Morphological and Anatomical Study of Anther Development and Pollen Pore Formation in Rhododendron pulchrum (Postprint)

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Abstract

In angiosperms, some taxa exhibit poricidal anther dehiscence, and the development of such anthers, formation of pollen pores, and pollen dispersal mechanisms are poorly understood. The anthers of Rhododendron plants generally dehisce via apical pores. This study investigated anther development, pollen pore formation, and pollen dispersal mechanisms in Rhododendron × pulchrum Sweet through anatomical observations and paraffin sectioning. The results showed: (1) The apical pore-forming region of R. × pulchrum anthers has a different tissue composition from the main body of the anther. The pore-forming region consists of parenchyma tissue originating from the meristematic tissue at the apex of the stamen primordium; at pollen maturity, these parenchyma cells degenerate to form the pollen pore. The main body of the anther develops from archesporial cells, which undergo multiple divisions and differentiations to form pollen sacs with multilayered anther walls. (2) The anther wall of R. × pulchrum fully develops from the microspore mother cell stage to the microspore tetrad stage, comprising 6–7 cell layers, including one epidermal layer, 2–3 endothecial layers, 1–2 middle layers, and one tapetal layer. The middle layer degenerates immediately after tetrad formation, while the tapetum degenerates and disappears before pollen maturation. At pollen maturity, the anther wall retains the epidermis and 2–3 layers of endothecium. (3) Unlike longitudinal dehiscence anthers, the endothecium of R. × pulchrum does not undergo fibrous thickening at pollen maturity; instead, it accumulates polysaccharides and thickens, exhibiting flexibility and elasticity. (4) During pollen development in R. × pulchrum, the four microspores produced by the microspore mother cell do not separate, resulting in mature pollen as tetrads with viscin threads between pollen grains. It is hypothesized that the multilayered and thickened endothecium in R. × pulchrum reduces the space within the pollen sac, thereby "extruding" the upper pollen through the apical pore. The viscin threads between pollen grains enable

insects to remove pollen as aggregated masses during pollination. The multilayered endothecium with polysaccharide accumulation represents an adaptation to apical pore dehiscence.

Full Text

Anther Development and Formation of Dehiscence Pore in *Rhododendron* × *pulchrum* Sweet

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Abstract

Poricidal dehiscence occurs in certain angiosperm lineages, yet the developmental processes underlying dehiscence pore formation and pollen release mechanisms remain poorly understood. The genus *Rhododendron* is characterized by apical pore dehiscence. This study investigated anther development and dehiscence pore formation in *Rhododendron* × *pulchrum* Sweet through microdissection and paraffin sectioning to elucidate the mechanism of pollen release. Our findings reveal: (1) The apical pore-forming region and the main body of the anther possess distinct tissue organizations. The pore-forming region consists of parenchyma tissue derived from the apical meristem of the stamen primordium, which degrades at pollen maturity to form the dehiscence pore. The anther body originates from archesporial cells that undergo multiple divisions to develop into pollen sacs with multilayered walls. (2) The anther wall fully differentiates during the microspore mother cell to microspore tetrad stages, comprising 6-7 layers: one epidermal layer, 2-3 endothecial layers, 1-2 middle layers, and one tapetal layer. The middle layers degenerate shortly after tetrad formation, while the tapetum disappears before pollen maturity, leaving only the epidermis and 2-3 endothecial layers at anthesis. (3) Unlike longitudinal dehiscence anthers, the endothecium in *R.* × *pulchrum* does not undergo fibrous thickening but instead accumulates polysaccharides, gaining thickness, flexibility, and elasticity. (4) The four microspores produced by each microspore mother cell remain united, developing into pollen tetrads connected by viscous threads. We propose that the thickened, multilayered endothecium reduces pollen sac volume, thereby "squeezing" the upper pollen tetrads out through the dehiscence pore, while inter-pollen viscous threads facilitate clumped pollen removal by insect pollinators. These multilayered, polysaccharide-accumulating endothecial characteristics represent adaptations to apical pore dehiscence.

Keywords: *Rhododendron*, *Rhododendron* × *pulchrum*, anther development, poricidal dehiscence, endothecium, parenchyma

Introduction

The anther is a crucial component of the stamen, containing both reproductive and nutritive tissues involved in pollen grain formation and release. Timely dehiscence of mature anthers is essential for pollen release, thereby ensuring successful pollination and fertilization (Ding et al., 2013). Among angiosperms, four anther dehiscence patterns exist: longitudinal, transverse, poricidal, and valvular. Longitudinal dehiscence predominates in most species (Endress, 1996). Transverse dehiscence occurs in some Malvaceae species (Ma, 2015) and is functionally similar to longitudinal dehiscence, merely oriented perpendicular to the anther' s longitudinal axis, leading some scholars to exclude it as a distinct type (Endress, 1996). Poricidal dehiscence appears in Ericaceae (Hermann & Palser, 2018), Melastomataceae (Renner, 1989; Cortez et al., 2014), some Solanaceae (Garcia et al., 2008), and certain Leguminosae (Marazzi et al., 2007). Valvular dehiscence characterizes Berberidaceae (Batygina, 2002), Hamamelidaceae (Huford & Endress, 1989), and some magnoliid groups (Huford and Endress, 1989).

Longitudinal dehiscence is the most common pattern, with well-understood processes and mechanisms. During anther development, the middle layers and tapetum degenerate sequentially, while the endothecium becomes fibrous. However, several unthickened cells at the junction between pollen sacs, termed lip cells, form the dehiscence slit at maturity (Zhou and Liu, 2004). Research indicates that slit formation results from programmed cell death regulated by multiple genes, leading to gradual degradation of specific cells (Reape et al., 2008).

Poricidal dehiscence is restricted to few lineages and thus remains understudied. Garcia et al. (2008) and Cortez et al. (2014) investigated poricidal anthers in Solanaceae (*Solanum*) and Melastomataceae (*Miconia*), respectively, finding that apical pore regions possess only a single cell layer that degrades or ruptures to form the pore, while surrounding anther walls are multilayered with distinct epidermal and endothecial layers. Hermann & Palser (2018) examined mature anthers across multiple Ericaceae taxa, revealing that apical pore regions lack epidermis and endothecium, instead comprising two parenchyma types—resorption tissue and collapse tissue.

These studies partially elucidate the anatomical structure and tissue composition of poricidal anther pores, but focus primarily on pre-dehiscence and dehiscence stages without revealing complete developmental trajectories. Critical questions remain unanswered: When do structural differences between pore and surrounding tissues originate? Do they derive from the same tissues? What are the developmental processes? How does pollen release occur after pore formation? Since pore formation constitutes part of overall stamen development and is intimately linked with anther wall development, comprehensive investigation of stamen ontogeny is necessary to fully understand apical pore development.

Ericaceae represents a model family for poricidal dehiscence, exhibiting typical apical pores, lateral pores, or slits (Hermann & Palser, 2018). The family

comprises approximately 125 genera and 4,000 species (Fang et al., 2005), with *Rhododendron* being the largest genus worldwide (~1,000 species), including 571 species in China, most of which are endemic (Ma et al., 2017). *Rhododendron* species possess typical apical pore dehiscence, yet stamen development remains poorly studied. Hermann & Palser (2000, 2018) briefly addressed one or two species without detailed developmental processes. In China, Zhang (2014) and Dai (2018) investigated stamen development in *Rh. simsii* but did not examine pore development. *Rhododendron* × *pulchrum* Sweet, a widely cultivated hybrid with abundant flowers and easy accessibility, provides an excellent model for investigating stamen development, pore formation, and pollen release mechanisms.

Methods

External Morphological Observation Flower buds, young flowers, and anthers at dehiscence were collected. Using a Nikon stereomicroscope, bracts, sepals, and petals were carefully removed with dissecting needles, leaving only the floral apex and stamens for observation and photography.

Histological Observation Paraffin sectioning was performed on flower buds and isolated stamens at various developmental stages. From February to June 2022, *R.* × *pulchrum* buds and flowers at different developmental stages were collected and fixed in FAA solution (50% ethanol:glacial acetic acid:formaldehyde = 90:5:5). Following dehydration through an ethanol series, conventional paraffin embedding was conducted. Sections were cut at 8 m thickness, stained with safranin-fast green, and mounted in neutral balsam according to standard plant histological methods (Li, 1987). Observations and photography were performed using an Olympus B51 microscope. Anther developmental types were classified according to angiosperm reproductive biology standards (Hu, 2005).

While most anther development studies employ transverse sections only, we conducted both transverse and longitudinal sections in multiple orientations to comprehensively observe pore initiation and development.

Results

External Morphological Development The complete developmental sequence from stamen initiation to maturity is illustrated in Figure 1 [Figure 1: see original paper]. When petal primordia became semicircular, stamen primordia initiated in a 2/5 phyllotactic pattern: five primordia emerged first in a circular arrangement, followed by a second whorl arising in the gaps between the first, ultimately forming a ring of ten stamen primordia (Fig. 1A–C). Primordia development was asynchronous; when the second whorl emerged, the first whorl had already differentiated into filament and anther (Fig. 1B).

Stamen primordia began as small, flattened protrusions (Fig. 1A) that rapidly differentiated into swollen anthers above and slender filaments below. At this

stage, anthers were basifixed with a hook-shaped filament attachment, growing horizontally (Fig. 1B). Subsequently, filaments elongated upward while anthers grew downward and expanded rapidly, transitioning from basifixed (Fig. 1B) to dorsifixed (Fig. 1C–E) with attachment points near the apex. As cells divided, anthers grew downward and outward, pollen sacs became turgid, and filaments became embedded between lateral pollen sacs (Fig. 1F–I). Coloration progressed from translucent (Fig. 1B–D) to white (Fig. 1E–F), then pink (Fig. 1G–I), finally becoming purple at maturity (Fig. 1J–L). Early anthers appeared rod-shaped on each side; during development, both pollen sacs on each side enlarged, creating longitudinal furrows (Fig. 1H–I, J, L), resulting in four distinct pollen sacs in the mature anther.

The pore-forming zone appeared early in development. When stamen primordia first differentiated anthers, two shallow depressions formed at the apex—the future pore-forming zones (Fig. 1B). These persisted throughout anther development. Initially colorless, the pore-forming zones appeared as mere depressions (Fig. 1D–F). As pollen sacs darkened, the zones became increasingly conspicuous, clearly differing in tissue composition from surrounding anther walls (Fig. 1G–I). At maturity, no longitudinal slits formed; only the apical pore-forming zone tissue degraded to create dehiscence pores (Fig. 1J–K), through which white pollen was "extruded" (Fig. 1L). Fine viscous threads connected pollen grains into clumps adhering around the pores (Fig. 1L).

Anther Wall and Pollen Development in Transverse Section Transverse sections revealed a tetrasporangiate anther. During early development, archesporial cells differentiated subepidermally at the four corners, appearing larger and more densely cytoplasmic than surrounding cells (Fig. 2A). Each archesporial cell underwent a periclinal division to produce primary parietal and primary sporogenous cells (Fig. 2B). The primary parietal cell divided to form secondary parietal cells (Fig. 2B), which underwent several periclinal and anticlinal divisions to create multilayered anther walls. Primary sporogenous cells divided repeatedly to form secondary sporogenous cells—densely cytoplasmic, tightly packed cells of triangular or quadrangular shape (Fig. 2C). At the secondary sporogenous cell stage, the anther wall differentiated into four layers: epidermis, endothecium, middle layer, and tapetum (Fig. 2C). Epidermal cells were large with thick walls and large vacuoles, while inner layers were smaller and more densely cytoplasmic (Fig. 2C).

Sporogenous cells developed into microspore mother cells, which remained tightly packed within the locule but were isolated by thick callose walls (Fig. 2D, F). Meiosis produced dyads and tetrads (Fig. 2D), occurring so rapidly that all three stages coexisted within a single locule (Fig. 2D, F). The anther wall was incomplete during the sporogenous cell stage; endothecial and middle layers continued dividing during meiosis, reaching full differentiation by the tetrad stage with 6–7 layers: one epidermis, 2–3 endothecial layers, 1–2 middle layers, and one tapetum (Fig. 2D–E). Epidermal cells were large with thick

walls; endothecial cells were smaller; middle layer cells were smallest and flattened (Fig. 2E). Tapetal cells were large, densely cytoplasmic, and closely associated with sporogenous cells while separated from middle layers (Fig. 2D-E), suggesting tapetal origin from sporogenous rather than parietal cells.

After tetrad formation, callose walls between tetrads degraded, allowing tetrads to separate (Fig. 2G), but the four microspores within each tetrad remained united, developing as pollen tetrads. Adjacent microspore walls fused to form compound pollen walls (Fig. 2G). Each microspore enlarged rapidly by absorbing nutrients from the tapetum, becoming uninucleate pollen that subsequently underwent mitosis to form bicellular pollen. Mature pollen exhibited granular exine ornamentation, completing the transformation from microspore tetrads to pollen tetrads (Fig. 2J).

Complex anther wall changes occurred during pollen tetrad development. The middle layer, present as two cell layers during meiosis, degenerated rapidly after tetrad completion, disappearing before tetrads dispersed. The tapetum, of the glandular type, degraded in situ to nourish developing microspores, which expanded into uninucleate then binucleate pollen (Fig. 2G). Simultaneously, the tapetum expanded outward, compressing the endothecium into a flattened layer appressed to the epidermis (Fig. 2G, J). At pollen maturity, residual tapetal tissue remained appressed to the endothecium, providing nutrients for endothecial re-development and polysaccharide accumulation (Fig. 2H, I). The final pre-dehiscence anther wall consisted of one epidermal layer and 2–3 endothecial layers thickened by polysaccharide accumulation, conferring flexibility and elasticity (Fig. 2H, I).

The tetrasporangiate anther appeared butterfly-shaped in transverse section (Fig. 2A), with two pollen sacs on each side. Initially, parenchyma separated the two sacs on each side (Fig. 2A). During development, middle layers and tapetum contributed to the septum (Fig. 2K). Subsequent degeneration of middle layers, tapetum, and finally the parenchymatous septum resulted in complete fusion of the two sacs on each side before dehiscence (Fig. 2L). Apical transverse sections differed structurally, lacking normal anther walls and consisting solely of parenchyma—the pore-forming region that degrades to form dehiscence pores (Fig. 3H).

Anther Wall Development in Longitudinal Section Longitudinal sections revealed that stamen primordia first differentiated a lower filament, while the upper apical meristem divided more rapidly on the outer side, causing the primordium apex to curve inward (Fig. 3A). The apical meristem continued dividing, with epidermis differentiating just below the apex and four meristematic zones containing archesporial cells appearing subepidermally (Fig. 2B). Archesporial cells underwent repeated divisions to form anther walls and sporogenous cells (Fig. 3C, D), while the apical meristem formed parenchyma (Fig. 3C, D). Since archesporial tissue originated below and grew downward from the meristem, parenchyma remained at the anther apex throughout develop-

ment (Fig. 3C, D). The anther divided into left and right portions growing downward and outward; filament growth separated the initial meristem into two apical parenchyma zones—the future pore-forming regions—positioned above each anther half (Fig. 3C, D, F, G, H). Initially, apical parenchyma was positioned above the small anthers (Fig. 3C, D), but as parenchyma division ceased while anther development continued, the parenchyma zone became proportionally smaller, forming a depression below the surrounding anther wall—the pore-forming zone (Fig. 3F, G, H). At pollen maturity, middle layers and tapetum degenerated, leaving epidermis and endothecium (Fig. 3I). The apical pore-forming zone, lacking anther walls and composed solely of parenchyma, degraded to form the dehiscence pore (Fig. 3I).

The tetrasporangiate anther ultimately produced only two dehiscence pores because the two pollen sacs on each side were separated by a parenchymatous septum sharing a common apical pore-forming zone (Fig. 3G). While transverse sections showed uniform structure except at apical pores (Fig. 3H), longitudinal sections revealed three variants: (1) sections avoiding filament and septum showing separate left and right pollen sacs (Fig. 3E); (2) sections through filament and septum showing predominantly septal parenchyma (Fig. 3F); and (3) sections through one side showing two sacs separated by septal parenchyma but sharing an apical zone (Fig. 3G). The septal parenchyma, initially cellular, gradually degraded (Fig. 3G), allowing the two sacs to coalesce, with apical parenchyma degradation forming a single pore (Fig. 3I). Apical transverse sections clearly demonstrated each side' s pore-forming parenchyma zone (Fig. 3H).

Discussion

Characteristics of Anther Development Previous studies on Ericaceae stamen development have identified common features including glandular tapetum, non-fibrous and non-banded endothecial thickening, and pollen tetrads (Matthews & Knox, 1926; Bowers, 1930; Copeland, 1943; Davis, 1966; Hermann & Palser, 2000; Gao et al., 2002). The Rhododendroideae subfamily is further distinguished by viscous threads between pollen grains (Matthews & Knox, 1926; Bowers, 1930; Copeland, 1943; Waha, 1984; Hesse, 1986; Clemants, 1995). Intergeneric variation primarily concerns anther wall structure, with reported layer numbers ranging from 2–4 (Chou, 1952; Batygina et al., 1963; Budell, 1964; Yakobson, 1968; Hermann & Palser, 2000), particularly regarding endothecial layers, with some authors suggesting incomplete endothecial differentiation (Chou, 1952; Davis, 1966).

Our observations confirm several previously reported features for Ericaceae, including glandular tapetum, non-fibrous endothecium, pollen tetrads, and interpollen viscous threads. However, anther wall development, particularly endothecial development, differs from earlier reports. We observed continuous endothecial development and transformation throughout all stages, with at least four distinct conditions: (1) a single small-celled endothecial layer during the sporogenous cell stage; (2) 2–3 endothecial layers during microspore meiosis, sub-

tended by two middle layers; (3) flattened endothecium compressed by outward-expanding tapetum after middle layer degeneration; and (4) polysaccharide accumulation and re-thickening before dehiscence. The reported variation in anther wall layers (2–4) among Ericaceae (Chou, 1952; Batygina et al., 1963; Budell, 1964; Yakobson, 1968; Hermann & Palser, 2000) may reflect either systematic differences among taxa or incomplete observation of developmental stages, as some authors acknowledged (Chou, 1952). Studies on *Rh. simsii* by Zhang (2014) and Dai (2018) also observed polysaccharide accumulation in mature endothecium, suggesting this is a stable characteristic.

Regarding tapetal origin, some studies suggest Ericaceae tapetum derives from parietal cells, while others propose sporogenous cell homology (Davis, 1966). In *R.* × *pulchrum*, early tapetum closely associates with sporogenous cells while separating from other wall layers, suggesting sporogenous origin, consistent with Zhang' s (2014) findings on *Rh. simsii*. Later tapetal displacement to appose the endothecium for nutritional support may explain alternative interpretations of parietal origin if only later stages were observed.

Previous studies documented anther orientation reversal in many Ericaceae, from basifixed initiation to dorsifixed maturity (Artopoeus, 1903; Matthews & Knox, 1926; Hermann & Palser, 2000). Our morphological observations confirm this phenomenon, with longitudinal sections revealing it results from archesporial tissue originating below and growing downward from the meristem.

Development of Dehiscence Pore and Pollen Release Mechanism
Combined morphological observations and transverse/longitudinal paraffin sections reveal the apical pore development process in *R.* × *pulchrum*: the apical pore-forming zone consists of parenchyma derived from the stamen primordium' s apical meristem, which degrades to form the dehiscence pore. The anther body originates from archesporial cells located below the meristem that grow downward, dividing and differentiating into pollen sacs. At pollen maturity, the anther wall retains only epidermis and 2–3 endothecial layers. Thus, the pore-forming zone and anther body diverge early in stamen development, confirming previous suggestions for Ericaceae (Artopoeus, 1903; Hermann & Palser, 2018).

In longitudinal dehiscence anthers, three processes coordinate dehiscence: tapetal degradation, endothecial fibrous thickening, and slit formation. Middle layers and tapetum degenerate sequentially, while endothecial inner tangential and radial walls develop fibrous thickenings except at unthickened "lip cells" (several cells in transverse section, forming longitudinal columns) that create the dehiscence slit (Ding et al., 2013; Zhou and Liu, 2004). Thus, lip cells differentiate from endothecial cells only at late developmental stages. In contrast, *R.* × *pulchrum*' s poricidal mechanism differs fundamentally in location, timing, and tissue origin.

Previous research on Ericaceae poricidal mechanisms implicated two

parenchyma types: resorption tissue accumulating calcium oxalate crystals in large sacs that rupture to form pores, and collapse tissue that simply shrinks (Artopoeus, 1903; Maclachlan, 1929; D' Arcy, 1996; Hammer & Palser, 2018). In *R.* × *pulchrum*, pore-forming cells neither accumulate crystals nor collapse but simply degrade, leading us to adopt the broader term "parenchyma."

While longitudinal dehiscence allows passive pollen release, apical pores require specialized mechanisms. *R.* × *pulchrum* exhibits several adaptive features: (1) 2–3 endothecial layers that avoid fibrous thickening and instead accumulate polysaccharides, maintaining wall integrity while increasing thickness, flexibility, and elasticity; (2) pollen tetrads with inter-pollen viscous threads. We propose that thickened, multilayered endothecium reduces pollen sac volume, "squeezing" upper pollen tetrads out through apical pores, consistent with our stereomicroscopic observations of pollen "extrusion." Viscous threads clump pollen, enabling bees to remove pollen masses, compensating for the apical pore position. Finally, the tapetum' s early nutritive support for microspores and later endothecial support represent additional developmental adaptations for apical pore dehiscence.

In summary, *R.* × *pulchrum* dehiscence pores originate from a specialized apical parenchyma zone that degrades to form pores. Multilayered endothecium with polysaccharide accumulation, pollen tetrads, and inter-pollen viscous threads represent adaptations promoting pollen release through apical pores.

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Note: Figure translations are in progress. See original paper for figures.