CV, Coefficient of variability (%); G1, Skewness; Sg1, Standard error of skewness; G2, Kurtosis; Sg2, Standard error of kurtosis; K-S T, Kolmogorov-Smirnoff test with Lilliefors significance correction for normalcy; Sh-Wilk, Shapiro-Wilk test for normalcy.

Table 7. Length and width (µm) of Cotyledonary stomata of *T. foenum-graecum*.

Statistics	Cotyledon dorsal		Cotyledon ventral	
	Length	Width	Length	Width
N	30	30	30	30
Mean	35.10	18.668	29.224	14.30
SE	0.69665	0.45178	0.64313	0.46158
CV%	10.87	13.26	12.05	17.68
Minimum	24.96	15.60	21.84	9.36
Maximum	40.56	23.40	37.44	20.28

REMARKS

Taken together the results, the micromorphology of *T. foenum-graecum* seedlings exhibited following features. 1) Periclinally convex epidermal cells with sinuous anticlinal wall (waviness of epidermal cells in terms of number of wave crests per cell averaging to 8.04 ± 0.106 (N = 215, CV = 19.33%). 2) There were two types of trichomes – simple non-glandular unicellular pustulate trichomes on cotyledons, petioles and lamina and margins of leaflets and capitate glandular trichome on basal hypocotyl. 3) Cotyledons and leaflets were amphistomatous and s*ensu* Kirkham's (2014) nomenclature, they were isostomatous. Stomata were elliptical to wide elliptical, oriented in various directions but sometimes aligned in their long axis. As per Ditcher's (1974) classification, there was only one type of stomatal complex in *T. foenum-graecum* i.e., anomocytic type. 4) *Sensu* Prabhakar's (2004) classification, there were four types of stomatal complexes – Anisocytic, tetracytic, staurocytic and anomocytic owing to the fact that Prabhakar considers NCs equivalent to SCs - an artificiality. A similar situation was observed by Khan and Zaki (2020) in *Helianthus annuus* var. US 666 wherein only anomocytic stomata (indistinct NCs) was identifiable on the basis of Ditcher's scheme, but four types of stomatal complexes were found based on Prabhakar's scheme – predominantly tetracytic, anisocytic, anomocytic and staurocytic. For clarity, definitions of stomatal complexes recognized in *T. foenum-graecum* as per two systems is given in Appendix I.

A great degree of stomatal diversity has been reported in papilionaceae by various authors. Tribe Trifolieae is reported to exhibit paracytic (more frequent on stem and petiole), anisocytic, anomocytic (more frequent on leaflets) and haplocytic stomata (Shah and Kothari, 1975) on the basis of classical stomatal identification keys. Among 40 papilionaceous species of tribe Hedysareae, the most frequent stomata were reported to be paracytic except *Zornia* where it was anisocytic and in *Aeschynomene*, *Alhagi* and *Taverniera* where it was anomocytic (Kothari and Shah, 1975). Taia (2004) described stomata in Trifolieae based on Dilcher (1974) terminology including nine species of *Trigonella*. He described paracytic, diacytic and anomocytic stomata in Egyptian species (Table 8). Mukhtar and Riaz (2021) have studied leaf architecture as an aid to delimit species of *Trigonella* in Pakistan. They reportedly followed Metcalfe and Chalk (1950) to delimit stomata. They described anisocytic stomata in three species of *Trigonella* viz. *T. foenum-graecum*, *T. gharuensis*, and *T. gracilis*, anomocytic stomata in ten species viz. *Trigonella* species such as *T. anguina*, *T. cachemiriana*, *T. corniculata*, *T. emodi*, *T. Fimbriata*, *T. hamosa*, *T. monocantha* subsp. *monocantha*, *T. incisa* ssp. *monocantha* and *T. podperae*, tetracytic stomata in *T. occulta* and paracytic stomata in *T. pubescence* (Table 5). The stomatotype identification in at least *T. foenum-graecum* by Mukhtar and Riaz (2021) appears to be faulty in view of indistinctness of stoma-abutting-cells. Anitha and Priyadarshini (2012) while evaluating *T. foenum-graecum* for its pharmacognostic characterization on the basis of stem and leaf, also mistakenly described it to have anisocytic stomata, in spite of the fact that neighbouring cells abutting pore were indistinct. On the other hand, Patil *et al.* (2015) characterized the aerial parts of this species to bear anomocytic stomata, although they have not clearly mentioned the scheme of stomatal classification followed in their studies. It may frequently be observed that many publications even never specify any key of stomatal classification followed in their studies and several workers do not strictly adhere to the scheme carefully. People need to be more careful while working for stomatal identification.

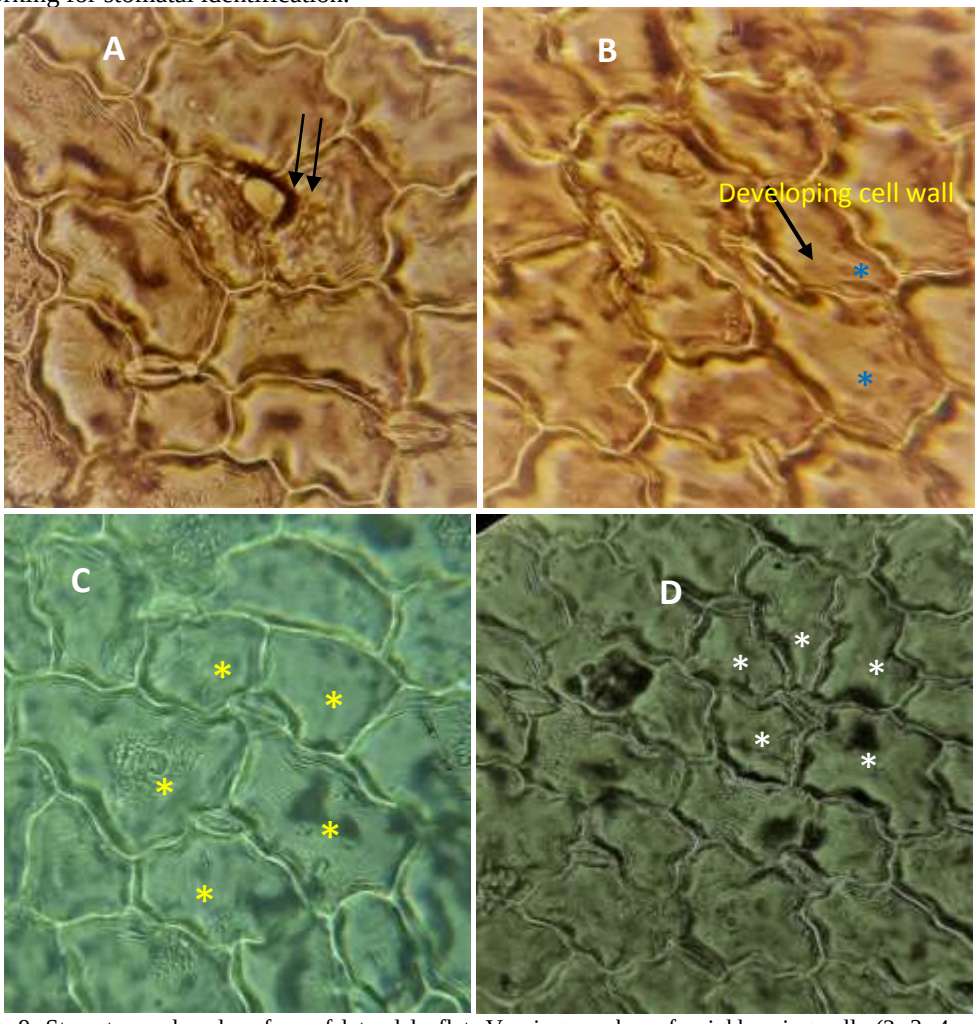

Fig. 9. Stomata on dorsal surface of lateral leaflet. Varying number of neighbouring cells (2, 3, 4 and 5) with common NCs between the stomata. A) A stoma without development of guard cells (shown by two arrows). B) A stoma abutted by two

cells may be expected to transform in three abutting NCs in view of a developing cell wall. C) Of the two adjacent stomata with common NCs - One stoma with 4NCs (two conjoint walls polar and other two lateral) and the other stoma with 3NCs. D) Five NCs apparent in a stoma (D).

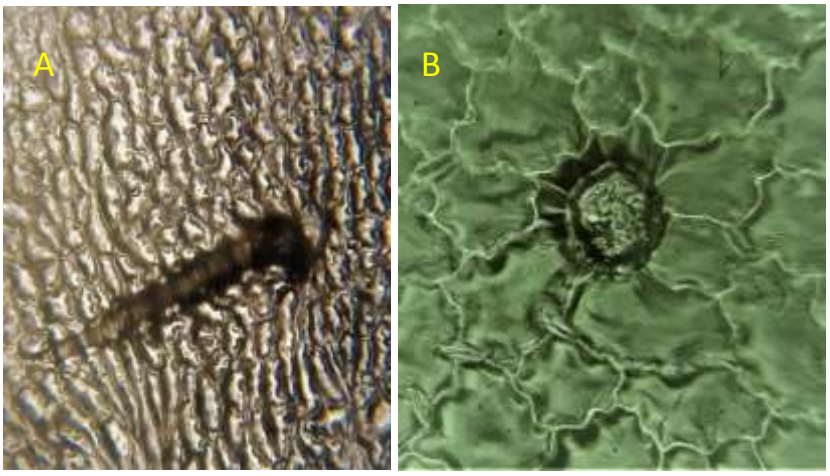

Fig. 10. Ventral surface of a lateral leaflet. A trichome (A) and Scar of a deeply broken trichomes clearly showing the basal pavement cells (B) – some stomata are also visible with 3 or 4 NCs. Epidermal cells are irregular to sinuous.

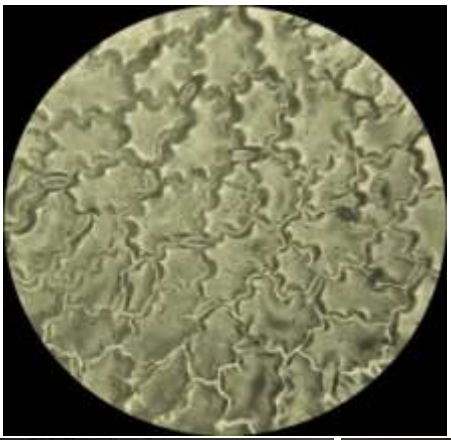

Fig. 11. Stomatal distribution on ventral surface of lateral leaflet. Neighbouring cells abutting stoma vary from three to five. The epidermal cells are sinuous in anticlinal contour. They are convex at periclinal surface and at margins their walls slope down to join the low-lying walls.

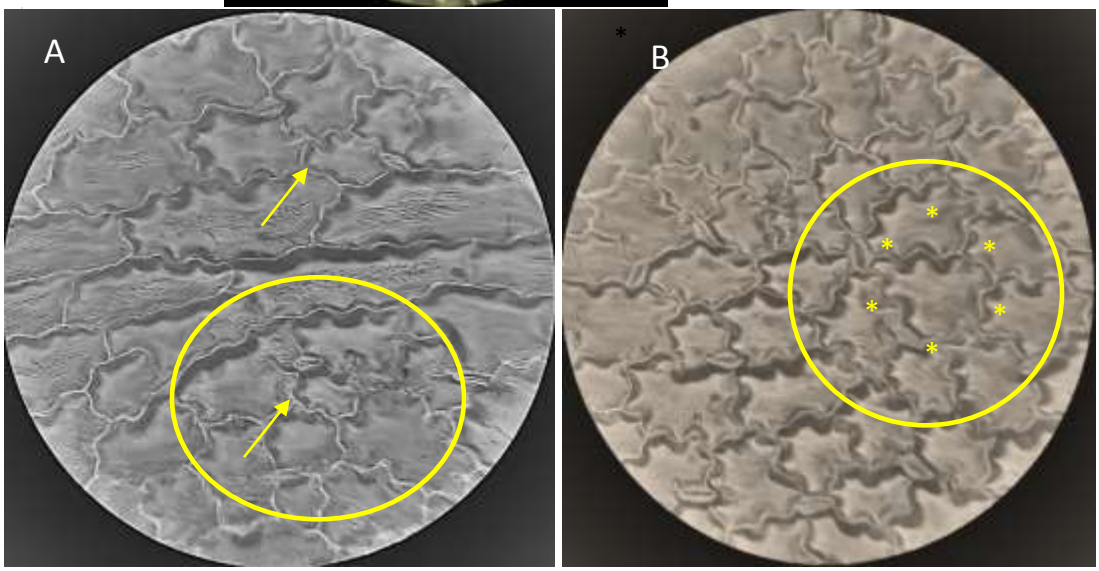

Fig. 12. A) Ventral surface of lateral leaflet showing vascular region composed of elongated cells with cuticular striations parallel in form of bands and stomata near vein with a developing cell walls (arrow-pointed). B) Ventral surface of a lateral leaflet showing a stoma abutted with six NCs.

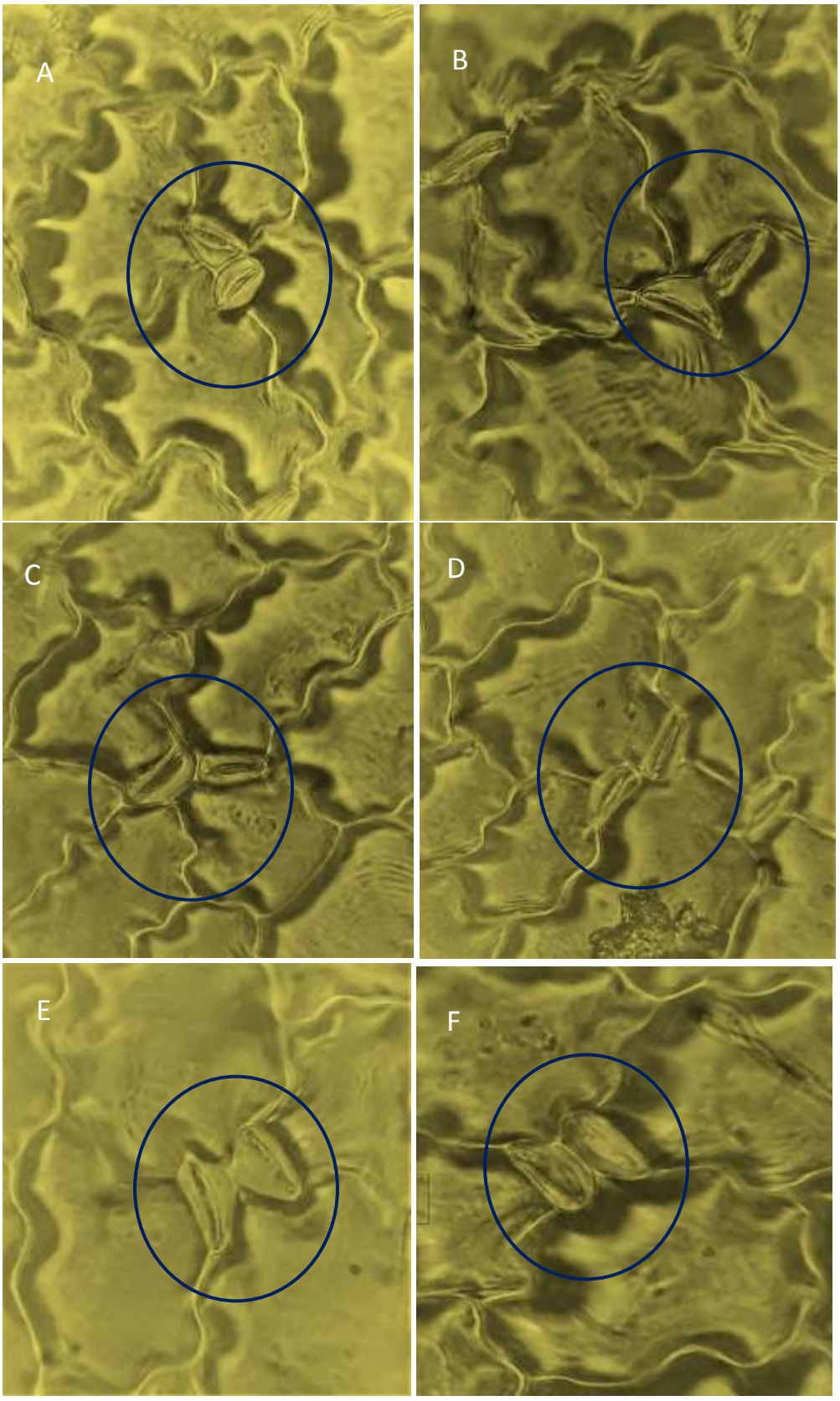

Fig. 13. Contiguous stomata on ventral surface of lateral leaflet of *T. foenum-graecum.*

The stomata lie at various angles to each other. They were juxtaposed (A), oriented at right angle (B), oriented at almost 180^{o} to each other (C), almost superimposed (D), lying by touching their lateral walls (E) and the two stomata lying like that of E but opposite with respect to their polarity. The contiguous pair at B is unique in the sense that one stoma shows its frontal (top) view and the other the lateral view.

The neighbouring cells in each case are sinuous at anticlinal contour and periclinally convex and laterally sloping down.

Magnification: 45 x 10x, zoom 4X.

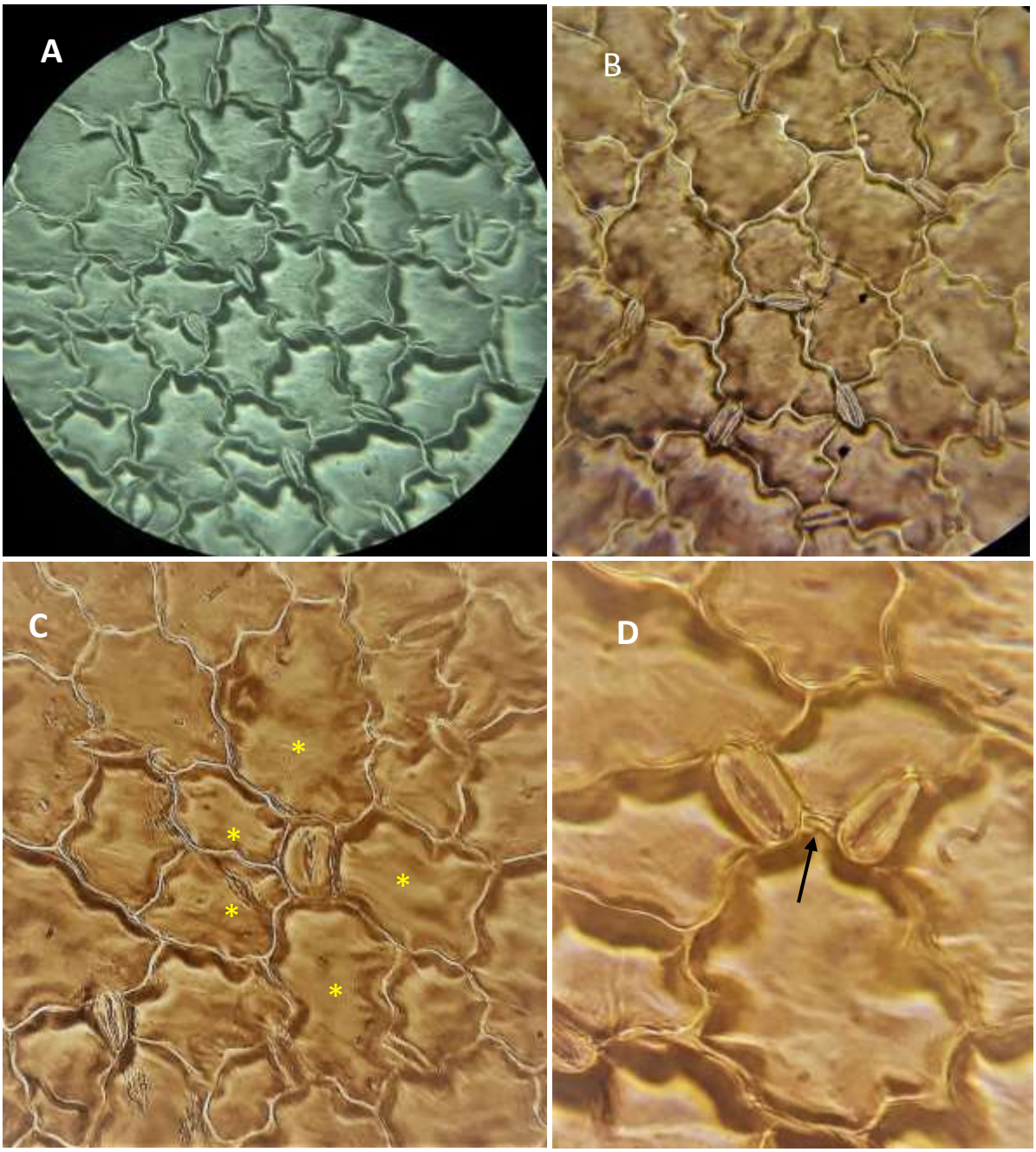

Fig. 14. Ventral surface of terminal leaflet showing stomata with three to five Neighbouring cells and sinuous type of epidermis (A and B), giant stomata with five NCs (C) and possibly a cytoplasmic connection (D, shown with an arrow) between two adjacent stomata – one with three NCs and the other with four NCs. Magnification: A, B and C, 4 x 10X and D, 45 x 10X, zoomed 1.6X).

Several terminologies in structural-morphogenetic classifications of stomata were given in classical publications of Metcalfe and Chalk (1950), Stace (1965), Tomlinson (1969), Van Cotthem (1970), Dilcher (1974), Wilkinson (1979), Carpenter (2005), etc. rendering more than 30 different patterns of stomata and subsidiary cells in vascular plants. All these systems gave emphasis on distinctiveness of epidermal cells surrounding the guard cells. Van Cotthem (1970) refers to cells abutting the stoma as neighbouring cells (NCs) if they are not different from the other epidermal cells in shape and size. Only if cells abutting guard cells differ from epidermal ground cells in shape and size, they are considered to be distinct and referred to as subsidiary cells (SCs) – a criterion followed in this paper. Indistinct surrounding cells render subsidiary cells as zero in number, a characteristic of anomocytic stomata (cf.

Van Cotthem, 1970; Dilcher (1974 or other similar classifications). Survey of literature reveals that the most widely used stomatal classificatory system is that of Metcalfe and Chalk (1950) or Dilcher (1974) and a great deal of data on stomatal complexes have accumulated over last many years on the basis of these schemes.

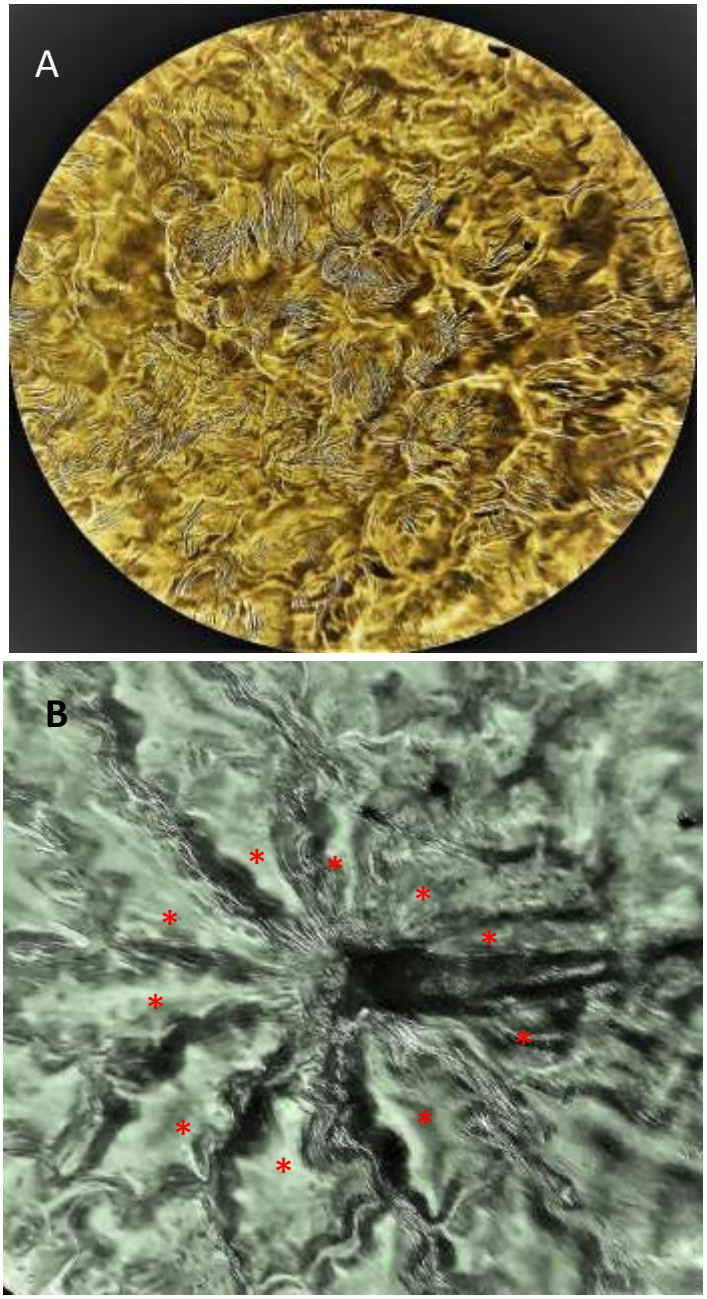

Fig. 15. Ventral surface of mature terminal leaflet. A) Thick cuticular sheet deposition and striations at some places masking stomatal and epidermal cellular integrity. B) A conical trichome showing ten basal pavement cells and the striations.

Table 8. Length and width (µm) of foliar stomata of *T. foenum-graecum.*

Statistics	Lateral leaflet				Terminal leaflet			
	Dorsal surface		Ventral surface		Dorsal surface		Ventral surface	
	Length	Width	Length	Width	Length	Width	Length	Width
N	30	30	30	30	30	30	30	30
Mean	22.880	10.53	23.400	10.972	22.568	10.680	21.43	10.58
SE	0.52535	0.37724	0.40279	0.32157	0.34869	0.37239	0.54930	0.2858
CV%	12.55	19.62	9.429	16.05	8.46	19.09	14.04	15.68
Minimum	18.77	7.80	18.72	6.24	18.72	7.80	17.16	7.80
Maximum	29.64	15.60	28.08	12.48	24.96	15.60	28.00	16.38

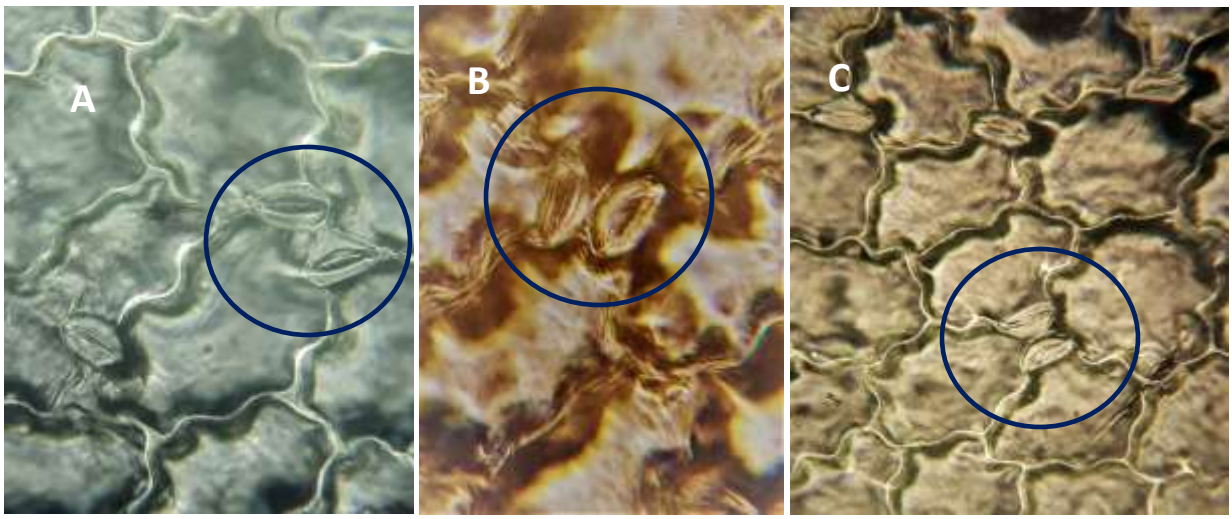

Fig. 16. Contiguous stomata on ventral surface of terminal leaflet.

Appendix I. Definitions of some stomatal complexes as per two systems for nomenclature of stomatal complexes.

Stomatal complex	Dilcher's (1974) Scheme	Prabhakar's (2004) Scheme
Anomocytic	Indistinct NCs*, variable in number, abutting the guard cells.	Four or more SCs or NCs or SCs + NCs abutting guard cells arranged in position other than tetracytic and staurocytic stomata.
Anisocytic	Three distinct SCs* (may be unequal in size.	Three SCs or NCs or SCs + NCs abutting guard cells variable in shape and size with one of them smaller (anisocytic), if one NC or SC is distinctly larger (anisotricytic) and if 3NCs or SCs or NCs +SCs are more or less equal in size (Isotricytic)
Tetracytic/ paratetracytic / Brachyparatetracytic	Four SCs abutting guard cells, Two smaller polar, two larger lateral in position are called paratetracytic whereas stomata with 4SCs - two larger polar and two smaller lateral are called as Brachyparatetracytic.	Four SCs or NCs or SCs + NCs abutting guard cells, variable in size and shape. Two polar and two lateral in position. It is called tetracytic which may be mono- or polycyclic.
Staurocytic	Four SCs* abutting guard cells, Two conjoint walls of SCs polar and two conjoint walls of SCs lateral	Four SCs or NCs or SCs + NCs abutting guard cells; Two conjoint walls are polar and two conjoint walls are lateral

*, sensu Van Cotthem (1970).

Prabhakar (2004) presented an over- simplified classificatory artificial approach on the subject and proposed eleven basic types of stomata on morphological basis of mature stomata not considering their ontogenetic pathways. 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 were considered subsidiary cells by virtue of their position, whether they differentiate from remainder epidermal cells or not – a diametrically opposite approach to the previous classical schemes. This system has been used by some workers owing to its simplicity (to cite few publications – Wang *et al.*, 2006; Chengqi *et al.*, 2007; Lüttage *et al.*, 2009; Huang *et al.*, 2010; Ekeke and Agbagwa, 2015; Khan and Zaki, 2019 a and b; Khan *et al.*, 2018, 2020). In his paper, Prabhakar described early history of stomatal nomenclature and admitted that the most widely accepted and used system in this regard was given by Metcalfe and Chalk (1950). He then, enumerated the mistakes made in identifying stomata (particularly paracytic stomatal complex) by several researchers and discussed shortly the views of scientists such as Metcalfe and Chalk (1950), Stace (1965), Cronquist (1968), Fahn (1969), Paliwal (1969a), Rajgopal (1973), Dilcher (1974), Cutter (1978), Esau (1979) and Wilkinson (1979). Then, he discussed subsidiary (distinct) cells and neighbouring cells (indistinct). He argued that the criteria to judge distinctness was left to subjective judgment of observers and

declared that the view of tracing evidence in herbarium and fossil specimens suffers from impracticability and suddenly came to conclude that the cells surrounding stoma (distinct or indistinct) should be regarded as subsidiaries by virtue of their location (opposite to the view of NCs of Van Cotthem (1970) and several classic and modern workers). In his scheme this criterion was, however, only applied to the first cycle of cells abutting guard cells. In second order cycle indistinct cells were not considered part of cycle and only distinct cells formed the basis of nomenclature as exemplified by several figures in his paper (e.g., Figs. 45-51, 71-78, 109, 111,121,133, 134, 140-142, 145,146, 157 170). The Prabhakar's system thus suffers from irrationality and artificiality and ignores information on stomatal morphogenesis, the valid base for taxonomic conclusions (Kondo, 1962; Pant, 1965; Stace, 1965, 1966, 1984; Maroti, 1966; Metcalfe and Chalk, 1950; Tomlinson, 1969, 1974; Van Cotthem, 1970; Fryns-Claessens and Van Cotthem, 1973; Dilcher, 1974; Wilkinson, 1979; Rasmussen, 1981, Willmar and Fricker, 1996, Carpentor, 2005). It also made the comparison of the results obtained impossible with the literature accumulated over more than 70 years regarding identification of stomatal complexes in countless taxa using the popular structural-morphogenetic classifications. Instead of improving the nomenclature system, it has oversimplified it to the level of unacceptability. It appears imperative that taxonomists should reach a consensus on use of a specific scheme that may facilitate comparison with the already accumulated literature over years. Of course, ontogenetic studies should take a great deal of labour and time. The scheme of Prabhakar (2004) makes it artificially easy to apply but renders the results not comparable to the vast amount of data accumulated on the subject on the basis of classical schemes. It is obvious that study of stomata should not ignore their ontogenetic and morphogenetic peculiarities. The study at seedling stage may provide information on stomatal ontogeny.

Prabhakar's basic approach of treating NCs equivalent to SCs raises certain very pertinent questions regarding their true anatomical, structural, morphogenetic, physiological and molecular status. A number of publications regarding molecular aspects of stomatal development, their structure and function have appeared in recent years (Nadeau and Sack, 2002; Mumm *et al.,* 2011; Vatén and Bergman, 2012; Peterson, 2013; Hashimoto-Sugimoto *et al.,* 2013; Higaki *et al.,* 2014; Gray *et al.,* 2020, Chowdhury *et al,* 2021). The basic molecular framework in *Arabidopsis thaliana* for cell fate and generation of cell polarity and key signals and receptors required to produce stomata in organized pattern and environmentally optimized numbers is well understood (Vatén and Bergman, 2012). Nadeau and Sack (2002) discussed development of stomata in *A. thaliana*. TOO MANY MOUTHS (TMM) is reported to function in receiving or transducing the cues to orient stomatal divisions. TMM also is a regulator of entry into stomatal pathways. FOUR LIPS (FLP) controls the number of symmetric divisions at the guard mother cell stage. Any mutations in TMM affects clustering of stomata and increased precursor cell formation and mutation in FLP results in many paired stomata in cotyledons of *Arabidopsis* (Yang and Sack, 1995).

Little progress has, however, been made toward understanding of SCs. The subsidiaries and their significance have recently been reviewed by Gray *et al.* (2020). It is evident from literature that taxonomists, anatomists, physiologists and developmental biologists have different perspective on definition of SCs. SCs are specialized cells and share mechanical linkage with guard cells and facilitate their movement by acting as reservoir of water and ions (Lawson and Matthews, 2020). Gray *et al.* (2020) have defined SCs in broader perspective as "*cells that are adjacent to guard cells (not necessarily touching) and are distinct from other epidermal cells*." The "distinction" is identified by unique morphology or unique molecular signature (genes or proteins expressed). The number of SCs and their morphology varies dramatically in morphology and ontogeny and so their functions – mainly three i.e., mechanical in facilitating guard cells movement, or as reservoir for water and ions and in some cases enhancing the unique morphology such as sunken stomata affecting gas exchange. Gray *et al.* (2020) have discussed three potential roles to SCs. – anatomical (to raise and lower guard cells relative to epidermal cells), mechanical (laterally moving guard cells overcoming large mechanical advantage to open stomata to full extent and molecular (Initiation of stomatal opening by the activation of Hþ-ATPases in the guard-cell plasma membrane and the role of the translocation factor of plasma membrane proton pump (PATROL 1).

Two molecular models of stomatal structure and functions have been studied.

1) In model species *A. thaliana* – subsidiary cells are subtle in morphology. The guard cells-abutting cells are unequal in size and variable in shape with two types of arrangements – Anisocytic in 30-60% cases (with one smaller SC) and anomocytic (irregularly shaped NCs) i.e., not every stomatal complex within the same *A. thaliana* leaf includes subsidiary cells (Nadeau and Sack, 2002). It was hypothesized that molecular markers in this case may be a good way to identify subsidiary cells. Gene specific expressions may be considered as evidence supporting their identity distinct from other epidermal cells, which may in turn be indicative of a unique function. The gene expression patterns of PATROL 1 (translocation factor of the plasma membrane proton pump ATPase) in *Arabidopsis* were studied via β-glucoronidase (GUS) reporter assay with PATROL 1 pro: GUS transgenic lines by Hashimoto-Sugimoto *et al.* (2013). GUS -positive cells were observed in small subsidiary cells adjacent to guard

cells. PATROL1 controls protein trafficking including that of the plasma membrane proton pump AHA1, so important for guard cell function (Hashimoto- Sugimoto *et al*., 2013). Higaki *et al.* (2014) investigated PATROL 1, as a translocation factor of the plasma membrane in guard cells and subsidiary cells in *A. thaliana*. Their epifluorescence microscopic studies suggested that PATROL 1 may contribute to stomatal movement by translocation of PMH+-ATPase in subsidiary cells. This clearly indicated that these cells have a unique molecular identity and should be considered part of the stomatal complex. Additional molecular markers of subsidiary cell fate should help to clarify if (and which) guard-cell adjacent cells have identities distinct from other epidermal cells.

2) In Z*ea mays* and other grasses subsidiary cells are always in pairs flanking the guard cells (paracytic arrangement). They are uniquely shaped, are more pectin-rich and are therefore readily identified. In this species, as early as 1970, Cl^- and K^+ shuttle between guard cells and subsidiary cells was demonstrated by Raschke and Fellows (1970). The subsidiary cells being morphologically obvious, were supposed to have potential molecular markers of their identification. A SWEET-family protein is expressed in subsidiary cells (Wang *et al.,* 2019). A gene encoding a specific Shaker-family potassium channel is also specifically expressed in maize subsidiary cells (Büchsenschütz *et al.,* 2005). Whether expression of these genes and subsidiary cell identity in general is conserved across plant species is unknown. Gray *et al.* (2020) have predicted that while some characteristics might be preserved, there is likely to be a large variation in the molecular components within subsidiary cells since they are varied in morphology, size, and ontogeny. A more thorough understanding of subsidiary cell function will help in to understand their significance in accurate classification. One thing is clear that we still need to explore if SCs are equivalent to NCs biologically.

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