Investigations into Phytoliths as Diagnostic Markers for the Grasses (Poaceae) of Punjab

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Abstract Grasses are known to accumulate amorphous silica (SiO2·nH2O) within and between cells as silica bodies of characteristic shapes. The position and type of the host cells are the characters that seem to control their shape and size. The present study was carried out to assess and utilize the diagnostic potential of phytolith types in the identification of grass taxa at sub-familial, tribal, generic and specific levels. Clearing solution method was employed for locating the position of phytoliths within and between cells. Dry and wet ashing methods were subsequently employed for their isolation. Scanning Electron Microscopy was performed to study the ultra-structural features of phytoliths. Micromorphometric measurements of phytoliths were carried out with the help of image analysis software (Image J 1.46r). The study has brought out diagnostic potential of phytolith types for characterization of grasses of Punjab plains. For example, hat shaped phytoliths were identified as the diagnostic marker type for Digitaria ciliaris (Retz.) Koeler. However, full taxonomic potential of phytolith types for characterization of taxa can be realized only after further analysis of their physical properties and chemical architecture.

Keywords Phytoliths, Grasses, Bilobate, Taxa, Diagnostic

1. Introduction

Phytoliths are amorphous silicon dioxide (SiO2·nH2O) deposits formed in specific intercellular and intracellular locations in several groups of vascular plants, notably the grasses [1-3]. They show a range of distribution in plant body but leaf epidermal cells present the most common location for the formation of phytoliths [4-6]. Silica deposits in plants have been attributed several biological functions ranging from mechanical strength and resistance to grazing [7], to disease control [8-9], alleviation of abiotic stress from metal toxicity, salinity, drought and high temperature [9-11]. They have been reported to regulate transpiration rates and reduce heat load of the foliage and other overground parts of the plant body [12-14]. But taxonomic characterization, identification and classification of plant taxa is an area of research wherein phytolith analysis has proved most useful. Apart from taxonomic diagnosis, phytoliths have provided useful evidences in preparing calendars of the use of grain crops in historic and prehistoric agriculture [15-17]. Distribution of phytoliths in soil has been utilized for the reconstruction of paleoclimatic regimes in the geological past [1]. Identification of plant species from micro-fossils is another use of phytolith analysis [18].

Vascular plants take up silica as monosilicic acid (H2SiO4), from the soil in considerable quantities and deposit it as phytoliths in the vegetative and reproductive parts [19]. Grasses constitute a taxonomic group where silica deposits have been widely studied and documented. Silica constitutes up to 5-20 percent of their shoot dry weight [20]. They are reported primarily in epidermal long cells, trichomes (hairs), specialized silica short cells, and as fillings within the bulliform cells of leaves and protective covers of the spikelets (glumes) & florets (lemmas) and the caryopsis [4, 21-23]. Owing to characteristic structure and shapes, phytoliths have found an increasing role in taxonomic diagnosis of grasses [14, 24].

Metcalfe [25] recognized that shape of phytoliths is a useful character for plant identification. Later on, utility of phytoliths in identification of grass species has been demonstrated and put to use. Ollendorf et al. [26] demonstrated the use of phytolith types for distinguishing Arundo donax L. from Phragmites communis (cav) Trin. ex Steud, two gaint reed grasses that present difficulties in taxonomic separation and identification. Subsequently, phytoliths have been utilized as additional evidences for taxonomic diagnosis of grass species [27-29] and races [30].

The increasing role of phytoliths in species diagnosis and classification in grasses is attributed to two main reasons. First, the nearly ubiquitous presence of phytoliths in grass species make them a universal and reliable character for characterization of grass species. Second, utilization of phytoliths which are mainly present in leaf epidermis and vegetative parts of the plant body helps in characterization
and identification of grass species from foliage and the vegetative parts and reduces dependence on the fertile parts (the inflorescence, spikelets and florets) which are employed almost exclusively in conventional systems of grass diagnosis. The present study aims at characterization of phytoliths as diagnostic markers for forty eight grass species of the area of study.

2. Materials and Methods

The grass species of the present study were collected from the Punjab plains in the North-Western Himalayan region. Physiographically, it is a plain region with an average elevation of 234 m asl. Mean annual rainfall is about 90-115 cm which is received mostly during the rainy season. Annual mean maximum and mean minimum temperatures are 31.3°C and 13.25°C respectively. Whole plant specimens were collected at flowering stage, cut to size and preserved in 70% ethanol at 4°C.

2.1. Phytolith Analysis

In situ location of phytoliths in epidermal cells were determined by the clearing solution method of Krishnan et al. [5]. Leaf segments were throughly washed and immersed in a clearing solution of Lactic acid and Chloral hydrate (3:1) kept at 70°C for 2 days. Cleared segments were mounted in fresh solution and observed under light microscope.

The method of Carnelli et al. [31] was followed for dry ashing of the material. The material was rinsed and cut into small pieces and heated to ashes in porcelain crucibles in a Muffle Furnace maintained at 470°C for 48 hours. The crucibles were taken out, cooled and the contents transferred to test tubes. Sufficient amount of Hydrogen peroxide (30%) was added to submerge the contents and the test tubes were kept at 80°C for 1 hour. Test tubes were taken out from the incubator; the mixture was decanted and rinsed twice with distilled water. Hydrochloric acid (10%) was added to the pellet followed by incubation again for 1 hour. Thereafter, the mixture was washed with distilled water and centrifuged at 7500 rpm for 10 minutes. The supernatant was decanted and the pellet was washed twice with distilled water. The centrifugation process was repeated four times till the pellet was clear. Finally, the pellet was dried for 24 hours at 60°C to a powder form. In this form, the material was taken in small bits and mounted on glass slides in DPX for optical microscopy. Olympus Micro Image Projection System (MIPS-USB 0262) was used for microphotography. Photographs were taken at a uniform magnification for ease of comparison. Phytoliths were classified into types and subtypes according to the International Code of Phytolith Nomenclature [32].

2.2. Morphometry and Statistical Analysis

Morphometric measurement of various types of phytoliths was done with Image J software (version 1.46r.). It is user-friendly software that allows measurements of overall size and other dimensional aspects of microscopic objects from their microphotographs. In the present study, twenty phytoliths of each type from different grass species in the sample were photographed with the help of a Micro Image Projection System (Olympus) and stored in separate computer files. Thereafter, dimensions of phytoliths were recorded with the help of the Image J software. After loading the software, images of phytoliths were retrieved into the current memory (RAM) of the computer. The software records dimensions as the cursor is dragged along the dimensions (length and breadth) in the images of the objects photographed. The perimeter was recorded by drawing an outline of phytoliths with the cursor. The software not only records perimeter but also calculates other morphometric parameters viz., aspect ratio, surface area, roundedness and solidity. In the present paper, we have included data on the surface area (µm²) and perimeter (µm) only. Mean and Standard Error of the various parameters was calculated with the help of PAST software. The level of significance of difference in the sizes of various types of phytoliths was tested with the help of χ² test and the table of critical values.

2.3. Scanning Electron Microscopy

Details of shape and surface features of phytoliths were studied with the help of Scanning Electron Microscopy. Dry ash was spread uniformly over the stubs with the help of double-sided adhesive tape. The stubs were put under a stereo scope for uniform spreading of the ash. Silver paint was applied on the edges of the stub. The samples were dried overnight at 40°C. Next day, stubs were coated with graphite using a vacuum evaporator (JEOL-JEE-4X). They were subsequently coated with gold by a sputter coater (POLARON) and imaged under SEM (ASID) at an accelerating voltage of 40KV.

3. Results & Discussion

Data on the presence/absence and morphometric measurements of various phytolith types in the grass species of the present sample are presented in Table 1. The values in the body of the table refer to surface area and perimeter of various phytolith types seen in the forty eight species belonging to 39 genera, 10 tribes and 6 subfamilies of the grass family Poaceae. It emerges from the table that phytoliths exhibit considerable variation with respect to shape, size and distribution. In this spectrum of variation, some phytolith types have emerged as diagnostic markers for subfamilies, tribes and genera in the present sample.

3.1. Bambusoideae

Bambusoideae exhibited a diversity of phytoliths types. The most common types were bilobates with variation in the size of lobes and the length and width of the shank (Figure...
In an earlier study, Lu and Liu [33] investigated phytoliths in this subfamily and found that bilobates along with saddles were the most frequent types and could therefore be utilized as the marker type for the subfamily. Our finding that bilobates and saddles occur in all the three members, *Bambusa ventricosa* Schrad., *B. vulgaris* Nees and *Arundinaria falcata* Nees of the subfamily in our collection lends further credence to the diagnostic importance of these types. Bilobates and saddles are formed in the epidermal short cells whose lumen is completely infilled with silica. The other types encountered in the subfamily were the dendrites in all the three members of the subfamily, trapezoids in *Bambusa ventricosa* Schrad., and *Arundinaria falcata* Nees., Scutiform and sinuate elongate in  *Bambusa vulgaris* Nees. (Figure 12a, 12c)

Light Microscope Photographs (Figure. 1-6): Epidermal layer showing bilobate phytoliths (1a-d), quadrilobate (2a, b), sinuate elongate (3a, b), saddle (4a-b), polylobe (trilobate) (5a, b), dendritic (6a-d), oval & rondel (6e) types of phytoliths. [Bar= 10μm]

*Bambusa vulgaris*=Bv;  *Arundinella nepalensis*=An;  *Oryza sativa*=Os;  *Bothriochloa pertusa*=Bp;  *Hordeum vulgare*=Hv;  *Paspalum flavidum*=Pf;  *Avena sativa*=As;  *Echinochloa crusgalli*=Ec;  *Saccharum bengalense*=Sb;  *Triticum aestivum*=Ta;  *Sorghum halepense*=Sh;  *Sporobolus diandrus*=Sd

3.2. Ehrhartoideae
**Oryza sativa** L. the only member of this subfamily in our sample showed cross-shaped, bilobate, quadrilobate and trapezoidal phytoliths. Of these types, bilobates are considered as the diagnostic types for the subfamily Panicoideae. However, presence of other types viz., cross shaped and quadrilobate helps in discriminating the oryzoid from the panicoid grasses. Earlier studies have shown that cross-shaped types and bilobate are the main phytoliths in this species [34]. In our study, bilobate phytoliths were seen in epidermal long cells above leaf veins in rows (Figure 1c).

### 3.3. Pooideae

Pooideae members revealed considerable diversity in shape and size of phytoliths. Within the subfamily, tribe Triticeae included the dendritic, trapezoid and sinuate elongate types in both the members (*Hordeum vulgare* L. and *Triticum aestivum* L.). These types are derived from epidermal long cells in both costal and intercostal regions of adaxial and abaxial epidermis (Figure 6a, 6b). The average size of sinuate elongate types were 2053 µm² and 2030 µm² in *Hordeum vulgare* L. and *Triticum aestivum* L. respectively (Tables 1). *Ball et al.* [30] reported phytoliths of similar shape in these species. The author found that trapezoid phytolith is the diagnostic type for *Triticum aestivum* L. along with the dendritic and sinuate elongate. We have also recovered these types in our collection. Other less frequent types viz., smooth elongate and quadrilobate phytoliths in *Hordeum vulgare* L. were also seen in the present studies (Figure 2a, 14m).

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**Light Microscope Photographs (Figure 7-13):** Epidermal layer showing cross shaped phytoliths (7a), Trapezoid (8b), nodular bilobate (9a, b), rondels (10a, b), clavate (11a-c), scutiform (12a-c) and rectangular (13a, b).[Bar= 10μm]

*Brachiaria reptans*=Br; *Panicum antidotale*=Pan; *Imperata cylindrica*=Ic; *Saccharum bengalense*=Sb; *Sorghum halepense*=Sh; *Arundinella nepalensis*=An; *Setaria verticillata*=Sv; *Saccharum bengalense*=Sb; *Dichanthium annulatum*=Dan; *Bambusa vulgaris*=Bvl; *Lolium tamulentum*=Lt; *Phragmites australis*=Pau

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**Plate II**
The tribe Poeae also revealed a range of phytolith morphotypes in *Avena sativa* L. The most frequent shapes are sinuate elongate and dendritic (Figure 3a, 3b) with mean surface area reaching up to 1189 µm² (Table 1). These types are deposited in epidermal long cells with the processes of the dendritics being the result of silicification of intercellular connections [35]. *Lolium temulentum* L., *Phalaris minor* Retz. and *Poa annua* L. also revealed similar types with mean surface area reaching up to 1839 µm² (Table 1). *Poa annua* L. is the only member of the tribe which showed oval and rectangular shaped phytoliths which can serve as a diagnostic marker for the species. The other types were saddle and rondel in *Avena sativa* L., (Figure 4a, 4b), scutiform and elevat in *Lolium temulentum* L. and cross shaped in *Phalaris minor* Retz. and *Poa annua* L. However, these types were smaller in size.

### 3.4. Arundinoideae

*Arundo donax* L. and *Phragmites australis* (cav) Trin. ex Steud have been reported to yield a range of phytolith types [36-37]. In the present investigations, dumbell and dendritic were found to be characteristic of *Arundo donax* L. whereas *Phragmites australis* (cav) Trin. ex Steud produced trapezoids, bilobates and elongate types (Figure 8a, 14k). Differences in the phytoliths were revealed between these species by Dore [38] and Piperno [39]. Besides the usual dumbbell and dendritic types, *Arundo donax* L. bore sinuate elongate, smooth elongate and quadrilobate types whereas *Phragmites australis* (cav) Trin. ex Steud, showed the presence of smooth elongate and sinuate elongate types. The mean surface area of elongate types was remarkably lower in *Arundo donax* L. (928 µm²) as compared to those of *Phragmites australis* (Cav) Trin. ex Steud.(2972 µm²)

### 3.5. Chloridoideae

Saddle-shaped phytoliths are known to be the diagnostic for members of this subfamily [23, 40]. They are also named as battle axes with double edges [41-43]. Another type recovered in the present sample was the thin Chloridoid type, a comparately longer saddle shaped type. They may have a wrinkly or a non-wrinkly surface. In literature, this type is also known as the long saddle type or tall collapsed type [43]. In the present sample, only four of the nine chlorodoid members showed the saddle shaped type (Table 1). Similar trend was observed by Jattisha and Sobu [24]. In the present study it emerges that dendritic types seemed to characterize the subfamily instead of saddle by being present in all the members except *Desmostachya bipinnata* (L.) Stapf. The last named species is marked by the presence of rondel types which it shares only with *Sporobolus diander* (Retz.) P. Beauv. within the subfamily (Figure 6e).

One of the members of the tribe Cynodonteae viz., *Cynodon dactylon* (L.) Pers. produced bilobates, dendritic, lanceolate and saddle types (Figure 14c, 14e). These types were also reported by Chauhan et al. [14] in leaf blade, leaf sheath and culm of *Cynodon dactylon* (L.) Pers. with the help of Laser Induced Breakdown Spectroscopy (LIBS). *Tragus bifloris* Schultz., the other member of the tribe Cynodonteae in our sample shares the trapezoid and dendritic with *Cynodon dactylon* (L.) Pers. but is distinguished from it by the presence of oval and rectangular types.
Table 1. Data on the presence/absence and size dimensions of phytoliths in grass species of the present sample

| Name of the Species | Abbr. | Size Dimensions | Phytolith types and size dimensions (μm)² |
|---------------------|-------|-----------------|-----------------------------------------|
|                     |       | BL  | CL  | CR  | DT  | HT  | LCN | NB  | OVL | PL  | QD  | RD  | RT  | SD  | SE  | SmE | SQR | STF | TZ  |
| **Subfamily: Bambusoideae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| **Tribe: Bambuseae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Bambusa ventricosa Nees | Bv  | Area (mm²) | 419±14 |  -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 1176±235 |
|                      |      | Perimeter (μm) | -  | -  | 94±122 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 178±200 |
|                     |      | Patrinia type | 51.3±12.1 | 101.8±4.4 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 159±4.14 |
| Bambusa vulgaris Schrad. | Bvl  | Area (mm²) | 289±45 |  -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 128±11.7 |
|                      |      | Perimeter (μm) | -  | -  | 476±60 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 167.7±6.8 |
| Arundinaria falcata Nees | Af  | Area (mm²) | 196±26 |  -  | 1291±477 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 1407±340 |
|                      |      | Perimeter (μm) | -  | -  | 1300±63 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 167.7±6.8 |
| **Subfamily: Ehrhartoideae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| **Tribe: Oryzeae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Oryza sativa L. | Os  | Area (mm²) | 167±13 |  -  | 208±28 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 113±04 |
|                      |      | Perimeter (μm) | -  | -  | 212±19 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 60±7.4 |
| **Subfamily: Pooideae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| **Tribe: Triticeae** |       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Hordeum vulgare L. | Hv  | Area (mm²) | 185±19 |  -  | 2155±683 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 1345±160 |
|                      |      | Perimeter (μm) | -  | -  | 205±228 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 229±15 |
|                      |      | Patrinia type | 1826±248 | 248±6.55 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | 167.7±6.8 |
| Species           | Tribe | Genus | Area (cm²) | Perimeter (cm) |
|-------------------|-------|-------|------------|----------------|
| *Triticum aestivum* |       |       | 1684±484   | 266±65.4       |
| *Avena sativa*    |       |       | 670±163    | 162±30.5       |
| *Lolium temulentum* |       |       | 134±09     | 183±05         |
| *Phalaris minor*  |       |       | 267±08     | 186±20.8       |
| *Poa annua*       |       |       | 206±10.8   | 115±11.4       |
| *Arundo donax*    |       |       | 155±35     | 1290±213       |
| *Phragmites australis* |     |       | 360±24     | 95±12.1        |

**Subfamily:** Arundinoideae

**Tribe:** Arundineae

| Species           | Tribe | Genus | Area (cm²) | Perimeter (cm) |
|-------------------|-------|-------|------------|----------------|
| *Poa annua*       |       |       | 1189±252   | 275±2.36.6     |
| *Lolium temulentum* |       |       | 341±57.3   | 67±6.4         |
| *Phalaris minor*  |       |       | 341±57.3   | 67±6.4         |
| *Poa annua*       |       |       | 216±9.2    | 1183±266       |
| *Arundo donax*    |       |       | 217±102    | 1290±213       |
| *Phragmites australis* |     |       | 217±102    | 1290±213       |

**Subfamily:** Chloridoideae

**Tribe:** Cynodonteae
## Investigations into Phytoliths as Diagnostic Markers for the Grasses (Poaceae) of Punjab

| Tribe: Eragrostideae |  |  |
|---------------------|--|--|
| **Cynodon dactylon** | (L.) Pers | Cd |
| Area | 114±11 | 157.6±12.7 |
| Perimeter | 38.8±3.8 | 74.10±16.6 |
|  | 43.9±20.5 |
| **Tragus Aflatus** Schult. | Tb | 365±64 |
| Area | 88.96±5.3 | 24.3±2.1 |
| Perimeter | 98.76±9.37 |
|  | 137.6±9.18 |
| **Tribe: Eragrostideae** |  |  |
| **Dactyloctenium aegypticum** (L.) Willd. | Da | 117±03 |
| Area | 1506±428 | 54.8±1.2 |
| Perimeter | 41.2±1.7 | 75.2±0.8 |
|  | 48.5±4.1 |
| **Tribe: Paniceae** |  |  |
| **Elusine indica** (L.) Gaertn. | Ei | 175±19 |
| Area | 880±190 | 207.3±20 |
| Perimeter | 66.2±8.5 | 140.5±22 |
|  | 185.7±23.8 |
| **Sporobolus diandrus** (Retz.) P. Beauv. | Sd | 157±25 |
| Area | 1023±389 | 108±10 |
| Perimeter | 37.9±2.2 | 108±0.3 |
|  | 37.9±2.2 |

### Table of Measurements

|  | Area | Perimeter |
|---|---|---|
| **Cynodon dactylon** | 114±11 | 38.8±3.8 |
| **Tragus Aflatus** Schult. | 365±64 | 24.3±2.1 |
| **Dactyloctenium aegypticum** (L.) Willd. | 117±03 | 38.8±3.8 |
| **Elusine indica** (L.) Gaertn. | 175±19 | 41.2±1.7 |
| **Sporobolus diandrus** (Retz.) P. Beauv. | 157±25 | 40.5±1.7 |

### Tribe: Eragrostideae

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|                          |          |          |          |          |          |          |
|--------------------------|----------|----------|----------|----------|----------|----------|
| **Buchetia rapoosa**    | **L.**  | **Stepf.** | **Bren** | **Area** | 137.36±14.52 |          |
| **Cenchrus setigerus**   |          |          |          |          | 345.8±11.03 |          |
| **Digitaria abrudens**   |          |          |          |          | 0464.0.01 |          |
| **Bracharia ramosa**     |          |          |          |          | 212.36±0.85 |          |
| **Digitaria ciliaris**   |          |          |          |          | 219.26±18.66 |          |
| **crusgalli**            |          |          |          |          | 319.87±4.90 |          |
| **Cheimopygos**          |          |          |          |          | 519±67.27 |          |
| **colonum**              |          |          |          |          | 72.14±10.57 |          |
| **Claytonia**            |          |          |          |          | 102±8.145 |          |
| **C.**                   |          |          |          |          | 106±2.25 |          |
| **Vahl.**                |          |          |          |          | 617±69.57 |          |
| **Conodiocourtia Vahl.** |          |          |          |          | 464±273 |          |
| **Dalh.**                |          |          |          |          |          |          |
| **Chrysopogon**          |          |          |          |          | 1582±3183.5 |          |
| **serrulatus**           |          |          |          |          |          |          |
| **(Retz.) Koeler**       |          |          |          |          |          |          |
| **Cr.**                  |          |          |          |          |          |          |
| **Veldkomp.**            |          |          |          |          |          |          |
| **areae**                |          |          |          |          |          |          |
| **Ef**                   |          |          |          |          |          |          |
| **Digitaria ciliaris**   |          |          |          |          |          |          |
| **(Retz.) Koeler**       |          |          |          |          |          |          |
| **Dc**                   |          |          |          |          |          |          |
| **Echinochloa colona**   |          |          |          |          |          |          |
| **(L.) Link.**           |          |          |          |          |          |          |
| **Ut**                   |          |          |          |          |          |          |
| **Echinochloa**          |          |          |          |          |          |          |
| **crusgalli**            |          |          |          |          |          |          |
| **(L.) P. Beauv.**       |          |          |          |          |          |          |
| **Ets**                  |          |          |          |          |          |          |
| **Echinochloa**          |          |          |          |          |          |          |
| **etaphytrum**           |          |          |          |          |          |          |
| **(Hoffm.) W.D. Clayton**|          |          |          |          |          |          |
| **El**                   |          |          |          |          |          |          |
| **Echinochloa**          |          |          |          |          |          |          |
| **etaphytrum**           |          |          |          |          |          |          |
| **(Hoffm.) W.D. Clayton**|          |          |          |          |          |          |
| **Pan**                  |          |          |          |          |          |          |
| **Panicum etaphytrum**   |          |          |          |          |          |          |
| **Froel.**               |          |          |          |          |          |          |
| **Pam**                  |          |          |          |          |          |          |
| **Panicum maximum**      |          |          |          |          |          |          |
| **Jacq.**                |          |          |          |          |          |          |
| **Perimeter**            |          |          |          |          |          |          |
| **Area**                 |          |          |          |          | 107±0.7 |          |
| **Perimeter**            |          |          |          |          | 1936±0.0 |          |
| **Pan**                  |          |          |          |          | 289±742.5 |          |
| **37.66±3.60**           |          |          |          |          | 147±7.5 |          |
| **47.06±1.91**           |          |          |          |          | 188.9±10.2 |          |
| **28.30±1.87**           |          |          |          |          | 114±32.6 |          |
| **1586±11.6**            |          |          |          |          |          |          |
| **56.3±5.2**             |          |          |          |          | 112±2.2 |          |
| **67.6±6.4**             |          |          |          |          | 119±2.9 |          |
| **78.1±2.5**             |          |          |          |          | 234±60.2 |          |
| **113±16.7**             |          |          |          |          | 73.7±2.2 |          |
| **199±13**               |          |          |          |          | 53±144 |          |
| **108±11**               |          |          |          |          |          |          |
| **88.8±20.3**            |          |          |          |          | 102±8.145 |          |
| **106±2.25**             |          |          |          |          |          |          |
| **58±14**                |          |          |          |          |          |          |
| **37.5±19**              |          |          |          |          | 112±2.2 |          |
| **106±2.25**             |          |          |          |          | 119±2.9 |          |
| **68±9.2**               |          |          |          |          | 1936±0.0 |          |
| **255±47**               |          |          |          |          | 112±2.2 |          |
| **389±8.03**             |          |          |          |          | 119±2.9 |          |
| **289±742.5**            |          |          |          |          | 73.7±2.2 |          |
| **234±60.2**             |          |          |          |          | 1936±0.0 |          |
| **519±67.27**            |          |          |          |          | 72.14±10.57 |          |
| **102±8.145**            |          |          |          |          |          |          |
| **464±273**              |          |          |          |          |          |          |
| **1582±3183.5**          |          |          |          |          |          |          |
| **174.76±5.80**          |          |          |          |          |          |          |
| **1042±704.85**          |          |          |          |          |          |          |
| **147±5±377**            |          |          |          |          |          |          |
| **107±0.7**              |          |          |          |          | 1936±0.0 |          |
| **289±742.5**            |          |          |          |          | 73.7±2.2 |          |
| **107±0.7**              |          |          |          |          | 1936±0.0 |          |
| **289±742.5**            |          |          |          |          | 73.7±2.2 |          |
| **234±60.2**             |          |          |          |          | 1936±0.0 |          |
| **519±67.27**            |          |          |          |          | 72.14±10.57 |          |
| **102±8.145**            |          |          |          |          |          |          |
| **464±273**              |          |          |          |          |          |          |
| **1582±3183.5**          |          |          |          |          |          |          |
| **174.76±5.80**          |          |          |          |          |          |          |
| Species                      | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  | Area  | Perimeter  |
|-----------------------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|-------|------------|
| Paspalum flavidum            | 116±18| 186±276    | 1369±288 | 227±10     | 121±19 | 264±17     | 227±28 |             |       |            |       |            |       |            |       |            |       |            |       |            |
| Paspalum paspaloids          | 3.18±1| 83.6±3.5   | 1299±631 | 89±02      | 45±20  | 91±13±4    | 77±14±2|             |       |            |       |            |       |            |       |            |       |            |       |            |
| Setaria verticillata         | 113±26| 270±40     | 197±54  | 73±05      | 113±25 | 809±181    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Arundinella nepalensis       | 166±43| 533±115    | 367±45  | 55±42      | 38±12  | 43±12±3    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Bothriochloa pertusa         | 261±16| 108±207    | 495±231 | 140±17     | 229±27 | 923±172    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Cymbopogon martinii          | 170±47|             | 155±280 | 135±223    | 144±147| 185±223    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Dichanthium annulatum        | 159±04| 110±259    | 268±45  | 253±26     | 84±12 | 243±197    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Dichanthium caricosum        | 133±17| 109±172    | 256±25  | 569±59     | 907±33 | 878±182    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Eulaliopsis binata           | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Heteropogon contortus        | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Eulaliopsis binata           | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Tabwa                      | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Eulaliopsis bicolor         | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Tabwa                      | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Eulaliopsis bicolor         | 199±18| 39.6±16.05 | 74.6±27.1| 162.6±12.6| 242±6.30| 54±4.74    |       |            |       |            |       |            |       |            |       |            |       |            |       |            |       |            |       |            |       |            |
| Species                        | Area   | Perimeter  | Area   | Perimeter  |
|-------------------------------|--------|------------|--------|------------|
| *Imperata cylindrica* (L.) P. Beauv. | 217±15 | 83.7±1.4   | 197±08 | 61.07      |
|                              | 77.9±0.9 |           | 124±05 | 48.06      |
| *Saccharum bengalense* Retz.  | 125±19 | 77.9±0.9   | 187±32 | 61.07      |
|                              | 184±6.6 |           | 241±68 | 77.9±0.9   |
| *Saccharum ravennae* L.       | 114±16 | 72.4±1.8   | 259±08 | 72.4±1.8   |
|                              | 50.3±6.6 |           | 187±32 | 72.4±1.8   |
| *Sorghum halepense* (L.) Pers. | 114±10 | 51.3±2.2   | 233±22 | 72.4±1.8   |
|                              | 72.4±1.8 |           | 223±22 | 72.4±1.8   |
| *Vetiveria zizanioides* (L.) Nash | 186±10 | 72.4±1.8   | 213±30 | 72.4±1.8   |
|                              | 72.4±1.8 |           | 213±30 | 72.4±1.8   |
| *Zea mays* L.                 | 245±15 | 72.4±1.8   | 257±90 | 72.4±1.8   |

*Mean±Standard error; The minus sign (−) indicates absence.*
Scanning Electron Micrographs (Figure 14a-n): Saddle (a) (Bar= 2.5μm), dendritic (b and c with Bar= 12μm & 10μm respectively), trapezoid (d) (Bar= 2μm), Saddle (e) (Bar= 10μm), bilobate (dumbell) (f,g) (Bar= 2 & 2.5μm respectively) with a narrow (f) and broad (g) shank, ovate (h) (Bar= 10μm), Polylobate (i) (Bar= 3 μm), hat-shaped (j) (Bar= 2μm), Sinuate elongate (k) (Bar= 20μm), Clavate (l) (Bar= 10μm), Smooth elongate (m)(Bar= 3μm) & Rectangular (n) (Bar= 2 μm).

Arundinella nepalensis=An; Dichanthium caricosum=Dca; Cynodon dactylon=Cd; Paspalum paspaloids=Pp; Digitaria abhuds=Dab; Cenchrus setigerus=Cs; Poa anuva=Pa; Digitaria ciliaris=De; Phragmites australis=Pau; Dicanthium annulatum=Da; Eleusine indica=El; Panicum antidotale=Pan

Within Chloridoideae, phytolith types help to characterize the tribes and genera [21]. Besides the dendritic shaped, all the seven species of the tribe Eragrastideae produced the bilobate phytoliths but showed difference in other types. Desmostachya bipinnata (L.) Stapf. is distinctly marked out by the presence of lanceolate and quadrilobate types of phytoliths which occur only in this species of the tribe Chloridoideae. Similarly, Sporobolus diander (Retz.) P.Beauv. is characterised by the presence of oval types which it shares only with Leptochloa chinensis (L.) Nees. The other congeneric species, Leptochloa. panicea (Retz.) Ohwi. did not show this type. However, these two species of the genus stood out from the rest of the tribe Eragrastideae in producing rectangular phytoliths which are absent in other members of the tribe. However these species of Leptochloa show a significant difference in size of rectangular types being much larger in Leptochloa panicea (Retz.) Ohwi. (P≤ 0.001). The trapezoidal types were present in both the species but showed a difference in size having significantly larger surface area in Leptochloa. panicea (Retz.) Ohwi. (P≤ 0.001). Similarly, Neyraudia arundinacea (L.) Henrard and Sporobolus diander (Retz.) P.Beauv. comprised a pair of species of the Eragrastideae that had the clavate types which were significantly smaller in the later named species (P≤0.05). The diagnostic significance of the morphometric data on phytoliths in taxonomic characterisation has been shown in several studies [24]. Some other types viz., rondel and clavate occurred in various combinations.
3.6. Panicoideae

Several studies have mentioned bilobate phytoliths as diagnostic of panicoid grasses [44, 45]. Bilobate types of phytoliths emerged as the most ubiquitous type in panicoid species of the present sample as well (Table 1). However within the subfamily, a tribe wise pattern of distribution of phytolith was observed.

The tribe Arundinellae represented in the present sample by a single member Arundinella nepalensis Trin. showed up bilobate as the most common type (1b). After the bilobate, the saddle and rondels was found to be most abundant and diagnostic type which could help to discriminate this species from other panicoid species (Figure 10b, 14a). Other less common types were clavate, dendritic and nodular bilobate.

In the tribe Paniceae, apart from the bilobates, hat-shaped emerged as the diagnostic type for Digitaria ciliaris (Retz.) Koeler (Figure 14j). Similarly, the saddle shaped were seen only in Panicum maximum Jacq. and cross shaped types emerged as characteristic of Brachiaria repens (L.) Gardn. & Hubb. (Figure 7a) in the present sample.

Andropogoneae, the third tribe of the subfamily Panicoideae, was represented by 12 species in the present sample. The bilobate types were present in all the species of this tribe. However, dendritic, clavate, and smooth elongate emerged as the other common types in the tribe. The trapezoidal phytoliths seem to differentiate the two species emerged as the other common types in the tribe. The sample. The bilobate types were present in all the species of this tribe ranged from 114±10 μm² in Saccharum revennae (L.) Pers. and Dichanthium annulatum (Forssk.) Stapf. On the other hand, species that bore narrow bilobates with thin shanks included Digitaria abludens (Roem & Schult.) Veldkamp, Dichanthium caricosum L.A. Camus, Dichanthium annulatum (Forssk.) Stapf and Echinochloa crusgalli (L.) P. Beauv. (Figure 14f).

3.7. Surface Features

Examination of surface features of various kinds of phytoliths through light and Scanning Electron Microscopy did not show up significant variation in surface ornamentation and ultra structure that could be utilized in diagnosis of grass taxa. However, SEM examination revealed that bilobate phytoliths could be put into distinct groups based on the differences in the shape of the outer margin. One group comprised of species with bilobates having flattened outer margin (Cenchrus setigerus Vahl, Setaria verticillata (L.) P. Beauv., Eriochloa fimbriata (Hosch.) W. D. Clayton, Imperata cylindrica (L.) P. Beauv., Sorghum halepense (L.) Pers. (Figure 14g). The other group bore a characteristic and diagnostic depression on the outer margin (Digitaria abludens (Roem & Schult.) Veldkamp, Arundinella nepalensis Trin., Bothriochloa pertusa (L.) A. Camus, Cymbopogon martini (Roxb.) Watson, Dichanthium annulatum (Forssk.) Stapf., Echinochloa crusgalli (L.) P. Beauv., Paspalidium flavidum (Retz.) A. Camus and Vetiveria zizanioides (L.) Nash) (Figure 14f). In recent literature, such types have been labeled as scooped bilobates [46] and diagnostic significance attached to such scooping of the bilobates. In a review of phytolith types in grasses, Rudall et al. [47] have concluded that this scooped subtype are diagnostic of Leersia-Oryza clade in particular and of the whole tribe Oryzae to a lesser degree. Apart from the shape of outer margin, scanning electron micrographs has clearly brought out the presence of a half lobe on one side of the central shank in some of the bilobate types. These are called nodular bilobate types (Cenchrus setigerus Vahl., Digitaria ciliaris (Retz.) Koeler, Dichanthium caricosum (L.) A. Camus and Sorghum halepense (L.) Pers. It emerges from the present studies that phytoliths in grasses display a wide range of variation in shape, size and ultrastructure which could be utilised for characterisation and diagnosis of grass taxa.

4. Conclusion

Characterization and identification of grasses is a tedious task partly because of a heavy dependence on reproductive parts which are not only available for a limited period in the phonological cycles of grasses but are also very small in size and have overlapping morphologies among taxa. Characters of vegetative morphology provide little help in conventional formats of grass description and identification. In this backdrop, phytoliths provide a potent character for taxonomic characterization and identification of grasses. Our
results from phytolith analysis of forty eight grass species reveal that characteristic combinations of phytolith types are diagnostic of subfamilies, tribes, genera and species comprising the present sample. The study has also brought to light some novel types such as the hat shaped phytoliths from *Digitaria ciliaris* (Retz.) Koeler. Surface features and the morphometric measurements provide additional parameters for characterization and diagnosis of grass taxa. Further studies on the phytoliths of grass species of the region would help in developing phytolith profiles of the leaf epidermis and other tissues and organs. It is only after such analysis that we would be able to develop phytolith signatures of grass species, genera and higher taxa. With these advances, species identification in grasses would be possible even in the vegetative phase. An epidermal peeling with a diagnostic phytolith signature would yield as important a character for species identification as the structure of the spikelet in the reproductive phase of the grass phenology.

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