Insights of morphology and functional pollen biology of medicinally important endangered plant species Rauvolfia serpentina (L.) Benth. ex Kurz.

SOUMITRA HALDER
Visva-Bharati University
SUBRATA MONDAL
subrata.mondal@visva-bharati.ac.in

Visva-Bharati University

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Insights of morphology and functional pollen biology of medicinally important endangered plant species *Rauvolfia serpentina* (L.) Benth. ex Kurz.

Soumitra Halder and Subrata Mondal*

Department of Botany, Visva-Bharati, Santiniketan-731235

sendmailtohalder@gmail.com, *submondal@rediffmail.com (Author for correspondence)

Abstract

Rauvolfia serpentina (L.) Benth. ex Kurz, a critically endangered medicinal shrub of the Apocynaceae family, is renowned for its bioactive indole alkaloids, particularly reserpine. Despite its ethnobotanical significance, the species faces severe threats from habitat loss, overexploitation, and reproductive constraints. This study investigates its floral biology, pollen viability, and *in vitro* germination to guide conservation and breeding strategies.

Floral phenology revealed year-round blooming, with peak flowering during late January–March and May–early July. The species exhibits actinomorphic, pentamerous, insect-pollinated flowers, with anthesis and anther dehiscence occurring between 5:30–8:30 AM. A high pollen-to-ovule ratio (600:1) and nectar secretion support an obligate outcrossing system.

Pollen viability, assessed through Fluorescein Diacetate (FDA), Acetocarmine, and 2,3,5-triphenyl tetrazolium chloride (TTC) assays, displayed a pronounced diurnal pattern. Peak viability (≥98%) occurred between 5:00–10:00 AM, followed by a sharp decline at post-noon (12:00 PM). FDA and TTC were more reliable indicators of metabolic viability than acetocarmine staining.

In vitro pollen germination using Brewbaker and Kwack medium demonstrated sucrose-dependent germination, with optimal performance at 25–35% sucrose. Maximum tube elongation (537.07 ± 66.23 µm) occurred at 35% sucrose. Germination was absent below 10%. Notably, polysiphonous pollen tubes- multiple tubes from a single grain- emerged under high sucrose and boron concentrations, indicating possible *in vitro*-induced plasticity.

This integrated analysis of floral traits, pollen viability, and germination physiology contributes valuable insight for developing targeted conservation, propagation, and genetic improvement strategies. These findings provide critical insights into the reproductive biology of *R. serpentina*, emphasizing the role of floral timing, metabolic integrity, and nutrient regulation. This knowledge is pivotal for refining conservation protocols and advancing genetic improvement efforts for this threatened status and rising environmental pressures.

Keywords: *In vitro* pollen germination, Pollen viability, Acetocarmine, FDA, TTC

1. Introduction

Rauvolfia serpentina (L.) Benth. Ex Kurz. is an evergreen, upright, semi-woody, glabrous, and perennial shrub with a maximum height of up to 60-80 cm (Akare et al., 2025; Debnath et al., 2024; Mukherjee et al., 2019; Sharma et al., 2022). The plant possesses semi-tuberous roots with pale to brown cork and elliptic to lanceolate or obovate leaves in whorls of three.

To honor German physician and botanist Leonhard Rauwolf (1535–1596), the generic name *Rauvolfia* was first proposed in 1703 by French botanist Charles Plumier (Plumier, 1703). *Rauvolfia* is native to tropical climates worldwide and includes about 110 species of trees and shrubs that range in size from tiny to large. Africa, Burma (Myanmar), Central and South America, India, China, Java, Japan, Malaysia, and Sri Lanka are only a few of the tropical and subtropical regions where the species is widely dispersed. Because of its critically endangered status in Northeast India, this species requires conservation measures that are both *in vitro* and *in vivo*. Its survival is seriously threatened by habitat fragmentation, pollution, and dwindling pollinator populations, all of which lead to reproductive failure and additional species endangerment in different countries. *Rauvolfia serpentina* is seriously threatened in India due to indiscriminate and extensive collection (Gupta, 1988) and is listed as endangered by the International Union for Conservation of Nature (IUCN) (Susila et al., 2013; Shitiz & Gupta, 2021). Understanding the reproductive cycle of such species is essential for guiding genetic improvement initiatives and developing effective conservation strategies.

Previous studies have addressed aspects of the reproductive biology of *Rauvolfia serpentina* (Akare et al., 2025), but a complete compilation of data- especially regarding pollen viability across the circadian period (5:00 AM to 5:00 PM)- remains limited. This unique dataset, along with stress-related observations, can significantly enhance our understanding of reproductive timing and environmental sensitivity in this endangered species.

In light of these needs, the present study aims to fill this gap by examining key reproductive traits of *R. serpentina*. Special focus is placed on *in vitro pollen germination, conducted using Brewbaker and Kwack's medium, which* played a crucial role in this research. Interestingly, the development of **polysiphonous pollen tubes** was observed, providing further insight into the reproductive physiology of the species.

This article seeks to provide a detailed synthesis of reproductive parameters and pollen performance in *R. serpentina*, offering a foundation for conservation efforts and future breeding interventions.

2. Materials and methods

The study was conducted following the methods outlined by Mathur and Mohan Ram (1986), and Reddi et al. (1980), to observe various phenological events in *Rauvolfia serpentina* plants growing in and around the University medicinal plant garden at Visva-Bharati, Santiniketan, Birbhum, West Bengal, India (87°41' and 87°42' east longitude, 23°40' and 23°42' north

latitude). Different populations of the plant taxa were selected through random sampling to study floral biology, breeding systems, and pollination under natural conditions.

Flowering Phenology: Flowering twigs were tagged to observe different phenological events, including the initiation of inflorescences, maturation of floral parts, flowering initiation, duration, intensity, anthesis, post-anthesis changes, stigma receptivity, and senescence. Phenological observations were made through regular field visits at both individual and population levels. Flowering twigs were tagged at the bud stage. Details of floral structure, such as the number of pistils and stamens per flower and their relative position, were observed under the stereomicroscope (Zeiss Primo Star). The morphometry of flowers such as pedicel, calyx, corolla, androecium, and gynoecium (n = 50)

Scanning Electron Microscopy (SEM) Observation

For SEM analysis, pollen grains from mature undehisced anthers were dusted over cover slip. The dusted material sputter-coated with a thin layer of gold was observed under a GEMINI®SEM 450 scanning electron microscope under varying magnifications. The morphological features, such as exine ornamentation, pollen shape, and aperture characteristics, were observed using a GEMINI (FESEM 450 8216010130) scanning electron microscope under varying magnifications. The terminology for pollen description follows the guidelines proposed by (Erdtman, 1952; Punt et al., 2007).

Estimation of Pollen Production

To assess total pollen production per anther, ten mature flower buds (collected prior to anther dehiscence) were selected. From each bud, five anthers were carefully dissected and vortexed in 100 µL of 0.5 M sucrose solution. The resulting pollen suspension was loaded into a hemocytometer, and the number of pollen grains within 25 central squares was counted. The total pollen count per anther was calculated using the formula:

(mean number of pollen grains per square × 10 × 0.1) / 5.

To estimate pollen grains per flower, the calculated number of grains per anther was multiplied by the average number of anthers per flower (Barrett, 1985).

Fluorochromatic Reaction (FCR) Test

The fluorochromatic reaction test (FCR) was conducted following the protocol of (Heslop-Harrison & Heslop-Harrison, 1970) to determine pollen viability at different time intervals. Pollen grains were collected at anthesis and subsequently tested at hourly intervals for up to five hours post-anthesis (Shivanna & Rangaswamy, 2012).

A stock solution of fluorescein diacetate (FDA) was prepared by dissolving 2 mg of FDA in acetone. The working solution was made by mixing FDA with 10% sucrose solution to prevent pollen bursting. Pollen grains were uniformly spread on a microscope slide containing the sucrose-FDA mixture and incubated in a humid chamber for 5–10 minutes. Observations were carried out using a fluorescence microscope.

Acetocarmine test

Pollen fertility was evaluated using the acetocarmine-glycerine staining technique as described by (Shivanna & Rangaswamy, 1992, 1993). This method assesses the presence of cytoplasm in intact pollen grains, with stained (deeply red) grains considered fertile.

TTC test

The tetrazolium method involves mounting pollen grains in a 2,3,5-triphenyltetrazolium chloride (TTC) solution and letting them sit in a dark, humid environment for an hour. The percentage of viable pollen is determined by counting the reddish-colored grains of viable pollen (Hauser & Morrison, 1964; Stanley & Linskens, 1974).

Studies on *in vitro* Pollen Germination

In vitro pollen germination was carried out using Shivanna and Rangaswamy's (1993) procedure. Media with different concentrations of sucrose (1–50%) and boric acid (25–300 ppm) were used to germinate pollen grains. Additionally, Brewbaker and Kwack's (BK) medium was optimized by varying the concentrations of its key components: sucrose, boric acid, calcium nitrate, magnesium sulfate, and potassium nitrate to determine the most suitable combination for promoting pollen tube growth.

The experiment was carried out under carefully monitored environmental conditions in a growth chamber, with the temperature maintained at 25 °C and relative humidity between 80–90%. Observations were recorded after three hours and subsequently at one-hour intervals throughout the five-hour incubation period to record the development of tube elongation and pollen germination under each treatment condition. Pollen germination percentage and pollen tube length were calculated following the standard procedure outlined by Shivanna and Rangaswamy (1992). A pollen grain was considered germinated when the pollen tube length was equal to or greater than the diameter of the pollen grain. The average tube length was measured for at least 20 germinated pollen grains per replicate. A pollen grain was considered germinated if the pollen tube was at least as long as the pollen grain diameter (Stanley & Loewus, 1964; Tuinstra & Wedel, 2000)

Pollen Germination Anomalies

In vitro experiments revealed an unusual phenomenon where a single pollen grain produced two pollen tubes (polysiphonous condition). To learn about any possible ramifications for the reproductive biology of the *R. serpentina*, this uncommon occurrence was recorded and examined.

Data analysis

For statistical analysis, percentages of pollen viability, size and germinability were first conducted at the significance level of $P < 0.05$. If some of the means were different, then comparison of means by Tuckey's multiple comparison test (TMRT) or HSD test was performed at $P < 0.05$.

Data analysis is performed using the Graphpad Prism (version 7.03).

Results:

Table 1. Floral Characteristics of *Rauvolfia serpentina* (L.) Benth. ex Kurz

Floral Character	Observation
Flowering period	Throughout the year, with peak flowering from late January to late March, and late May to the first week of July
Number of inflorescences per plant	16 ± 12
Number of flowers per inflorescence	60 ± 34
Flower type	Pentamerous, bisexual, actinomorphic
Flower length	2 ± .4 cm
Flower colour	White, often tinged with pink
Flower opening time (Anthesis)	05:30 h – 07:30 h
Nectar	Present
Number of anthers per flower	5
Anther dehiscence mode	Longitudinal slit
Anther dehiscence time	07:30 h – 08:30 h
Average number of pollen grains per anther	480 ± 25 (based on observations from 50 flowers of different plants)
Mean number of pollen grains per flower	2400 ± 56
Number of carpels per flower	2
Mean number of ovules per flower	3 ± 1
Pollen/ovule ratio	600:1
Pollen shape	Triangular in polar view
Pollen aperture	Tricolporate
Pollen size	70±65 µm
Flower duration	1 ± 1.5 days

Rauvolfia serpentina is a perennial, erect shrub that typically attains a height of 50–60 cm. Its vegetative growth accelerates during the monsoon months of June and July (Fig. 1a). The leaves are simple and arranged in whorls of three at each node (verticillate phyllotaxy). Each leaf is elliptic to lanceolate in shape, tapering to a pointed apex with slightly wavy margins. Leaves measure approximately 5–7 cm in length and 3–5 cm in breadth.

The inflorescence is a corymbose cyme (Fig. 1b), found at both terminal and axillary positions. On average, a plant produces around 16 ± 12 inflorescences, each bearing 60 ± 34 flowers. The

inflorescence consists of a primary peduncle giving rise to 4–6 secondary branchlets, with additional tertiary branches. Flowers are borne on both the secondary and tertiary branches. The peduncle and pedicel are initially green but turn bright red at the beginning of fruit development.

Flowers are pedicellate (Fig. 1c), hermaphroditic, actinomorphic, and pentamerous, measuring 2 ± 0.4 cm in length. They are white, often tinged with pink to violet, and contain 5 anthers per flower. Flower opening (anthesis) occurs between 05:30 AM and 07:30 AM, with anther dehiscence occurring shortly afterward between 07:30 AM and 08:30 AM through longitudinal slits. The *Rouvolfia* flower possesses a long corolla tube; its pollination is adapted to psychophilous, as insects with a long proboscis can access the nectar from the base of the corolla tube.

Each anther (Fig. 1d) produces approximately 480 ± 25 pollen grains, and the mean number of pollen grains per flower is 2400 ± 56. The flower has 2 carpels, each with 3 ± 1 ovules, giving a pollen-to-ovule ratio of 600:1. The pollen grains are triangular in polar view, tricolporate, and measure 70 ± 6.5 µm in diameter. Pollen grains are released as monads (Fig. 2a).

Scanning electron microscopy (SEM) analysis revealed that the pollen grains *of Rauvolfia serpentina* are 3-zonoporate, with three well-defined pores uniformly distributed across the pollen surface (Fig. 2a). The pollen grains exhibited an oblate-spheroidal shape with a mean polar axis of 61.61 ± 2.41 µm (n=12) and an equatorial diameter of 53.87 ± 4.09 µm, indicating moderate size variation among individual pollen grains. The average pore diameter measured 10.67 ± 2.34 µm, and the apertures appeared as circular to slightly elliptical openings (ellipsoidal) with smooth margins (Fig. 2d), suggesting efficient sites for pollen tube emergence. The exine surface of the pollen, as observed under high magnification, was finely granulate to microreticulate in pattern, consistent with previous reports in members of Apocynaceae (Tripathi et al., 2022). The morphological features, such as 3-zonoporate condition, distinct aperture zones, and well-developed exine ornamentation, support the entomophilous nature of the species, which often relies on aperture-based rapid pollen tube protrusion. Such structural attributes are very significant for understanding pollen viability, dispersal efficiency, and reproductive fitness in natural and stress-induced environments.

192

193 **Table 2**: Effect of Different Sucrose Concentrations on *In Vitro* Pollen Germination of *Rauvolfia serpentina* (L.) Benth. ex Kurz Using Brewbaker
194 and Kwack Medium

conc (%)	After 3hrs		After 4hrs		After 5hrs	
	Pollen Germination (%)	Mean tube length(µm)	Pollen Germination (%)	Mean tube length(µm)	Pollen Germination (%)	Mean tube length(µm)
1% + BK	-	-	-	-	-	-
2% + BK	-	-	-	-	-	-
5% + BK	-	-	-	-	-	-
8% + BK	-	-	-	-	-	-
10% + BK	68.17 ± 0.78^{bc}	93.18 ± 17.39^{c}	69.1 ± 1.7^{bc}	130.28 ± 22.46^{c}	70.1 ± 0.34^{bc}	173.02 ± 47.35^{c}
12% + BK	72.44 ± 0.07^{ab}	158.62 ± 16.73^{ab}	74.4 ± 5.4^{ab}	197.94 ± 30.77^{b}	78.2 ± 4.1^{ab}	336.50 ± 57.66^{b}
15% + BK	74.51 ± 0.11^{ab}	132.61 ± 21.29^{ab}	76.87 ± 1.2^{ab}	292.95 ± 57.33^{a}	78.6 ± 3.34^{ab}	446.68 ± 21.03^{a}
20% + BK	67.78 ± 0.15^{bc}	142.25 ± 22.12^{ab}	68.24 ± 2.5^{bc}	207.30 ± 43.77^{b}	79.7 ± 2.1^{ab}	309.35 ± 40.12^{b}
25% + BK	74.22 ± 0.16^{ab}	113.97 ± 15.81^{bc}	80.21 ± 5.4^{a}	271.28 ± 41.90^{a}	89.4 ± 1.4^{a}	458.82 ± 38.77^{a}
30% + BK	79.28 ± 0.22^{a}	177.54 ± 33.42^{a}	82.8 ± 1.7^{a}	260.77 ± 22.25^{a}	85.2 ± 1.1^{a}	536.19 ± 76.75^{a}
35% + BK	83.71 ± 0.45^{a}	143.13 ± 24.69^{ab}	84.1 ± 1.5^{a}	398.67 ± 45.53^{a}	84.5 ± 2.07^{a}	537.07 ± 66.23^{a}
40% + BK	72.47 ± 0.15^{ab}	61.93 ± 10.85^{d}	81.14 ± 4.2^{a}	137.25 ± 22.00^{c}	85.7 ± 1.8^{a}	197.82 ± 25.78^{c}
45% + BK	52.66 ± 0.12^{c}	67.09 ± 7.15^{d}	61.14 ± 3.67^{c}	88.22 ± 4.36^{d}	65.7 ± 1.8^{c}	127.07 ± 13.89^{d}
50% + BK	41.30 ± 0.08^{d}	68.57 ± 12.02^{d}	43.62 ± 1.07^{d}	83.30 ± 6.33^{d}	44.02 ± 0.65^{d}	117.38 ± 5.55^{d}

195 (Different letters indicate significant differences at $p < 0.05$ (Tukey's HSD). Treatments sharing at least one letter are not significantly different.)

The pollen grains were considered germinated in *in vitro* conditions when the pollen tube length exceeded the diameter of the pollen grain (Tuinstra & Wedel, 2000). In our observations, during the first two hours of incubation, the pollen tubes had just emerged and were relatively short, with some pollen grains showing delayed germination. The effect of varying sucrose concentrations on *in vitro* pollen germination and pollen tube growth of *R. serpentina* was evaluated using Brewbaker and Kwack (BK) medium supplemented with 1% to 50% sucrose. No pollen germination was observed at lower sucrose concentrations ranging from 1% to 8%, even after 5 hours of incubation.

Germination initiated at 10% sucrose concentration (Fig. 3a), with a pollen germination percentage of 70.1% (Table. 2)after 5 hours and an associated mean pollen tube length of 173.02 ± 47.35 µm. A progressive increase in both pollen germination percentage and pollen tube length was observed with further increases in sucrose concentration. The maximum pollen germination (89.4%) was recorded at 25% sucrose concentration after 5 hours (Fig. 3b). The highest mean pollen tube length (537.07 ± 66.23 µm) was observed at 35% sucrose at the same time point. Interestingly, while pollen germination remained high at 30% and 35%, the longest pollen tubes were noted at these levels. However, a decline in both pollen germination percentage and tube length was observed at sucrose concentrations beyond 35% (Fig. 3c). At 45% sucrose, (Fig. 2d) germination reduced to 65.7% with a tube length of 127.07 ± 13.89 µm, while at 50%, these values dropped further to 44.02% and 117.38 ± 5.55 µm respectively. The present study results indicate that sucrose concentration plays a critical role in regulating *in vitro* pollen germination and tube elongation in *R. serpentina*. Similar to previous reports on pollen physiology (Akare et al., 2025), pollen grains required a minimum threshold of sugar availability to initiate metabolic processes required for germination. The absence of germination at low sucrose concentrations (1–8%) highlights the species' requirement for a minimum carbohydrate supply to support pollen metabolism and pollen tube initiation. Similar findings have been reported in other members of Apocynaceae and in stress-sensitive species where pollen grains require external carbohydrate sources for germination. Pollen germination started at 10% sucrose and improved significantly up to 25–35%. Maximum tube length (537.07 µm) at 35% sucrose is likely due to enhanced turgor pressure and carbohydrate availability facilitating rapid tube elongation, as also observed in other medicinal species(Shi & Yang, 2010; Hu et al., 2024).

The Brewbaker and Kwack (BK) medium(Brewbaker & Kwack, 1963), used as the basal germination medium in this study, contains essential inorganic nutrients including Boric acid (200 ppm), Calcium nitrate (100 ppm), Magnesium sulfate (200 ppm), and Potassium nitrate (100 ppm). These components play key roles in pollen tube wall synthesis, membrane integrity, and metabolic regulation. Particularly, boron and calcium ions are known to stimulate pollen tube growth by regulating cytoskeleton organization and vesicle trafficking (Polster et al., 1992; Sidhu & Malik, 1986; Q. Wang et al., 2003) .The synergistic effect of BK salts and optimal sucrose concentration in this study reflects the need for both carbohydrate energy source and ionic balance for successful pollen germination(Khan et al., 2023).

Salts such as magnesium sulfate ($MgSO_4$), calcium nitrate ($Ca(NO_3)_2$ and potassium nitrate (KNO_3) play a pivotal role in regulating pollen germination and development of the pollen tube (Brandoli et al., 2024; Brewbaker & Kwack, 1963; Cai et al., 2022; Ghanta & Mondal, 2013; Pal & Mondal, 2016; Reddy, 1968; Saha, 2024). Calcium is an important divalent cation (Ca^{++}) in cellular metabolism and is critically important for the germination of pollen and the elongation of the pollen tube. Its role in establishing and maintaining plasma membrane integrity and permeability is well documented(Brewbaker & Kwack, 1963; Pertl-

Obermeyer et al., 2018; Steinhorst & Kudla, 2013; H.-J. Wang et al., 2010; Weigand, 2022). Calcium is also essential for the directional growth of pollen tubes (Derksen et al., 1995; Hepler et al., 2012; Holdaway-Clarke & Hepler, 2003; Pandian et al., 2020; Pierson et al., 1994; Steer & Steer, 1989). Calcium ion, modulates callose synthesis at the time of pollen tube extension via modulation of intracellular calcium concentrations experimentally supported according to (Bednarska, 1989) in *Oenothera biennis* L.and *Haemanthus albiflos* L.. In addition, the regulated transport of inorganic ions including Ca^{2+}, K^{+} across the pollen plasma membrane, has a marked effect on both germination and tube growth (Feijó et al., 2000). Exogenous addition of potassium (K^{+}) and Ca2+ has been reported improve germination rates and stimulate pollen tube elongation in *Arabidopsis thaliana* (L.) Heynh. (Wu et al., 2018; Fan et al., 2001). The results of the current study are consistent with those of previous studies, including (Ghanta & Mondal, 2013; Pal et al., 2017) that highlight the multifaceted regulatory roles of calcium, potassium, magnesium, and nitrate ions in the complex pollen physiology of pollen development and tube growth. The declining trend in germination and tube length at high sucrose levels (40–50%) likely reflects osmotic stress(Hebbar et al., 2018; Parrotta et al., 2018), which inhibits water uptake and disrupts pollen metabolic activities, a phenomenon reported in many species under hyperosmotic conditions (Hill et al., 2012).

Interestingly, under *in vitro* conditions in Brewbaker and Kwack (BK) medium containing 20% sucrose and 200 ppm boric acid, and through a similar combination of 15% sucrose + 200 ppm boric acid, pollen grains produced polysiphony, where more than one pollen tube emerges from a single pollen grain. In many cases two pollen tubes were produced from one grain; not as frequently three pollen tubes were produced from the pollen grain and all three germinal apertures.

Normally, a single pollen tube emerges from pollen grain during germination (Boavida et al., 2005; Rodriguez-Enriquez et al., 2013; Shivanna & Tandon, 2020; H.-J. Wang et al., 2010), however the occurrence of more than one pollen tube from a single pollen grain, although rare, has been observed under certain *in vitro* conditions ((Lang & Parrie, 1992; Stone et al., 2004); Stone et al., 2004). However, it is important to remember that pollen tube elongation *in vitro* cannot completely reflect the physiological conditions required for a successful *in vivo* double fertilization which overall, may not provide every signal and environmental cue (Stone et al., 2004). In the current study, we describe the first instance of multiple pollen tube emergence from single grains of *R. serpentina*, a threatened and medicinally important species. This phenomenon, called polysiphony was described under *in vitro* conditions in modified Brewbaker and Kwack medium with high concentrations of boric acid and sucrose. Stone et al. (2004) previously reported similar instances of polysiphony in Conospermum Species , and (Bodhipadma et al., 2022) reported similar findings in *Clitoria ternatea* L.(butterfly pea).Thus, the emergence of multiple pollen tubes from isolated grains under the stated conditions might indicate a threshold-dependent physiological perturbation which is revealing about the precise regulation of polarity during pollen germination (Chen et al., 2008; Fernando et al., 2005; Lelivelt, 1993; Mascarenhas, 1993).

While the biological relevance of this polysiphonous behavior is uncertain, it could be a physiological aberration associated with the *in vitro* culture conditions or a part of inherent developmental plasticity of the pollen grain. Future work concerning *in vivo* germination and fertilization assays could establish whether these multiple tubes had the ability to produce successful fertilization events on the stigma and in the ovule.

Fluorescein Diacetate (FDA) test

Fluorescein diacetate (FDA) testing of pollen from *R. serpentina* indicated a clear diurnal pattern of pollen viability over a 12-hour period from 5:00 AM to 5:00 PM (Fig. 5). Viability was highest during the early morning hours, peaking between 5:00 AM and 9:00 AM (≥98%–99.99%) (Fig. 5g), corresponding to the phases of flower pre-anthesis and anthesis. During this interval, pollen grains exhibited intense green fluorescence and distinct outlines under fluorescence microscopy, indicative of strong esterase activity and intact plasma membranes-hallmarks of viable pollen(Heslop-Harrison & Heslop-Harrison, 1970; Impe et al., 2020; Li et al., 2023; Trognitz, 1991). A gradual decline in viability was noted from 10:00 AM onward, with a sharp reduction post-noon. By 5:00 PM, viability dropped to the lowest recorded value (11.34 ± 6.55%). The pollen grains which contains fertile cytoplasm can only fluoresce with the FDA dye, therefore based on the results, we can conclude that the reduction in viability percent of the pollen in the FCR test could either be the result of non-fertile cytoplasm contained in the pollen.This evidence indicates the fleeting nature of pollen viability in *R. serpentina* and its vulnerability to daily variations in environmental conditions (e.g., temperature and humidity). Additionally, the results highlighted that the best time for collection and testing pollen viability would be during the first hours of anthesis (preferably before 10:00 AM). Notably, this pattern of early morning pollen viability by FCR test is also supported by similar findings in *Rauvolfia hookeri* (Ranjusha et al., 2013).

Acetocarmine test

The viability of the pollen grains obtained by acetocarmine assay showed that pollen collected during the hours between 5:00 AM and 7:00 AM were mostly undehisced but stained completely(Gins et al., 2022; Kucukrecep et al., 2020) (Fig. 6), indicating that some cytoplasmic integrity was preserved before full anthesis. During a time between 8:00 AM and 10:00 AM (Fig. 6ab), viability was constant and ranged from 95.00 ± 1.34% to 90.45 ± 2.30% indicating that viability during this time was high and was very active in terms of anthesis. The time at which pollen will have the most viability for the potential use in breeding programs or when used *in vitro*, was between 8:00 AM and 10:00 AM to collect pollen.

However, the results illustrated that a gradual decline in viability began just after 11:00 AM (88.85 ± 1.45%) and continued into the afternoon. By 1:00 PM (70.27 ± 1.30%), viability had significantly reduced and by 3:00 PM, viability had decreased to a low of 30.65 ± 3.70%. Even at 5:00 PM, there was still pollen that had at least some viability at a minimum of 10.34 ± 1.57%. After 12 PM (Fig. 6c), the staining became more variable indicating that at some point there may have been cytoplasmic degradation or metabolic decline.

While acetocarmine stained pollen is a good way to report on the presence of cytoplasm(Gins et al., 2022; Rathod et al., 2018; Shekari et al., 2016), it does not report directly on viability related to physiology or enzymatic function. So even though ovule and earlier morning pollen appear to be fully stained, they may not be metabolically functional each time(Skrzypkowski et al., 2023). This makes the case for the necessity for further viability tests such as FDA or

TTC, especially when pollen possilbe functions such as for potential fertilization or long-term storage are developed.

TTC test

The TTC(2,3,5-triphenyl tetrazolium chloride) assay revealed that pollen viability was highest during the early to mid-morning hours (7:00 AM-10:00 AM), coinciding with peak anthesis (Cook & Stanley, 1960; Lone et al., 2021; Sulusoglu & Cavusoglu, 2014). The TTC test is based on the activity of **dehydrogenase enzymes**, particularly **succinate dehydrogenase**, which reduce the colorless TTC to a red-colored **formazan** compound in metabolically active cells (Griffo, 2024). Pollen viability (Fig. 7a), as indicated by TTC reaction with dehydrogenase enzymes, increased steadily from early morning to mid-morning (7:00 AM-10:00 AM), followed by a sharp decline in the afternoon (Li et al., 2023). At **5**:00 **AM**, enzymatic activity was moderate (49.03 ± 2.4%) and increased up to **60.56 ± 2.0%** by **7**:00 **AM**. The highest viability was recorded at **9 AM (89.79 ± 1.90%)** and **10**:00 **AM (90.11 ± 4.3%) (Fig. 6b)**, reflecting peak dehydrogenase activity and metabolic performance in viable pollen. Pollen viability dropped sharply after the peak, decreasing to 78.85 ± 4.45% at 11:00 AM, and to 73.15 ± 3.17% at noon. Viability continued to drop to 68.20 ± 3.3% by 1:00 PM. Viability again continued to drop to 65.38 ± 3.27% by 2:00 PM. There was no detectable reduction in TTC to provide a net percent viability (0% viability) after 3:00 PM, providing further evidence that the dehydrogenase enzyme activity is inactive, and pollen is metabolically inactive. These findings show that the TTC assay is sensitive to enzymatic viability in pollen, and that pollen from *R. serpentina* remains metabolically active at the earliest time after anthesis. The viability decrease throughout the day highlights the importance that timing releases and collects pollen plays in the success of fertilization or preservation.

Conclusion:

The present investigation offers a comprehensive understanding of the floral biology, pollen morphology and physiology, and *in vitro* pollen germination responses of *Rauvolfia serpentina*- an endangered, medicinally important taxa of Apocynaceae. Detailed floral phenology revealed that the species revealed that flowering throughout the entire year. Flower anthesis and pollen viability occur in early morning hours (5:00–9:00 AM), closely aligned with anther dehiscence pattern and nectar secretion, suggestive of insect-mediated pollination strategies. The floral structure, including pentamerous, actinomorphic, bisexual flowers with a high pollen-ovule ratio (600:1), indicates an outcrossing tendency requiring efficient pollinator visitation for reproductive success.

In vitro pollen germination studies using Brewbaker and Kwack (BK) medium demonstrated the critical role of sucrose and ionic balance werw critical to the pollen tube growth. Optimal germination was observed at 25–35% sucrose, with maximum tube elongation at 35%, highlighting the species carbohydrate dependency. Beyond 35%, germination declined, likely due to osmotic stress. The BK medium constituents, especially calcium and boron, significantly influenced pollen physiology, corroborating their established roles in pollen tube guidance, integrity, and directional growth. The phenomenon of polysiphony i.e more than one pollen tubes arising from a single grain) observed under specific *in vitro* conditions (sucrose15%,20%) and boron (200ppm)) suggests a physiological perturbation that may reflect developmental plasticity or *in vitro* phenomenon, warranting further *in vivo* validation.

In so far as pollen viability assays (FDA, acetocarmine, and TTC) confirmed a distinct diurnal pattern, with peak metabolic viability occurring between 8.00–10.00 AM. FDA and TTC assays were better at assessing in detecting enzymatic and membrane integrity compared to acetocarmine, which mainly stains cytoplasmic content. The sharp decline in viability post-noon underscores the temporal sensitivity of pollen for successful fertilization or experimental use, suggesting early morning as the optimal window for collection and use in breeding or conservation programs.

In consideration of the overall, this study underscores the intricate regulation of pollen development and germination in *R. serpentina*, highlighting the crucial roles of environmental cues, metabolic timing, and nutritional conditions. These findings not only aid in optimizing germplasm preservation and pollination strategies but also offer a foundation for future work on reproductive resilience under abiotic stresses, including anthropogenic pollution and heavy metals effects, which leads to the threatened status of the species.

Figure 1 Rauvolfia serpentina **(L.) Benth. ex Kurz. (a) Habit of the plant showing overall growth form; (b) Flowering twig with multiple inflorescences; (c) A single flower exhibiting typical corolla structure; (d) Anther showing oblong shape and dithecous condition.**

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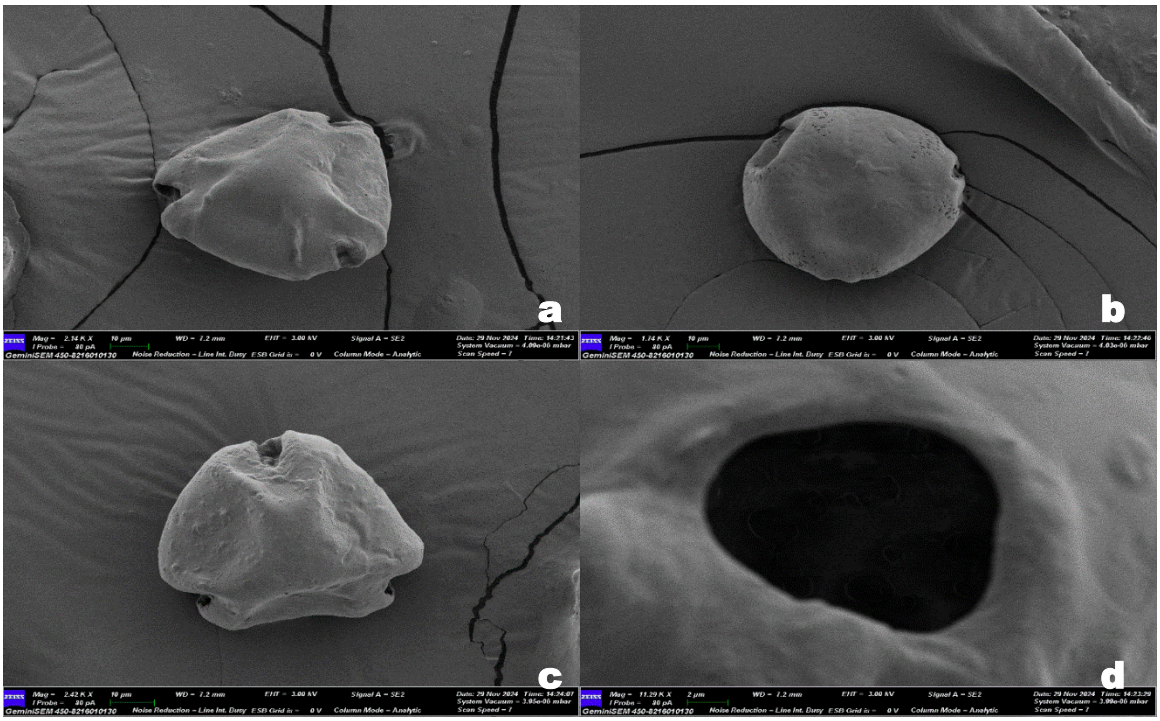

Figure 2. Scanning electron micrographs of *Rauvolfia serpentina* pollen grains. (a) Oblique view showing a trizonoporate pollen grain with three distinct apertures, characteristic of the species. (b) Polar view displaying the nearly spherical shape and symmetrical outline of the grain. (c) Equatorial view highlighting the overall morphology and surface texture of the exine with partial visibility of one aperture. (d) High-magnification image of a single aperture, revealing the detailed ultrastructure and the inner aperture zone, potentially associated with pollen tube emergence.

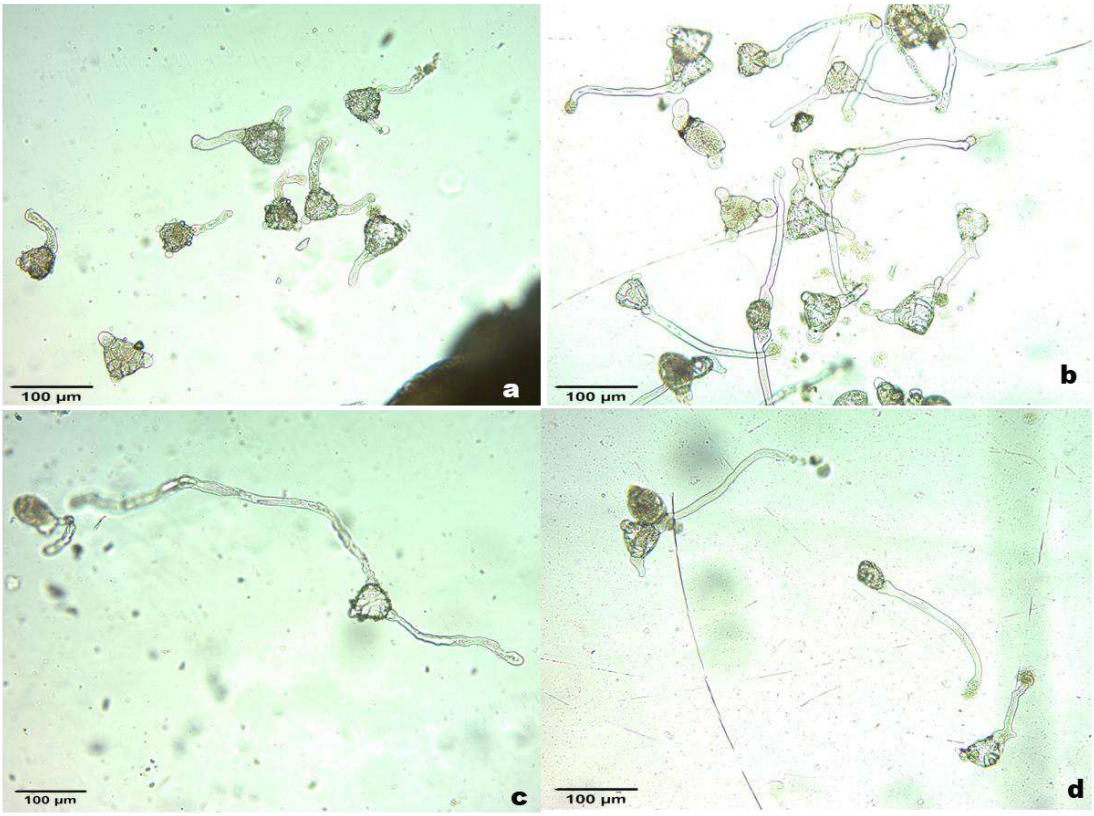

Figure 3: *In vitro* pollen germination test of *Rauvolfia serpentina* on Brewbaker and Kwack (BK) medium with varying sucrose concentrations (a) Germination at 10% sucrose concentration; (b) At 25% sucrose concentration; (c) At 35% sucrose concentration, showing the highest mean pollen tube length (537.07 ± 66.23 µm after 5 hours of incubation.; (d) At 45% sucrose, where pollen germination reducued

Figure 4: Polysiphonous pollen tubes observed in *Rauvolfia serpentina.*
Multiple pollen tubes emerging from a single pollen grain were recorded under *in vitro* germination conditions, indicating polysiphonous development.

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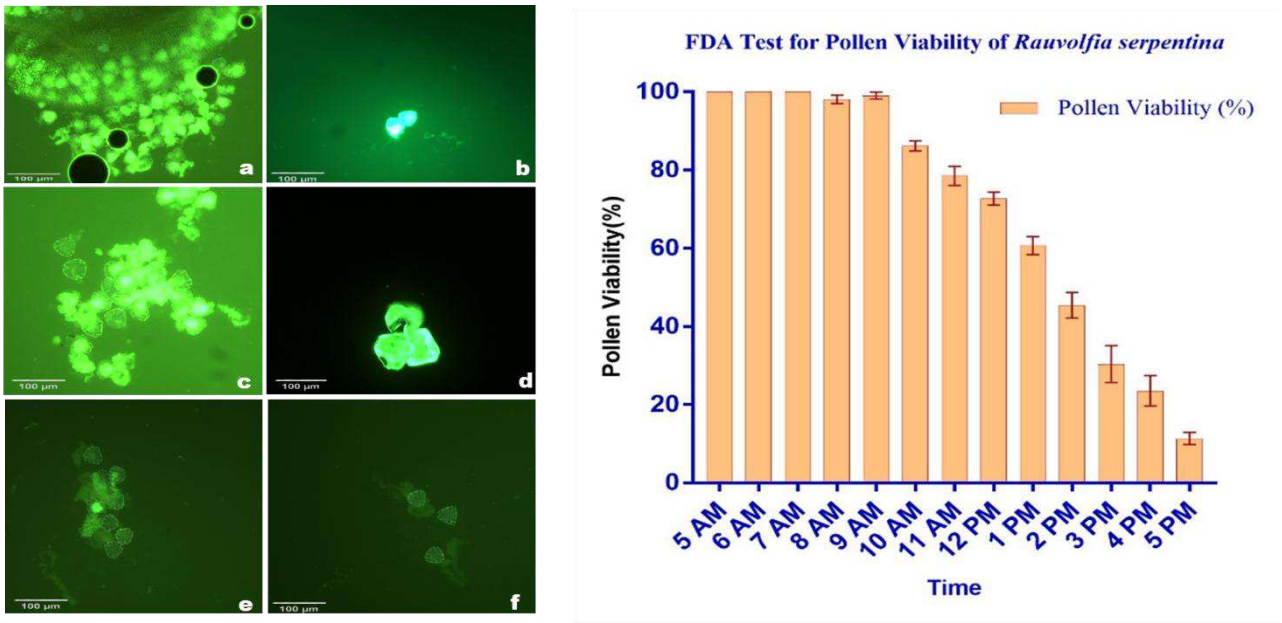

Figure 5: Fluorescein diacetate (FDA) test for pollen viability (a) Pollen grain collected before dehiscence (5:00–7:00 AM); (b) Pollen showing optimum high fluorescence indicating maximum viability (fluorescence observed using blue excitation filter); (c) Fluorescent pollen grain under UV light (8:00 AM); (d) Pollen at 9:00 AM showing high fluorescence; (e) Pollen at 12:00 PM showing reduced fluorescence, suggesting decreased viability; (f) Pollen at 3:00 PM with minimal fluorescence, indicating low viability. (g) Fluorescein diacetate (FDA) test graph showing diurnal variation in pollen viability of Rauvolfia serpentina from 5:00 AM to 5:00 PM.

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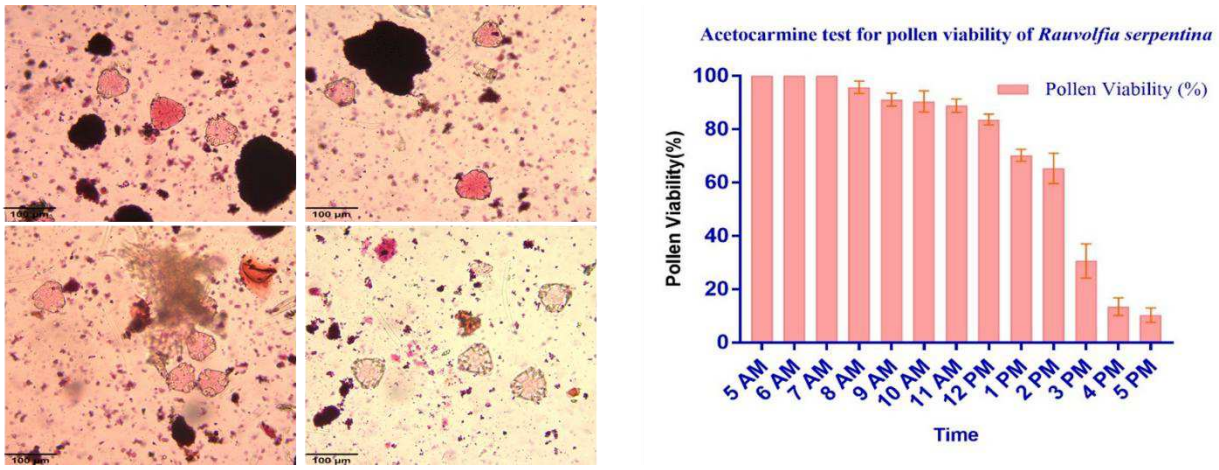

Figure 6: *Acetocarmine test for pollen viability of Rauvolfia serpentina.*

Viable pollen grains stain dark red, whereas non-viable grains remain faintly stained or unstained. The test shows a gradual decline in viability throughout the day:(a) 7:00 AM – majority of pollen grains take up the stain, indicating high viability;(b) 8:00 AM – slightly reduced staining intensity; (c) 12:00 PM – moderate viability with fewer darkly stained grains; (d) 4:00 PM – mostly faintly stained or unstained grains, indicating low viability; f) Graph showing the percentage of pollen viability from 5:00 AM to 5:00 PM.

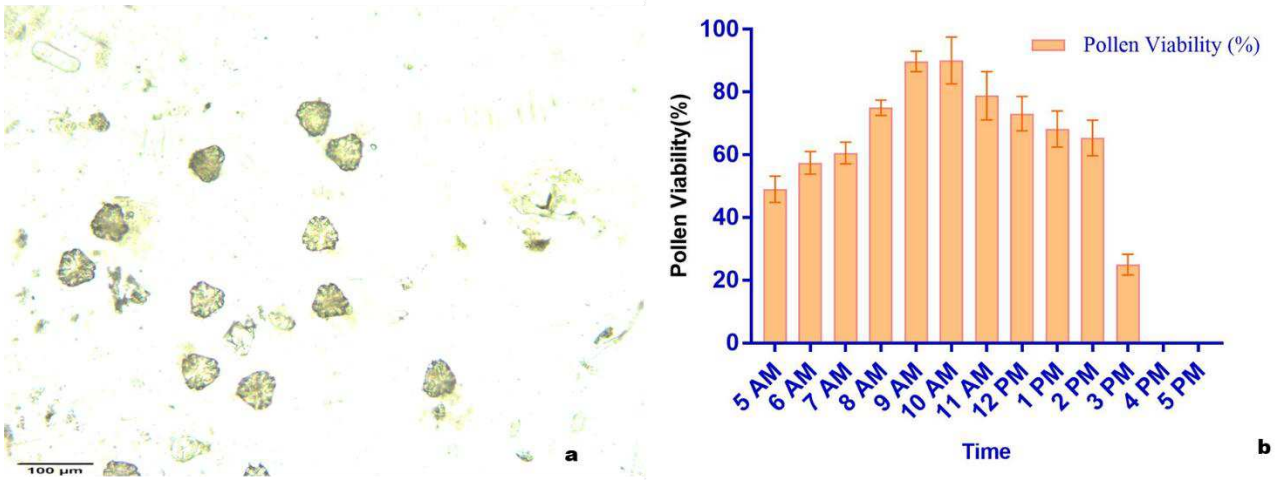

Figure 7: TTC test for pollen viability of *Rauvolfia serpentina.*
(a) Microscopic view showing dark red-stained viable pollen grains, indicating high viability; (b) Graph showing variation in pollen viability percentage from 5:00 AM to 5:00 PM, based on acetocarmine staining results.

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Declaration of Interest Statement:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics, Consent to Participate, and Consent to Publish declarations:

The plant material (Flowers/buds) of *Rauvolfia serpentina* (L.) Benth. ex Kurz. used in this study were collected from cultivated plants maintained in our institutional garden. The collection and use of plant material complied with local and national guidelines for research on plant resources in India. No specific permits or licences were required for this study.

Data Availability Statement:

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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