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Morphology, Reproductive Biology and Anatomy of the Foxtail Orchid, *Rhynchostylis retusa* (L.) Blume

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ABSTRACT

Background: *Rhynchostylis retusa* (L.) Blume, commonly known as the fox-tail orchid, is a promising epiphytic native species for breeding programs. Its' population is diminishing in the native habitat due to unscrupulous overexploitation and habitat destruction. Hence, conservation of this species attains prime importance. To achieve the foregoing task, phenotypic traits in relation to adaptation are to be elucidated.

Methods: In the present investigation, the morphological characters were documented and the correlation between each trait was determined. Furthermore, the anatomy of leaves and roots was documented, as was the stomatal structure and distribution on the abaxile and adaxile surfaces of the leaves. Biometric features of floral characters and reproductive biology such as anthesis, stigma receptivity, pollen production per pollinium, pollen viability, pollination mechanisms, self and cross-compatibility; male sterility, *etc*. were studied and documented. Observations on capsule development, nature of seeds, seed viability, *etc*. were also determined.

Result: The seed viability tests revealed that about 40% of the seeds are viable. The observations gathered during this investigation can be used to develop conservation, sustainable utilization and genetic improvement strategies for this crop.

Key words: Anatomy, Morphology, Orchids, Reproductive biology, *Rhynchostylis retusa.*

INTRODUCTION

Rhynchostylis retusa is commonly known as the "fox-tail orchid," since the pendant inflorescence looks like a fox's tail. It is an epiphytic orchid comprising only four species, which are distributed in the Indian subcontinent: China, Indochina, Malaysia, Indonesia and the Philippines (Higgins, 2013). It is the state flower of Assam and has cultural importance in northeast India. Due to its exquisite flowers arranged in racemose inflorescences, this species tops the list of important Indian wild orchids. Furthermore, it has been used as a medicine in Indian traditional systems of treatment. Native habitat destruction and climate change harm its population in the wild. Moreover, the foregoing factors affect highly cross-pollinated orchids since they adversely affect pollination services and plant communities where orchids exist as epiphytes. It is listed in Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora, also known as the Washington Convention). The generic name comes from two Greek words, "Rhynchos," meaning "beak" and "stylis," meaning "column." When observing a fully opened flower, the anther cap resembles a bird's head and the tip of the anther cap resembles a bird's beak. The overall appearance is similar to that of a bird in flight. "Wonderful flowers are nature's art, a thing of pure beauty." Despite their horticultural and commercial importance, a thorough examination of basic plant information is lacking. It is well acknowledged that systematic documentation of data on morphometry, reproductive biology and anatomy is a prerequisite for identifying desirable traits for breeding, elucidating evolutionary significance, examining species interrelationships and assessing the adaptability of any crop, particularly orchids. In the absence of floral parts and damaged vegetative parts, anatomical data will aid in confirming plant taxonomic identity. In these backdrops, the present investigation was aimed at evaluating the morphometric, anatomical and reproductive biology of the most promising native orchid species, *R. retusa*.

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MATERIALS AND METHODS

The study was carried out during 2021-2023 at the Saraswathy Thangavelu Extension Center of KSCSTE-JNTBGRI, Puthenthope, Thiruvananthapuram, in collaboration with the College of Agriculture, Vellayani, Thiruvananthapuram.

Plant morphology

The *Rhynchostylis retusa* plants collected from the Kulathupuzha forest area in 2018 were established on teak, mango and coconut palm tree trunks at the Saraswathy Thangavelu Extension Centre of KSCSTE-Jawaharlal Nehru Tropical Botanic Garden and Research Institute Puthenthope, Thiruvananthapuram, situated at 8°34′51″ N latitude and 76°50′3″ E longitude. The plant materials chosen were healthy, insect and disease free and grew in natural conditions without the use of chemical or biophysical treatments. The observations of morphometric characters such as number of roots, root length, stem length, stem girth, internodal length, number of leaves per plant, leaf length and width, inflorescence length, number of flowers per inflorescence, capsule length and width were made during the period from April 2022 to March 2023 and the correlation of all the characters was statistically analyzed.

Root anatomy

Medium-sized, recently harvested *R. retusa* velamen roots were cut into extremely thin cross sections by hand, stained in safranin for 30 to 90 seconds and then used for microscopic examinations.

Leaf anatomy

The epidermal layer of fresh orchid leaves was peeled off from both the adaxial and abaxial sides and stained with 0.1% safranin stain before being mounted on a glass slide with an overlying coverslip. These specimens were observed under a compound microscope. Thin cross sections of leaves were also taken by hand, as mentioned earlier and stained using safranin (0.1% w/v) for 60-90 seconds and observed under a microscope.

Acetocarmine stain (45% w/v) was used to stain pollinium. Images were taken under a compound light microscope (Leica LAZ 500).

Floral morphology and reproductive biology

The nature of inflorescence, floral characters, mode of pollination, anthesis and floral biology were examined, as these are important parameters for adopting suitable genetic improvement. Pollen viability and stigma receptivity were determined by adopting controlled artificial pollination mechanisms.

Pollinium, pollen morphology and mechanism of pollination

Pollen production in pollinium was measured using a hemocytometer. Pollinium was collected in an Eppendorf tube and crushed well. The prepared pollen suspension was gently filled in the two chambers under the coverslip of the hemocytometer and the pollen grains were observed under the microscope and counted in four sets of sixteen corner squares and in the center square of 25 subsquares and the average pollen grain load present in each pollinium was calculated. Pollen morphology was also examined after staining with acetocarmine. The pollination mechanism was also observed and documented in the data.

Fruit morphology

Fruit types and morphological characteristics were investigated. The length was measured excluding the pedicel and the width was measured at the region of maximum diameter.

Seed characterization

Seed characters and seed viability were determined by adopting a staining technique and making observations through a microscope. Seed volume, embryo volume and percent air space in the seed were estimated as per the method suggested by Arditti *et al.* (2000).

Seed volume (µm^3)

Based on seed length and width, seed volume was calculated for fusiform-shaped seeds as below:

$$SV = 2[(SL/2)(1.047)]$$

Where:
SV= Seed volume.
SL= Seed length.
SW= Seed width.

Embryo volume (µm^3)

Based on seed length and width, seed volume was calculated for prolate spheroids as follows:

$$EV = 4.19\ (EL/2)$$

Where:
EV= Embryo volume.
EL= Embryo length.
EW= Embryo width.

From seed volume and embryo volume, the percentage of air space is calculated as:

$$\%\ \text{air space} = (SV\text{-}EV/SV) \times 100$$

Where:
SV= Seed volume.
EV= Embryo volume.

Seed viability was assessed using a modified version of the Evans blue test (Batty *et al*., 2001).

Estimation of seed viability

The seeds were soaked in distilled water for 16 hours, after which the water was discarded and the seeds were suspended in Evans blue solution (1% (w/v) for one hour. Finally, the seeds were thoroughly washed several times in distilled water to remove excess stain and the seeds were placed on a microscopic glass slide and viewed under a compound light microscope. Seeds with unstained embryos were considered viable and those with blue-colored embryos were considered non-viable.

Seed viability percentage =

$$\frac{\text{Total number of unstained seeds}}{\text{Total number of seeds}} \times 100$$

Statistical analysis

Correlation analysis was carried out for different plant morphometric traits.

RESULTS AND DISCUSSION

Plant morphology

Rhynchostylis retusa is a monopodial orchid with an epiphytic habit (Fig 1a). Like other epiphytic orchids, it has clinging roots for anchoring, absorbing roots that penetrate the bark humus and aerial roots that hang free in the air and help in the absorption of moisture and nitrogen. The roots contain chlorophyll and are believed to be capable of photosynthesis. The hanging roots are also green, especially in areas close to the growing tip, with a thick layer of cuticle on the outer surface. The roots acquire nutrition for the plant from decaying matter on the bark surface and other organic material like algae, insect excrement, *etc*. The velamen radicum increases the surface area for the absorption of nutrients. The root surfaces are both smooth. The hanging roots are rounded, while the roots that pass through the host bark are flatter on the side of attachment to the tree (Fig 3a). Roots show one or more branching patterns as they grow. The average number of roots per plant was 7.2 and the average root length was 60.01 cm.

The stem is rigid and short, exhibiting a monopodial growth habit. The number of leaves on the stem depends on the age of the plant. The internodal region is invisible due to the presence of the encircling leaf sheath. Leaves are alternate and the base of the stem is supported by clinging roots to the host plant. The average length of the stem was 15.28 cm, the average stem girth was 3.62 cm and the internodal length was 1.34 cm, respectively.

Leaves are distichous, arching, dark green, fleshy, leathery and thick, which is an adaptation to the epiphytic growth habit (Fig 1a and b). They are long, with an average length of 25.43 cm and an average width of 2.59 cm, lanceolate, smooth and entire. Leaves are deeply channeled, keel-shaped, almost curved and glabrous. A didentate apex with unequal size, *i.e*., one is longer and broader than the other. A third, smaller protrusion can also be seen (Fig 1c). Higgins (2013) observed that the leaf apex of the species was highly variable. This feature may help in genus identification when the plant is not in bloom. The shape and size of the leaf tip help drain rainwater efficiently. Rapid removal of water droplets from leaf surfaces during heavy rains is very important for plants to avoid mechanical damage. The correlation of morphometric traits are depicted in Table 1 and Fig 2.

Root anatomy

The cross-section of the root anatomy is depicted in Fig 3. The aerial roots undergo considerable modification to allow water absorption through a special compact multicellular layer called the velamen, which is composed of dead cells that are silvery-grey, white, or brown in colour with fibrous thickening. Aerial roots exhibited drought-resistant characteristics. Next to the velamen tissue is the exodermis, which is single-layered and consists of thick-walled cells. But some of the cells of the exoderm are thin-walled and called passage cells. Water passes through these cells from the velamen to the cortex. The cortex is several layers thick and made of parenchymatous, oval-shaped cells with many intercellular spaces. Some of the cells of the cortex are pigmented. Endodermis is the innermost cortical layer, consisting of barrel-shaped cells with thickened walls. A few endodermal cells opposite the protoxylem are thin-walled and called passage cells. The single-layered pericycle follows the endodermis and consists of thin-walled cells. Many vascular bundles are radial in organization in the root; that is, vascular bundles are exarch and polyarch. Xylem elements alternate with small patches of phloem. Some of the cells of the xylem and phloem parenchyma become thick-walled and lignified around and in between the phloem and xylem bundles. This represents conjunctive tissue. The innermost pith is well-developed and composed of thin-walled, round, parenchymatous cells with many intercellular spaces. The anatomical features are in agreement with the earlier reports (Thangavelu, 2017).

Leaf anatomy

The leaves of *Rhynchostylis retusa* are amphistomatic, where stomata are present on both the upper and lower epidermis, unlike most orchids, in which stomata are found only on the lower epidermis. The stomata on the lower surface are more numerous than those on the upper surface. The stomata are small in size, distributed over the expanded lamina surface and never show a tendency to group. Most

Fig 1: a. *Rhynchostylis retusa* habit, b. a showing shoot tip, c Leaf tip dentation.

of the guard cells are at the same level as the epidermis and the stomata are observed sunken or below the epidermal layer on the adaxial side. The substomatal chamber is small and oval in shape. The inner walls of the guard cells are highly thickened. As reported earlier (Das *et al*., 1992), the guard cells are surrounded by four epidermal cells, two of which are elongated and parallel to the long axis of the guard cells and two narrow rectangular cells; this is a unique identifying feature of *R. retusa*. Dressler (1993) described this type of arrangement as a paracytic stomatal structure. The average number of stomata on the lower and upper leaf surfaces was 10.60 and 6.2 per 500 m^2, respectively. These are scattered and not equidistant from each other. On the adaxial side, the stomata were observed to be sunken. This feature, along with the high leaf thickness and epiphytic habitat, points towards xerophytic adaptation in *R. retusa* (De, 2020). The microscopic observation of stomatal distribution is depicted in Fig 4.

The cross-section of a leaf under a microscope is depicted in Fig 4. The leaf cuticle is smooth to ridged along the contours of the epidermal cells, present on both adaxial and abaxial surfaces. Epidermal cells are single-layered, isodiametric parenchymatous cells with few intercellular spaces. The hypodermis is also single-layered, with spaces between cells filled with depositions that are brightly stained. Mesophyll cells are very irregular in shape and some of them have raphides. Vascular bundles are collateral. Both the xylem and phloem are embedded in sclerenchymatous tissues, as reported earlier (Kousalya *et al*., 2017).

Floral morphology and reproductive biology

Rhynchostylis retusa is a seasonal orchid that blooms from May to August, when the southwest monsoon rains are over. Higgins (2013) reported that the flowering period is determined by climatic conditions. *R. retusa* blooms only once a year. Some plants do not flower every year. There may be one or more inflorescences per plant. Inflorescences emerge from the base of leaves on the lower part of the stem during previous seasonal growth. The plant bears a pendant inflorescence, with an average length of 33 cm, arising from the leaf axil. The average number of flowers per spike is 62 and they are in clusters. Flowers are shortly pedicellate; the average length of the pedicel is 0.87 cm and the distance between the flowering nodes is 0.84 cm. Flowers are small, complete, zygomorphic, showy, fragrant, trimerous, irregular, hermaphrodite and epigynous. Sepals and petals are similar, spreading, white and often mottled with purple or purple blush. On the outside, it is white with a shade of purple. When individual flowers are observed, the sepals are larger than the lateral petals and the labellum is broader. Each flower is 1-2 cm in size and spirally arranged on the spike. The third petal, known as the labellum or lip, is adnate to the column, purple-colored, concave and folded inward and upward. It is single-lobed, completely purple on the inside and white towards the lip. It is spurred at the base

Table 1: The correlation between different morphometric traits.

	Root (cm)		Stem (cm)		Inter-nodal length	No. of leaves	Leaf (cm)		Inflore-scence length (cm)	No. of flowers	Capsule (cm)	
	Number	length	length	girth			length	width			length	width
No. of roots	1											
Root length (cm)	-0.41	1										
Stem length (cm)	-0.31	-0.63	1									
Stem girth (cm)	-0.41	-0.37	0.92*	1								
Internodal length (cm)	-0.20	-0.79	0.95*	0.80	1							
Number of leaves	-0.68	0.43	0.39	0.56	0.10	1						
Leaf length (cm)	-0.33	0.60	-0.20	-0.17	-0.43	0.67	1					
Leaf width (cm)	-0.53	0.80	-0.35	-0.26	-0.55	0.63	0.93*	1				
Inflorescence length (cm)	-0.58	-0.15	0.86	0.92*	0.66	0.80	0.18	0.08	1			
Number of flowers	-0.91*	0.07	0.62	0.71	0.55	0.59	-0.003	0.15	0.75	1		
Capsule length (cm)	-0.99 ***	0.33	0.41	0.52	0.29	0.70	0.27	0.45	0.66	0.95*	1	
Capsule width (cm)	-0.15	-0.20	0.41	0.20	0.28	0.47	0.66	0.41	0.43	0.07	0.15	1

***Correlation is significant at 0.001 level (two tailed).
**Correlation is significant at 0.01 level (two tailed).
*Correlation is significant at 0.05 level (two tailed).

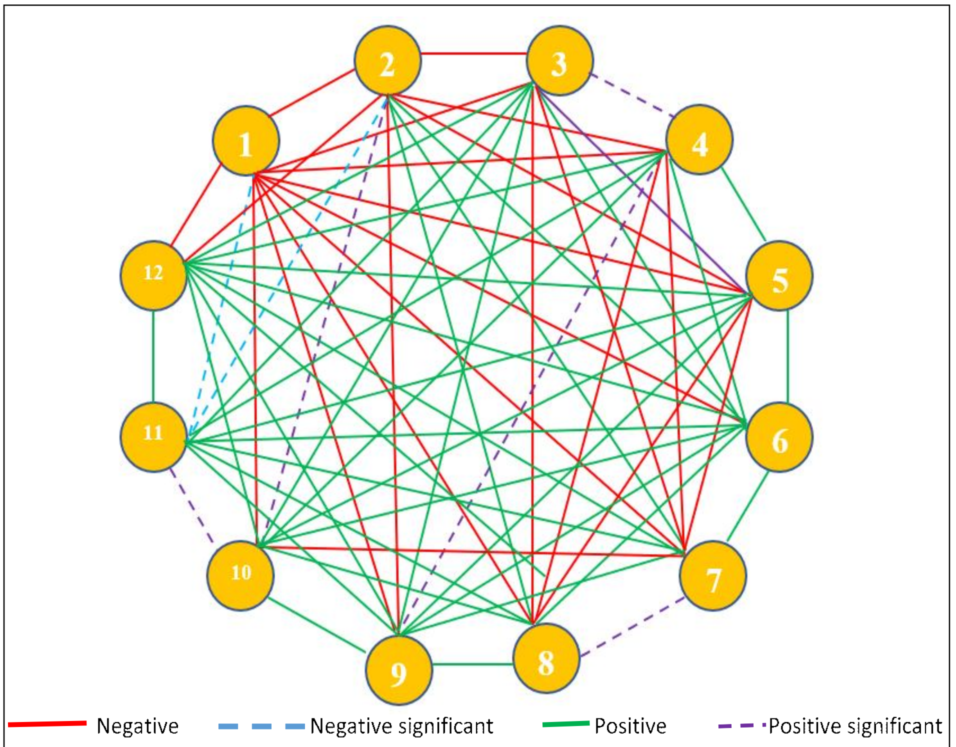
and the labellum is shorter in length than the spur. The spur is cylindrical and less than a centimeter long. The pedicel is white or purplish white and about 1 cm long. Attractive flowers emit a pleasant fragrance, which therefore attracts insects too. The blooms can last up to one month. The flowers do not bloom in a regular pattern from the proximal to the distal end of the racemose inflorescence. In a flower, the lateral sepals are the first to open, unfolding a day before anthesis. The inferior ovary is unilocular and the placenta is parietal. As a depression below the level of the column, stigma is a sticky, shining pad. It is positioned between the pollinia and ovary in the column, closer to the pollinia in distance than the ovary, but separated from the pollinia by the rostellum.

Pollinium, pollen morphology and pollination mechanism

Anthesis of the flowers occurs in the evening and early morning, from 5 pm to 8 am. The white background of the flowers can attract and enhance insects during dark hours, pollinators and other possible insect pests with nocturnal habits. Stigma receptivity lasts for 4-5 days following anthesis and reduces over time. The initial two to three days after anthesis are the peak time of stigma receptivity. Pollinia (Fig 5) are easily attached to the stigma by the viscidium during high stigma receptivity. The stigmatic surface is sunken and padded with sticky fluid. This period was observed to be before noon or before 10.30 to 11 AM. A single pollinium, or a part of it, is enough to effect pollination.

Pollination, if effected can be noticed within a day. Visible signs are stigmatic closure, the flower begins to fade and wilt, sepals and petals will wither and dry, the stigmatic end of the column bulges as reported earlier (Arditti, 1971), the labellum retracts closer to the column, the proximal end of the pedicel, *i.e*., the ovary, turns a light greenish colour. Two pollinia are found in one flower, which is subglobose, pale yellow in colour and has a smooth, soft texture. The diameter of the pollinia is 788.21 µm (Fig 5), the pollen load per pollinium is an average of 20,600,0 and the pollen fertility percentage was 99.6%. Pollen averaged 37.62 m in length and 24.34 m in width (Fig 5i and 5i). The exine, which is smooth and thin and the intine, which is thick, form the pollen's two-layered wall. The pollen grain is obovoid to triangular in shape, mostly seen as a tetrad, but a triad is also observed.

Self-pollination as well as cross-pollination (geitonogamy) occur in nature. The presence of hermaphrodite flowers ensures self-pollination. Scented nectar-producing showy flowers increase insect attraction, resulting in allogamy. Generally, it is classified as a cross-pollinated crop based on various floral mechanisms. Major pollinators are insects like honey bees, ants, flies, *etc*. Pollinator bees carry pollen from a flower and may deposit it on the stigma of another



1. Number of roots, 2. Root length (cm), 3. Stem length (cm), 4.Stem girth (cm), 5. Internodal length (cm), 6. Number of leaves, 7. Leaf length (cm), 8. Leaf width (cm), 9. Inflorescence length (cm), 10. Number of flowers, 11. Capsule length (cm), 12. Capsule width (cm)

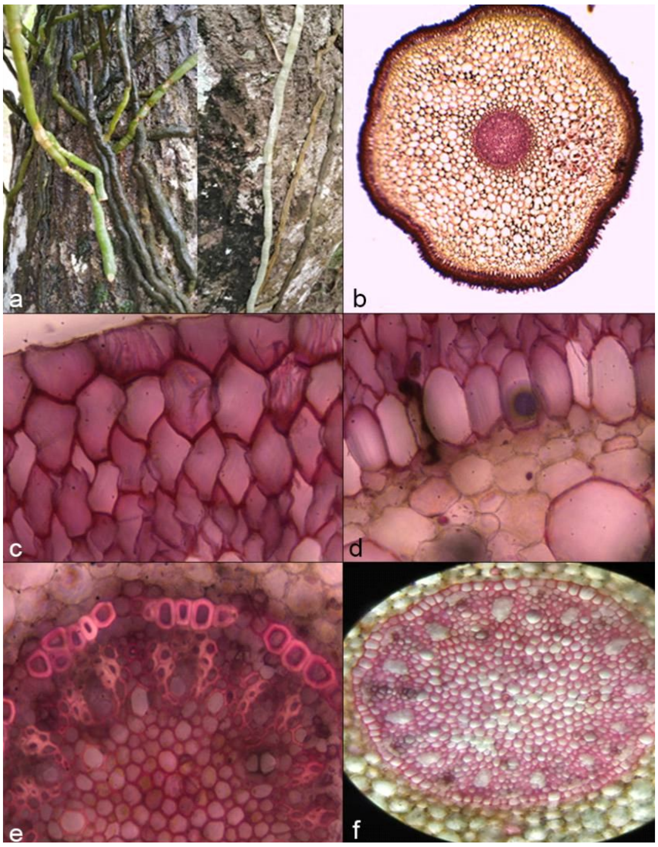
Fig 2: Correlation between different morphometric traits, flower in the same inflorescence or in a different inflorescence of the same plant. Buragohain *et al*. (2014) reported that although the flowers of *R. retusa* are regularly foraged by various insects, for example, ants, beetles, bees and butterflies, for sweet nectar, two species of carpenter bees of the genus Xylocopa were biotic pollinators. It is cross-compatible with other orchid genera, such as *Aeridis* (Seeja, 2018). Pollinia may also be transferred from a different species to the stigma of *R. retusa*, leading to allogamy. The initial post-fertilization changes of the flowers are depicted in Fig 6a.

Fruit morphology

The capsules of *Rhynchostylis retusa* are like a three-sided prism, with a maximum diameter at the distal end. The average size of a capsule is 2.18 cm in length and 0.86 cm in width (Fig 6b). The capsules are green as they develop; they gradually increase in size and the hairs inside start to develop after three months. There is a greater increase in the diameter of the fruit than in its length between four and five months. Between five and a half and six months after the fruit sets, it matures and turns yellow, completely packed with dust-like seeds embedded in thin white hairs. Then, by the seventh month, the capsule dries up, turns brown and shrivels and millions of seeds escape through the grooves running longitudinally. Grooves are prominent and dehiscence occurs along the underside.

Seed characterization

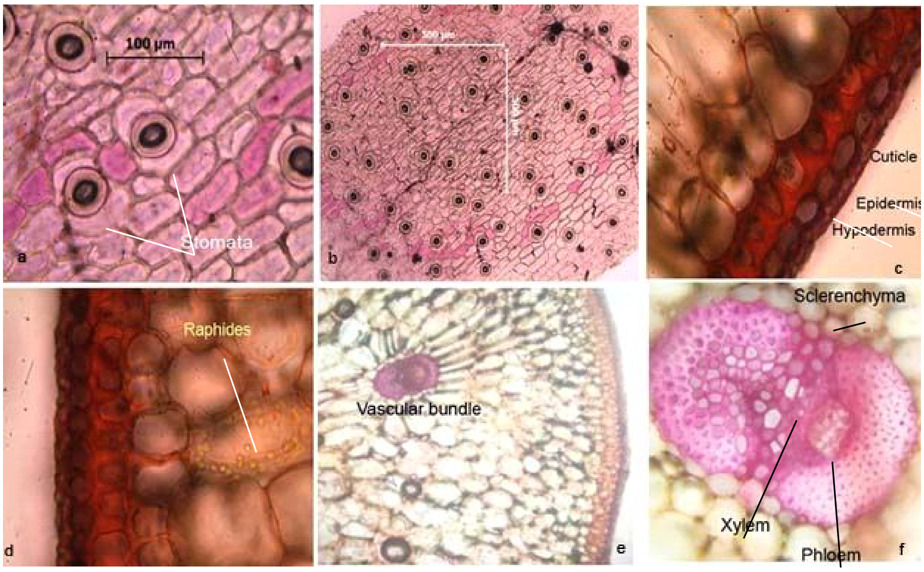
When the capsule is cut, the seed mass embedded in white fibres is seen. The microscopic observation of the seeds revealed that they are a simple fusiform-shaped structure consisting of a seed coat and embryo. The microscopic observations of the 100-day-old seeds and 145-day-old seeds are depicted in Fig 6c,d,e,f and g respectively. It has an average length of 264.20 µm, a width of 48.54 µm and a seed length/width size of 5.59 µm Fig 6i). The embryos are spherical in shape and occupy most of the volume of the seed coat or are smaller. It has an embryo in the center or on one side. It has an average length of 53.26 µm and a width of 26.73 µm. According to Healey *et al*. (1980) the embryos in orchids tend to be uniform in size within a genus, but embryo size varies from genus to genus. In fact, Augustine *et al*. (2001) admit that there can be considerable variation in orchid seed and embryo size. Therefore, the measurement of embryo volume is of taxonomic importance, along with the morphological observation of seed characters. The average seed volume was 164296.95 µm^3, the embryo volume was 19279.25 µm^3 and air space made up 87.70%



a. Velaman root, b. C.S. of root, c. root cells with fiber, d. velamen tissues, e, f. vascular bundles showing xylem, phloem and endodermis.

Fig 3: Root morphology and anaotamy.

of the total seed volume. The testa, or seed coat, is reticulated and ornamented with air spaces. Mature testa cells are transparent and lignified to varying degrees. Longitudinal elevated ridges are formed on the flat, smooth surface of the testa cells. These longitudinal ridges, running close enough to each other, are slightly twisted and

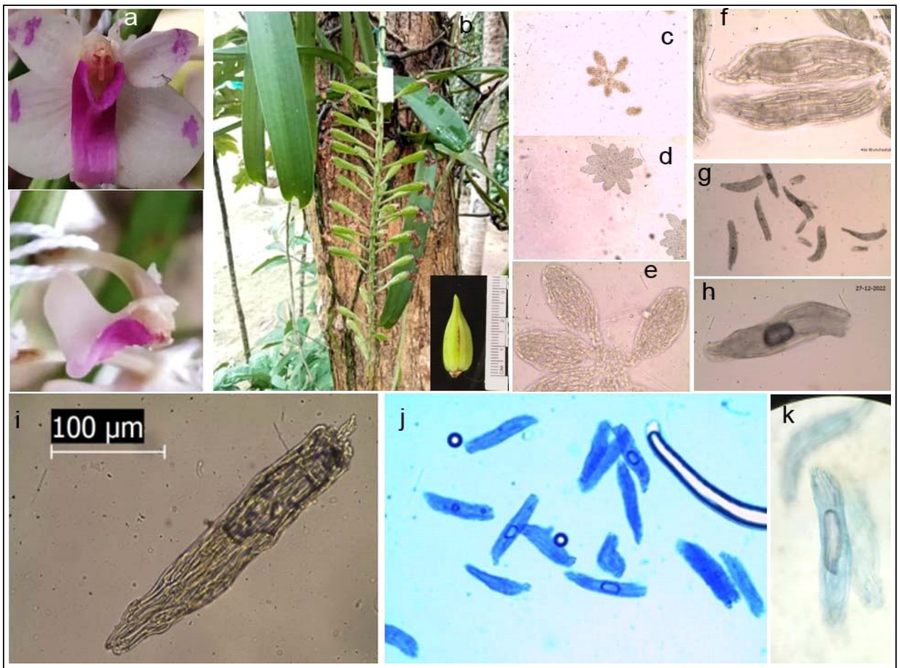


a. Stomatal distribution on the abaxial side of the leaf, b. Stomata per unit area, c. a portion of the leave showing cuticle, epidermis, hypodermis and deposition in between hypodermis, A portion of the leaves CS showing raphides deposition, e. leaves CS showing vascular bundle, f. Enlarged view of Vascular bundule showing sclernchymatous bundle cap, xylum and phloem

Fig 4: Leaf anatomy.

a. Rhynchostylis retusa bearing inflorescence, b. Inflorescence showing flower opening during initial stage, c. Inflorescence with 90% opened flowers, d. Inflorescence showing flower opening complete on next day, e. Single flower with purple spotted marking, f. Single flower without purple colour marking, g. Dissected flower parts, h. Pollinia, Iand j Microscopic observation of pollen grain.

Fig 5: Morphology of floral parts.



a and b. Floral changes after pollination, c. Plant bearing capsule and a single mature capsule, c,d ande. Immature seeds from 100 days old capsule, f. Seeds from 145 days old capsule, g. A microscopic view of mature seeds, h. enlarged microscopic view of a single seed, i. Seed measurement, j. Viability test-stained in Evan's blue.

Fig 6: Fruit development and seed characters.

interconnected horizontally by similar ridges. In some seeds, the testa cells are too low at one end to form a sufficiently open space. Even within a single fruit, the embryo colour can range from brown to yellow to golden yellow to blue. The average seed viability was found to be 39.72 per cent. The viable embryos didn't stain (Fig 6 j and k). Aerosols in the seeds of orchids are of great morphological interest and are important for successful germination. In fact, very light and buoyant seeds with a greater percentage of airspace may get dispersed over wide geographical locations, while those with minimal airspace may get confined to a few narrow local patches (Augustine *et al*., 2001). Therefore, if such a species becomes endangered, conservation measures are difficult. During maturation, there is an increase in the percentage of air space in the seeds.

Increased aeration is due to the increased cell length of the testa and not to an increased number of cells in the seed coat (Swamy *et al*., 2004). This variation in seed space with ontogeny might be the reason why the embryonic region cannot be seen with a definite boundary under the light microscope in immature seeds.

The morphometric, anatomical and reproductive biology of this endangered species can be utilized for practical conservation and sustainable utilization, particularly for its genetic improvement as well as the generation of new commercially viable hybrids.

CONCLUSION

Rhynchostylis retusa, known as the fox-tail orchid, faces threats from overexploitation and habitat destruction, highlighting the importance of its conservation. This study documented the orchid's morphological, anatomical, and reproductive characteristics, finding a seed viability rate of approximately 40%. These insights are crucial for crafting conservation strategies, promoting sustainable use and enhancing genetic improvement programs, ultimately supporting the preservation and future breeding of this valuable species.

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Conflict of interest

All authors declared that there is no conflict of interest.

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