**Pollen micro-morphometry of two endangered species of *Rauvolfia* L.(Apocynaceae) from the Indo-Gangetic plains of Central India using LM, CLSM and FESEM**

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**ABSTRACT**

*Rauvolfia* belongs to the family Apocynaceae and encompasses herbs or shrubs with leaves in whorls of 3 or 4. Itis an endangered plant of the tropics and sub-tropics. We present a preliminary study and analysis of the morphological details of the pollen of two extant species of *Rauvolfia* (*R. serpentina* (L.) Benth. ex Kurz and *R. tetraphylla* L.) from the Ganga plain using a light microscope (LM), confocal laser scanning microscope (CLSM) and field emission scanning electron microscope (FESEM). The critical point drying method (CPD) was adopted to test the pollen size difference from the conventional acetolysis method (ACE). The pollen morphology of *R. tetraphylla* differs from *R. serpentina* by several specific traits. Its pollen grain is 3-colporate; oblate to oblate-spheroidal; sexine punctate to obscure and mostly as thick as nexine; a distinct thickening is present around the ectocolpi. The study shows that *R. serpentina* can be distinguished with *R. tetraphylla* by its pollen shape, size, sexine ornamentation (particularly the presence and absence of punctae/perforations in the mesocolpial region) and length of the ectocolpi thickening. The pollen shape and aperture number are more or less common features in *Rauvolfia* spp., but the presence and absence of a punctate pattern at the mesocolpial position marks the primary difference between the two species. The *t*- test was applied to observe the statistical significance of pollen morphological data of both species. This study provides a source of information for systematic and conservation purposes and provides a baseline to facilitate palynological studies of past vegetation and palaeoenvironments.

**KEYWORDS**: Pollen morphology; *Rauvolfia serpentina*; *R. tetraphylla*; Endangered; Ganga Plain; India.

1. **Introduction**

The botanical investigations of the reproductive parts of endangered plant species are of significance in adopting propagation measures for long term survival (Garg & Rao 1996). Pollen morphology is directly correlated with the pollination mechanism (Garg & Rao 1997; Rao et al. 1999) as the ornamented pollen tends to be more easily trapped on pollinating insect's body parts, compared to smooth pollen grains. Similarly, pollen germination on the stigmatic surface is directly dependent on pollen wall thickness, and the number of apertures (Walker & Doyle 1975). The investigations of pollen morphology in endangered species of *Rauvolfia (R. serpentina* and *R. tetraphylla*) is therefore significant in providing insight on pollination limitation if any, during vector transfer of pollen to the stigma. This is critical for successful germination and reproduction for species and their long term survival or may be one of the possible causes of a species rarity. While pollen strata and the surface pattern is indicative of pollen trapping (sticking) and loading capacity of the vectors, the germinal apertures are the regions of pollen tube growth on the stigma and provide information on germination success and/or failure.

The pollen micro-morphometric analysis under LM and FESEM are always crucial for accurate identification and critical to be able to identify the fossil counterpart in the sediment for the reconstruction of palaeoflora vis à vis past climatic changes. These pollen micro-morphometric characters could also aid in facilitating the conservation, establishment and multiplication (through plant reproductive processes) of both the species of *Rauvolfia* ex situ and/or in situ as well as aiding in the authentication and standardization of drugs prepared from them.

In developing countries, medicinal plants are attaining greater importance in the primary health care of individuals and communities. For the proper identification and standardization of crude drugs, accurate anatomical and morphological description of the botanical sources of these drugs is necessary, and this description must take into account all the diagnostic features for an accurate species identification including the pollen micro-morphometry (Fazal et al. 2013). Despite the high interest in these two endangered species no extensive studies have been made on their pollen morphology especially in *R. tetraphylla* and both species lack accurate information about their pollen micro-morphometry. Thus, the present study aims to provide accurate information regarding the pollen morphology in *R. serpentina* and *R. tetraphylla* and provide an auxiliary tool for the correct identification of these two endangered species which are used for medicinal purposes. The distribution of these two species of *Rauvolfia* within India is provided in Fig. 1.

Pollen morphology is of fundamental significance in the wider applications of palynological science, especially for the recognition and identification of isolated pollen grains, such as those recovered from the atmosphere, honey or sedimentary deposits. In addition, palynology serves as an important source of phylogenetic information and has made a tremendous contribution to the systematics and phylogeny of angiosperms based on evolutionary trends in pollen wall architecture (Wodehouse 1936; Erdtman 1952; 1957; 1971; Nair 1965; Walker & Doyle 1975). Recently a number of papers on pollen morphology of various taxa have documented its contribution to plant systematics (Moon et al. 2008; Tripathi et al. 2017; 2019; Farooqui et al. 2019; Gasparino et al. 2021; Hu et al. 2021). Moreover, pollen morphological data in combination with other botanical data offers one of the most promising means of understanding the ecology and demography of a species (Rasoloarijao 2019). For example, the exine morphology of pollen is a prominent feature with characteristic variations between species or among its variants which can be identified through FESEM studies.

*1.1. Description of family Apocynaceae and the studied species of Rauvolfia with their medicinal importance*

Apocynaceae Juss., is positioned in Gentianales (asterids, lamiids) and includes about 400 genera and 5000 species located in tropical and subtropical regions around the world (Stevens 2001 onwards; Endress 2004; APG IV 2016). The family is currently divided into five subfamilies: Rauvolfioideae (cosmopolitan; 10 tribes/83 genera; 915 species), Apocynoideae (cosmopolitan; eight tribes/80 genera; 822 species), Periplocoideae (Old World; 33 genera), Secamonoideae (Old World; eight genera) and Asclepiadoideae (cosmopolitan; four tribes/172 genera) (Endress & Bruyns 2000; Middleton 2007; Endress et al. 2014; Morales et al. 2017). The Apocynaceae has been the subject of several taxonomic, evolutionary and phylogenetic analyses (Endress & Stevens 2001; Endress et al. 2007; Ionta & Judd 2007; Simoes et al. 2007; Wyatt & Lipow 2007; Rapini 2012; Endress et al. 2014; Morales et al. 2017; Fishbein et al. 2018), many of which have included descriptions of pollen morphological characteristics (Nilsson 1990; Nilsson et al. 1993; Verhoeven & Venter 1998; Furness 2007; Van Der Ham et al. 2010; Van der Weide & Van der Ham 2012).

*Rauvolfia* (Rauvolfioideae, Apocynaceae) is a genus of evergreen trees and shrubs. It has approximately 85 species, with distribution throughout the world including Europe, Africa, Asia, Australia, and Central and South America (Mabberley 2017). The species of *Rauvolfia* are mainly known for the phytochemical ‘Resperine’ which has been widely used as a hypertensive drug (Sahu 1983). Various parts of these plants are used to treat human ailments (Dutta & Virmani 1964; Ebadi 2007; Sihag & Wadhwa 2011), within alternative systems of medicine. The uses of herbal medicine for the treatment of diseases and

infections are a safe and traditional therapy (Najafi & Deokule 2010).

Out of the six Indian species of *Rauvolfia* (*R. serpentina, R. tetraphylla, R. hookeri, R. micrantha, R. verticillata* and *R. sumatrana*), only two species, *R. serpentina* and *R. tetraphylla*, occur in the Gangetic plains of Central India. Both are considered highly medicinal and are regularly used in pharmaceutical industries for drug preparation. Species overexploitation has resulted in their near extinction in the wild and the dwindling population of *R. serpentina has* already resulted in its listing in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The other four species of *Rauvolfia* fall outside the present study range but are also limited in population size. The distribution and the flowering period of the Indian species of *Rauvolfia* are provided in table 1.

*Rauvolfia serpentina*,the Indian snakeroot or devil pepper, is native to the moist, deciduous forests of Southeast Asia, including India, Burma, Bangladesh, Sri Lanka, and Malaysia. It is used to treat a variety of nervous disorders in traditional medicine (Ayurveda) especially in Kerala (Blackwell 1990). It is rapidly declining in its natural habitat due to multiple ecological and biological reasons. These factors include its restricted distribution, small endemic populations, pollination limitation, poor seed viability and severe anthropogenic pressure on forest land. All of these have contributed to its decline throughout its range and it is now considered to be rare and endangered (Sihag & Wadhwa 2011). Seed propagation is considered to be the best method for raising the species commercially, though seed production is highly variable and low (Bhadwar et al. 1956).

*Rauvolfia tetraphylla* is commonly known as still tree or devil-pepper (Rao 1956). The plant is native to [Mexico](https://en.wikipedia.org/wiki/Mexico), [Central America](https://en.wikipedia.org/wiki/Central_America), [West Indies](https://en.wikipedia.org/wiki/West_Indies), and northern [South America](https://en.wikipedia.org/wiki/South_America). It is now naturalized throughout the tropics including [Australasia](https://en.wikipedia.org/wiki/Australasia), [Indochina](https://en.wikipedia.org/wiki/Indochina), and [India](https://en.wikipedia.org/wiki/India) (Vinay et al. 2016). It has been cultivated widely as both an ornamental and for use in traditional medicine (Mahalakshmi et al. 2019). Whole plant as well as different parts viz. root, fruit and leaves of *R. tetraphylla* are widely used in traditional medicine (in various forms such as paste, powder, decoction and juice), particularly for the treatment of snake bite. In Bogota, Colombia, the plant is used as an antidote and for blood pressure (Bussman et al. 2018). The leaves of *R. tetraphylla* are used ethnomedicinally by the Peasant community of San Jacinto, Northern Colombia to relieve tension (Bonzani 1999).

1. **Material and methods**
   1. *Flower characters and general methodology*

*Rauvolfia serpentina* usually grows to a height between 60 and 90 cm. (Pl. 1, fig. 1). It is an erect, glabrous, perennial shrub, with white or pinkish or red coloured flowers, about 5 mm in size, occurring in whorls arranged in cymose corymb inflorescence, pedicellate, calyx glabrous, lanceolate, corolla tube swollen a little above the middle and elliptic oblong rounded at the apex of flower bud (Pl. 1, figs. 1-3) and with a stalk diameter of 3.5 mm. Flowering and fruiting occurs from March to May. *R. tetraphylla* grows as a bush or small tree (Pl. 1, fig. 4). Its flower is white to slightly pinkish and more or less similar to that of *R. serpentina* except the size is slightly smaller, around 4 mm, but the stalk size is the same (Pl. 1, figs. 4-6). Flower specimens of *R. serpentina* and *R. tetraphylla* were obtained from the herbarium of the Botanical Survey of India, Allahabad (BSA). The fresh flowers of these two species of *Rauvolfia* were also collected from three different sites in central India, Botanical Garden of the BSA, Uttar Pradesh; Amarkantak Biosphere Reserve and Rewa District of Madhya Pradesh for comparison and accurate pollen morphometrical analysis. Pollen grains were extracted and prepared for the morphological observation using the standard acetolysis method (ACE) (Erdtman 1960). The critical point drying method (CPD) was also adopted in the present study to check any size differences in pollen (Moon et al. 2008). Fresh and rehydrated samples were dehydrated in an acetone series and then immersed in carbon dioxide before CPD (using CPD 030, BAL-TEC instrument) followed by the FESEM procedure.

*2.2. Pollen observation under different microscopic techniques*

Pollen observation has often relied on the use of techniques based on light microscopy (LM), field emission electron microscopy (FESEM), and more recently, confocal laser scanning microscopy (CLSM). Despite its limitations, conventional light microscopy remains the basic tool for palynological investigations. The highest resolution obtained through FESEM (Taylor 1999), also results in the destruction of the sample and limits its use to rare materials, particularly fossil forms. Therefore the use of alternative techniques such as CLSM is preferable (Shute et al. 1996; Hochuli & Feist-Burkhardt 2004; Tripathi et al. 2019). The latter is a powerful imaging technique that allows both high resolution optical sections and three-dimensional FESEM-like reconstructions and is non-destructive to the specimen.

*2.3. Light microscopy (LM)*

For the ACE method, the fresh polliniferous material (flower) was kept in water and gently teased out into a plastic centrifuge tube. The materials were then sieved through 150 µm mesh (0.146 µm pore size), and the filtrate was then centrifuged to decant the water. The final material was mounted on a glass slide in glycerine jelly and sealed with paraffin wax. The labelled slides are deposited in the museum of the Birbal Sahni Institute of Palaeosciences (BSIP), Lucknow, India (BSIP slide no. 17067-17068). No glycerol was used as in the FESEM studies, as it fills the entire lumina of a brochi making it difficult to see the density of the columella which is one of the important characteristics of the sexine for pollen identification. However, for the detailed 3-dimensional and LO-analysis of pollen through LM, temporary slides were prepared in glycerol. The morphological characters of pollen of the species of *Rauvolfia* were identified by referring to the published literature (Gupta & Sharma 1986; Nayar 1990). Pollen morphological terminology was adopted from Erdtman (1952) and Punt et al. (2007). The pollen photographs were taken using Olympus DP 25 digital camera attached to Olympus BX 61 microscope (Pl. 2). The mean of P and E and the ratio P/E (for determining the pollen shape) along with other primary, secondary and tertiary pollen characters were measured using 40 pollen grains for each species. Pollen morphometric analysis, including ectocolpi number and length, endopore length and breadth, length and breadth of ectocolpi thickening, pollen polar and equatorial length and exine thickness of *R. serpentina* and *R. tetraphylla* are provided in table 2. Additional quantitative pollen characters like apocolpus side (AS), polar area indices (PAI), equatorial diameter in polar view (EDPV), width of ridge (WR) and width of valley (WV) were also examined as part of the comparison of the two species of *Rauvolfia* (Table 3).

*2.4. Field Emission Scanning Electron Microscopy (FESEM) for the pollen treated with ACE*

FESEM study was carried out at BSIP, Lucknow. The pollen grains were prepared following Erdtman (1943). Surface cleaning of the pollen was initially achieved through the ACE process. The dried samples were then mounted on adhesive carbon tape placed on the copper stubs. The carbon tape is conducive so the charging effect is minimized. These prepared stubs were then coated with palladium alloy with a JEOL JEC 3000FC auto fine coater. The coating was done for 70 seconds in a nitrogen environment to make the sample’s surface conductive. These stubs are observed under a high vacuum field emission electron microscope, JSM 7610f JEOL model (FESEM). The images are taken at the desired magnification at 5 KV with LEI (lower electron imaging) and subsequently photographed (Pl. 3).

*2.5. Field Emission Scanning Electron Microscopy (FESEM) for the pollen treated with CPD*

For checking the pollen size difference from ACE treated pollen, the CPD method was adopted. Initially the anthers were picked from the dry flowers and the anther tip was removed using a sharp edge blade to undergo rehydration. The anthers remained for one hour in the wetting agent. The rehydration was followed by the dehydration process in a graded acetone series, the material was then critical point dried using CPD-030, BAL-TEC instrument (Schols et al. 2004; Song et al. 2017). The dried anthers were then mounted on stubs with double adhesive carbon tape and following the procedure mentioned above for ACE treated pollen.

*2.6. Confocal Laser Scanning Microscopy* (*CLSM*)

To examine the submicron level morphological characters of the pollen of the species of *Rauvolfia*, the Confocal Laser Scanning Microscopy (CLSM) was performed by using standard protocols (Schopf et al. 2006) at the Birbal Sahni Institute of Palaeosciences (BSIP), Lucknow. Three-dimensional confocal fluorescence imaging was obtained on a Leica TCS SP8 Confocal Laser Scanning biological microscope system equipped with two Melles Griot lasers, a 488 nm 20 mW-output argon ion laser and a 633 nm10mW-output helium–neon laser (Melles Griot, Carbsbad CA). The images were acquired using a 100× oil-immersion objective (numerical aperture = 1.4). The observations were made with fluorescence-free microscopy immersion oil. Filters were used in the light-path of the system to remove wavelength <510 nm (for 488 nm laser excitation) from the kerogen- derived fluorescence emitted by the specimens. Identical parameters for each acquisition were used, to enable comparisons of the registered autofluorescence. Sets of the acquired image were subsequently processed and examined on LAS-X imaging software.

To obtain an overall view of pollen surface patterning and shape for each species, an image of a whole grain surface was collected at a confocal zoom magnification setting of X 2 (94 nm/ pixel). Image noise was reduced by averaging some individual frames. The channels providing the best result for each pollen grain were selected and the signal-to noise ratio improved by time averaging over eight repetitions for each optical section. The distances between each section were chosen to take into account the size of the specimen and the required level of precision (0.2–0.5mm). Pseudocolours were assigned to each type of pollen to differentiate species and varieties.

*2.7. Statistical significance*

Data obtained after the analysis of pollen morphological characters were statistically determined through *t-*test analysis using SPSS (11.5.0, USA) software for the pollen morphological characters of *R. tetraphylla* and *R. serpentina*. A probability of *p*-value ≤0.05 was taken to indicate statistical significance (Table 4).

*2.8. Vouchers of the studied species of Rauvolfia*

*Rauvolfia serpentina*: Ballia, near Dahreti, 3.1.1997, R.C. Srivastava 60165 (BSA); Balrampur, west Sohelwa, 10.1.2007, K.K. Khanna 68726 (BSA); Allahabad, BSI garden, 26.9.1966, R.B. Bose & H.S. Pandey 11408 (BSA); Sitapur, Elysia, 2.12.2004, B.K. Shukla 61278 (BSA); Amarkantak, 3.1.2005, S.L. Bondya & A.N. Shukla 62107 (BSA).

*Rauvolfia tetraphylla*: Allahabad, Chatham Lines, 22.10.1964, T. Rajgopal 6157 (BSA); Mirzapur, 8.12.1961, U.C.Bhattacharyya 18325 (BSA); Lucknow, near State Bank, 25.5.1956, R. Patil 254 (BSA).

**3. Results:**

*3.1. Pollen morphology of R. serpentina*

Pollen 3-colporate; Oblate-spheroidal, 47.75x52 µm (ACE), 52.94 x 51.33 µm (CPD); Ectocolpi length 36.29 µm, a distinct thickening is present at either side of ectocolpi; Endopore lalongate, 2.0 x 6.5 µm; Exine 3.26 µm, sexine as thick as nexine, distinct, punctate/perforated at mesocolpial region between the ectocolpi (Pl. 2, figs. 1-4; Pl. 3, figs. 1-3; Pl. 4, figs. 1-2; Pl. 5, figs. 4-6; Table 2).

*3.2. Pollen morphology of R. tetraphylla*

Pollen 3-colporate; Oblate, 20.93x35.81 µm (ACE); 25.53 x 38.89 µm (CPD); Ectocolpi length 15.67 µm, a distinct thickening is present around the ectocolpi; Endopore lalongate, 1.8x5.5 µm; Exine 2.28 µm, sexine as thick as nexine, not distinct, psilate (Pl. 2, figs. 5-6; Pl. 3, figs. 4-6; Pl. 4, figs. 3-4; Pl. 5, figs. 1-3; Table 2).

*3.3. Comparison of some additional quantitative pollen characters in R. serpentina and R. tetraphylla*

The AS, EDPV, WR and WV are higher in *R. serpentina* compared to *R. tetraphylla*. The length of AS is 41.46 and 29.31 µm in *R. serpentina* and *R. tetraphylla*, respectively. The EDPV is recorded to be 61.46 µm in *R. serpentina*, which is almost double the diameter of *R. tetraphylla* (35.98 µm). The PAI of *R. tetraphylla* (0.81µm) is greater than *R. serpentina* (0.67 µm). The WR and WV are greater in *R. serpentina* than *R. tetraphylla*, based on the relatively bigger pollen size of the former (Table 3 & 4).

*3.4. Statistical significance*

The *t*-test analysis reveals the significant variations in the pollen morphological characters in the two species of *Rauvolfia* (p≤0.05) collected from the same climatic conditions. It was observed from the above analysis that ectocolpi length, ectocolpi thickening length, equatorial length, equatorial diameter in polar view, width of valley, width of ridge, apocolpus side and polar/equatorial axis varied significantly in the two species.

**4. Discussion**

*4.1. Pollen unit and shape*

In our study, both *R. serpentina* and *R. tetraphylla* have oblate-spheroidal and oblate shapes, respectively in most of their monads (Pl. 2, fig. 1; Pl. 3, fig. 1). However, a spheroidal shape was reported in *R. tetraphylla* collected at the Ballygunge Science College Campus, Kolkata (Bose et al. 2012). Rao & Shukla (1975) discussed the pollen morphology of *R. serpentina* and considered the oblate shape as characteristic of the species. Erdtman (1952) considered the oblate amb as typical of the single discussed species, *R. verticillata*, but it was later observed in different species of *Rauvolfia*. Our pollen shape results for *R. tetraphylla* and *R. serpentina* matches the pollen characteristics of other species of *Rauvolfia* from India and abroad, where oblate-spheroidal and oblate are the common shapes observed in most species of *Rauvolfia* (Erdtman 1952; Rodrigues et al. 2016). It was also observed that the pollen shape of both the studied species of *Rauvolfia* does not alter after the two different chemical treatments (ACE and CPD), however, the minute pollen size alterations were observed.

*4.2. Size of Monad*

Erdtman (1952) in an extensive pollen morphological study suggested that the size range of Apocynaceae pollen varies from 20-25 to 75-110 µm. In our present morphological study, *R. serpentina* exceeds the range of polar length and is among the largest in pollen size (47.75x 52 µm) (Pl. 1). However, *R. tetraphylla* (20.93 x 35.81µm) lies in the middle of the pollen size range for the Apocynaceae. Punt & Hoen (1995) showed that the grains of the same pollen type had different sizes after two different treatments (glycerine jelly and silicone oil). In our study the pollen size of *R. serpentina* and *R. tetraphylla* was obtained by the ACE method. But pollen size after CPD treatment indicated minute size differences, where pollen size of *R. serpentina* (52.94 x 51.33 µm) and *R. tetraphylla* (25.53 x 38.89 µm) are relatively greater compared to ACE treated pollen, but not to the extent of causing misidentification. It is clear from the above observation that in *Rauvolfia* species, pollen size may be considered as a decisive factor for the discrimination of different species and thus, could play a complementary role in pollen identification.

*4.3. Aperture*

Pollen grains possess a 3-colporate aperture at the angles of the planes (angulaperturate). The ectocolpi is surrounded by a distinct thickening which is not a common feature in angiosperms (Pichon 1948; Pasha & Roy 1980; Koch et al. 2018). The ectocolpi thickening is a very important distinguishing feature for sorting different species of *Rauvolfia*. The length of ectocolpi thickening is longer in *R. serpentina* (15.54 µm) forming a concave margin at either side of the ectocolpi and is shorter in *R. tetraphylla* (9.94 µm) with a ‘u’ shaped margin flowing parallel around the ectocolpi. It is interesting to note that the ectocolpi thickening in *R. tetraphylla* is not distinctly visible under FESEM but is distinct and clearly visible under LM studies. Ectocolpi is thick in *R. tetraphylla* compared to *R. serpentina*. The margin is thick and this character is very well observed in FESEM. The ectocolpi number is always three in both species of *Rauvolfia.* However, tetra-ectocolpi was reported in *R. sprucei* (Erdtman 1952). The margin of the ectocolpi is more or less smooth in *R. serpentina* but indistinct in *R. teraphylla*. The pollen of *Rauvolfia* is always compound with lalongate endopore which is very clear under FESEM. The alignment of ectocolpi and thickening around it in the pollen monad is very unique and has not been observed in other angiosperm pollen. The endopore is sunken in both species. However, their superficial size could be measured under FESEM. Therefore, ectocolpi length, endopore size and length of ectocolpi thickening plays a vital role in distinguishing the pollen of these two species of *Rauvolfia* and may be useful for distinguishing other species of the genus as well.

*4.4. Exine character*

The exine stratification is taxonomically useful for distinguishing genera or even species of Apocynaceae (Huang 1989). Sexine in most of the studied species of *Rauvolfia* was found to be psilate, but rarely scrobiculate as in *R. verticillata* (Erdman 1952). Under FESEM and CLSM it was observed that the sexine is punctate/perforated in *R. serpentina* and is limited to mesocolpial region. As the arrangement of the punctate pattern could only be seen under FESEM, therefore, we examined the detail of the characters of the punctate surface using FESEM. In *R. serpentina*, the exine is broad and thick, which could be easily differentiated into a sexine and nexine layer. The tectum of sexine has a plain surface lacking ridges and furrows. Towards the ectocolpi the sexine layer looks slightly thin and makes a wavy structure. The sexine and nexine have a similar thickness forming a continuous layer. In *R. tetraphylla*, the tectum has a smooth surface and the sexine and nexine could not be differentiated.

*4.5. Implications of additional quantitative pollen characters*

Besides the main diagnostic pollen characters, the additional quantitative pollen morphological characters like AS, PAI, EDPV, WR and WV were examined in *R. serpentina* and *R. tetraphylla*  as auxiliary parameters to complement the main pollen morphometrical characters in the two studied species of *Rauvolfia*. All these quantitative pollen characters were recorded to be greater in *R. serpentina* except the polar area indices (PAI) which is greater in *R. tetraphylla*, thus supporting our comparative interpretation.

Most of the main and additional pollen morphological characters can be observed and studied under LM, but for the examination of endopore size, sexine ornamentation and exine thickness, it is necessary to use FESEM and CLSM analysis. Moreover, for the palaeoecological implications, the pollen morphometric analysis under LM was always crucial to identify and count the fossil counterpart in the sediment.

*4.6. Practical aspects of the pollen micro-morphometry of the two endangered species of Rauvolfia*

*Rauvolfia* flowers are pollinated inadvertently by the lemon butterfly, *Papileo demoleus* L. (*R. serpentina*) and by honeybees (*R. tetraphylla*) during their flights between different flowers (Wadhwa & Sihag 2012; Sharma 2020). Their smooth pollen surface are indicative of insufficient pollen trap (sticking) on the insect's ventral body parts, which could be an additive factor resulting in unsuccessful pollination, the primary cause of loss of species multiplication (through plant reproductive processes). Pollen morphometric parameters are therefore, suggestive of adopting alternative reproductive strategies for successful pollen transfer to the stigma, mainly in endangered species, for their conservation, establishment and multiplication *ex situ* and/or *in situ*, coupled with authentication and standardization of drugs prepared from their flowers. Such information is also relevant in the determination of palaeoflora of fossil pollen recovered.

**5. Conclusions**

Pollen characters are useful in solving complicated problems of interrelationships

between various taxa and assessment of their status in the classification, particularly with reference to the families, subfamilies, tribes, genera, species, and subspecies.

The result of our detailed study of pollen morphology and morphometry of *R. serpentina* and *R. tetraphylla* using LM, CLSM and FESEM improved our understanding of species characters useful in identification and taxonomic characterization. Pollen morphotypes similar to *Rauvolfia* have been recorded from time to time in Quaternary sediments of India. The Government of India has banned the export of *R. serpentina* to prevent over- exploitation from the wild and eventual extirpation. Because of its wide use in medicines and the ban on its export, the adulteration of this drug has increased in recent years (Jyothi et al. 2013). Thus, demonstrating the need for palynologists to study the minute pollen morphological detail to systematically clarify, resolve and validate queries regarding precise identification and taxonomic characterization. The pollen grains represent an important diagnostic characteristic in *Rauvolfia* because they are described as having thickenings around the ectocolpi, a characteristic not found in the closely related genera. Our data confirm this information for the two species analyzed. In addition, we found a difference in the surfaces of the pollen grains, with perforations occurring only along the mesocolpial region of *R. serpentina*. The differences in the surfaces of the pollen grains, with the presence of perforations along the mesocolpial region of *R. serpentina* and the confirmation of thickenings around the ectocolpi, show the importance of pollen characteristics in identifying taxonomically useful characteristics for the delimitation of genus or species. The major differences and recommendations from the pollen micro-morphometry of two species of *Rauvolfia* are:-

1. The basic ornamentation of sexine differentiates *R. serpentina with R. tetraphylla* under FESEM*.*
2. *R. serpentina* and *R. tetraphylla* both bear oblate-spheroidal and oblate shapes respectively in most of their monads. Due to the distinct monad shape, it is possible to easily identify them to genus and species level.
3. A distinct thickening is present at either side of the ectocolpi which is the characteristic morphological feature of *Rauvolfia* pollen.
4. *R. serpentina* has the largest pollen size (47.75x 52 µm). However, *R. tetraphylla* (20.93 x 35.81µm) lies in the mid-range of Apocynaceae pollen size.
5. The CPD treated pollen size of *R. serpentina* (52.94 x 51.33 µm) and *R. tetraphylla* (25.53 x 38.89 µm) are slightly larger compared to ACE treated pollen.
6. *Rauvolfia* was found to bear psilate exine, rarely scrobicuate in some species such as *R. verticillata* .The punctate/ perforated pattern in *R. serpentina* was observed in the mesocolpial region under FESEM.
7. Alignment of ectocolpi and the thickening around it is a very unique morphological character and has not been observed in any other angiosperm pollen. A sunken endopore is present in both of the studied species of *Rauvolfia*.
8. It was observed from the *t*-test analysis that the main pollen morphological characters like ectocolpi length, ectocolpi thickening length, equatorial length and polar/equatorial axis varied significantly along with some additional quantitative characters like EDPV, AS, WV and WR in the two species of *Rauvolfia*.
9. The LM could not provide any micro-morphometric details of exine features, therefore, FESEM and CLSM study of these prime pollen characters are necessary for understanding the taxonomic characterization at the species level.
10. For palaeoecological studies, the pollen morphometric analysis under LM is always crucial to identify and count the fossil counterpart in the sediment.
11. Pollen micro-morphometric characters could also aid in the conservation, establishment and multiplication of both the species of *Rauvolfia* *ex situ* and/or *in situ* as well as helps in the authentication and standardization of drugs prepared from them.

**Acknowledgements**

I sincerely thank Director, BSIP for his kind permission for the collaboration and publication of this research work (BSIP/RDCC/Publication No. 04/2020-21)under BSIP, Lucknow and BSI, Allahabad joint collaboration. I would like to express my deep sense of gratitude to Dr. Matthew J. Pound, Handling Editor and the anonymous reviewers for their suggestions which helped me to improve this MS with full dedication. ST thanks Dr. James B. Riding, Editor-in-Chief, Palynology for encouragement. Thanks are due to Dr. H. Gregory McDonald for his constant support. I like to thank Dr. Subodh Kumar for the FESEM studies.

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**Legends:**

Figure 1. Distribution of *Rauvolfia serpentina* and *R. tetraphylla* in different states of India.

Plate 1. 1. A small twig of *Rauvolfia serpentina* with mature bud, 2. Multiple buds of *R. serpentina* showing the cymose inflorescence with ripe black berries, 3. A twig of *R. serpentina* with complete flowering, about 5mm (flower) and 3.5 mm stalk 4. A complete mature plant of *R. tetraphylla* with fruits, 5&6. Fruit of *R. tetraphylla* i.e red berry that turns black on ripening.

Plate 2. LM micrographs, 1. Polar view of *Rauvolfia serpentina* with distinct thickening around the ectocolpi, 2. Equatorial view of *R. serpentina*, 3. A group of *R. serpentina* in polar view, 4. Polar view of *R. tetraphylla*, 5. Equatorial view of *R. tetraphylla*.

Plate 3. FESEM micrographs, 1. Polar view of *Rauvolfia serpentina* showing obscure sexine pattern, 2. Enlarged view of mesocolpial region in *R. serpentina* showing perforations, 3. A view of punctate sexine at mesocolpial region in *R. serpentina*, 4. Polar view of *R. tetraphylla*, 5. Enlarged view of ectocolpi region of *R. tetraphylla* in polar view, 6. Enlarged view of sexine showing psilate pattern.

Plate 4. FESEM micrographs after CPD treatment, 1&2. Pollen of *Rauvolfia serpentina* showing the three anastomosing ectocolpi , 3. Pollen of *R. tetraphylla* showing clear thread like ectocolpi, 4. *R. tetraphylla* showing clear endopore image.

Plate 5. CLSM micrographs, 1. False colour composite showing the wall thickness of *Rauvolfia tetraphylla,* 2. Polar view of *R. tetraphylla* showing ectocolpi and obscure sexine pattern, 3. Cross section of pollen body showing the internal pattern of aperture in *R. tetraphylla*, 4. The distinct striations on surface of *R. serpentina* pollen, 5. Side view of *R. serpentina* pollen showing distinct aperture and thickening around it, 6. False colour composite of *R. serpentina* showing ectocolpi thickening in front and distinct striations in polar view.

Table 1. Distribution and phenology of *Rauvolfia species* within India.

Table 2. Analysis of major pollen morphological characters, including ectocolpi number and length, endopore length and breadth, length and breadth of ectocolpi thickening, pollen polar and equatorial length and exine thickness of *Rauvolfia serpentina* and *R. tetraphylla.*

Table 3. Measurements of the additional quantitative pollen characteristics in *Rauvolfia serpentina* and *R. tetraphylla*.

Table 4. The *t*-test analysis of overall pollen morphological characters of *Rauvolfia tetraphylla* and *R. serpentina.*