IJNLS
INTERNATIONAL JOURNAL OF NATURE AND LIFE SCIENCES
Research article

International Journal of Nature and Life Sciences (IJNLS)
https://www.journalnatureandlifesci.com
e-ISSN: 2602-2397; ISSN: 2602-2478
Vol. 1(2), December 2017, pp. 72-82

Comparative leaf and pollen micromorphology on some Grasses taxa (Poaceae) distributed in Pakistan

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Received: 15 September 2017, Revised: 06 October 2017, Published Online: 30 December 2017

Abstract
Six grass species of *Lamarckia aurea* (L.) Moench, *Lolium persicum* Boiss. & Hohen. ex Boiss, *Poa annua* L, *Festuca arundinaceae* Schreb, *Aegilops cylindrica* Host and *Hordeum murinum* L. were investigated for their foliar micromorphological and palynological characters through light and scanning electron microscope for their taxonomic importance. Different foliar micromorphological characteristics i.e. silica bodies, macrohairs, microhairs, stomatal number, size, stomatal shape, stomatal density (SD), epidermal cell number, epidermal cell density (ECD), subsidiary cells, short and long cells were examined and observed on both abaxial and adaxial surfaces. The pollen characters i.e. pollen type, polar and equatorial view and diameter along with some other characters were also observed. These foliar morpho and palynological characters were found significant in the delimitation of these species.

Key words: Foliar epidermal morphology, Palynology, Delimitation, Grasses, Pakistan.

1. Introduction

The family Poaceae is one of the largest among angiosperms families and ranks 1st in abundance, 3rd in number of genera after Asteraceae and Orchidaceae and 5th in number of species after Asteraceae, Orchidaceae, Leguminoseae and Rubiaceae (Ahmad et al., 2011). This family comprises about 11.000 species and 700 genera, 60 tribes and six sub families are widely distributed around the world (Clayton and Renvoize, 1986; Chen et al., 2006). The family is represented in Pakistan by 158 genera and 492 species in 26 tribes and five sub families (Cope, 1982). A leaf of grasses consists of leaf sheath, lamina and a colourless membranous or hairy ligule at the junction of sheath and lamina. Before the later part of the 19th century and the classical taxonomical studies were only based on the reproductive features of the plants species as floral characters were considered to provide the most valuable characters to taxonomic affinities but the grasses do not flower for a greater part of their life cycle and the modern studies also suggest that they are not sufficient for the complete systematics and phylogenetic relationships. Their for other characters (embryology, anatomy, cytology, physiology, palynology and phenology) are also significant, in these characters the foliar epidermal studies has of second importance used in the taxonomic studies of a number of families after cytology (Baranova, 1972; Strivastava, 1978; Stace, 1984; Nwokeocha, 1996). The foliar epidermal features are very significant in systematics and used in the characterization within subfamilies and tribes (Palmer et al., 1981, 1985, 1986, 1988; Renvoize, 1982 a & b, 1983). Many microscopic features of the leaf epidermis, including; intercostal long and short cells, stomatal cell type and shape, type of papillae, prickle hairs, macro- and microhairs, hooks, margins and silica bodies are taxonomically informative and used in the classification of grasses (Avdulov, 1931; Prat, 1932; Metcalfe, 1960; Ellis, 1979; Petronela and Nevena, 2010). Clayton and Renvoize (1986) to solve the taxonomic problems in poaceae used the foliar micromorphological characters. Islam et al., (2009) investigated the epidermal features of a rice cultivar leaf and described the leaf surfaces of the taxa of the

family using commonly used anatomical diagnostic characteristics, such as stomatal aperture type and number, hair type and size, prickle density and size, long and short cell properties, and silica body density). Leaf is the most widely used vegetative organ for identification in plant taxonomy and microscopic features such as epidermal cells, stomata, cuticle, surface contours, roughness and ornamentation are in use since the beginning of the last century (Desai and Raole, 2013). Ahmad et al. (2012) also found during the leaf epidermal studies of salt rage grasses that the size of stomatal complex, long cells, shape of subsidiary cells, silica bodies are found to be important in differentiation of different taxa. Palmer and Gerbeth-Jones (1986, 1988) have described the East African grasses in different publications for specific tribes by using scanning electron microscope. The palynology word is derived from Greek "Palynein" meaning dust or flour. In recent palynology is used in many field including geology, criminology, medicines, paleobotany and allergy prohibitions. But it has the significant uses in the taxonomy and phylogenetics of plants (Huang, 1972). Different palynalogists worked on the taxonomic significance of palynological characters in different groups of plants from time to time and found it a very useful character for identification of plant species (Wodehouse, 1935; Tschudy and Scott, 1969; Faegri et al., 1989). From most of the previous literature it was noticed that the pollens of grasses are mostly spheroidal to ovoid, circular and monoporate (Salgado-Labouriau and Rinaldi, 1990; Skvarla et al., 2003). Ahmad et al. (2011) observed different palynological features of 5 species of chloride for its taxonomic utility. Perveen and Qaiser (2012) also observed the palynological features of 54 grass species and found these characters very useful for delimitation of complex grass species. Although some work exists on the significance of SEM and LM studies in identification of plants species (Khan et al., 2017), but more comprehensive work is needed.

2. Material and Methods

The six grass species along with floral parts were collected from Malakand Agency during May to November 2016 for SEM and Light micromorphological studies. The collected plant specimens were pressed, documented, dried, identified and mounted on standard herbarium sheets. The voucher specimens were deposited in the QAU, Herbarium.

2.1. Leaf epidermal and paly-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 glycerine jelly and stained by Delafield's Haematoxylin. For preparation of pollen slides anthers were taken from the collected florets and placed on the slide. 45% acetic acid were added and crushed with the help of glass rod. The pollens were distributed with the help of stirred needle, covered with a cover slip and sealed by using transparent nail polish.

The peels and pollen slides were examined under a compound microscope (Model: Nikon ECLIPSE E200) and micro photographs were taken by polarize camera DCM35 350k pixel USB2.0. Different epidermal and palynological characters were studied at different magnifications (10X, 40X and 100X objectives). Pollen fertility was calculated by;

Pollen fertility test (%) = number of stained pollen / Total number of pollen × 100

2.2. 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. For Palynological studies pollen were taken from anthers after crushing them in 45% acetic acid and one or two drops were mounted of metallic 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.

2.3. 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)

3. Results and Discussion

The species showed diversity in foliar micro morphological and palynological features.

3.1. Stomatal complex and trichomes

All species were amphistomatous i.e. bears stomata both surfaces with only paracytic type of stomata in all species on both surfaces. In all species the lower surfaces of the leaves were observed to have high stomatal and epidermal densities than upper surfaces. *Festuca arundinacea* with highest stomatal density on lower (308.51 mm^{-2}) surface, followed by *Lamarckia aurea* and *Aegilops cylindrica* (202.13 mm^{-2}) each and *Hordeum murinum* (117.02 mm^{-2}). While *Lamarckia aurea* (234.04 mm^{-2}) has the highest stomatal density on upper surface followed by *Aegilops cylindrica* (127.66 mm^{-2}). The stomatal index of the varied from 11.43 to 34.52 and a general pattern of high stomatal indexes were observed on lower surfaces of the leaf than upper surfaces. *Festuca arundinacea* with highest stomatal index on lower surfaces, while *Lamarckia aurea* and *Poa annua* with stomatal index of 27.50 each were on upper surface (Table 1). The largest and smallest stomata were observed on lower surface which varied in size from 45.70×28.56 µm (*Lamarckia aurea*) to 25.72×13.88 µm (*Festuca arundinacea*). The subsidiary cells were found parallel shaped in all species except in *Festuca arundinacea* and *Hordeum murinum* having low domed subsidiary cells (Figure 1). The guard cells were of dumb bell shaped in all species. According to

Hetherington and Woodward (1987) dumb bell shaped stomata of grasses are generally believed to represent a more evolutionary advanced form than their kidney shaped counter parts. The rectangular long cells were common in all species. Round shaped silica bodies were observed in all species except *Aegilops cylindrica* and *Lamarckia aurea* with rectangular silica bodies. Hooks and prickles were very common, mostly occurring on lower surfaces but also observed on the upper surfaces as well. Macrohairs, microhairs and papillae were not very common. Microhairs are absent in all species that is characteristic of subfamily Pooideae (Prat, 1936; Watson et al., 1985; Amarasinghe and Watson, 1990). The foliar epidermal micro morphological characters i.e. epidermal cells, stomata, trichomes, silica bodies, papillae and hairs provide extensive taxonomic data related to grasses and proved to be an important tool in delimitation of taxa in many plant families (Khan et al., 2017). Particularly subfamilies and tribes during last century it is become the second most important character that has been investigated in many families of Angiosperms through both light and Scanning Electron Microscope (Metcalfe, 1960, Prat, 1936; Stebbins 1956; Ellis, 1979; Palmer and Tucker, 1981, Palmer et al., 1985; Mejia – Saules and Bisbey, 2003). Bibi et al. (2007) and Ahmad, (2009) also found the leaf epidermal characters importance and useful in the systematics of grasses at tribes level.

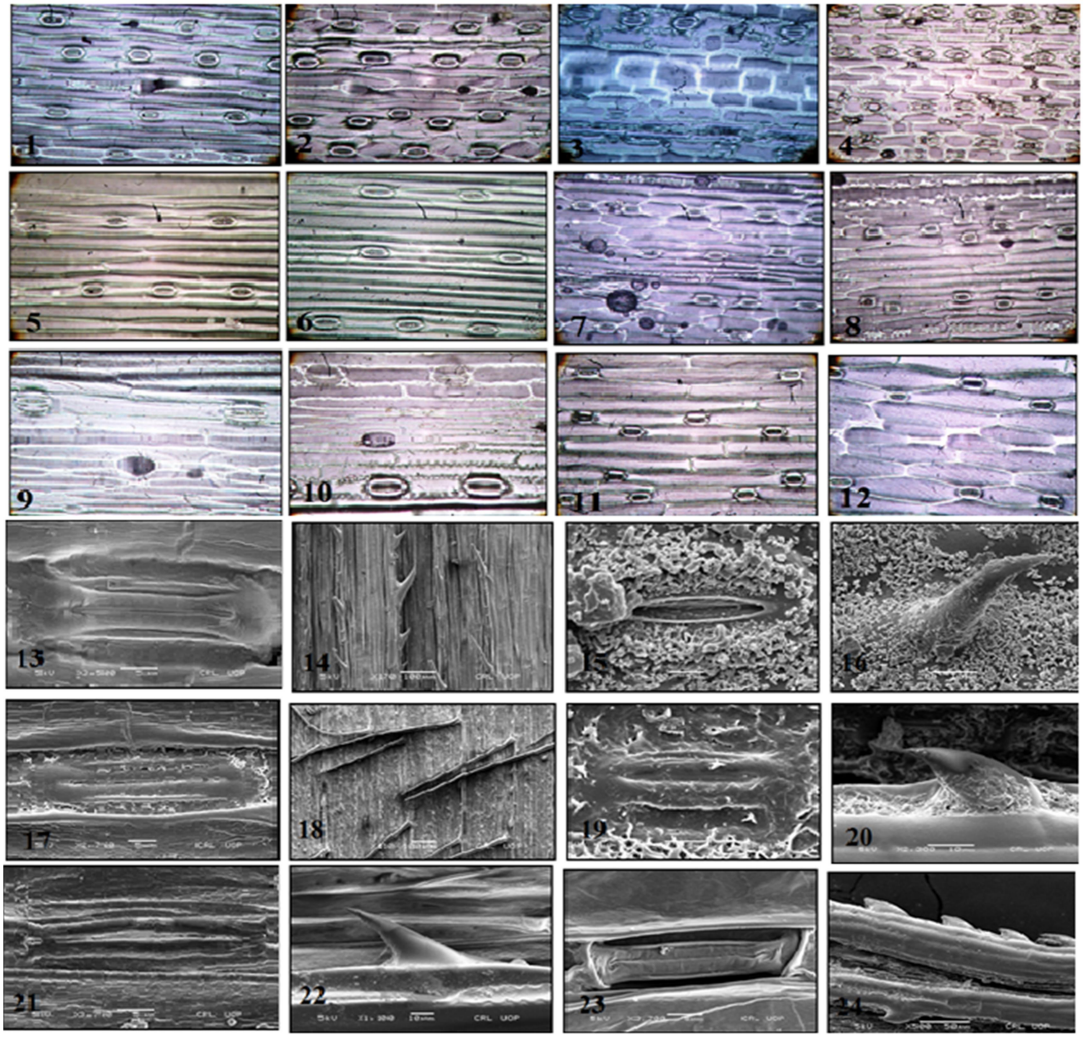

Figure 1. Different foliar micromorphological characters stomata, trichomes, silica bodies can be seen on both abaxial and adaxial surfaces at light (40X) and SEM in *Aegilops cylindrica* (1,2, 13 and 14), *Festuca arundinaceae* (3, 4, 15 and 16), *Hordeum murinum* (5, 6, 17 and 18), *Lamarckia aurea* (7, 8, 19 and 20), *Lolium persicum* (9, 10, 21 and 22) and *Poa annua* (11, 12, 23 and 24) .

3.2. Diversity in palynological features

The pollens were circular in polar view and Prolate, oblate or Spheroidal in equatorial view. All the pollens were monad type with scabrate sculpturing (Figure 2). *Festuca arundinaceae* was observed with maximum polar diameter 36.11µm (27-38µm) and *Poa annua* with lowest 22.53 µm (20.5-24 µm). *Aegilops cylindrica* has the highest equatorial diameter 27.2 µm (26.5-28.5 µm) and *Hordeum murinum* has the minimal 21.7 µm (21-23 µm). Meo (1999) in his investigation reported the polar diameter for *P. annua* 24.13 µm and equatorial diameter as 24.88 µm which very close to our results (Table 3). The polar-equatorial ratio was also maximum for *Festuca arundinaceae* (1.42), followed by *Hordeum murinum* (1.14), *Lolium persicum* (0.99), *Aegilops cylindrica* (0.98), *Lamarckia aurea* (0.96) and minimum for *Poa annua* (0.85). All the pollens were also monoporate and the colpi diameter were maximum for *Lolium persicum* 3.12 µm (2.85-3.75 µm) and minimum for *Aegilops cylindrica* 1.25 µm (0.85-1.4 µm). *Poa annua* pollens has the most thick exine 1.61 µm (1.2-18.5 µm), followed by *Lamarckia aurea* 1.51µm (0.9-1.75 µm), *Lolium persicum*1.25µm (0.85-1.5 µm), *Festuca arundinaceae* 1.23µm (0.95-13.5 µm), *Hordeum murinum* 0.77µm (0.6-0.95 µm) and *Aegilops cylindrica* 0.74 µm (0.55-0.9 µm). *Poa annua* has the most fertile pollens (92.02%) and *Hordeum murinum* has the lowest pollen fertility (85.32%). Khan et al. (2017) investigated the gymnosperm pollen flora of Pakistan using light and scanning electron microscope method and found these palyno-morphological significant in taxonomic identification of species.

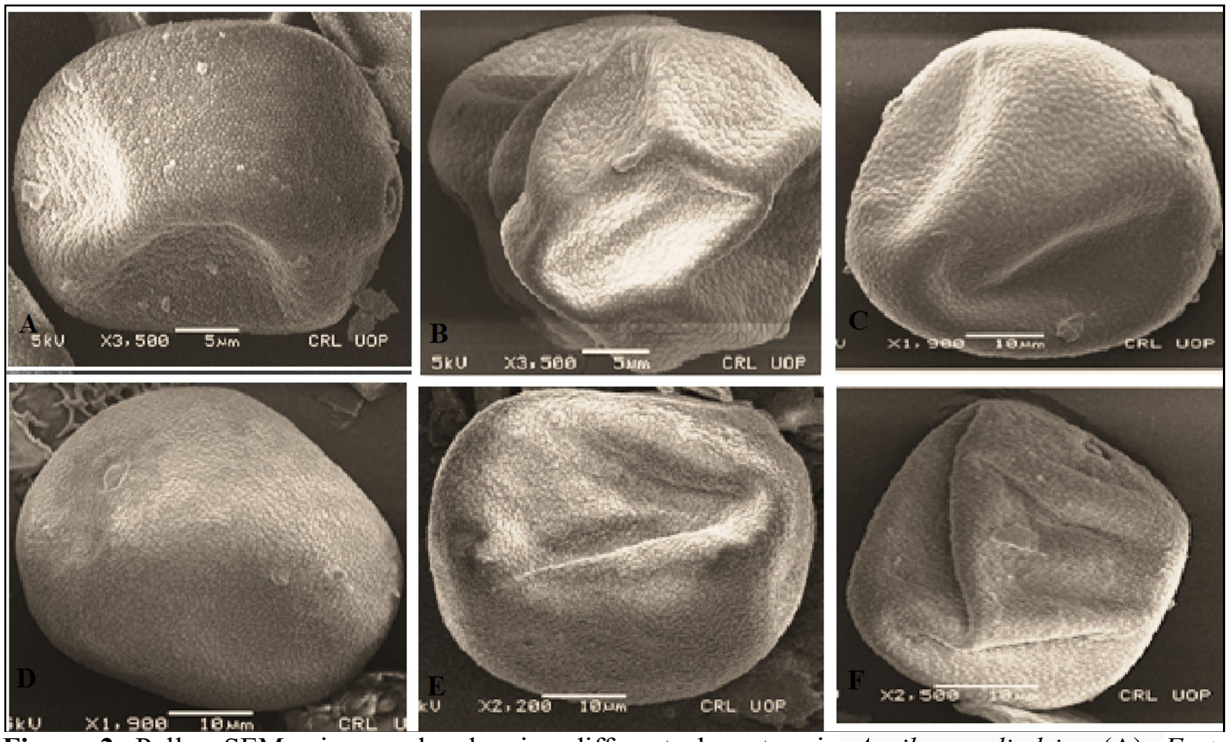

Figure 2. Pollen SEM micrographs showing different characters in *Aegilops cylindrica* (A), *Festuca arundinaceae* (B), *Hordeum murinum* (C), *Lamarckia aurea* (D), *Lolium persicum* (E) and *Poa annua* (F).

Key based on palynological and foliar epidermal morphological characters

1. + Silica bodies rectangular, pollen shape in equatorial view prolate.....2
   - Silica bodies rounded pollen shape in equatorial view other than prolate.....3

2. + Long cell sinuous, macrohairs present, P/E ratio 0.98.....*Aegilops cylindrica*
   - Long cell straight, macrohairs absent, P/E ratio 1.51.....……..*Lamarckia aurea*

3. + Subsidiary cells low domed, pollen shape in equatorial view spheroidal.....4
   - Subsidiary cells parallel, pollen shape in equatorial view oblate.....5

4. + Long cell rectangular, micro hairs present, P/E ratio 1.42..... *Festuca arundinaceae*
   - Micro hairs present, P/E ratio 1.14.....*Hordeum murinum*
   - Prickle absent, stomatal index greater than12.20.....3

3. + Long cell wall slightly sinuous, macrohairs absent, P/E ratio 1.25.....*Lolium persicum*
   - Long cell wall straight, macrohairs absent, P/E ratio 0.85.....*Poa annua*

4. Conclusions

The Scanning electron and light microscopy foliar epidermal and Palynological studies were found very helpful in the identification at species levels. The key based on silica bodies, microhairs, macrohairs, hooks, papillae, prickles, stomatal index and stomatal densities are useful in the robust identification of closely related species.

Table 1. Showing the upper and lower foliar epidermal features of the grass species.

Epidermal surface/ characters		*Lamarckia aurea*	*Lolium persicum*	*Poa annua*	*Festuca arundinacea*	*Aegilops cylindrica*	*Hordeum murinum*
Abaxial	**Stomatal complex**						
	Stomatal length	33.66 µm	34.75 µm	30 µm	25.72 µm	35.10 µm	35.58 µm
	Stomatal width	18.41 µm	20.85 µm	11.25 µm	13.88 µm	10.75 µm	18.90 µm
	Stomatal type	Paracytic	Paracytic	Paracytic	Paracytic	Paracytic	Paracytic
	Stomata number	22	5	11	8	12	10
	Number epidermal cell	58	36	29	46	35	39
	Stomatal density mm^{-2}	234.04	53.19	117.02	85.12	127.66	106.38
	Epidermal cell density mm^{-2}	617.02	382.98	308.51	489.36	372.34	414.89
	Stomatal index	27.50	12.20	27.50	14.82	25.53	24.41
	Number of stomatal rows between two costal zones	5	2	5	4	3	3
	Subsidiary cell shape	Parallel	Parallel	Parallel	Low domed	Parallel	Low domed
	Guard cells shape	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell
	Silica bodies and short cells						
	Silica bodies shape	Rectangular	Rounded	Rounded	Rounded	Rectangular	Rounded
	Costal silica bodies	Present	Present	Present	Present	Present	Present
	Intercostal silica bodies	Absent	Absent	Absent	Absent	Absent	Absent
	Short cell wall shape	Round	Round	Round	Round	Round	Round
	Long cells						
	Size	79× 8 µm	233×16 µm	79×7 µm	123×15 µm	134×17 µm	167× 22 µm
	Shape	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular
	Long cell wall shape	Straight	Slightly sinuous	Straight	Straight	Straight	Straight
	Number of rows of long cells between two costal zones	7	5	6	4	7	13
	Macrohairs	Absent	Absent	Present	Present	Present	Present
	Microhairs	Absent	Absent	Absent	Present	Absent	Absent
	Hooks	Present	Absent	Absent	Present	Present	Present
	Prickles	Present	Present	Absent	Absent	Present	Present
	Papillae	Present	Absent	Absent	Absent	Present	Present

Table 1 (continued **)**

Epidermal surface/ characters		*Lamarckia aurea*	*Lolium persicum*	*Poa annua*	*Festuca arundinacea*	*Aegilops cylindrica*	*Hordeum murinum*
Adaxial	**Stomatal complex**						
	Stomatal length	45.70 µm	32.5 µm	34.91 µm	30.60 µm	36.25 µm	45.70 µm
	Stomatal width	28.56 µm	10.5 µm	7.78 µm	14 µm	17.5 µm	21.28 µm
	Stomatal type	Paracytic	Paracytic	Paracytic	Paracytic	Paracytic	Paracytic
	Stomata number	19	4	8	29	19	11
	Number epidermal cell	62	31	33	55	52	32
	Stomatal density mm^{-2}	202.13	42.55	85.11	308.51	202.13	117.02
	Epidermal cell density mm^{-2}	659.58	329.79	351.06	585.12	553.19	340.43
	Stomatal index	23.46	11.43	19.51	34.52	26.76	25.58
	Number of stomatal rows between two costal zones	4	2	4	5	4	3
	Subsidiary cell shape	Parallel	Parallel	Parallel	Low domed	Parallel	Low dome
	Guard cells shape	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell	Dumb bell
	Silica bodies and short cells						
	Silica bodies shape	Rectangular	Rounded	Rounded	Rounded	Rectangular	Rounded
	Costal silica bodies	Present	Present	Present	Present	Present	Present
	Intercostal silica bodies	Absent	Absent	Absent	Absent	Absent	Absent
	Short cell wall shape	Round	Round	Round	Round	Round	Round
	Long cells						
	Size	128× 12 µm	115×14 µm	260×26 µm	86×9 µm	124×13 µm	202×19 µm
	Shape	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular	Rectangular
	Long cell wall shape	Straight	Slightly sinuous	Sinuous	Sinuous	Sinuous	Sinuous
	Number of rows of long cells between two costal zones	6	7	8	3	9	9
	Macrohairs	Absent	Absent	Absent	Absent	Present	Present
	Microhairs	Absent	Absent	Absent	Absent	Absent	Absent
	Hooks	Present	Present	Present	Present	Present	Present
	Prickles	Present	Present	Present	Present	Present	Present
	Papillae	Present	Absent	Absent	Absent	Present	Present

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

Parameter	***Lamarckia aurea***		***Lolium persicum***		***Poa annua***		***Festuca arundinaceae***		***Aegilops cylindrica***		***Hordeum murinum***	
Abaxial	Stomata	Epidermal cells	Stomata	Epidermal cells	Stomata	Epidermal cells	Stomata	Epidermal cells	Stomata	Epidermal cells	Stomata	Epidermal cells
Mean	21.6	58.4	4	36.6	9.2	28.4	6.8	48.2	9.8	32.2	9	39.4
Variance	1.04	5.04	0.4	4.24	1.36	5.84	1.36	6.96	2.96	7.76	0.8	3.44
Standard deviation	1.02	2.25	0.63	2.06	1.17	2.42	1.17	2.64	1.72	2.79	0.89	1.86
Co-efficient of variance	0.05	0.04	0.15	0.06	0.13	0.09	0.17	0.06	0.18	0.09	0.10	0.05
Standard error	0.51	1.12	0.32	1.03	0.58	1.21	0.58	1.32	0.86	1.39	0.45	0.93
Adaxial												
Mean	17.6	62	3.4	29.2	7	32.6	26.8	55.8	18.2	54.6	9.4	32
Variance	0.64	8.4	1.04	19.76	0.8	4.24	2.16	1.76	1.36	4.64	1.04	7.2
Standard deviation	0.8	2.90	1.02	4.45	0.89	2.06	1.47	1.33	1.17	2.15	1.02	2.68
Co-efficient of variance	0.05	0.05	0.30	0.15	0.13	0.06	0.06	0.02	0.06	0.04	0.11	0.08
Standard error	0.40	1.45	0.51	2.22	0.45	1.03	0.74	0.66	0.58	1.08	0.51	1.34

Table 3. Different palynological character of grass species.

S#	**Species names**	**Pollen type**	**Equatorial view**	**Polar view**	**Equatorial diameter (µm)**	**Polar diameter (µm)**	**P/E ratio**	**Exine thickness (µm)**	**Sculpturing**	**Colpi diameter (µm)**	**Pollen fertility(%)**	**Flowering period**
1	*Lamarckia aurea*	Monad	Prolate	Circular	24.2 (23.5-25)	23.2 (22.5-24)	0.96	1.51 (0.9-1.75)	Scabrate	2.97 (2.5-3.45)	89.75	July-August
2	*Lolium persicum*	Monad	Oblate	Circular	25.8 (24-27)	25.73 (24-27)	0.99	1.25 (0.85-1.5)	Scabrate	3.12 (2.85-3.75)	91.35	March-April
3	*Poa annua*	Monad	Oblate	Circular	26.4 (25.5-27.5)	22.53 (20.5-24)	0.85	1.61 (1.2-18.5)	Scabrate	2.13 (1.85-2.25)	92.06	March-November
4	*Festuca arundinaceae*	Monad	Spheroidal	Circular	25.4 (23-28)	36.11 (27-38)	1.42	1.23 (0.95-13.5)	Scabrate	2.72 (2.45-3)	84.05	May-July
5	*Aegilops cylindrica*	Monad	Prolate	Circular	27.2 (26.5-28.5)	26.71 (25-28.5)	0.98	0.74 (0.55-0.9)	Scabrate	1.25 (0.85-1.4)	91.45	March-May
6	*Hordeum murinum*	Monad	Spheroidal	Circular	21.7 (21-23)	24.75 (23.5-26)	1.14	0.77 (0.6-0.95)	Scabrate	1.73 (0.9-2.55)	85.32	April-July

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