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COMPARATIVE FOLIAR MICRO MORPHOLOGICAL STUDIES OF TRIBE ARUNDINEAE, ARISTIDEAE AND CHLORIDEAE FROM MALAKAND AGENCY, KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract

Six (6) species of three tribes Arundineae (*Arundo donax* L. & *Phragmites karka* (Retz.) Trin. ex Steud.), Aristideae (*Aristida adscensionis* L. & *Aristida cyanantha* Nees ex Steud.) and Chlorideae (*Cynodon dactylon* (Linn.) Pers.& *Tetrapogon villosus* Desf.) were investigated for their foliar micro-morphological variation and its taxonomic significance through Light and Scanning Electron microscope. The three tribes has a great diversity in the micro-morphological features i.e. stomatal type number, size, shape, stomatal density (SD), silica bodies, macrohairs, micro hairs, epidermal cell number, epidermal cell density (ECD), subsidiary cells, prickles, hooks, papillae, short and long cells on both abaxial and adaxial surfaces. In species of Arundineae paracytic stomata were observed, in Aristideae tetracytic type of stomata were observed, while in Chlorideae both paracytic and teracytic stomata were seen. The Arundineae has maximum stomatal number (Mean 21.4 to 46.2) and epidermal cell (Mean 51.6 to 77.2). These foliar micro-morphological characters were found significant in the delimitation and systematics of these species and can be used as tool in the identification and assessment of taxonomic problems at species and tribe levels.

Key words: Poaceae, Stomata, Taxonomy, micromorphology, SEM, Malakand Agency.

Introduction

The foliar epidermal studies are important for classification, delimitation and for sorting out the evolutionary and phylogenetic problems (Stace, 1984; Chaudhri *et al*., 2014). Morphological characters play significant role in the identification of all levels of taxonomic ranks (families, tribes, genera and species etc.) but as grasses do not flower for a greater part of their life cycle, the foliar epidermal studies are useful in the complete systematics and phylogenetic relationships of the taxa and second in importance after cytology (Stace, 1984; Nwokeocha, 1996; Nazir *et al*., 2013). The Poaceae (Gramineae) commonly known as grass family, which comprises about 11,000 species and 700 genera, 60 tribes and six sub families are widely distributed around the world (Ullah *et al.,* 2015; Chen & Peterson, 2006). The family is represented in Pakistan by 158 genera and 492 species in 26 tribes and five sub families (Cope, 1982). The epidermis consists of various types of functionally specialized cells and play vital role in restricting water loss, regulate gaseous exchange, defense, attract pollinators, photosynthesis, transpiration, respiration, mechanical strength and flexibility. Palmer and Tucker (1981) also observed that foliar epidermal features were useful in the systematics and characterization within subfamilies and tribes. Many leaf epidermal characters such length and shape of epidermal cells, stomata, stomatal type, papillae, prickle hairs, macro- and micro hairs, hooks, margins and silica bodies are taxonomically informative and can be used as an important tool in the delimitation of grasses (Prat, 1932; Metcalfe, 1960; Ellis, 1979; Petronela & Nevena, 2010). Watson & Dallwitz (1992) reported detailed description of the leaf epidermis in numerous taxa, pointing out the significance of these characters in the systematics of the Poaceae. The anatomical and morphological studies are useful for solving taxonomic problems of monocots (Bibi *et al.,* 2015; Gillani *et al.,* 2003). The foliar micro-morphology in recent years becomes more significant due to their specificity at subfamily, tribe and generic level (Desai & Raole, 2013). Palmer & Gerbeth-Jones (1986, 1988) have investigated the East African grasses foliar micro-morphological by SEM in different publications for specific tribes. Desai & Raole (2013) carried out studies of leaf micro-morphological features .i.e. epidermal cells, stomata, cuticle, surface contours, roughness and ornamentation, of 14 species at generic level belonging to 4 tribes of Bambusoideae and Pooideae from Gujarat, India. Shaheen *et al*., (2011) also found the foliar micro-morphological variation useful in the systematic delimitation of problematic taxa like *Setaria* spp. In the current study we investigated the variation foliar epidermal anatomy in three tribes of grasses and its implication in the identification of these complex tribes.

Material and Methods

The species of tribe Arundineae, Aristideae and Chlorideae were collected from Malakand Agency during March 2014 to April 2015. The Malakand agency with a total area of 952 square kilometers is situated in the Northern area of Pakistan in the KP province and lies

between 34^0 $22^/$ to 34^0 $41^/$ North latitudes and 71^0 $37^/$ to 72^0 $14^/$ East longitudes. The climate of the area is dry sub-tropical. The soil of Malakand is sandy-loam with gravel layers/loams developed from old/sub-recent piedmont materials. Five species from each were collected and observed for SEM and Light micro-morphological studies. The collected plant specimens were pressed, documented, dried, identified and mounted on standard herbarium sheets. The voucher specimens were deposited in the Herbarium of Centre of Plant Biodiversity and Botanical Garden, University of Peshawar (UPBG).

Leaf micro morphological studies: Leaf samples were prepared according to the modified method of Cotton (1974) who followed Clark (1960) technique. Fresh leaves were taken from vigorously growing plants and immersed in water for 2 hours in order to prevent them from drying. Epidermal strips were removed from both upper and lower epidermis from these leaves by simple peeling method, nail polish or by scrapping method. Sometime the dense venation leaves were fixed in formalin acetic acid, alcohol and IAA, having ratio of 1:1:3 (Farmer's fluids) and then immediately it was stored in 70% alcohol. For microscopic study the slides were prepared, both for adaxial and abaxial surfaces. Five samples of both adaxial and abaxial surfaces were prepared for each species and observed for different parameters. The epidermal peels were mounted in a glycerin jelly and stained by Delafield's Haematoxylin. These stained peels were examined under a compound microscope (Model NIKON ECLIPSE E200) and micro photographs were taken by polarize camera DCM35 350k pixel USB2.0 installed in Laboratory of Centre of Plant Biodiversity, University of Peshawar. Different micro-morphological characters of both abaxial and adaxial surfaces were studied at different magnifications (40X and 100X objectives). Measurements of all the epidermal components were done by standard micrometry. Terminology and classification for the epidermal cells are followed after Metcalfe (1960, 1971).

Scanning electron microscopy: For Scanning Electron Microscopy (SEM) the leaves of the dried, preserved specimens of grasses were taken. A section from the upper middle portion of mature undamaged leaf was cut for study. In case where the epidermis has a heavy coat of epicuticular wax was removed from both upper and lower surface of the section by soaking the material for 12 to 24 hours in Xylene. Two pieces of leaf were taken and mounted on stubs with double coated scotch tape. The one piece of leaf was mounted on stub from lower side in order to exposed upper surface and other piece was staked on stubs from upper side in order to exposed lower surface in stubs. The specimens were sputter-coated with gold-palladium and then observed under Scanning Electron Microscope (Model JEOL JSM-5910) installed Central Resource Library (CRL) Department of Physics University of Peshawar. The photographs were taken using Polaroid P/N 665 film. Each specimen was analyzed at the microscope using a standard check sheet of diagnostic features (stomata, macro-hairs, micro hairs).

Statistical analysis

a. Stomatal Index:

$$S.I = \frac{S}{E+S} \times 100$$

S.I= Stomatal Index.

S= No. of stomata per unit area.

E=No. of epidermal cells per unit area.

b. Stomatal density and epidermal cell density: The stomatal density was calculated according to the methods outlined by Ceulemans *et al*., (1995) and Teng *et al*., (2006). The numbers of stomata were counted in each field (0.0940 mm^2). The stomatal density (SD) and ECD are expressed as "the number of stomata and epidermal cells per unit leaf area". The SD and ECD were based on the observation of three samples.

c. Mean:

$M = \sum(X)/N$ ($\sum X$= Sum of observations, N= number of observations)

d. Standard deviation:

$S = \sqrt{S^2}$ (S^2= Variance of observations)

e. Variance:

$S^2 = \sum(X-M)^2/n-1$ (n= number of observations)

f. Co-efficient of variance:

CV= Sd/X (Sd= Standard deviation)

g. Standard Error:

SE= $S/\sqrt{n}$ (Sd= Standard deviation, n= number of observations).

Results and Discussion

Both Foliar epidermal surfaces of the collected six species of three tribes i.e. Arundineae, Aristideae and Chlorideae were investigated by light and scanning electron microscopy for variation in epidermal characters. In tribe Arundineae paracytic, in Aristideae tetracytic, while in Chlorideae both paracytic and tetracytic types of stomata were observed (Table. 1). In

all three tribes the lower surfaces have high number of stomata than upper surfaces. *Arundo donax* with highest Stomatal density on lower (521.28 mm^{-2}) surface, followed by *Phragmites karka* (446.81 mm^{-2}) and *Tetrapogon villosus* (260.64 mm^{-2}). *Arundo donax* (411.70 mm^{-2}) also has the highest stomatal density on upper surface followed by *Cynodon dactylon* (297.87 mm^{-2}) and *Phragmites karka* (255.32 mm^{-2}) as shown in Figure. 2. Number of stomatal rows between two costal zones varied between 1 to 3on both surfaces. *Arundo donax* and *Phragmites karka* with highest stomatal index on both surfaces (Table. 1). The largest and smallest stomata were observed on upper surfaces which varied in size from 27×10 µm (*Phragmites karka*) to 6.39×4.25 µm (*Aristida cyanantha*). The subsidiary cells were found triangular shaped in all species except in *Tetrapogon villosus* having high domed subsidiary cells (Figure. 6). The leaf epidermal studies showed that both species of tribe Arundineae has paracytic type of stomata with triangular shaped subsidiary cells, dumb bell shaped silica bodies. Micro hairs were present in *Phragmites karka* but not seen in *Arundo donax*. The micro hairs were also not observed in *Arundo donax* by Metcalfe (1960) and Ahmad (2009). Palmer & Tucker (1981), Renvoize (1986), Nazir *et al*., (2013) and Desai & Raole (2013) also have similar work and reported both *Arundo donax* and *Phragmites karka* had differences in leaf epidermal features i.e. long cells and macrohairs. The leaf epidermal studies of Aristideae showed the presence of tetracytic type of stomata, triangular subsidiary cells, and saddle shaped silica bodies. Papillae were absent, while hooks, prickles and micro hairs were present on both surfaces. Tateoka *et* a*l.,* (1959), Clifford & Watson (1977) and Ahmad (2009) reported similar epidermal features and while Metcalfe (1960) observed the presence of saddle shaped silica bodies and absence of micro hairs in their studies. The leaf epidermal studies of tribe Chlorideae revealed the presence of paracytic and tetracytic type stomata, rounded silica bodies, absence of macrohairs and presence in *Tetrapogon villosus* and *Cynodon dactylon*. These features were similar to the results of Prat (1934, 1961), Metcalfe (1960) and Ahmad (2009). Based on these foliar epidermal character a taxonomic key and dendrogram (Fig. 1) using past software (3.14).

Table-2: Showing the statistical co-relations of number of stomata and epidermal cells of upper and lower foliar epidermises of the species of Tribe Poeae and Triticeae.

Parameter	*Arundo donax*		*Phragmites karka*		*Aristida adscensionis*		*Aristida cyanantha*		*Cynodon dactylon*		*Tetrapogon villosus*	
Abaxial	**Stomata**	**Epidermal cells**	**Stomata**	**Epidermal cells**	**Stomata**	**Epidermal cells**	**Stomata**	**Epidermal cells**	**Stomata**	**Epidermal cells**	**Stomata**	**Epidermal cells**
Mean	35.8	51.6	21.4	53.2	18	78.2	9.8	106.2	25.8	94.8	20.6	64.6
Variance	6.56	23.44	5.84	13.36	5.2	24.56	2.96	19.36	2.16	2.16	4.24	4.64
Standard deviation	2.86	4.84	2.42	3.66	2.28	4.96	1.72	4.4	1.47	1.47	2.06	2.15
Co-efficient of variance	0.07	0.09	0.11	0.07	0.13	0.06	0.18	0.04	0.06	0.02	0.10	0.03
Standard error	1.28	2.42	1.21	1.83	1.14	2.48	0.86	2.2	0.74	0.74	1.03	1.08
Adaxial												
Mean	46.2	76.2	40	77.2	18.2	76	20.6	74.4	13.4	87	22	83.2
Variance	4.56	12.16	5.2	28.16	2.96	18	3.44	11.04	3.44	4	2	3.76
Standard deviation	2.14	3.49	2.28	5.31	1.72	4.24	1.86	3.32	1.86	2	1.41	1.94
Co-efficient of variance	0.05	0.046	0.06	0.07	0.10	0.06	0.09	0.05	0.14	0.02	0.06	0.02
Standard error	1.07	1.74	1.14	2.65	0.86	2.12	0.93	1.66	0.93	1	0.71	0.97

Conclusions

Significant variation micro-morphological characters like silica bodies, micro hairs, macrohairs, hooks, papillae, prickles, stomatal index and stomatal densities were observed that were of vital value in the identification and delimitation of closely related species of these species. The Scanning electron and light microscopy micro-morphological studies were found very helpful in the identification and classification of the species at the tribal and species levels.

Table 1. Showing the Foliar epidermal features of the species of Tribe Arundineae, Aristideae and Chlorideae.

Epidermal surface/ characters		Arundineae		Aristideae		Chlorideae	
		Arundo donax	***Phragmites karka***	***Aristida adscensionis***	***Aristida cyanantha***	***Cynodon dactylon***	***Tetrapogon villosus***
Abaxial				**Stomatal complex**			
	Stomatal length	19.46 µm	27 µm	8.67 µm	6.39µm	23.22 µm	17.25 µm
	Stomatal width	7.36 µm	10 µm	5.65 µm	4.25µm	8.6 µm	5 µm
	Stomatal type	Paracytic	Paracytic	Tetracytic	Tetracytic	Paracytic and Tetracytic	Paracytic and Tetracytic
	Stomata number	38	23	19	11	27	23
	Number epidermal cell	56	55	79	110	95	64
	Stomatal density mm^{-2}	411.70	255.32	233.40	113.48	297.87	244.68
	Epidermal cell density mm^{-2}	601.06	595.75	840.43	1180.85	1021.28	680.85
	Stomatal index	40.43	29.49	19.39	9.10	22.13	26.44
	Number of stomatal rows between two costal zones	2	2	2	3	2	1
	Subsidiary cell shape	Triangular	Triangular	Triangular	Triangular	Triangular	High dome
	Guard cells shape	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell
				Silica bodies and Short cells			
	Silica bodies shape	Dumb bell	Dumb bell	Saddle	Saddle	Rounded	Rounded
	Costal silica bodies	Present	Present	Present	Present	Present	Present
	Intercostal silica bodies	Absent	Absent	Absent	Absent	Absent	Absent
	Short cell wall shape	Sinuous	Sinuous	Round	Round	Sinuous	Sinuous
				Long cells			
	Size	134×8 µm	118×12 µm	84×7µm	32×7 µm	32×8 µm	62×10 µm
	Shape	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular
	Long cell wall shape	Thin sinuous	Coarsely sinuous	Sinuous	Thin sinuous	Sinuous	Sinuous
	Number of rows of long cells between two costal zones	3	6	4	4	6	2
	Macro hairs	Absent	Present	Absent	Absent	Absent	Absent
	Micro hairs	Absent	Present	Present	Present	Present	Absent
	Hooks	Present	Present	Absent	Absent	Absent	Absent
	Prickles	Present	Present	Present	Absent	Present	Present
	Papillae	Absent	Absent	Absent	Absent	Present	Present
Adaxial				**Stomatal complex**			
	Stomatal length	22.6 µm	20.64 µm	8.75 µm	7.80µm	26.6 µm	16.32 µm
	Stomatal width	8.4 µm	12.04 µm	4.65 µm	5.51 µm	12 µm	6.93 µm
	Stomatal type	Paracytic	Paracytic	Tetracytic	Tetracytic	Paracytic and Tetracytic	Paracytic and Tetracytic
	Stomata number	48	41	21	23	15	24
	Number epidermal cell	72	77	75	79	86	82
	Stomatal density mm^{-2}	521.28	446.81	234.04	234.04	168.09	260.64
	Epidermal cell density mm^{-2}	765.96	819.15	797.87	836.88	943.62	878.72
	Stomatal index	40.00	34.75	21.88	22.55	14.85	22.64
	Number of stomatal rows between two costal zones	2	3	3	2	2	1
	Subsidiary cell shape	Triangular	Triangular	Triangular	Triangular	Triangular	High dome
	Guard cells shape	Concave	Concave	Dumb bell	Dumb bell	Dumb bell	Dumb bell
				Silica bodies and Short cells			
	Silica bodies shape	Dumb bell	Dumbbell	Saddle	Saddle	Rounded	Rounded
	Costal silica bodies	Present	Present	Present	Present	Present	Present
	Intercostal silica bodies	Absent	Absent	Absent	Absent	Absent	Absent
	Short cell wall shape	Sinuous	Sinuous	Round	Round	Sinuous	Sinuous
				Long cells			
	Size	143×8 µm	136× 9 µm	135×6 µm	68×14 µm	35×15 µm	92×17µm
	Shape	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular
	Long cell wall shape	Thin sinuous	Coarsely sinuous	Sinuous	Sinuous	Sinuous	Sinuous
	Number of rows of long cells between two costal zones	5	6	8	4	6	3
	Macro hairs	Present	Absent	Absent	Absent	Absent	Absent
	Micro hairs	Absent	Present	Present	Absent	Present	Absent
	Hooks	Present	Present	Present	Absent	Absent	Absent
	Prickles	Present	Present	Present	Present	Present	Present
	Papillae	Absent	Absent	Absent	Absent	Present	Present

Key based on foliar micro morphological characters of leaf abaxial surface

1	+ Paracytic type of stomata on both surface	2
	- Stomatal type other than Paracytic on both surface	3
2	+ Long cells thin sinuous, micro hairs absent	*Arundo donax*
	- Long cells coarsely sinuous, micro hairs present	*Phragmites karka*
3	+ Tetracytic type of stomata on both surface	4
	- Paracytic and tetracytic type of stomata on both surface	5
4	+ Silica bodies saddle shaped, hooks present	*Aristida adscensionis*
	- Hooks absent, short cells with sinuous walls	*Aristida cyanantha*
5	+ Subsidiary cells triangular, micro hairs present	*Cynodon dactylon*
	- Micro hairs absent, Subsidiary cells high domed	*Tetrapogon villosus*

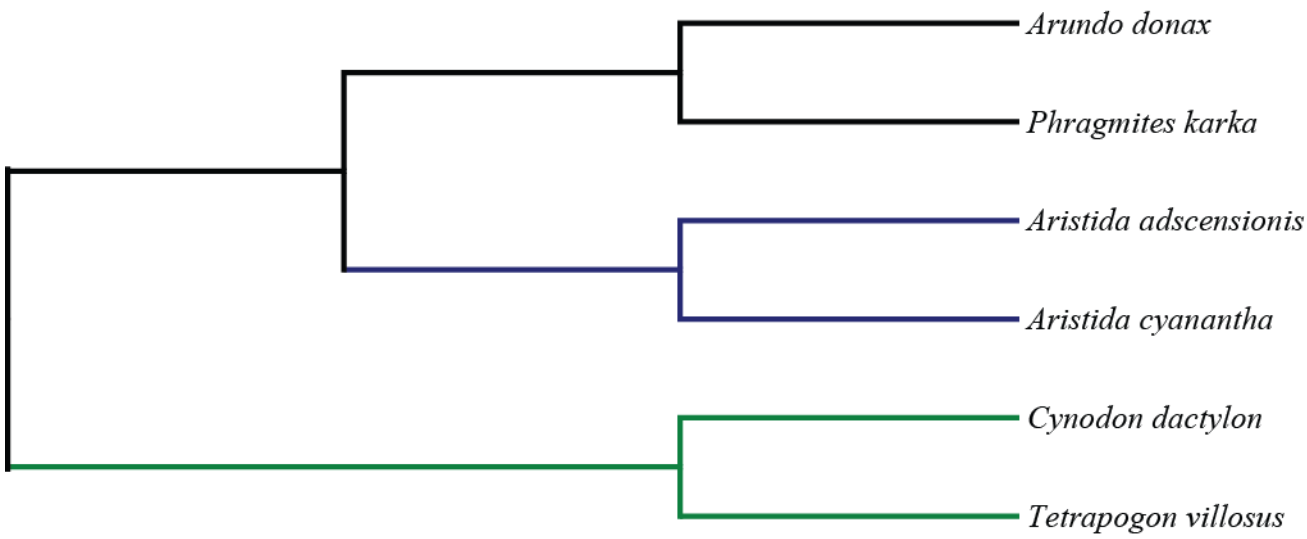

Fig. 1. Showing the relationship among the species of the three tribes.

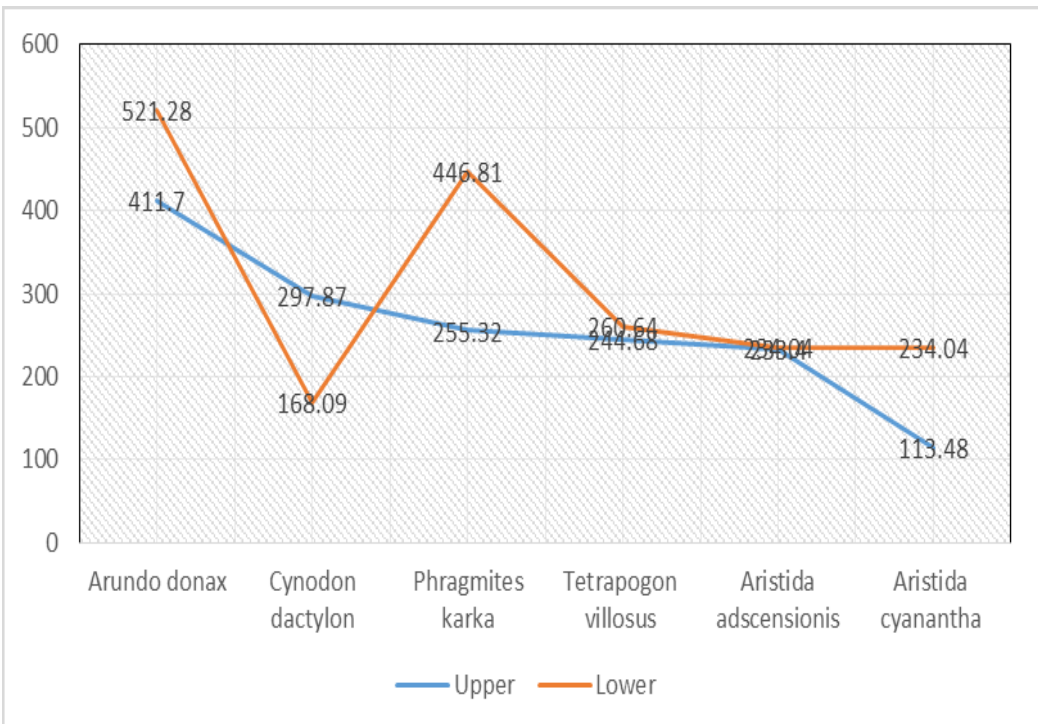

Fig. 2. Showing the pattern of stomatal densities between upper and lower surfaces.

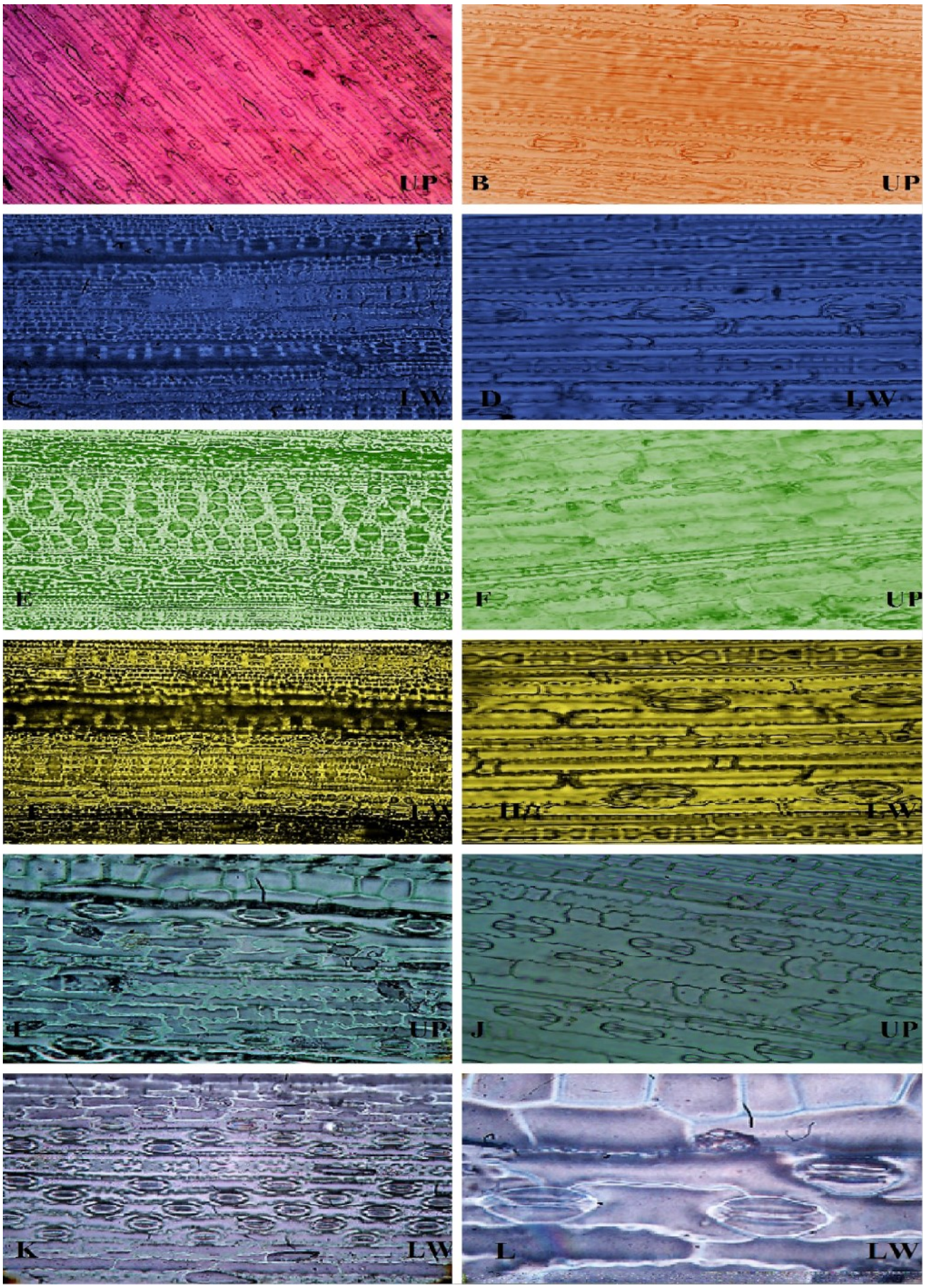

Fig. 3. Representing different micro-morphological characters of *Aristida adscensionis* (A-D) *Aristida cyanantha* (E-H) and *Arundo donax* (I-L) at 40X, 100X of upper and lower surfaces.

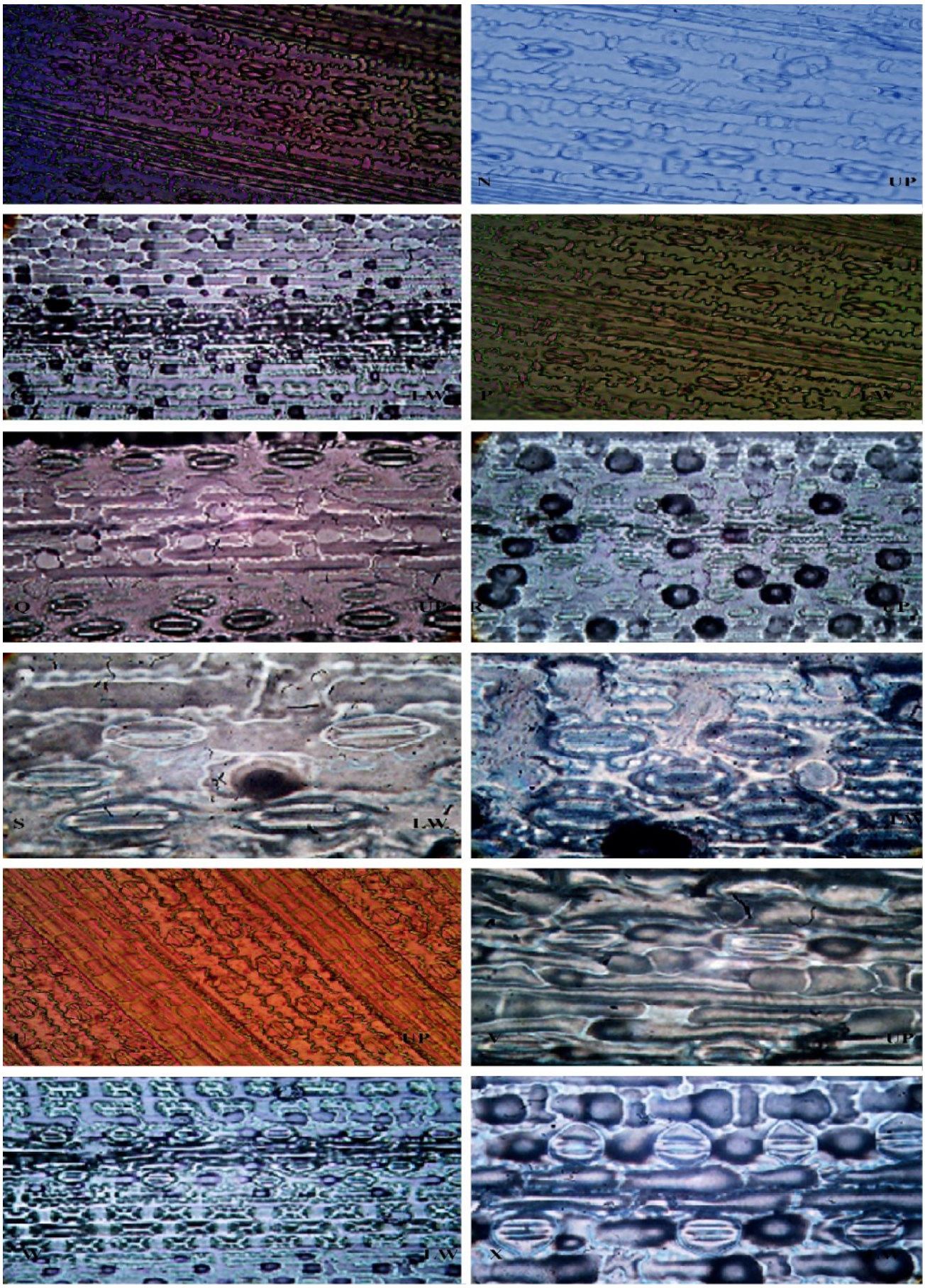

Fig. 4. Showing stomata, micro hairs, prickles, silica bodies, papillae of *Cynodon dactylon* (M-P) *Phragmites karka* (Q-T) and *Tetrapogon villosus* (U-X) at 40X, 100X of both upper and lower surfaces.

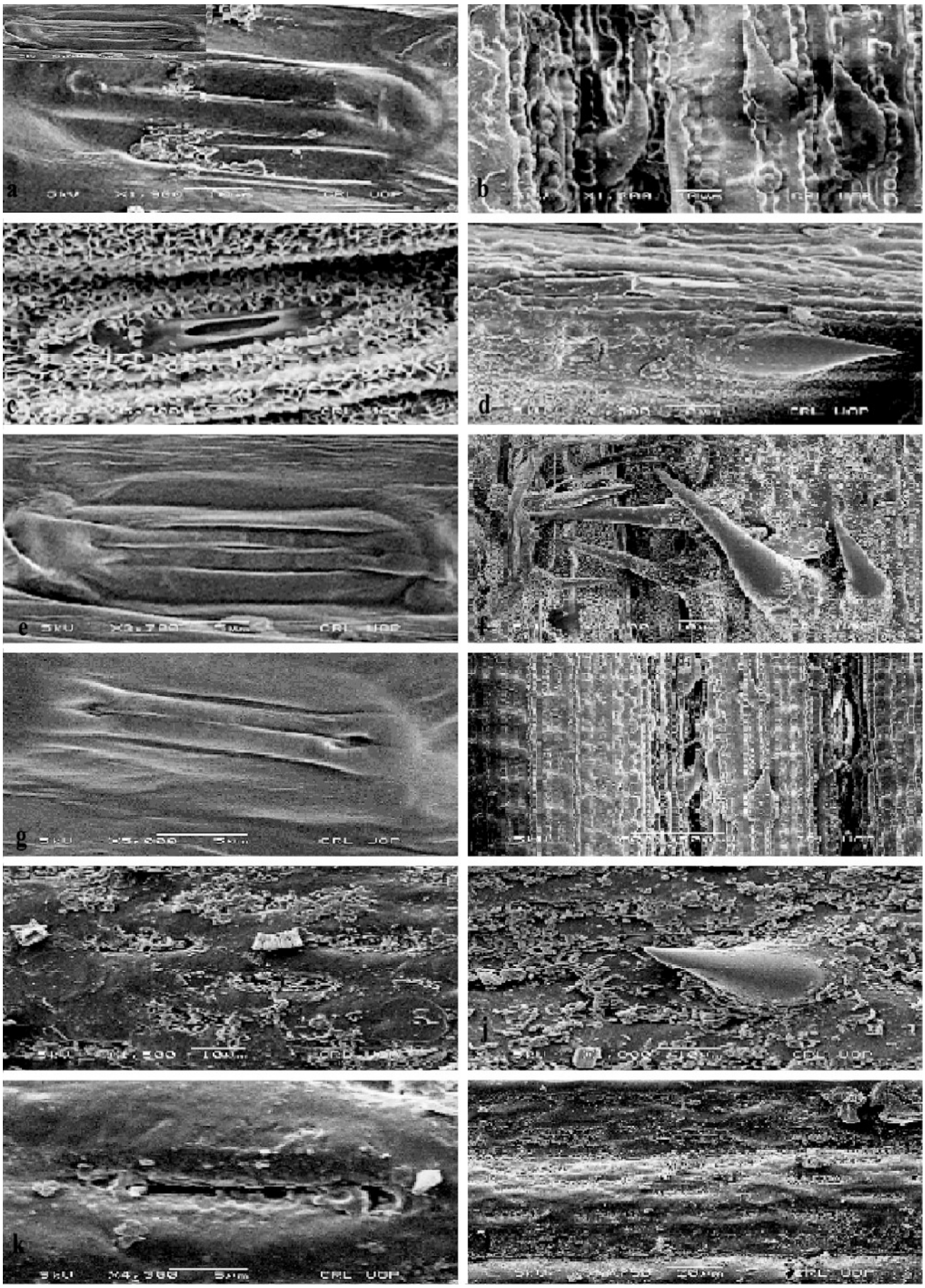

Fig. 5. SEM micrographs of *Aristida adscensionis* (a-d) *Aristida cyanantha* (e-h) and *Arundo donax* (i-l) showing various micro-morphological characters at both upper and lower surfaces.

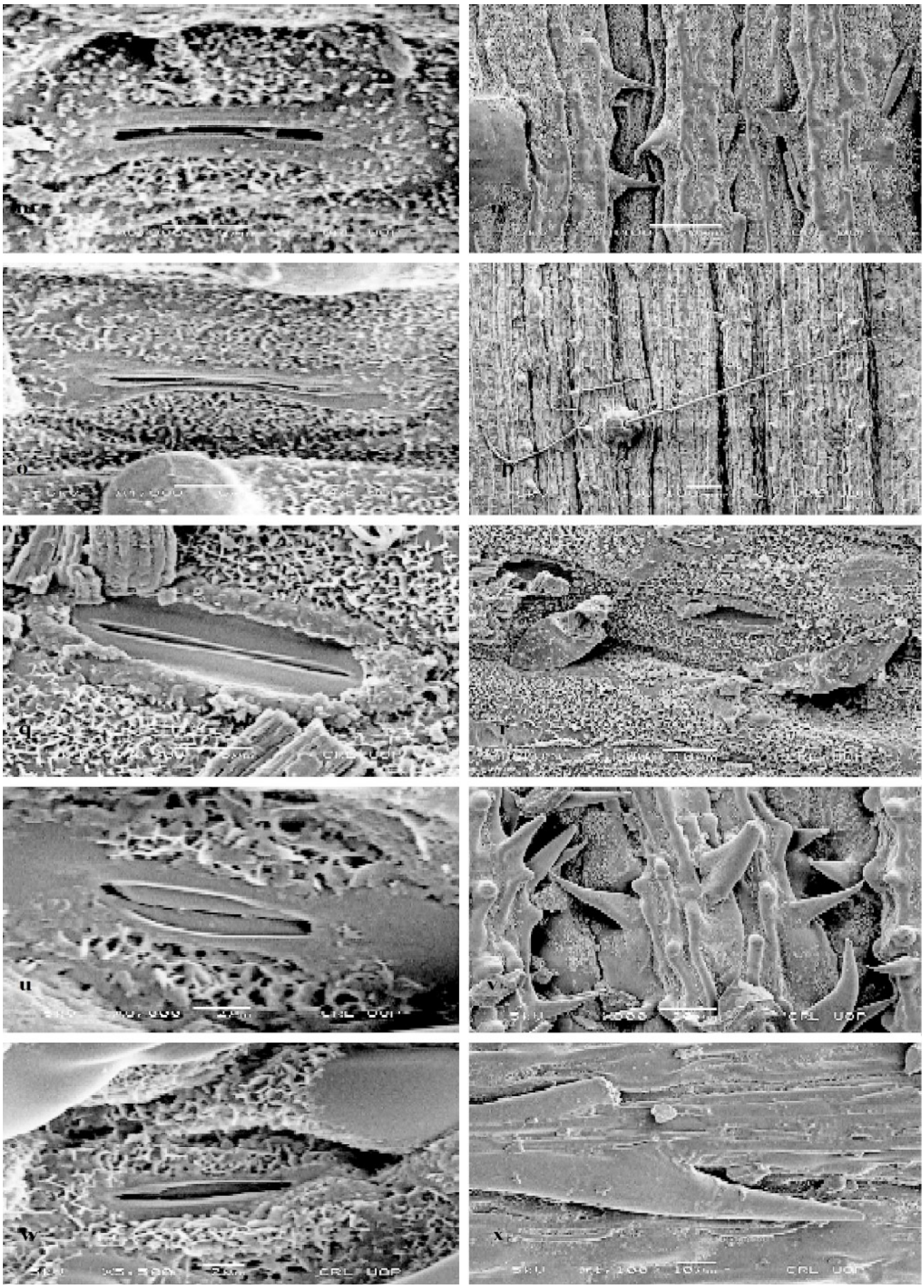

Fig. 6. Showing stomata, micro hairs, macro hairs, prickles, papillae of *Cynodon dactylon* (m-p) *Aristida cyanantha Phragmites* (q-t) and *Tetrapogon villosus* (u-x) at both surfaces.

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