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Observations on the gynoecial pathway for pollen tube growth in sweet lowbush blueberry (*Vaccinium angustifolium* Ait.)

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Summary

Gynoecial structure in sweet lowbush blueberry, *Vaccinium angustifolium* Ait., was investigated in order to characterize the pollen tube pathway in order to provide a framework for further studies on pollination and fungal infection. Closed flower buds and pollinated open flowers were collected from managed lowbush blueberry fields in Colchester County, Nova Scotia, Canada. Following chemical fixation, the tissue samples were examined histologically using light and scanning electron microscopy. The continuous pathway was characterized by a fluted, exudate-filled stylar canal that connects the wet stigmatic surface with the exudate covered surface of the ovarian placentae. Following pollen deposition and germination, tubes growing along the pathway eventually arrive at the micropyles of the anatropous ovules; ovule penetration by pollen tubes and fertilization of the female gametophytes ensue. The pollen tube pathway of this taxon conforms to the general pattern reported from other ericalean taxa.

Introduction

Among the Ericaceae, *Vaccinium* is a highly polymorphic genus containing species that are typically acidophilic, predominantly outcrossing, and distributed throughout many regions of the arctic, temperate and tropical zones (CAMP, 1942, 1945; VANDER KLOET, 1988). In eastern North America, the wild blueberry industry depends on the successful management of the sweet lowbush blueberry, *Vaccinium angustifolium* Ait. This economically important woody perennial species spreads vegetatively by the formation of rametes. When sexually mature, erect shoots produce numerous inverted pedunculate, racemose inflorescences (CAMP, 1945). Each inflorescence bears several complete, actinomorphic, pentacyclic flowers characterized by an urceolate perianth configuration (PALSER, 1961). During anthesis, the sympetalous corolla will recurve at the tips to expose the papillate stigma and the porocidal tips of the mature anthers (VANDER KLOET, 1988). The floral morphology, nectar production and pendulous orientation of the blossoms of sweet lowbush blueberry are consistent with other entomophilous species of this genus (WOOD, 1961; WOOD, 1962; HALL et al., 1971).

The syncarpous, pentacarpellate gynoecium typical of the genus *Vaccinium* consists of a single stigma attached to an elongate, hollow style subtended by a pentalocular ovary with axile placentation and numerous ovules (BELL and BURCHILL, 1955; MUNOZ and LYRENE, 1985). The gynoecium is usually surrounded by ten stamens attached to the base of the corolla near the nectariferous tissue located on the upper ovarian wall; this configuration in conjunction with the position of the poricidal anther tips in relation to the stigma indicate mechanical pollen transfer by insect sonication (MCGREGOR, 1976; JACQUEMART and THOMPSON, 1996). When released from the anthers, *Vaccinium* pollen is of the dry type, covered with pollenkitt and dispersed in tetrads held together by viscin threads (HESLOP-HARRISON and SHIVANNA, 1977; PACINI, 2000). Each individual pollen grain is bicellular and tricolporate with pollen germination occurring two to three hours following deposition on a receptive wet stigma (MUNOZ and LYRENE, 1985; VANDER KLOET, 1988). Following successful pollination, pollen tube growth through the various gynoecial tissues may take three to four days before reaching the ovule-bearing placentae within the ovary (BELL, 1957).

Typically in angiosperms, determination of pollen compatibility and the nutritional support of pollen tube growth are mediated by the gynoecial tissues (HERRERO and HORMAZA, 1996). In addition, recent research summarized by LORD and RUSSELL (2002) indicates that pollen tubes in some way become predisposed to receive and follow molecular signals originating from inside of the ovules during their passage through the stigma and style. Thus, an essential pathway for pollen tube growth and guidance is established extending from the stigmatic surface to the ovular micropyle within the ovary. Certain environmental stresses such as frost, however, may interfere with compatible pollen tube growth in sweet lowbush blueberry due to differences in tissue sensitivities along this same gynoecial pathway. Even before anthesis, ovule bearing placentae may sustain damage severe enough to interfere with normal fruit set of this economically important wild species (OLSON and EATON, 2001).

In addition, gynoecial tissues of sweet lowbush blueberry are susceptible to infection by the fungus *Monilinia vaccinii-corymbosi* which transforms a normally fleshy fruit into a pseudosclerotium called a mummy berry (HILDEBRAND and BRAUN, 1991). Fungal access to the inner ovarian tissues is achieved when fungal infection hyphae from germinating conidia on the stigma grow in conjunction with pollen tubes down the style and enter the ovary; in effect the pollen tube pathway functions as the gynoecial infection pathway for this economically important disease (SHINNERS and OLSON, 1996). Given the economic importance of this species, there is relatively little information available concerning the basic micromorphology of compatible pollen tube growth so critical for fruit production. This study, therefore, uses light and scanning electron microscopy to describe the gynoecial pathway for pollen tube growth in the sweet lowbush blueberry from eastern Canada.

Materials and methods

Flower buds of sweet lowbush blueberry, *Vaccinium angustifolium* Ait., were collected in May and June in 2000 and in 2001 in fields in Kemptown and Debert, Colchester County, N.S., Canada. For convenience, the following developmental stages were determined and followed during the study: (1) young bud – very little of the corolla is visible; (2) petal elongation – the petals elongate but stay tightly closed; (3) petal spread – elongation is completed and the flower bud is just opening at the tips of the petals; (4) open flower – the flower is at anthesis and the petals are open and distinctively recurved with the stigma protruding and exposed; (5) petal drop – the corolla and stamens have senesced and dropped off.

The flower buds were chemically field fixed using either 3% GA (glutaraldehyde) or FAA (Formalin-acetic acid-alcohol) for both light

(LM) and scanning electron microscopy (SEM). Selected gynoecial tissue samples were dissected and placed in a fresh fixative. For SEM the fixed tissue samples were washed several times in phosphate buffer (pH 6.8) and then postfixed in 2% osmium tetroxide followed by three washes in phosphate buffer. Then tissue samples were dehydrated in an ethanol series to anhydrous ethanol. Dehydrated specimens were critical point dried and coated with platinum/gold using a Samsputter 2a sputter coater. The observations were conducted using a Bausch and Lomb Nanolab 2000 SEM at a voltage of 15 kv.

For LM histological examination the dehydrated specimens were subjected to a propylene oxide series, infiltrated with Spurr's resin (SPURR, 1969) and sectioned on a Sorvall MT 6000 ultramicrotome between 0.5-1.0 µm. For general observation, certain sections were mounted on gelatin-coated slides and stained with azure II (1%) methylene blue (1%). To determine the presence of insoluble polysaccharides in the selected plastic sections staining with periodic acid-Schiff's reagent was carried out. Selected sections were subjected to a saturated solution of DNPH (2,4 dinitrophenyl-hydrozine) and 1% periodic acid prior to usage of Schiff's reagent (O'BRIEN and MCCULLY, 1981). Then slides were counter stained in 1% aniline blue black to identify the proteins (FISHER, 1968). Additional plastic sections were stained with 1% sudan black B solution to detect lipids (BRONNER, 1975). The stained sections were then mounted with SP15-500 permount (Fisher Scientific) (BERLYN and MIKSCHE, 1976; O'BRIEN and MCCULLY, 1981). Observations were carried out using a Leitz Diaplan Photomicroscope.

For aniline blue-UV induced fluorescence microscopy to identify callose associated with pollen tube walls, certain specimens of the field fixed plant material were selected and hand sectioned. Sectioned tissue samples were stained in a solution of 0.05% aniline blue in 0.15 M K_2HPO_4 at pH 8.6. Observations were made using an Olympus BH2-RFL Photomicroscope with UVFL objectives and barrier filter L-435.

Results

The essentially capitate stigma of *Vaccinium angustifolium* is of the wet type, having five lobes separated by five main grooves radiating outward from a central depression at the center of the stigma; the distal end of each groove terminates with a short bifurcation (Fig. 1A). All five lobes appear slightly raised and rounded near the outer rim of the stigma giving the surface an overall convex contour. Although histologically the stigma is composed of parenchymatous ground tissue, the surface is characterized by low papillate cells that become inundated with an exudate before anthesis (Fig. 1B, D). Post-fixation, plastic embedded sections of the papillate surface stained with Sudan Black B demonstrate a lipidic component to the exudate. Below the papillate surface are several layers of elongated cells characterized by large interstitial spaces also filled with lipidic exudate (Fig. 1C). The intercellular spaces of these cells lead directly into the fluted, hollow canal in the center of the style. Following successful pollination, pollen tetrads deposited on the stigma appear embedded in the lipidic exudate (Fig. 2A, B). Individual pollen grains in each tetrad are tricolporate and two celled. Upon germination, pollen tubes emerge from adjacent grains in the tetrad and grow down through the exudate in between the cells of the stigma (Fig. 2C). The numerous pollen tubes pass through the exudate filled intercellular spaces of the stigma cells and converge near the entrance to the upper stylar canal (Fig. 2D).

The five grooves separating the lobes of the stigma are continuous with five hollow clefts of the stylar canal that appear overall star-shaped in cross section (Fig. 3A). The hollow canal is lined by an epithelial transmitting tissue and is filled with a mucilaginous secretion. The parenchymatous ground tissue that makes up the bulk of the style is vascularized by the five bundles. Each of these vascular bundles is situated adjacent to the tip of the cleft of the canal. The junction between the stylar canal and upper ovary is continuous; each hollow cleft of the canal is directly connected to an ovarian locule through a thin cleft forming a conduit between adjacent locular septae in the upper ovary (Fig. 3B). The pollen tubes enter the mucilage filled clefts of the stylar canal and proceed to grow downward toward the base of the stylar canal (Fig. 3A); pollen tubes from each canal cleft will enter an ovarian locule through the associated conduit (Fig. 3B).

The parenchymatous ovary is characterized by the five locules with axile placentation and numerous ovules. The upper region of each placenta has a small cleft that is continuous with the cleft in between the associated upper ovarian septae. The placental cleft opens onto the surface of the placenta. The surface of each ovule-bearing placenta is composed of cells that are also papillate in appearance (Fig. 4A, B). The surface of placenta is covered by a mucilaginous secretion that appears similar to and continuous with that observed in the stylar canal. The micropyle of each anatropous ovule is oriented toward the placental surface (Fig. 4A, B). The mature ovules are unitegmic and contain a Polygonum-type female gametophyte (Fig. 5A). Once in the locule, pollen tube growth proceeds across the placental surface toward the anatropous ovules (Fig. 4C). The numerous pollen tubes eventually form a tortuous mat on the surface of each placenta (Fig. 4D). The pollen tubes grow among the ovular micropyles; only one pollen tube will penetrate a micropyle of a single ovule in order to facilitate the fertilization events (Fig. 5B).

Discussion

Although there is a great deal of variation in the morphological details, members of the Ericales have gynoecia composed of a clearly defined stigma, style and ovary (CRONQUIST, 1981). According to the characteristics of the receptive surface for pollen capture, HESLOP-HARRISON and SHIVANNA (1977) categorize ericalean stigmas as belonging to the wet-type producing an exudate on a receptive surface with or without low to medium papillate cells. The overall appearance of the stigma from the closely related Monotropaceae, Pyrolaceae and Ericaceae varies and may be described as funnel-like, truncate, obtuse or capitate with lobes (CRONQUIST, 1981; PALSER et al., 1992; OLSON, 1991, 1994). Among the Ericaceae, stigma structure of the genus *Rhododendron* is the most thoroughly documented (WILLIAMS et al., 1982; PALSER et al., 1992). In *Rhododendron*, each truncate stigma is characterized by an overall lobed appearance created by several main grooves that radiate outward from a central depression; each of the grooves, however, is further subdivided by a network of numerous laterally branching short grooves (PALSER et al., 1992). Although the wet receptive surface is nonpapillated, the numerous surface grooves reflect a system of exudate filled convoluted furrows that extend to the stylar canal.

The overall shape of the stigma in the ericaceous *Vaccinium angustifolium* is similar to that in *Rhododendron*; the stigmas of both species are essentially convexly capitate and lobed. A contrast to the numerous surface grooves of *Rhododendron*, however, there are only five large grooves that radiate from a central depression and each of these terminates at its distal end in a bifurcation. Although the receptive surface of the stigma in both genera is covered with an exudate, *Rhododendron* is nonpapillate in contrast to the low papillate cells observed in *V. angustifolium*. In addition, the stigmatic cells below the receptive surface of *V. angustifolium* separate from one

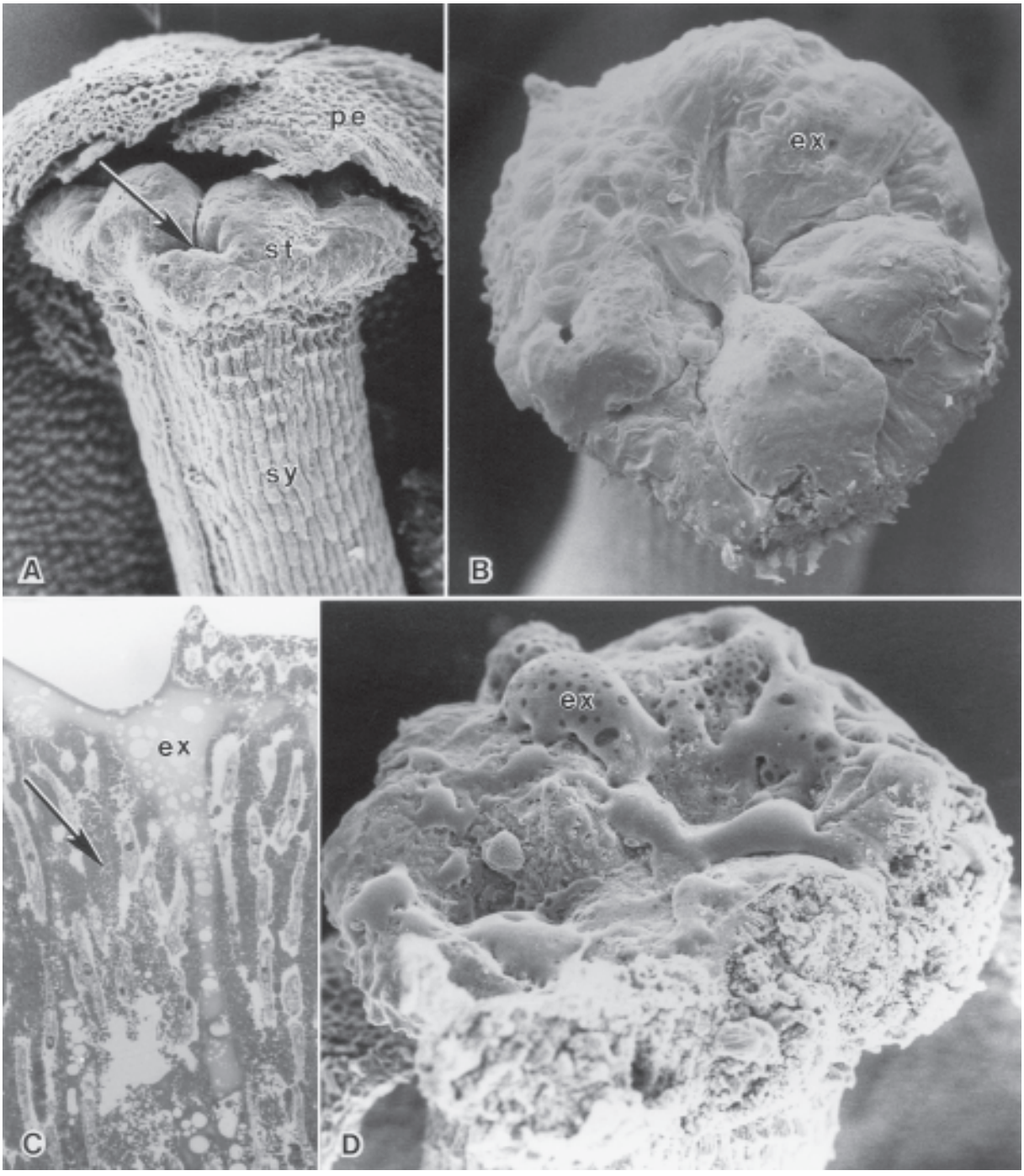

Fig. 1: Stigmas of *Vaccinium angustifolium*. A. SEM of a stage 1 stigma with arrow indicating the bifurcation at the distal end of a groove. x 382. B. SEM of a stage 2 stigma and the appearance of the exudate. x 428. C. Resin-embedded longitudinal section through a stage 3 stigma. Arrow indicates the lipidic exudate filling the space between papillated cells (stained with azur II/methyl blue). x 307. D. SEM of a stage 3 stigma. Stigmatic surface is covered with copious exudate. x 615.
Figure abbreviations: pe, petal; st, stigma; sy, style; ex, exudate; po, pollen tetrad; ol, ovary locule; ov, ovule; sc, stylar canal; pl, placental tissue; ow, ovary wall.

another forming a network of exudate filled interstitial spaces that may be described as an transmitting tract.

Stigmatic exudates are presumably involved with pollen adhesion, hydration, germination and the eventual penetration of the gynoecial tissues by the pollen tubes (KNOX, 1984; HERRERO and HORMAZA, 1996; LORD and RUSSELL, 2002). In *Vaccinium angustifolium*, NOORMETS and OLSON (2002) conducted receptivity tests on fresh stigmata using a technique adapted and modified from DAFNI (1992) based on the detection of peroxidase activity. The first appearance of the exudate coincides with detectable peroxidase activity in stage one (young bud) of individual flower development. Although the stigmatic exudate of *Vaccinium angustifolium* was not analyzed chemically in the present study, basic histochemical staining identifies lipids as a major constituent. WOLTERS-ART et al. (1998) investigated

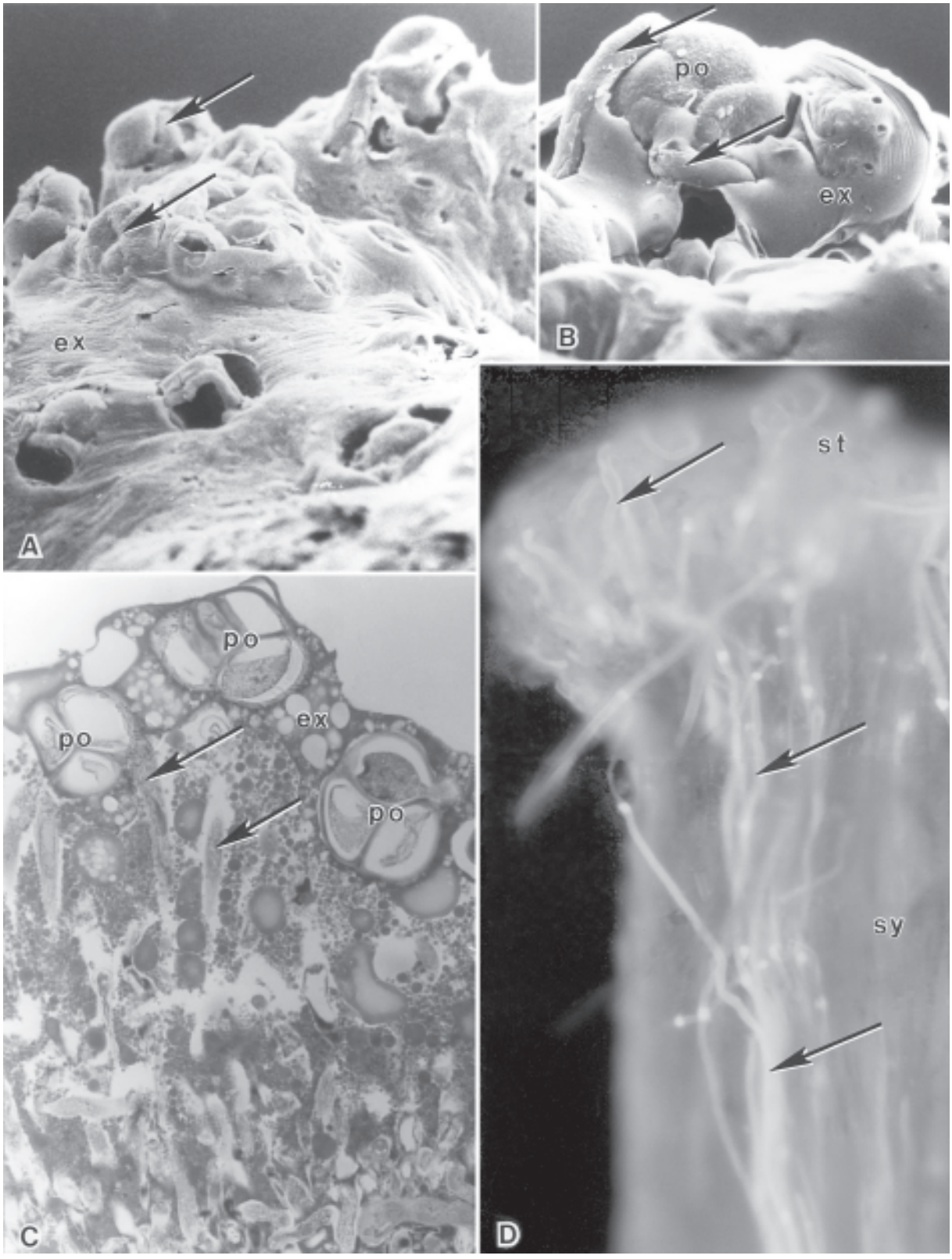

Fig. 2: *V. angustifolium* stage 4 stigmas with pollen tetrads. A. SEM of a stigma with pollen tetrads embedded in the surface exudate. Arrows indicate pollen tetrads. x 1800. B. SEM of a stigma showing the emergent pollen tubes penetrating the exudate. Arrows indicate the pollen tubes. x 2995. C. Resin-embedded longitudinal section trough a pollinated stigma. Pollen tetrads embedded in the exudate. Arrows indicate the pollen tubes growing in between the stigmatic surface cells. x 410. D. Aniline blue fluorescent micrograph of a longitudinal free-hand section through the stigma and stylar canal. Arrows indicate the numerous pollen tubes. x 165.

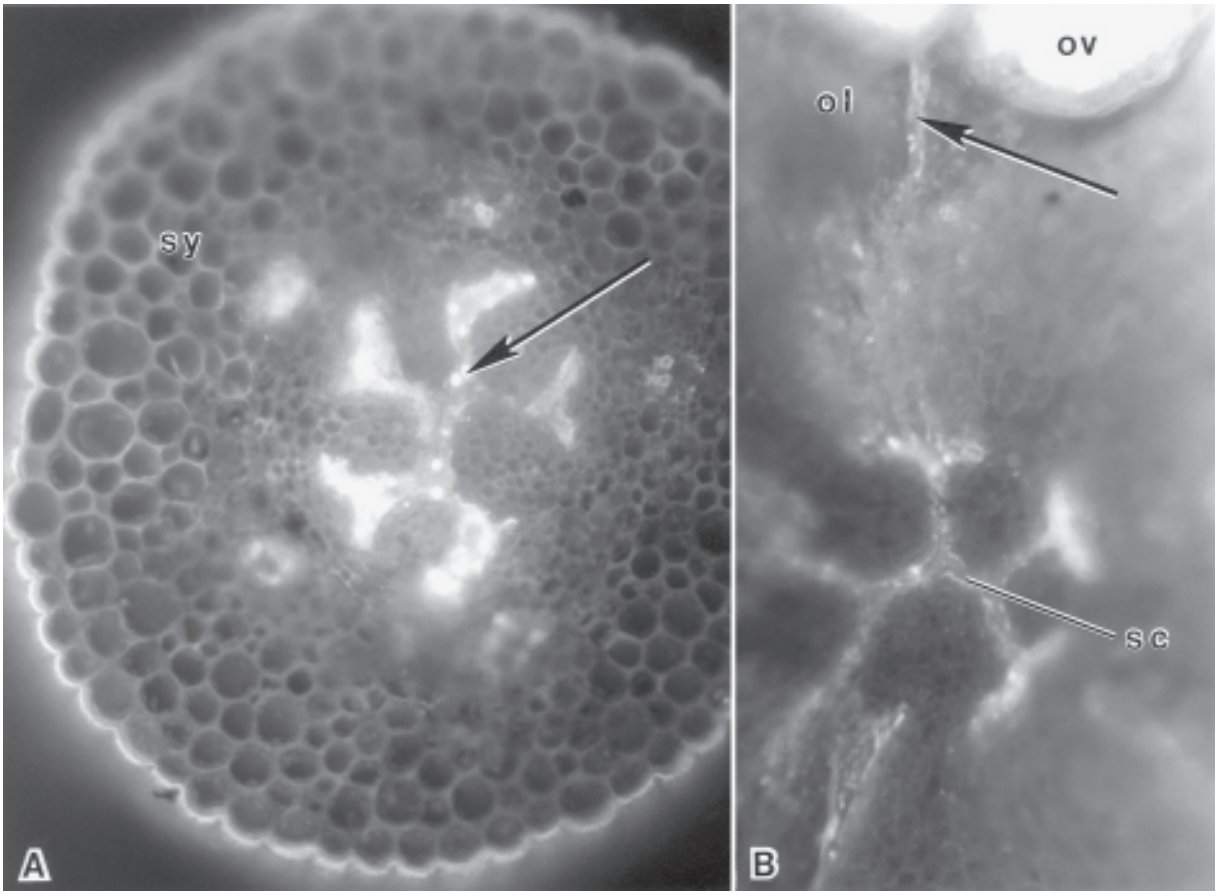

Fig. 3: *V. angustifolium*. A. Aniline blue fluorescent micrograph of a free-hand cross section through a stage 4 stylar canal. Arrow indicates the pollen tubes. x 206. B. Aniline blue fluorescent micrograph of a free-hand cross section through the junction between the stylar canal and upper ovary at stage 5. Arrow indicates the connecting conduit. x 206.

the possible role lipids play in the complex pollen-stigma interactions. Their experiments and careful analyses indicate that lipids are a crucial factor in establishing an external water gradient required for water uptake by both the pollen grains and the pollen tubes. It seems reasonable to suggest that lipids on the stigmatic surface of *V. angustifolium* may also help direct pollen tube penetration and growth in the gynoecial tissues for similar reasons.

Previous observations on the stylar structure in certain members of the Ericales reveal a common pattern; the stylar canal is hollow, fluted and filled with a mucilaginous substance presumably derived from an epithelial transmitting tissue that lines the canal. Among those members of the Pyrolaceae and Monotropaceae that were studied, the number of mucilage filled clefts in the fluted canal is five and reflects the number of ovarian locules (PYYKKÖ, 1968; OLSON, 1991, 1994). In the Ericaceae, the number of mucilage filled stylar clefts varies from five to ten among the various species of *Rhododendron* but also reflects the number of ovarian locules (WILLIAMS et al., 1982; PALSER et al., 1992). In *Vaccinium elliotii, V. corymbosum* and their hybrids, there are five stylar clefts that become filled with a mucilaginous secretion (MUNOZ and LYRENE, 1985). As expected, the observations of the style in *V. angustifolium* conforms to the previously established pattern characterized by five mucilage filled stylar clefts that are aligned with a corresponding ovarian locule.

At the style-ovary junction in members of the Ericales, each cleft of the stylar canal is restricted to a narrow cleft between the upper ovarian septae that forms a conduit into each ovarian locule. These septal clefts are continuous with an associated cleft in the placental tissues bearing the ovules. In various genera studied from the Ericaceae, Monotropaceae and Pyrolaceae, the clefted placental tissue is relatively massive and covered with a mucilaginous secretion; each locular region has the appearance of containing two protruding, ovule bearing placental lobes (PYYKKÖ, 1968, 1969; PALSER, 1992; OLSON, 1991, 1994). In *Vaccinium angustifolium*, the ovary is clearly divided into five locules separated by complete septae with axile placentation throughout the ovary. There is also a short cleft in the upper placenta that is aligned with the corresponding conduit connecting the stylar canal with the upper locule. The opening into each locule of *V. angustifolium*, therefore, leads directly to the secretion-coated surface of the ovule bearing placenta. Herein lies the significance of the structural alignment between the stylar canal and the ovarian locules in all ericalean taxa studied including *V. angustifolium*.

Observations on the gynoecial pathway for pollen tube growth in *Vaccinium angustifolium* are in accordance with previous descriptions from other ericalean species (WILLIAMS et al., 1982; MUNOZ and LYRENE, 1985; PALSER et al., 1992; OLSON, 1991, 1994). In the aforementioned studies, the pollen tubes follow an unobstructed pathway characterized by continuous secretions along the entire journey from the stigma to the ovules. The numerous pollen tubes growing from the stigmatic surface, through the stylar canals, into the locules and along the placental surfaces in *V. angustifolium* remain in direct contact with gynoecial secretions until they penetrate the ovular micropyles.

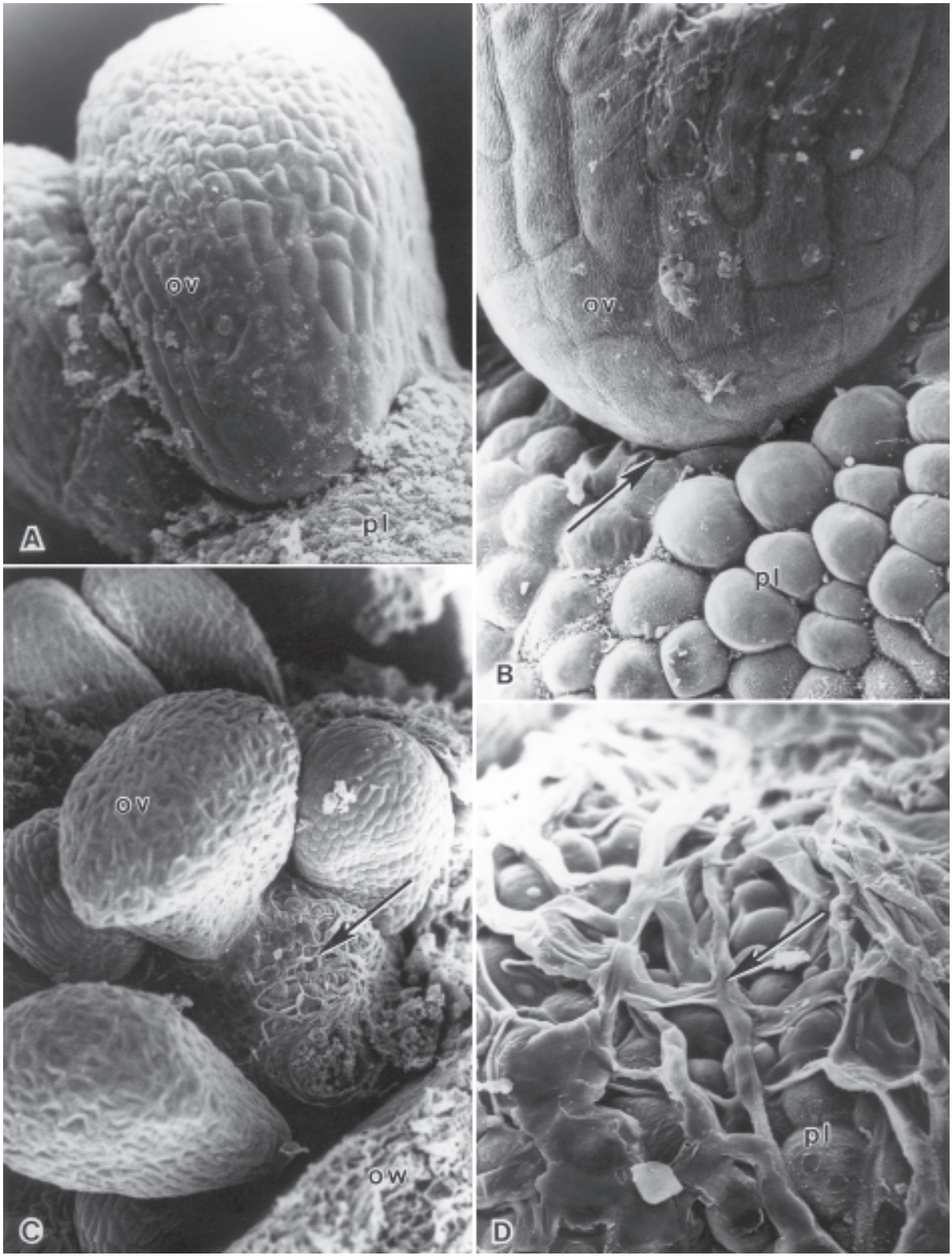

Fig. 4: *V. angustifolium*. A. SEM of anatropous ovules at stage 4. x 1270. B. SEM of ovule and papillated placental surface at stage 4. Arrow indicates the micropylar opening near the placental surface. Exudate is removed during the chemical preparation. x 2408. C. SEM of the ovules and pollen tubes on a placental surface at stage 5. Arrow indicates a tortous mat of pollen tubes. x 505. D. SEM of the pollen tubes at stage 5 with arrow indicating collapsed pollen tubes. x 2535.

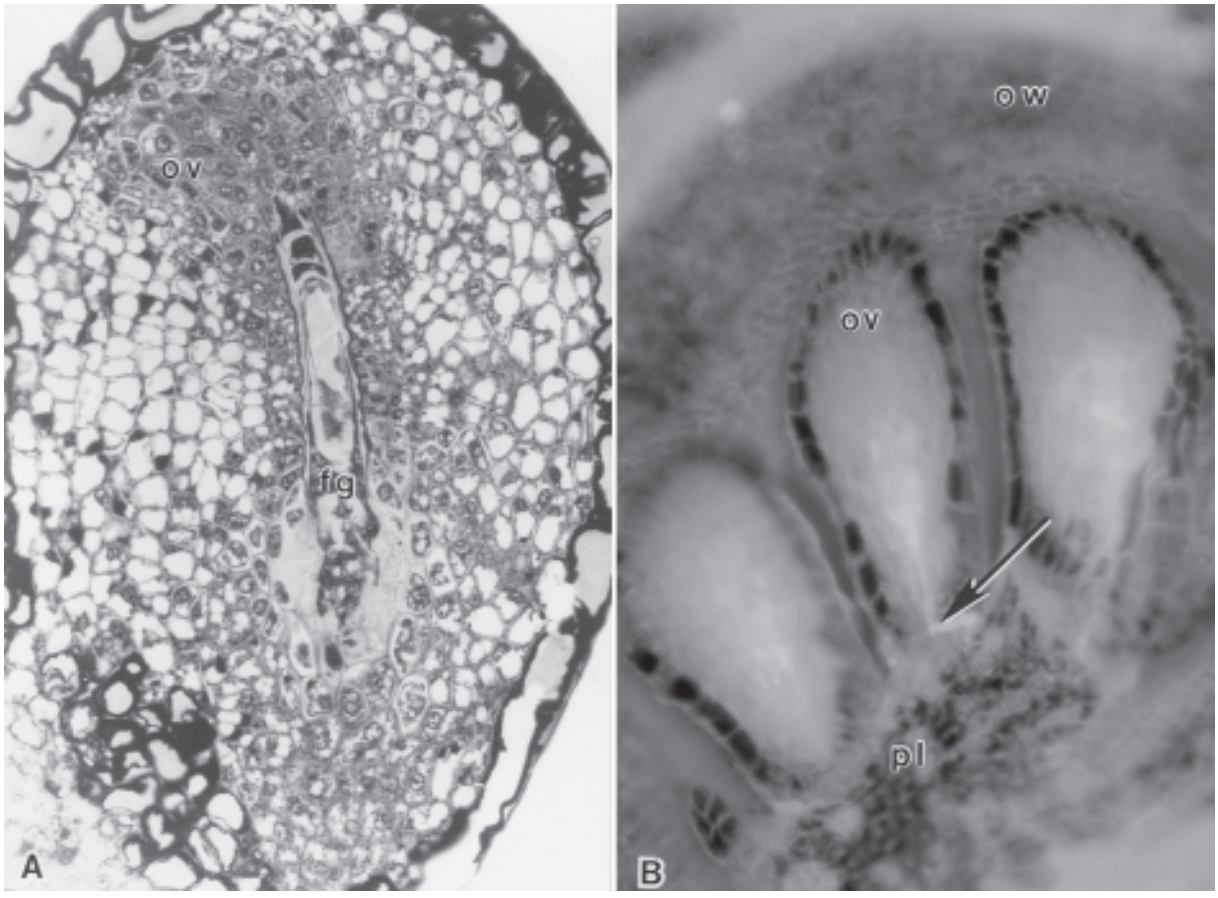

Fig. 5: *V. angustifolium*. A. Resin-embedded longitudinal section through a stage 4 ovule. Note the female gametophyte. x 410. B. Aniline blue fluorescent micrograph of a longitudinal free-hand section of a stage 5 ovary. Arrow indicates a single pollen tube entering the micropyle of an ovule. x 206.

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