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RESEARCH ARTICLE

A SCANNING ELECTRON MICROSCOPY STUDY OF POLLEN GRAINS

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Abstract

A simple, quick and non-destructive method of preparing fresh pollen grains for scanning electron microscopy (SEM) studies was used in this investigation. The pollen grains of seven taxa belonging to seven families were studied; six were dicots, namely *Delonix regia*, *Helianthus annuus*, *Justicia spicigera*,*Kigeliaafricana*,*Lagerstroemia speciosa*and *Portulaca umbraticola*,and one a monocot*Hymenocallis littoralis*. Fresh pollen grains were dehydrated in acidified 2, 2-dimethoxypropane (DMP) followed by criticalpoint drying in carbon dioxide (CO_2). In the preliminary study the acidified DMP treated pollen grains were mounted in glycerine and observed under the light microscope. The DMP direct method preserves the original shape of the pollen grains and dissolves the pollen coat thereby enabling observation of the surface ornamentation of the exine. The high magnifications of the SEM provided detailed information about the fine structure or micromorphology of the pollen surface. In the taxa studied the pollen are aperturate and are shed as monads.Among the taxa studied, *P.umbraticola* was apolar, *H.littoralis* was heteropolar,and the remaining five were isopolar. The exine was reticulate in *D. regia*, *H.littoralis*, *J.spicigera*, and *K.africana*; echinate in *H. annuus*and*P.umbraticola*; andfossulate, perforate in *L. speciosa*.

Introduction:-

Pollen grains are produced in the anthers by a process called microsporogenesis which results in the production of four haploid microspores through meiotic division of the pollen mother cells. Pollen is called two-celled if it contains the vegetative cell and generative cell, and three-celled if the generative cell divides to form two sperm cells. Pollen is a very fine usually yellow coloured dust that is carried to other plants by wind, insects, or other agents. The study of pollen morphology is called palynology. In higher plants, the most complex wall system is probably the pollen wall(Shivanna, 2003).A mature pollen grain wall (sporoderm)is double-layered; the exospore or exine is the tough resistant outer layer and the endospore or intineis the delicate innerlayer. The exine provides protection to pollen grains and is variously sculptured. The exine is mainly made up of sporopollenin which is an acetolysis- and decay-resistant biopolymer (Bhojwani *et al*., 2015; Halbritter *et al.*, 2018).Distinct orbicular granules of sporopollenin called Ubisch bodies are produced by the tapetum, especially in plants with secretory tapeta (Punt *et al*., 2007).

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The pollen grains produced by some angiosperms are sticky because of the pollen coat material which is derived from the tapetum; the pollen coat material is referred to as pollenkitt or tryphine.Tryphines are more heterogenous than pollenkitts in their chemical composition (Dickinson & Lewis, 1973; Pacini&Hesse, 2005).Pollenkitts are coloured and serve many functions including holding the pollen together in the anther so that dispersal occurs in clumps, maintaining sporophytic proteins involved in pollen-stigma interaction and recognition within cavities in the exine, facilitating pollination by attracting and adhering to insects and animals, and protecting from ultraviolet radiation, water loss, exocellular enzymes and hydrolysis (Pacini&Hesse, 2005; Halbritter *et al*., 2018).Tryphine is present only in the Brassicaceae, and the tryphine that coats the pollen grains of *Raphanus* is responsible for the self-incompatibility system (Pacini&Hesse, 2005; Dickinson & Lewis, 1973).

The pollen grains of different taxa vary in shape, size, polarity, apertures and surface ornamentation. Various terms have been used to describe the exine sculpturing. The exine is thin or absent at apertures. The NPC system describes the number, position and character of apertures in pollen grains (Erdtman, 1966; Saxena, 1993).The features of the pollen grains are genetically determined and are not affected by environmental conditions; and palynologyis a valuable tool in supporting cladistic and multidisciplinary studies in tracing the evolutionary history of taxa, and in detection of adulteration in crude drug preparations (Gonçalves-Esteves *et al*., 2022; Ragho, 2020). It is easy to collect and store pollen grains for a considerable length of time. Their storage under viable conditions facilitates their study and use throughout the year(Shivanna& Rangaswamy, 1992).

The light microscope allows the study of the morphology of pollen grains. However, the presence of surface coating does not allow a detailed study of the apertures and the exine. Pollen grains are opaque and highly refractive, and the depth of focus of light microscopes is limited; therefore, good images of pollen surface structure cannot be obtained. The scanning electron microscope (SEM) provides a far better depth of focus than a light microscope,and a detailed and accurate picture of the pollen surface can be obtained (van Laere*et al*., 1969). Acetolysis is a standard procedure for studying pollen grains under the light microscope and is suited for pollen grains with thick and stable exines; the method is also used for SEM studies. However, acetolysis can damage pollen with fragile exines and also the aperture membrane(Halbritter *et al*., 2018).

The conventional method for preparing pollen samples for SEM studies is laborious and time-consuming involving aldehyde fixation, dehydration, drying and sputter coating often altering the morphology of the pollen grains. Contrarily, the DMP (2, 2- dimethoxypropane) direct method is an easy and quick method by which fresh pollen as well as pollen from herbarium specimens can be studied by the SEM; the method preserves the surface details of the pollen, and permits the study of pollen that are fragile or with heavy coating on the exine (Halbritter*et al*., 2018; Halbritter, 1998). In the present investigation the fresh pollen grains of seven taxa; six dicots and one monocot, have been studied by the DMP direct method.

Materials and Methods:-

Plant materials studied

Angiosperms that were flowering during the period of study and easily available in and near the collegecampus were chosen for thestudy (Table 1).

Pollen collection

Fresh pollen was collected from dehisced or ready-to-dehisce anthers of flowers/florets on a butter paper. The pollen was scooped or dusted from the anthers using a pair of forceps and needle and mixed to obtain a homogenous sample.

Light microscopy

A drop of glycerine was taken on a microslide. A small sample of the pollen was dispersed in the glycerine drop using a needleand a coverslip was lowered carefully. The preparation was observed under a binocular compound light microscope, first using a 4X objective, and then under 10X and 40X.A cell phone camera was used to take pictures of the pollen grains.The morphological features of the pollen grains were noted.

Table 1:- The experimental materials.

S. No.	Common name	Scientific name	Family
1	Gul mohur/ royal poinciana	*Delonix regia*(Bojer ex Hook.) Raf.	Fabaceae

2	Sunflower	*Helianthus annuus* Linn.	Asteraceae
3	Beach spider lily	*Hymenocallis littoralis* (Jacq.) Salisb.	Amaryllidaceae
4	Mexican honeysuckle	*Justicia spicigera*Schltdl.	Acanthaceae
5	Sausage tree	*Kigeliaafricana*(Lam.) Benth. [synonym *K. pinnata* (Jacq.) DC.]	Bignoniaceae
6	Queen's crape myrtle	*Lagerstroemia speciosa*(L.) Pers.	Lythraceae
7	Wingpod purslane	*Portulaca umbraticola*Kunth	Portulaceae

Effect of acidified 2, 2- dimethoxypropane (DMP) on pollen

DMP [2, 2-dimethoxypropane or acetone dimethyl acetal, $[(CH_3)_2C(OCH_3)_2]$ is an organic compound and an alkylating reagent. DMP reacts with water to form acetone and methanol, thereby dehydrating the material. Acidified DMP solution was prepared by adding one drop of 0.2 M HCl to 30 mL of DMP (Halbritter, 1998). Acidified DMP was taken in a cavity block and freshly collected pollen was transferred into thecavity block. The lid was placed on the cavity block. The pollensamples were treated with acidified DMP for up to 40 minutes. The pollen inDMP were observed 10, 20, 30, and 40 minutes from transfer to DMP by taking out a small sample on a microslide and mounting in glycerine. The microslide was observed under a binocular compound light microscope.The treatment period when the exine could be seen with maximum clarity was chosen as the optimal treatment time which ranged from 20 to 40 minutes.Representative photographs were taken using a cell phone camera.

Scanning electron microscopy

The pollen samples were treated with acidified DMP for the duration standardized for the specific material. Followingtreatment with acidified DMP the dehydrated pollen grains were transferred to 100% acetone.Pollengrains in 100% acetone were poured into the white porous pots with the help of a micropipette using 10 microlitre (µL) tips.Then the white porous pots were put into the pellet. The pellet was then placed in the cavity of the Critical Point Drying (CPD) machine (model no. Leica EM CPD300) which was filled with acetone.The CPD process took around 1 hour and 15minutes.After completion of the CPD process, the dried pollen grains were brushed out on a carbon tape fixed on metal cylinders.The next step was gold coatingwhich was done using the Ion Coating Machine (model no. Jeol JEC-3000F). Thereafter the metal cylinders were transferred to the vacuum chamber of the SEM and the pollen grains were scanned using the SEM (model no. Jeol JSM 6610). The pollen grains were observed under different magnifications and photographs were taken.The terminology used in the present investigation is based on available literature (Halbritter *et al*., 2018; Punt *et al*., 2007; Erdtman, 1966; Saxena, 1993;https://research.fit.edu/media/site-specific/researchfitedu/appliedbiogeography/documents/protocols/Quick-Reference-Glossary-with-Illustrations.pdf).

Results and Discussion:-

Effect of acidified DMP on pollen

Under the light microscope morphological features of the pollen could be studied, namely the shape, aperture, and exine to some extent. The exine patterning of the pollen grains became clear and distinct on treatment with acidified DMP (Figure 1). DMP dissolved the pollen coat thereby allowing observation of the exine.The features of the pollen grains based on light and electron microscopy observations are in Table 2.In all the seven taxa studied the mature pollen grains are shed as individual units or monads, and the pollen grains are aperturate. The pollen grains were ellipsoidal in *H.littoralis*, cylindrical in *J.spicigera*, and spheroidal or nearly spheroidal in the remaining five taxa (Figure 1; Table 2). The pollen of *D. regia*, *H. annuus*, *J. spicigera*,*K.africana*and *L. speciosa*were isopolar, whereas of *H.littoralis* was heteropolar. Apolar pollen grains were observed in *P.umbraticola*.Apolar pollen have been reported in *Plantago*(Punt *et al*., 2007)and in ten Hawaiian *Portulaca*species, including two unknown taxa (Kim, 2013).

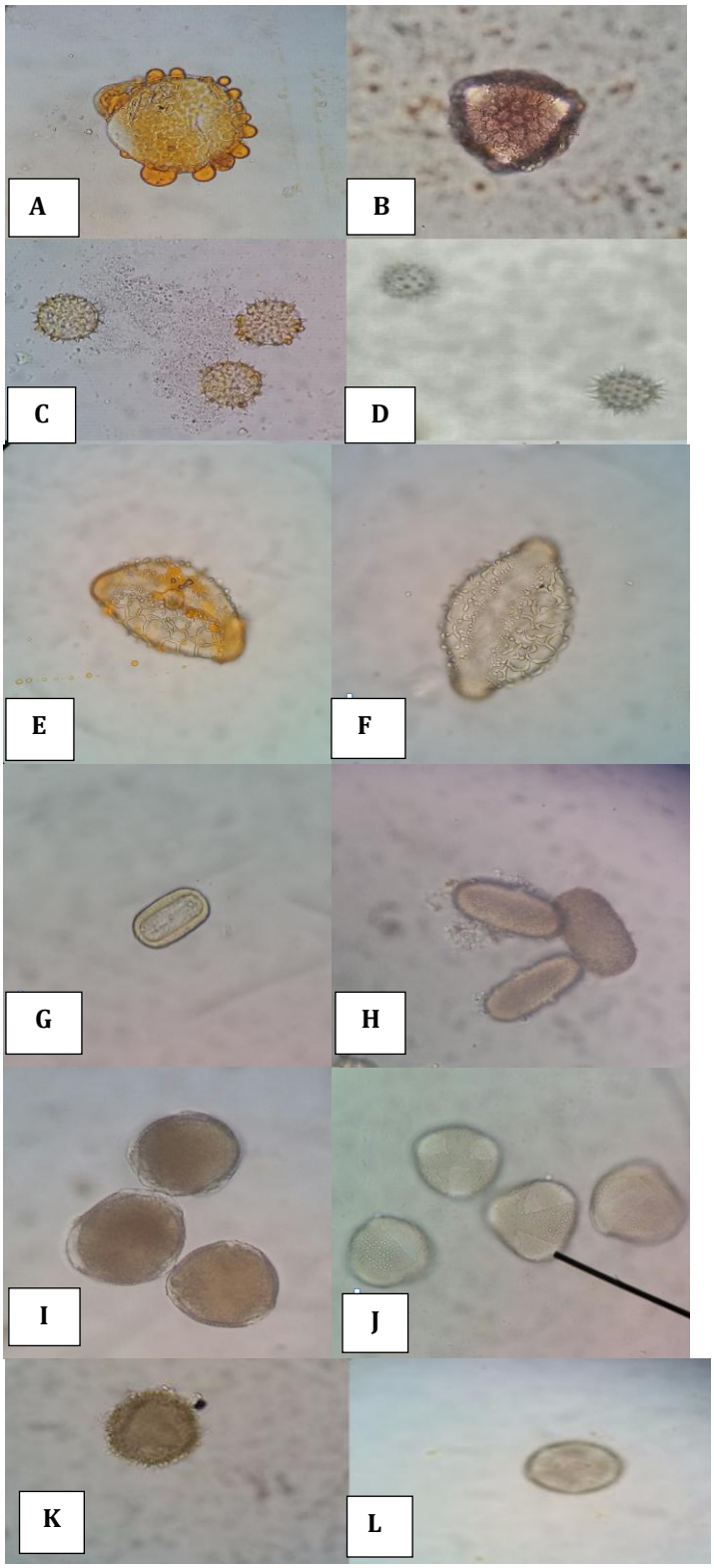

Figure 1:- Light micrographs of pollen grains: *Delonix regia* A. In glycerine, B. In acidified DMP; *Helianthus annuus*C. In glycerine, D. In acidified DMP; *Hymenocallislittoralis*E.In glycerine, F. In acidified DMP; *Justiciaspicigera*G. Inglycerine, H. In acidified DMP; *Kigeliaafricana* I. In glycerine, J. In acidified DMP; *Portulaca umbraticola*K. In glycerine, L. In acidified DMP.

Scanning electron microscopy

The DMP direct method has several advantages over traditional preparation protocols, including a short preparation time of less than 2 hours from treating the pollen to obtaining the scanning electron micrograph and excellent preservation of the size and shape of pollen grains.The high magnifications of the SEM enabledan accurate study of the micromorphology of the pollen surface (Figures 2-8).

H. littoralis pollen had a single elongated distally located aperture (monosulcate, Figure 4B),*J. spicigera* was dicolporate (Figure 5B), *K. africana* was tricolpate (Figure 6C), *D. regia*(Figure 2C), *H. annuus* and *L. speciosa* (Figure 7A,B) were tricolporate, and *P. umbraticola* (Figure 8A) was pantocolpate. Most monocotyledons and some primitive dicots show monocolpate pollen. Our observations on the pollen including the exine ornamentation of *D. regia*, *H. annuus* and *H. littoralis* are in agreement with earlier reports (Halbritter & Weis, 2016; Halbritter *et al*., 2020; Halbritter *et al*., 2021); on pollen of *J. spicigera* correspond to the work on *J. carnea* (Halbritter, 2015); on *K. africana* to previous research work (Ugbabe*et al*., 2013; Heigl, 2021); on *L. speciosa* correspond to the work on *L. indica* (Halbritter*et al*., 2021); and on *P. umbraticola* to the work on *P. grandiflora* (Halbritter, 2016). Table 2 gives a compilation of the features of the seven taxa studied.The exine ornamentation was reticulate in *D. regia*, *H. littoralis*, *J. spicigera*, and*K. africana*(Figures 2D, 4A,C, 5C,D, 6C,D);echinate in *H. annuus*and*P. umbraticola*(Figures 3C,D, 8C,D);andfossulate, perforate in *L. speciosa*(Figure 7D).

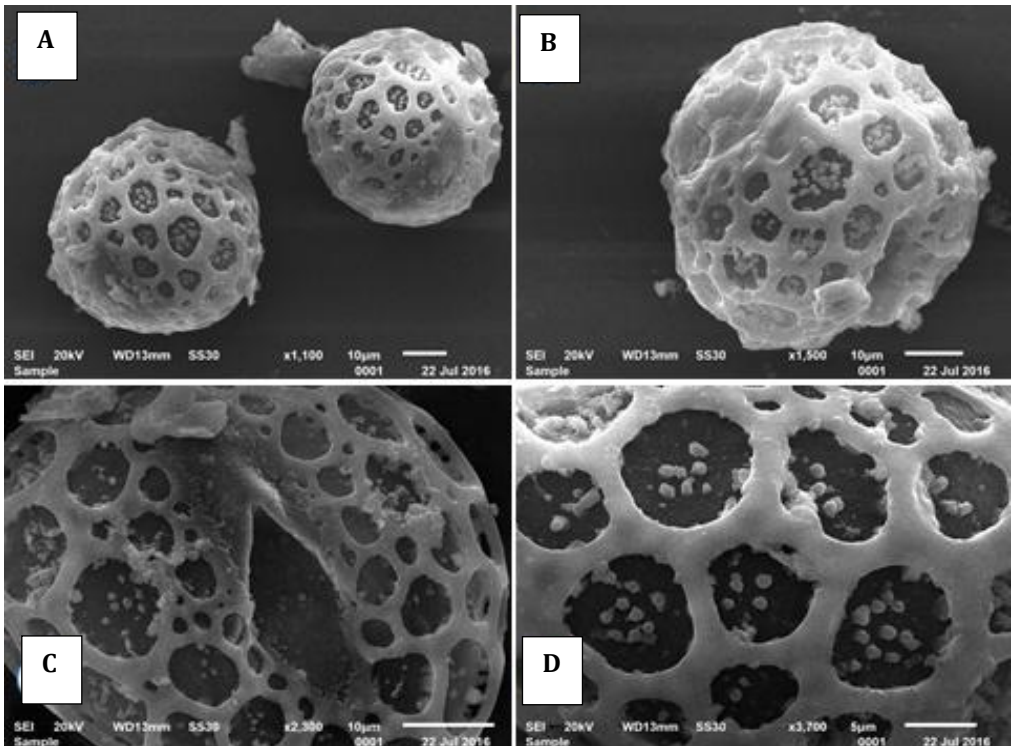

Figure 2:- Scanning electron micrographs of pollen of *Delonix regia*.A colporus is distinct in C, and the columellae and reticulate exine sculpturing are clear in D. The Bar represents 10 μm in A, B and C; and 5 μm in D.

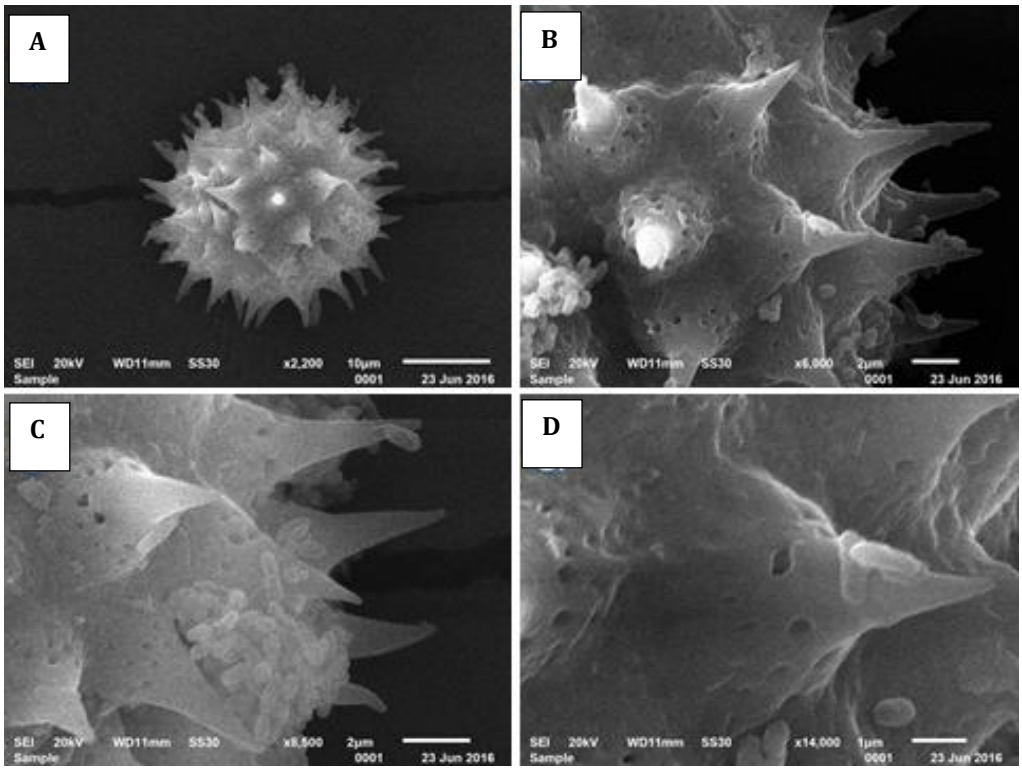

Figure 3:- Scanning electron micrographs of pollen of *Helianthus annuus*.The echinate, perforate condition is evident in B, C and D.The Bar represents 10 μm in A, 2 μm in B and C, and 1 μm in D.

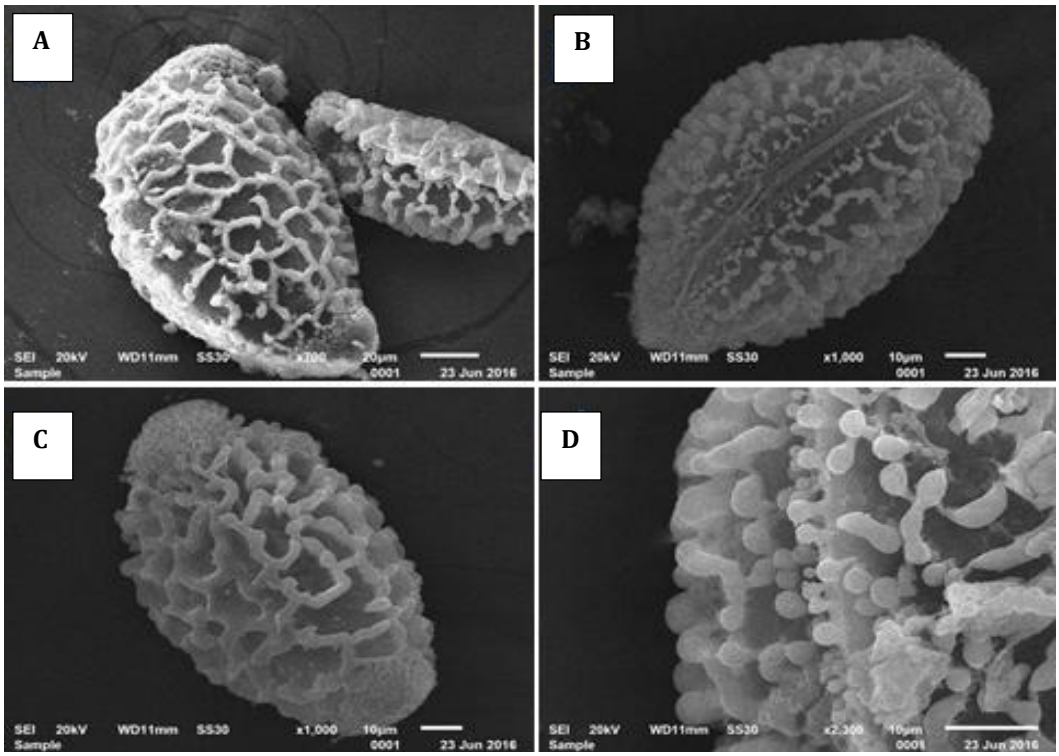

Figure 4:- Scanning electron micrographs of pollen of *Hymenocallis littoralis*. The reticulate surface is clear in A and C, and the clavaeare evident in D. In B the sulcus, is clear. The Bar represents 20 μm in A; and 10 μm in B, C and D.

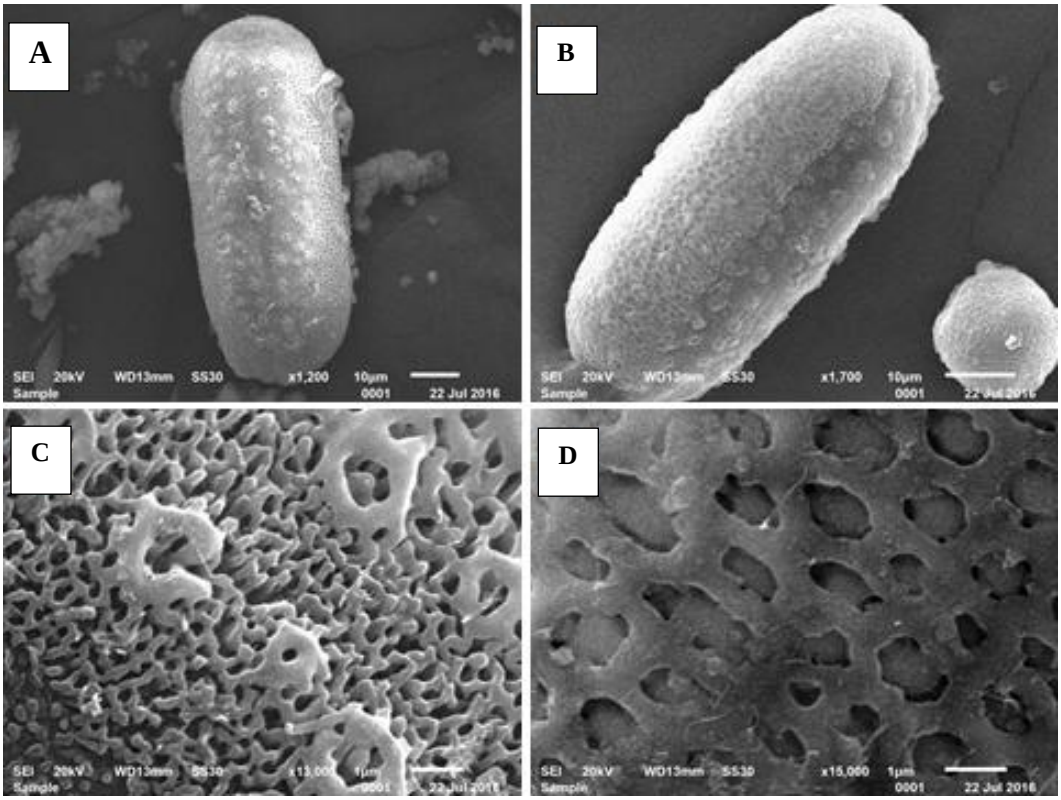

Figure 5:- Scanning electron micrographs of pollen of *Justicia spicigera*.The perforate, areolate, reticulate surface is clear in C and D. An aperture is evident in A and B. The Bar represents 10 μm in A and B; and 1 μm in C and D.

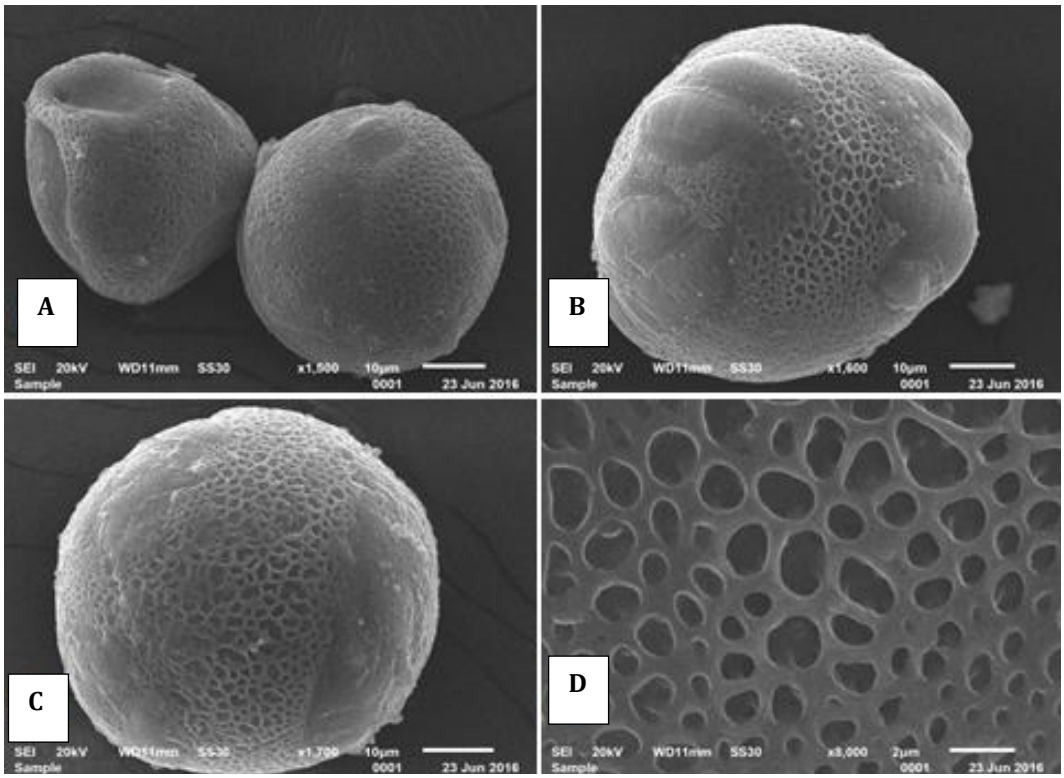

Figure 6:- Scanning electron micrographs of pollen of *Kigeliaafricana*.The reticulate surface is evident in C and D.The Bar represents 10 μm in A, B and C, and 2 μm in D.

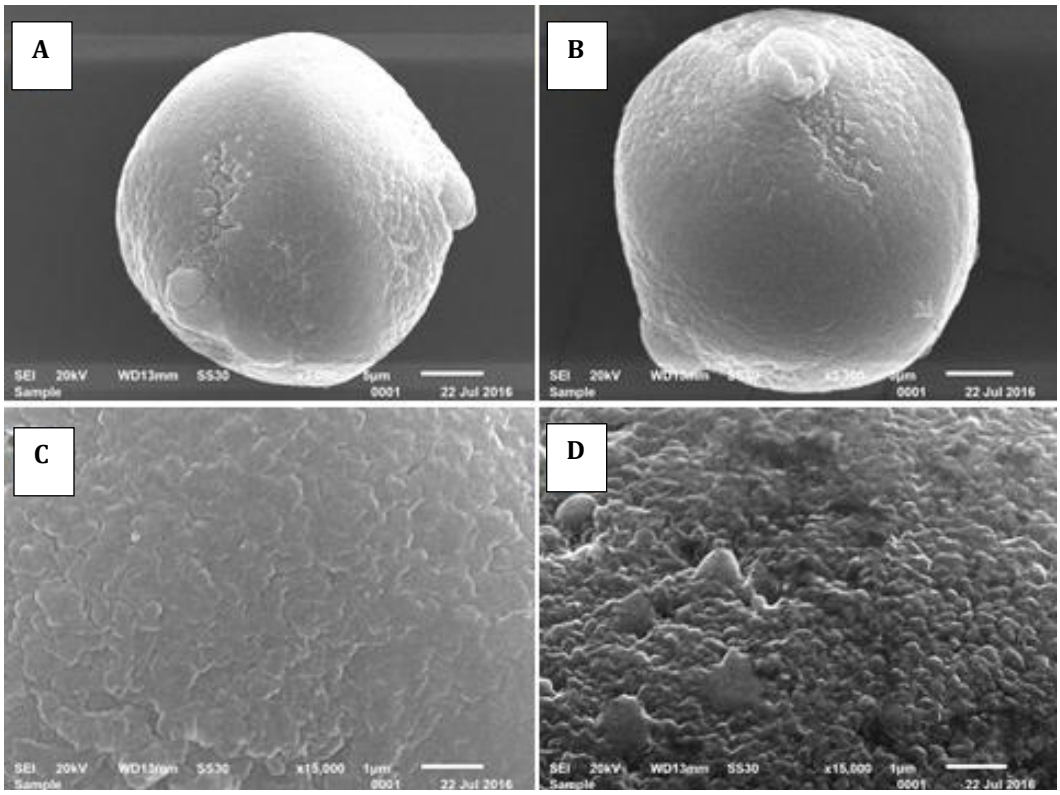

Figure 7:- Scanning electron micrographs of pollen of *Lagerstroemia speciosa*.Two apertures are clear in A and B.The fossulate, perforate surface is seen in D.The Bar represents 5 µm in A and B, and 1 µm in C and D.

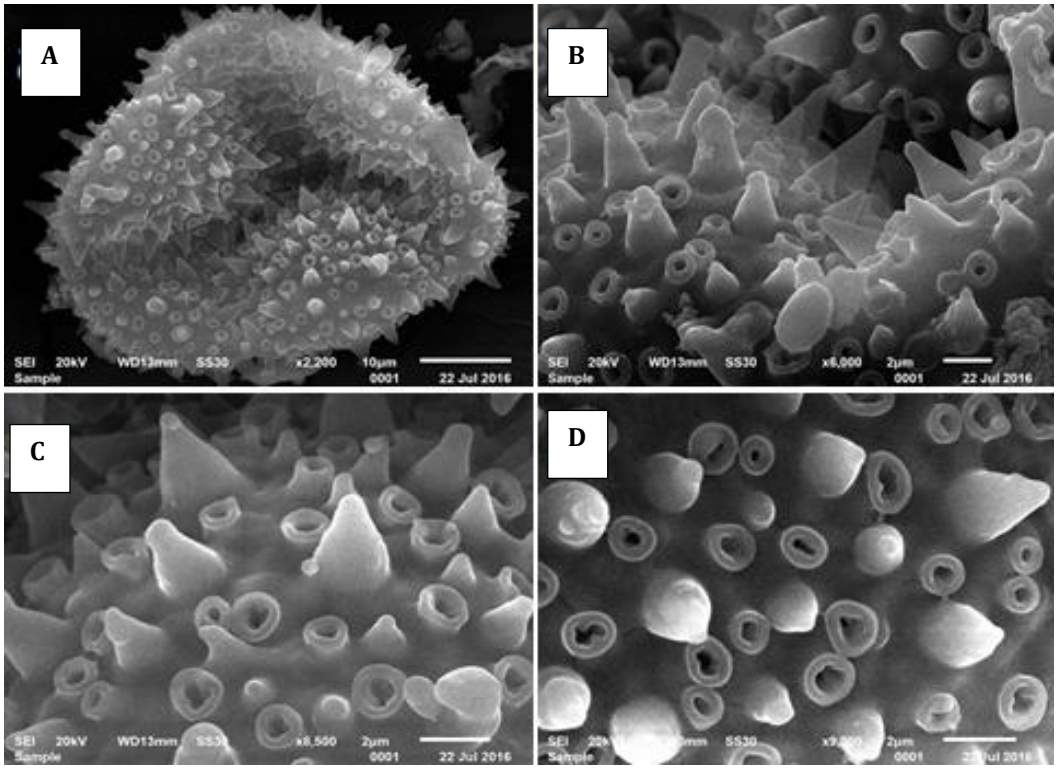

Figure 8:- Scanning electron micrographs of pollen of *Portulaca umbraticola*.The echinate, perforate surface is very clear in C and D. The Bar represents 10 µm in A;and 2 µm in B, C and D.

Conclusions:-

The DMP direct method offers many advantages and the surface architecture of pollen grains can be studied easily in a short time. Fresh as well as pollen from herbarium samples can be used. The pollen grains of seven angiosperm taxa belonging to seven families were studied by the DMP direct method. The method preserved the exine and fine structural details of exine patterning could be observed.

Table 2:- The features of the pollen grains of the seven taxa studied based on light and scanning electron microscopic observations.

Taxon	Pollen unit	Shape*	Polarity*	Aperture number & condition*	Exine ornamentation**
Delonix regia	Monad	Spheroidal	Isopolar	3, tricolporate	Reticulate, free-standing columellae
Helianthus annuus	Monad	Spheroidal	Isopolar	3, tricolporate	Echinate, perforate
Hymenocallis littoralis	Monad	Ellipsoidal	Heteropolar	1, monosulcate	Reticulate, clavate
Justicia spicigera	Monad	Cylindrical	Isopolar	2, dicolporate	Perforate, areolate, reticulate
Kigeliaafricana	Monad	Spheroidal	Isopolar	3, tricolpate	Reticulate
Lagerstroemia speciosa	Monad	Nearly spheroidal	Isopolar	3, tricolporate	Fossulate, perforate
Portulaca umbraticola	Monad	Spheroidal	Apolar	6 or more, pantocolpate	Echinate, perforate

*The shape, polarity, and aperture number and features are based on our observations as well as the literature consulted.

**Based on light and scanning electron microscopic observations. The terminology used is according to the literature (Halbritter *et al*., 2018; Punt *et al*., 2007; Erdtman, 1966; Saxena, 1993;https://research.fit.edu/media/site-specific/researchfitedu/appliedbiogeography/documents/protocols/Quick-Reference-Glossary-with-Illustrations.pdf).

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