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Histochemical Localization of Polysaccharides During Pollinium Development in Cottonia Peduncularis (Lindl.) Rchb.f. (Orchidaceae)

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Abstract

Cottonia peduncularis (Lindl.) Rchb.f., is monotypic and rare epiphytic orchid endemic to peninsular India, demonstrates remarkable structural and physiological adaptations to arboreal habitats. The present study investigates the histochemical localization of total insoluble polysaccharides during pollinium development and relates these findings to its adaptive morphology and reproductive ecology. Flower buds at various developmental stages, collected from Dandeli and Haliyal forests of Karnataka, were fixed in FAA, sectioned (6-8 µm), and subjected to Periodic Acid–Schiff's (PAS) staining following standard microtechniques. Both early and late sporogenous cells exhibited strong PAS reactivity, indicating high polysaccharide accumulation. Meiocytes retained PAS-positive reactions in cytoplasm and nucleoli throughout meiosis, followed by the formation of tetrahedral, isobilateral, linear, and inseparable tetrads through successive cytokinesis. A progressive increase in PAS positivity was observed in the cytoplasm of tetrads and microspores, reaching maximum intensity in the two-celled stage, particularly in the pollen wall. The tapetum is the secretory type, showed strong PAS reaction and gradual degeneration during tetrad formation, contributing polysaccharide-rich material to developing pollen. These results suggest the essential role of carbohydrates as an energy source during meiosis, wall differentiation, and pollen maturation. The findings provide insight into the biochemical basis of reproductive adaptation in *C. peduncularis*, linking its histochemical dynamics with ecological and evolutionary specialization within the Orchidaceae.

Keywords: Orchids, Pollinia, Histochemistry, Polysaccharides.

Introduction

Orchids, constituting the 'Orchidaceae' the second-largest family of flowering plants after Asteraceae with over 700 genera, 26,000-30,000 species comprising nearly 10% of angiosperms, and more than 100,000 registered hybrids, represent an exceptionally unique and diverse lineage within angiosperms [1], [2]. This family is of profound ecological, economic, and conservation significance, underscoring its evolutionary prominence through exceptionally high speciation rates [3]. This vast family exhibits extraordinary morphological and ecological versatility, enabling the colonization of diverse niches from lithophytic, terrestrial, and mycoheterotrophic to predominantly epiphytic habitats under challenging environmental conditions [4]. Critically, orchids are delineated by two fundamental growth architectures: monopodial, driven by a single apical meristem that sustains indefinite stem elongation alongside sequential leaf

production; and sympodial, characterized by the iterative development, flowering, and replacement of lateral shoots adaptations that enhance resilience and reproductive efficiency in varied environments [5]. The family Orchidaceae exhibits highly specialized vegetative and reproductive traits [4]. Orchids display remarkable diversity in floral morphology, pollination mechanisms, and reproductive strategies [6], together with exceptionally high speciation rates. These characteristics establish orchids as a premier model system for studies on plant evolution, adaptation, and coevolution with pollinators. A hallmark of this specialization is the formation of pollinia compact masses of pollen grains coherently bound together by a viscin matrix and attached to specialized structures such as the caudicle and retinaculum. This architecture ensures precise and efficient pollen transfer between flowers, thereby enabling highly specific pollinator interactions and substantially reducing pollen wastage [7], [8].

The pollinium represents the specialized functional unit of pollen transfer unique to orchids, critically underpinning reproductive success by enabling precise pollinator-mediated dispersal and minimizing pollen wastage. Its intricate cellular organization and biochemical composition dictate essential traits such as pollen viability, germination capacity, desiccation tolerance, and adhesion to pollinators [7]. These adaptations align with orchids' extraordinary floral and pollination specializations. Although histochemical studies in plants have identified major biomolecular classes including proteins, carbohydrates, lipids, and phenolic compounds each imparting distinct physiological roles the detailed localization of these compounds during pollinium development remains scarce, particularly in species like *Cottonia peduncularis*.

Proteins function as reserve nutrients and regulatory enzymes indispensable for pollen germination and tube growth [9]; lipids provide primary energy reserves while conferring surface hydrophobicity to enhance dispersal and pollinator adherence [10] and carbohydrates are vital for pollen wall sculpturing, energy metabolism, and cell-cell recognition [11]. Recent advancements in green nanotechnology suggest that these physiological barriers can be further reinforced by biogenic nanomaterials; for instance, pullulan-based zinc oxide (ZnO) nanocomposites have been shown to provide exceptional UV-filtering and antimicrobial protection, creating a robust shield against environmental pathogens [12]. Similarly, copper oxide (CuO) nanoparticles synthesized from medicinal plant extracts exhibit potent antioxidant activity and significant free radical scavenging potential, offering a novel approach to mitigating oxidative stress in reproductive tissues [13]. Furthermore, phenolic compounds confer protection against ultraviolet radiation, oxidative stress, and microbial pathogens, playing a crucial role in the structural integrity of the pollen wall [14].

Cottonia peduncularis (Lindl.) Rchb.f. exemplifies the extraordinary evolutionary adaptations within Orchidaceae. This rare and endemic epiphytic orchid, primarily distributed across the Western Ghats of India and parts of Sri Lanka, is renowned as the "bee orchid" for its sophisticated floral mimicry: the labellum strikingly resembles a female bee, luring male bees into pseudocopulatory pollination [15]. This represents a pinnacle of plant-pollinator coevolution that demands precise morphological and biochemical alignment in reproductive structures like the pollinium for specificity and success. Alarmingly, despite such intricate specializations, the histochemical aspects of pollinium development and differentiation in Orchidaceae remain woefully underexplored, with virtually no dedicated studies on species like *C. peduncularis*. This glaring knowledge gap imperatively necessitates rigorous histochemical investigations to map the biochemical architecture of orchid pollinia. Such *in situ* techniques uniquely pinpoint the localization, synthesis, storage, transport, and metabolism of key substances within cells and tissues,

yielding unparalleled insights into the physiological underpinnings, developmental trajectories, and functional adaptations of orchid reproductive tissues [16].

Therefore, this study aims to meticulously characterize the spatiotemporal distribution of polysaccharides a critical class of macromolecules involved in cell wall formation, energy storage, and cell-cell recognition during the developmental stages of pollinia in *Cottonia peduncularis* using an array of histochemical techniques. This approach not only elucidates the physiological foundations of pollen maturation but also illuminates the evolutionary innovations underpinning Orchidaceae's specialized pollination mechanisms. Despite its profound ecological and evolutionary significance, *Cottonia peduncularis* remains strikingly underexplored in terms of the biochemical composition and histochemical organization of its pollinium. Prior research has overwhelmingly prioritized morphology and palynology, with negligible attention to the spatial distribution of key biochemical constituents, despite their pivotal roles in pollen viability, desiccation tolerance, and pollinator adhesion [7]. Thriving in epiphytic niches subject to extreme fluctuations in humidity, temperature, and light, this species demands such insights to unravel adaptive responses influencing pollen development and reproductive success. The present study addresses this critical lacuna through *in situ* histochemical localization of pollinium development and differentiation, targeting carbohydrates and related metabolites essential for wall sculpturing, energy metabolism, and structural integrity. Furthermore, the present investigation aims to unravel the molecular mechanisms underlying the synthesis and regulation of these compounds by employing transcriptomic analyses, thereby revealing candidate genes pivotal for carbohydrate metabolism during distinct developmental stages of microspores and megaspores.

Materials and Methods

Collection of Plant Material

Flower buds at various developmental stages were collected from *Cottonia peduncularis* Rchb.f. plants growing in their natural habitats in the forested regions of Haliyal and Dandeli, Uttara Kannada district, Karnataka, India. Plant identity was confirmed based on morphological characteristics described in regional floras [44] and by comparison with authenticated herbarium specimens housed in the Herbarium of the Department of Botany, Karnatak College, Dharwar (Voucher S. No. 211016). Buds spanning the full range of floral development from early to mature stages were selected to facilitate a detailed examination of pollinium formation, differentiation, and maturation. Immediately upon collection, samples were fixed in the field to prevent tissue degradation and preserve cellular integrity for subsequent histochemical analysis.

Fixation and Preservation

Freshly collected flower buds were fixed in FAA fixative for 24-48 hours at room temperature [17]. This FAA fixation optimally preserved both cellular architecture and biochemical components, especially the polysaccharide materials in the pollen and supporting tissues. Following fixation, the tissues were washed in 70% ethanol to remove excess fixative and then stored therein until further processing.

Dehydration and Embedding

The fixed tissues underwent graded dehydration in a series of ethanol solutions (50%, 70%, 90%, and absolute ethanol, each for 24 h), followed by stepwise transitions through ethanol:n-butanol mixtures and pure n-butanol [18]. Dehydration was confirmed when the tissues appeared translucent without signs of

shrinkage. The dehydrated tissues were then infiltrated with paraffin wax using a standard schedule, involving multiple changes of molten paraffin at 58°C in a thermostatically controlled oven to ensure thorough penetration. Finally, the samples were embedded in oriented paraffin blocks to facilitate transverse sectioning of the developing pollinia.

Sectioning and Mounting

The paraffin-embedded blocks were sectioned using a semi-automatic microtome. Thin serial sections, ranging from 6–8 µm in thickness, were carefully cut [19]. The flattened sectioned ribbons were mounted onto clean glass slides pre-coated with a thin layer of gelatin adhesive to ensure proper adherence during subsequent staining procedures. The mounted slides were dried at room temperature.

Histochemical Analysis

Insoluble polysaccharides essential macromolecules underpinning cell wall formation, structural integrity, and developmental adaptations in orchid pollinia were precisely localized using the highly specific Periodic Acid–Schiff's reaction. This gold-standard histochemical technique selectively oxidizes vicinal diols in neutral polysaccharides (e.g., cellulose, hemicellulose, and starch), yielding a distinctive, stable magenta coloration for superior contrast and accurate spatiotemporal mapping in plant reproductive tissues [20]. The protocol followed Khasim's established method: paraffin-embedded sections were deparaffinized in xylene and rehydrated through a descending graded ethanol series to distilled water. Sections were oxidized with 1% periodic acid for 10–15 minutes to generate aldehyde groups, rinsed thoroughly in distilled water, and incubated in Schiff's reagent for 15–20 minutes in the dark [21]. Slides were then washed in running tap water for 10 minutes to develop the magenta stain, dehydrated in an ascending ethanol series, cleared in xylene, and mounted in DPX for microscopic examination. This rigorous approach ensures reliable detection of insoluble carbohydrate reserves critical for pollinium maturation and desiccation tolerance.

Microscopic Observation

The stained sections were examined under a compound light microscope equipped with bright-field illumination. Observations at various magnifications assessed the distribution and localization of polysaccharides within developing pollinia. This qualitative evaluation relied on PAS staining intensity as an indicator of relative polysaccharide abundance; regions exhibiting strong magenta coloration were noted as sites of high accumulation [20]. Microphotographs of representative sections were captured using a light microscope and appropriately labeled. Comparative staining intensities across developmental stages were used to infer shifts in biochemical composition during pollinium differentiation and maturation.

Light microscopy investigations of pollinium development in *Cottonia peduncularis*, consistent with earlier studies on orchid embryology [22], revealed distinctive structural and biochemical features. The pollinium wall is of the monocotyledonous type and comprises four distinct layers [23]. Sporogenous cells differentiate into pollen mother cells, while the tapetum is glandular (secretory) in nature [24]. Cytokinesis proceeds successively, yielding inseparable tetrads [8]. The locule develops successively through densely packed sporogenous cells, meiocytes, tetrads, microspores, and pollen grains, with pollinia released at the two-celled stage. Archesporium and sporogenous tissues exhibit rich PAS-positive material in both cytoplasm and nucleoli. During meiotic development, PAS staining intensifies in the cytoplasmic content

surrounding thin chromosomes. Young pollinial wall layers display abundant PAS content, which diminishes with maturation, particularly at the periphery of the pollinium locule [25]. The tapetum remains PAS-rich from inception until disintegration even in tapetal cells derived from the connective. Notably, rich PAS-positive staining in the endothecium facilitates the differentiation of endothecial thickenings in mature pollinia [26]. Collectively, these findings elucidate the dynamic biochemical and developmental processes underpinning reproductive tissue differentiation in *C. peduncularis*.

Figure 1. a & b: Habit, c: Developmental stages of flower buds, d: Single flower showing bee-shaped labellum with dark magenta mid lobe and yellow lateral lobes, e: Pair of pollinia attached to a short caudicle and viscid gland.

Results and Discussion

Distribution of Polysaccharides

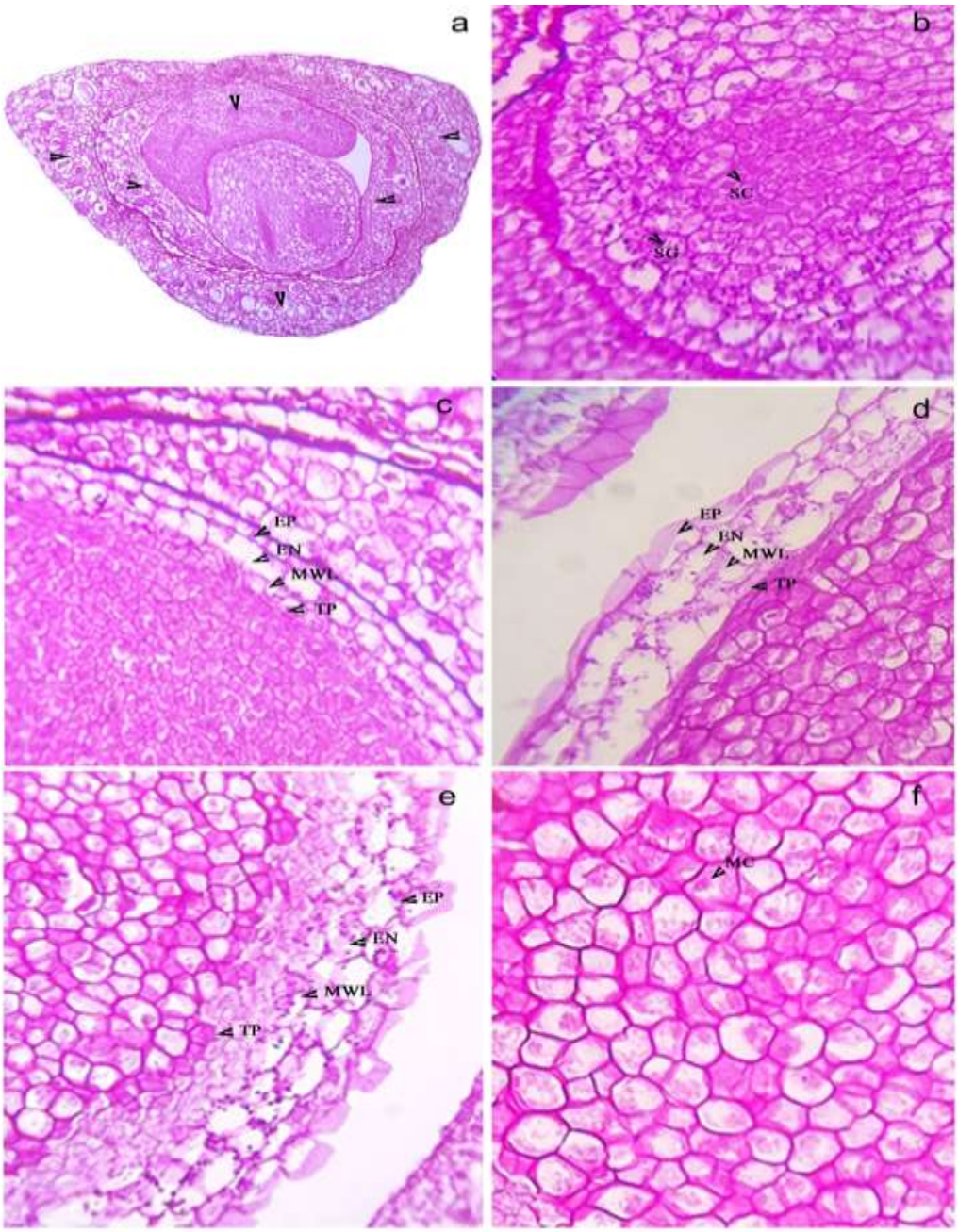
The differentiation of pollinial tissues is likely influenced by the presence and distribution of polysaccharides, as carbohydrate dynamics are central to anther development [27]. The young primordia

of pollinia consist of a mass of cells that develop and differentiate, exhibiting uniform polysaccharides predominantly in the cell walls and cytoplasm. At the hypodermal region, archesporial cells differentiate, characterized by PAS-positive storage materials, dense cytoplasm, and prominent nuclei (Fig. 2a & b), a pattern consistent with high metabolic activity observed in early orchid anther development [28].

The sporogenous cells exhibit strong PAS-positive staining, although their cytoplasm does not synthesize PAS-positive storage materials. The nuclei react more intensely to the PAS test (Fig. 2c), indicating high transcriptional activity associated with presynthetic stages [29]. The sterile septum contributes by serving as a storage tissue for starch, providing energy for pollinium development, a physiological role critical for sustaining microsporogenesis [30]. During meiosis, meiocytes display a slightly increased polysaccharide content in the cytoplasm. As meiosis progresses, no conspicuous changes in polysaccharide distribution are observed in the fully differentiated sporogenous tissues. At this stage, the parietal layers show variations in their polysaccharide content, mirroring the behavior of neighboring sporogenous tissues. These cells accumulate starch reserves, with PAS-positive material also observed in the cytoplasm (Fig. 2c). A notable feature during meiosis is the deposition of more PAS-positive material around meiocytes, suggesting that extracellular deposition is linked to the meiotic process and the formation of the callosic wall [31] (Fig. 2d & e).

PMCs increase in size, accompanied by notable changes in cytoplasmic division (Fig. 2f & g). Microspores in tetrads react strongly to the PAS test (Fig. 2k). The PAS-positive layer holding microspore tetrads together persists until microspores mature into pollen grains (Fig. 2k–m), a characteristic adaptation in Orchidaceae known as the "common wall" or "viscin matrix" which ensures mass pollen transfer [7]. Both generative and vegetative nuclei react strongly to the PAS test, although no PAS-positive storage is observed in these cells (Fig. 2m). The pollen wall is thin and PAS-positive (Fig. 2m). Pollen grains at the outer periphery of the pollinium develop considerably less PAS-negative wall material (Fig. 2l).

Pollen grains located in the interior of the pollinium do not develop an exine but exhibit distinct PAS-positive layers in the intine (Fig. 2l), a phenomenon typical of aggregated pollen where internal walls remain pectocellulosic to maintain cohesion [7]Yeung et al. 2018). In *Cottonia peduncularis*, pollen is shed at the 2-celled stage during sporogenous cell development, consistent with the majority of orchid species [22]. Endothecial thickenings, which differentiate at the mature stage in *Cottonia*, are also observed in many orchid species; the endothecial cells enlarge and form PAS-positive fibrous thickenings (Fig. 2h) essential for anther dehiscence [26]. As the pollinium matures, the tapetal layer nearly disintegrates, and at maturity, the wall layers become completely devoid of storage polysaccharides and degenerate entirely (Fig. 2h-j), marking the programmed cell death required for pollen release [24].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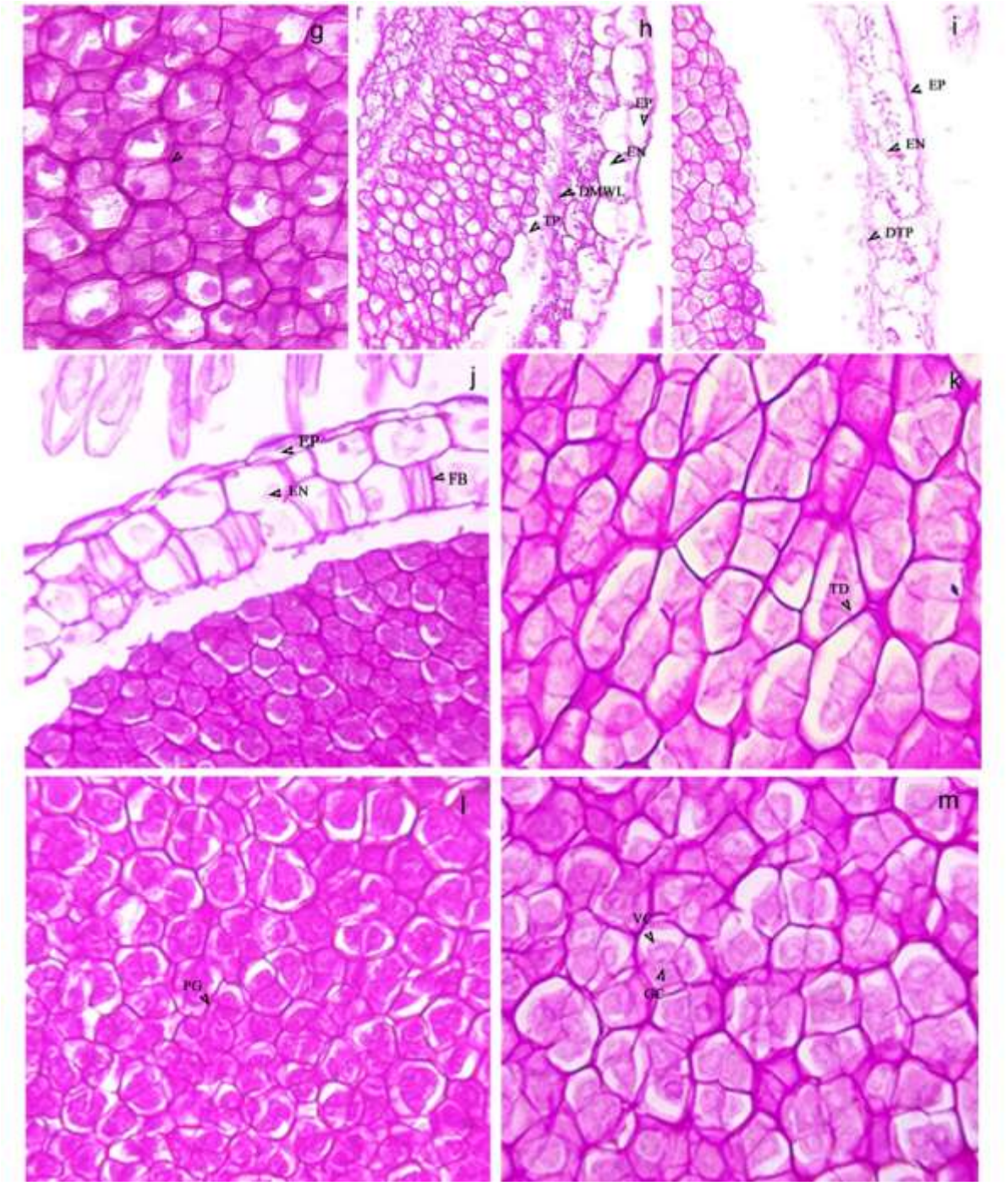

Figure 2. a-T.S of Pollinia of *Cottonia* tested for polysaccharides with PAS method shows, black arrow head;Tapels (10x X 40x), b-Archesporial cells showing PAS+ve cell wall, thin walled and sparsely distributed starch grains in parietal layers (10x X 40x), c-Wall layers of sporogenous tissue and tapetum are thin walled cells & are PAS+ve, the remaining wall layers are having thick cell walls & PAS+ve (10x X 40x), d & e-Wall layers of Pollen mother cell, contain sparsely cytoplasmic polysaccharides. f-Pollen mother cells showing rounded with rich PAS+ve content in the cytoplasm (10x X 40x). g-Division of meiocytes (10x X 40x), h-Degeneration of middle wall layers (10x X 40x), i-Degeneration of Tapetum layer, devoid of PAS+ve (10x X 40x), j-Fibrous thickenings of the endothecium are PAS+ve (10x X 40x), k-Tetrads (10x X 40x), l-Pollen grains shows the cytoplasm & nuclei are rich in polysaccharides (10x X

40x), m-Mature pollen grain ; the vegetative cell and generative cell showing rich content of PAS in the cytoplasm (10x X 40x).
Abbreviations: EP-Epidermis, EN-Endothecium, MWL-Middle wall layers, TP-Tapetum, SC-Sporogenous cells, SG-Starch grains, TD-Tetrad, DMWL-Degenerated middle wall layers, DT-Degenerated tapetum, FB-Fibrous bands, PG-Pollen grains, VC-Vegetative cell, GC-Generative cell.

Differentiation and Sporogenous Tissue Development

The differentiation and development of pollinial tissues in *Cottonia peduncularis* exhibit marked similarities with other orchid species, such as a monocotyledonous-type pollinium wall comprising four layers, glandular tapetum, successive cytokinesis yielding inseparable tetrads, and pollinia release at the two-celled stage [22]. However, sporogenous cells in *C. peduncularis* are distinctly characterized by high metabolic activity evidenced by strong PAS-positive staining in cytoplasm and intensely reactive nucleoli-without any detectable storage grains. This absence sharply contrasts with *Calanthe*, where transient PAS-positive storage grains accumulate in sporogenous cells [32], underscoring species-specific adaptations in energy mobilization that rely instead on adjacent tissues like the starch-storing sterile septum to fuel meiosis.

Meiosis and Meiocyte Characteristics

Meiocytes in *Cottonia peduncularis* are characterized by prominent nucleoli, dense cytoplasm rich in PAS-positive material, and intensified polysaccharide staining in both cytoplasm and intensely reactive nuclei during meiotic progression. A distinct PAS-positive layer is deposited extracellularly around meiocytes, further surrounding them and linking directly to the meiotic process, as observed in archesporium, sporogenous tissues, and neighboring parietal layers. This configuration aligns with the glandular tapetum and high metabolic activity typical of orchids [24]. Meiotic division proceeds *via* successive cytokinesis, yielding inseparable tetrads, a predominant pattern across Orchidaceae, in contrast to the simultaneous type limited to taxa like *Cypripedium*, *Paphiopedilum*, and *Epipactis* [22].

Microspore Tetrads and Organization

Following meiosis, the microspore tetrads in *Cottonia peduncularis* observed in both linear and isobilateral configurations exhibit strong PAS-positive staining in their cytoplasm and a persistent PAS-positive layer that binds them together, reflecting abundant polysaccharides. This enriched polysaccharide profile, coupled with dense cytoplasm and prominent nuclei, equips young microspores with robust metabolic reserves and structural cohesion essential for rapid subsequent development into cohesive massulae [33]. Unlike in genera such as *Neottia*, *Spiranthes*, *Cypripedium*, and *Cephalanthera*, where tetrads readily separate into individual pollen grains, *Cottonia* tetrads remain inseparably united a defining trait conserved across the majority of Orchidaceae species, facilitating efficient pollination *via* pollinia [7], [8].

Male Gametophyte Development

In the developing microspores of *Cottonia peduncularis* tetrads, both the cytoplasm and nuclei exhibit strong PAS-positive reactions for polysaccharides, mirroring the enriched profile observed in young microspores with dense cytoplasm and prominent nuclei though no storage grains are evident. During male gametophyte development, the microspore nucleus migrates to a peripheral position before

undergoing the first mitotic division, which proceeds almost synchronously across all microspores within the pollinium. This highly coordinated synchrony is facilitated by cytomictic channels interconnecting protoplasts, ensuring uniform progression to the characteristic 2-celled pollen stage at shedding [34]. In stark contrast, asynchronous division in *Pterostylis* has been directly attributed to the absence of these channels, conclusively highlighting their pivotal role in synchronizing male gametophyte ontogeny across Orchidaceae [33].

Pollen Morphology and Wall Structure

In *Cottonia peduncularis*, the exine is poorly developed or absent, particularly in interior pollen grains of the pollinium, which lack an exine but exhibit distinct PAS-positive layers in the intine. Outer peripheral pollen grains develop considerably less PAS-negative wall material, resulting in a thin, distinct PAS-positive pollen wall overall comparable to the intine and lacking ornamentation [35]. This pattern of strong intine reactivity and reduced exine mirrors observations in *Acacia* and underscores adaptive modifications in Orchidaceae for cohesive pollinia formation [36]. Such intraspecific variations in pollen wall structure, number, and shape are well-documented across angiosperms, enhancing pollination efficiency *via* inseparable tetrads [8]. At maturity, *Cottonia* pollen is shed at the characteristic 2-celled stage, with a round generative nucleus akin to that in *Rhynchostylis* [22]; this morphology aligns with detailed reports by Shivanna and Tandon 2014 [37].

Tapetum

In *Cottonia peduncularis*, the tapetum is glandular and of the secretory type, remaining uninucleate throughout development a configuration conserved across the vast majority of Orchidaceae species [22] and critically essential for its nutrient-provisioning role. This secretory function compensates for the absence of self-storage reserves in both tapetum and sporogenous tissues. Critically, tapetal cells exhibit absolutely no synthesis or accumulation of storage PAS-positive grains at any stage, sharply distinguishing them from adjacent sporogenous tissues' high metabolic activity (marked by intense cytoplasmic PAS reactivity and prominent nucleoli, yet also lacking storage grains) and strikingly contrasting with transient storage grains observed specifically in sporogenous cells of taxa like *Calanthe* [30].

This specialized adaptation ensures efficient material supply during meiosis-when meiocyte metabolic activity is temporarily suspended by callose envelopes *via* retained biochemical contents and subsequent ultimate disintegration at pollinium maturity. As the tapetal layer nearly disintegrates, it releases products directly to fuel microspore development amid degenerating wall layers [24]. Although binucleate tapetal cells occur in select orchid taxa such as *Paphiopedilum* [38], *Cottonia's* uninucleate type obviates multi-layering. This stands in distinct contrast to non-orchid pollinia-formers such as *Asclepias curassavica*, *Pergularia*, and *Calotropis*, where tapeta differentiate additional layers and contribute significantly to pollen wall materials [39] a feature absent in *Cottonia*, underscoring its streamlined secretory function without additional structural contributions.

During meiosis, when metabolic activity is partially suspended in the meiocytes due to the isolation by the callose envelope, the tapetum being an immediate neighboring tissue with high retained biochemical content functions to supply the required materials [31]. The degenerated products are generally utilized by the developing microspores. In *Cottonia*, the tapetum appears not to contribute PAS-positive wall precursors, unlike in plants where the differentiation of complex pollinia involves massive sporopollenin transfer. Where the pollinia are formed in Asclepiadaceae, the tapetum often becomes multi-layered, as

seen in *Asclepias*, *Pergularia*, and *Calotropis* [40]. Therefore, while the tapetum is imperative for nutrition, its role in the production and transportation of pollen wall material in *Cottonia* is reduced compared to these multi-layered systems.

Development of the Pollinial Wall and Epidermis

The development of the pollinial wall in *Cottonia peduncularis* conforms to the typical monocotyledonous type, featuring a persistent epidermis overlying the endothecium. In some orchid species, epidermal disorganization at maturity exposes the endothecium as the primary structural layer; however, *Cottonia's* epidermis remains intact throughout development, preserving pollinial integrity. This condition directly aligns with reports from multiple orchid taxa documented by [22], [41] and integrates seamlessly with the post-tapetal degeneration of fibrous endothecial thickenings and middle wall layer dynamics observed herein [26].

Middle Wall Layer and Starch Dynamics

In *Cottonia peduncularis*, the solitary middle wall layer constitutes the principal storage depot for polysaccharides during early sporogenous cell development, critically compensating for the absence of reserves in the neighboring secretory tapetum and sporogenous tissues. As development advances, these cells elongate tangentially and flatten markedly. Postmeiosis, degeneration ensues with swift mobilization and depletion of storage contents, directly fueling pollen development [27]. This precise dynamic mirrors well-documented patterns across Orchidaceae, where premeiotic starch surges in the endothecium and two to three middle wall layers, followed by sharp postmeiotic reduction [29], [42]. These reserves are thus indispensable for forging the robust pollinial wall, as confirmed by its intense PAS reactivity.

Endothecial Differentiation and Tapetal Influence

A distinctive hallmark of *Cottonia peduncularis* microsporangium development is the remarkably early differentiation of fibrous endothecial thickenings, which are indispensable for upholding pollinial integrity until their strategic final degeneration. This pattern sharply contrasts with numerous angiosperm species such as *Asclepias curassavica*, *Pergularia*, and *Calotropis* where such thickenings emerge only after tapetal degeneration [40]. This temporal separation provides compelling empirical support for the well-established hypothesis that functional tapetal cells actively suppress endothecial differentiation *via* persistent biochemical inhibition, likely mediated by phytohormonal or enzymatic signals [43].

Tapetal breakdown thus releases this inhibition, permitting rapid thickening maturation, as unequivocally demonstrated by the precise temporal coordination observed across Orchidaceae, including *Cottonia*. Here, endothecial maturation meticulously follows middle wall layer depletion and tapetal disintegration mirroring the postmeiotic starch mobilization and degeneration dynamics documented in the middle wall layer ensuring an efficient transition from nutrient storage to robust structural reinforcement vital for cohesive pollinia formation. Although specific molecular pathways remain to be fully elucidated, this tightly orchestrated sequence is robustly corroborated by post-tapetal endothecial dynamics in diverse orchid taxa, unequivocally affirming the tapetum's pivotal regulatory influence [22], [41]. This conserved mechanism is crucial for synchronizing anther dehiscence timing, a process highly susceptible to disruption by environmental perturbations that impair tapetal function.

Conclusion

The present study provides a comprehensive histochemical characterization of the distribution and relative concentration of insoluble polysaccharides during the development of *Cottonia peduncularis* pollinia. Our qualitative analysis reveals that carbohydrates play a multifaceted role as both structural components and primary energy resources throughout microsporogenesis and pollen maturation.

The dynamic variations observed in PAS-positive staining intensity reflect critical metabolic transitions. The strong reactivity within cell walls, callosic tetrads, and intine layers underscores the intensive utilization of polysaccharides for wall differentiation and the maintenance of pollen viability. Notably, the absence of sustained cytoplasmic starch reserves, combined with transient tapetal activity, suggests a highly efficient metabolic allocation a physiological strategy potentially adapted to its specific epiphytic habitat.

Furthermore, the differentiation of the pollinial wall and the subsequent development of fibrous endothecial thickenings highlight the precise coordination between tissue degeneration and structural maturation. Ecologically, the reproductive success of *C. peduncularis* is underpinned by these histochemical shifts, which ensure the functional competence of the mature pollinium. Given its specialized pollination strategy *via* bee mimicry and its status as a rare monotypic orchid, these findings offer vital insights into the reproductive biology and adaptive physiology of the species. Collectively, this study contributes to a deeper understanding of orchid reproductive evolution and provides a biological basis for the conservation of this unique orchid taxon.

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