Protoplasma (2015) 252:1313–1323
DOI 10.1007/s00709-015-0764-y

ORIGINAL ARTICLE

Pollen development in *Rhododendron* in relation to winter dormancy and bloom time

Olga E. Mirgorodskaya · Nuria K. Koteyeva · Alexandra V. Volchanskaya · Evgeny A. Miroslavov

Received: 9 December 2014 / Accepted: 19 January 2015 / Published online: 3 February 2015

Abstract Microsporogenesis and microgametogenesis of *Rhododendron ledebourii* (semi-deciduous), *Rhododendron luteum* (deciduous), and *Rhododendron catawbiense* (evergreen) were studied by light and electron microscopies in order to determine the stages of pollen development in relation to period of winter dormancy and bloom time throughout an annual growth cycle. Development of generative organs starts in June in *R. ledebourii* and in July in *R. luteum* and *R. catawbiense* and reaches completion about 11 months later. *R. luteum* and *R. catawbiense* microspores undergo meiosis at the end of the August and spend winter at the vacuolization stage. Mitosis with the formation of bicellular pollen grain occurs shortly before flowering at the beginning of June. *R. ledebourii* develops two types of flowers which differ in the timing of microgametogenesis. The first type is characterized by early microspore meiosis and mitosis leading to development of bicellular pollen grains by the end of August, and is prone to fall blooming during warm autumn temperatures. Microspores of the second flower type have a more prolonged vacuolization stage with mitosis and subsequent bicellular pollen grains occurring in November. By winter, flower buds in *R. ledebourii* are more advanced developmentally than in *R. catawbiense* and *R. luteum*, and bloom about 1 month earlier. The different strategies of pollen development identified both within and between these three *Rhododendron* species were recognized which are not associated with leaf drop during winter but appear to be related to the time of spring flowering and the frequency of autumn flowering.

Keywords *Rhododendron* · Pollen development · Tapetum · Winter dormancy

Handling Editor: Peter Nick

Electronic supplementary material The online version of this article (doi:10.1007/s00709-015-0764-y) contains supplementary material, which is available to authorized users.

O. E. Mirgorodskaya · N. K. Koteyeva (✉) · A. V. Volchanskaya · E. A. Miroslavov
V.L. Komarov Botanical Institute of Russian Academy of Science, 2, Prof. Popov St., 197376 St. Petersburg, Russia
e-mail: knouria@mail.ru

Introduction

Developmental events through the male gametophyte (or pollen) formation are highly similar between flowering plants indicating their conserved evolutionary character with conserved genetic control (Blackmore et al. 2007; Borg et al. 2009; Ma 2005; McCormick 2004; Wilson and Zhang 2009). However, having mainly the same sequence of structural and cytomorphological events, pollen development has very variable duration of each stage from several weeks in herbaceous annuals (Bedinger 1992; Owen and Makaroff 1995; Vinckier et al. 2012; Zhang et al. 2011) to about 1 year in woody and some spring-blooming herbaceous perennials from temperate climate (El-Ghazaly and Grafström 1995; Jacobs and Lersten 1994; Jansson and Douglas 2007; Julian et al. 2011; Khodorova et al. 2010; Miroslavov et al. 2008). The extended flower development is related mostly to adaptation to seasonal climate with period of winter dormancy. Chilling requirement for proper flower development and dormancy release is a well-known phenomenon for many temperate zone perennials (Atkinson et al. 2013; Perry 1971; Samish 1954). Despite extensive research of flower ontogenesis in temperate climate plants, an understanding of the mechanisms involved in temperature-dependent flower development is still limited (Van der Toorn et al. 2000). It has been demonstrated that various factors contribute to proper

flowering in response to cold, such as hormonal status (Rietveld et al. 2000), water balance (Van der Toorn et al. 2000; van Kilsdonk et al. 2002), and features of reserve substance transport (Kamenetsky et al. 2003; Khodorova et al. 2010; Lambrechts et al. 1994; Marquat et al. 1999). Pollen development, which is temperature-sensitive (Barton et al. 2014; De Munk 1973; Hak and Russell 2004; Khodorova et al. 2010; Ohnishi et al. 2010), is a good system for studying structural reproductive development in the context of seasonal temperature changes.

The phenology and biology of flowering in different *Rhododendron* species has been studied extensively (Aleksandrova 1989; Babro et al. 2007; Bell and Burchill 1955; Malciūtė et al. 2011; Schneider 1972; Willingham 1976) with special attention to freezing tolerance (Ishikawa and Sakai 1981; Pellett 1987) or to recent climate changes (Ellwood et al. 2013). In most *Rhododendron* species originating from temperate climates or high altitudes, inflorescence and flower differentiation occur in the late summer followed by dormancy during the cold season, and it is a well-known chilling requirement to overcome dormancy and proper spring flowering in *Rhododendron*; however, regulating mechanisms (including the timing of flower initiation and development) are less characterized.

Pollen development in *Rhododendron* has been studied using light microscopy, showing that meiosis and tetrad formation occur before the onset of winter dormancy, and pollen of some species and hybrids overwinters at the late vacuolization stage of development (*Rhododendron canadense*, Bell and Burchill 1955; Leach hybrid rhododendrons, Stowe et al. 1989; *Rhododendron luteum*, *Rhododendron schlippenbachii*, Shamrov and Babro 2008; *R. luteum*, Mirgorodskaya et al. 2011). However, it is not known whether this strategy of pollen development is common for all *Rhododendron* species growing in temperate climate, and how it is related to unproper autumn blooming or to timing of spring bloom. Furthermore, there is little information on the precise sequence of events at the ultrastructural level and the timing of the structural changes involved.

About 25 *Rhododendron* species have been introduced to Botanical Garden of Komarov Botanical Institute (St. Petersburg, Russia) where the low temperature period lasts for up to 5 months. Most of them originated from the Russian Far East, Caucasus, and Altai. For several years, there has been a steady increase of average autumn temperatures, and as a result, certain *Rhododendron* species are flowering during early autumn more frequently than they used to do in the past. The taxa used for the present investigation differing in winter leaf drop and frequency of autumn flowering were semi-deciduous *Rhododendron ledebourii* which blooms at fall, deciduous *R. luteum*, and evergreen *Rhododendron catawbiense. R. ledebourii* (combined with *Rhododendron dauricum* in some systems) is endemic to Altai, Sayan Mountains in Russia (Semenjuk 1976). The area of wild distribution of *R. luteum* includes eastern part of Middle Europe, Balkan Mountains, Asia, and subalpine zone of the Caucasus. The origin of *R. catawbiense* is the eastern part of the North America from Virginia to Georgia, Tennessee, and Alabama (Aleksandrova 1989).

The purpose of this study has been to characterize the microsporogenesis and microgametogenesis in three *Rhododendron* species along with the flower initiation and development, and to compare the advantages of different pollen development strategies to avoid frost damage. In this study, the special attention was devoted to structural features of tapetum function supporting pollen development. Comparative analysis allows to study relationships between winter resting stage of microspore development and the frequency of autumn blooming and the time of spring blooming.

Materials and methods

Flower buds of *R. catawbiense* Michx. (evergreen), *R. luteum* L (deciduous), and *R. ledebourii* Pojark (semi-deciduous) (Ericaceae) were studied in the Botanical Garden of the Komarov Botanical Institute of the Russian Academy of Sciences (St. Petersburg, Russia). The study was done in an over a 3-year period (August 2010 to November 2013); Supplementary Fig. 1A shows the fluctuations of average air temperatures during the winter time in comparison with climatic norm for St. Petersburg region, and Supplementary Fig. 1B represents fluctuations of daily temperatures during autumns under interest. Samples for light (LM) and transmission electron (TEM) microscopy were collected twice per month. Anthers were fixed for 48 h in 2 % (*v*/*v*) paraformaldehyde and 2 % (*v*/*v*) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) followed by 12 h of post-fixation at 4 °C in 2 % OsO_4. After dehydration in a 30, 50, 70, and 96 % (*v*/*v*) ethanol series followed by 100 % acetone, samples were infiltrated in Epon-Araldit M resin (Fluka, Buchs, Switzerland). Cross sections were made on a Reichert Ultracut E ultramicrotome (Reichert-Jung GmbH, Heidelberg, Germany). Semi-thin sections (1 μm) for LM were stained with 0.1 % (*w*/*v*) toluidine blue and observed with an Axio Scope1 equipped with an Axio Cam RMc5 (Zeiss, Germany). Thin sections (70 nm) for TEM were stained with 4 % uranyl acetate and Reynold's lead citrate and examined using Hitachi-H600 (Tokyo, Japan) transmission electron microscope.

For scanning electron microscopy (SEM), flower buds and anthers were fixed using the above protocol, postfixed in 2 % OsO_4, then dehydrated in an ethanol series to 100 % ethanol, critical-point dried, placed onto specimen mounts, sputter-coated with gold, and examined using a JEOL JSM35C (Tokyo, Japan) scanning electron microscope.

For the investigation of pollen germination, the stamens of *R. ledebourii* flowers were collected near the time of anther opening during the autumn and spring blooming. Pollen germination ability was tested in 1 % sucrose (*w*/*v*) at 25 °C during 2–3 h and observed with an Axio Scope1 equipped with an Axio Cam RMc5 (Zeiss, Germany).

Results

Morphology and timing of flower initiation Early stages of flower initiation and anther development are presented for three *Rhododendron* species in Fig. 1. Flower initiation occurs in June in *R. ledebourii* and in July in *R. catawbiense* and *R. luteum* (Table 1), with similar morphogenetic events. *R. ledebourii* usually has two (up to five) separate flower buds sited close to each other at the apical part of branches (Fig. 1a) while *R. catawbiense* and *R. luteum* are characterized by 15–25 flowers initiated per inflorescence (Fig. 1b). Flower initiation in inflorescence occurs in acropetal direction, from the base to the tip, as a result, different stages of flower development can be found in one axis (Fig. 1b).

The sequence of flower development in all three species starts from almost simultaneous initiation of sepals followed by petals and stamens at the periphery of receptacle in alternate pentagonal whorls (Fig. 1a, b). *R. luteum* forms one whorl of five stamens, while in *R. ledebourii* and *R. catawbiense*, two whorls of stamens are formed sequentially (Fig. 1c). Morphologically differentiated stamens are observed in early July for *R. ledebourii* (Fig. 1c) and in August for *R. catawbiense* and *R. luteum* (Table 1). At this stage, anthers contain sporogenous tissue (Fig. 1d).

Sporogenous tissue stage In all studied species, sporogenous tissue is located within four loculi of anthers (Fig. 1d) and consists of large polygonal cells that are undifferentiated microsporocytes (Fig. 1f). Future tapetum is represented by one cell layer at the periphery of the anther loculus followed by few middle layers, endothecium, and epidermis towards the outside of the anther wall (Fig. 1e). The tapetal cells have rectangular form and are smaller sized than microsporocytes. At the level of ultrastructure microsporocytes and tapetal cells are meristematic cells having a nucleus at the center, abundant cisterns of rough endoplasmatic reticulum (RER), few

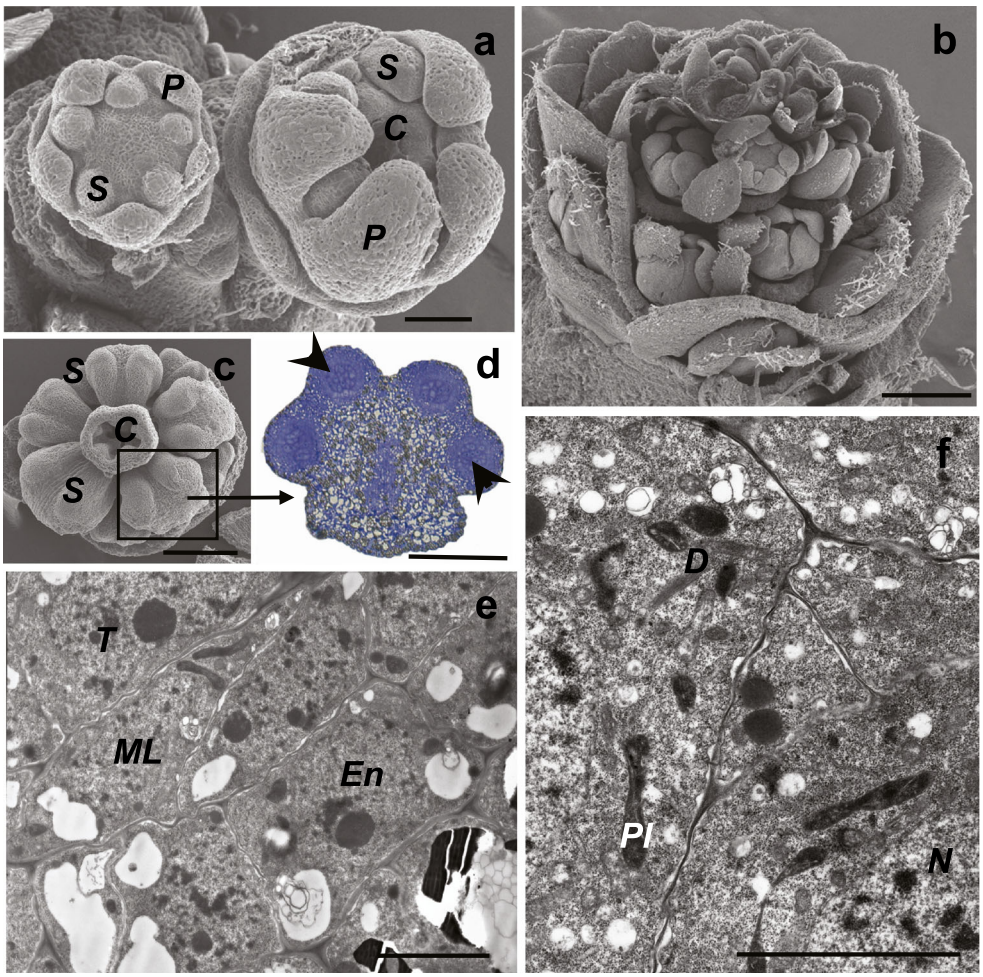

Fig. 1 Flower initiation and early stages of microsporangia development in *Rhododendron* species. **a** Developing flowers of *R. ledebourii* with sepal, petal, and stamen primordia alternate wholes (at the *left*) and more pronounced stage (at the *right*) in the end of June. **b** Developing inflorescence of *R. catawbiense* at the middle of July showing acropetal character of flower initiation and differentiation. **c**, **d** Differentiated anthers in *R. ledebourii* flowers (**c**) and their cross section with sporogenous tissue within the loculus, pointed by *arrowheads* (**d**) at the early July. **e**, **f** Cross section of anther wall showing three developing layers (**e**) and details of ultrastructure of undifferentiated microsporocytes (**f**) in *R. luteum* at August. *C*, carpel; *D*, dictyosomes; *En*, endothecium; *ML*, middle layers; *N*, nucleus; *P*, petal; *Pl*, plastid; *S*, stamen; *T*, tapetum. *Scale bars*: **a**–**c**—100 μm; **d**—50 μm; **e**, **f**—5 μm

Table 1 Time scale of pollen development according to the 3 years of observation (2010–2013) in three *Rhododendron* species

	Flower initiation	Stage of sporogenous tissue	Meiosis and tetrad stage	Stage of vacuolization	Stage of bicellular pollen grain	Mature pollen grain
R. ledebourii (apical / subapical flowers)	June	July	Late July	Early August August–November	Late August November–April	September–November May
R. catawbiense	July	August	Late August–early September	September–April	Middle of May	Late May–early June
R. luteum	July	August	Late August–early September	September–April	Middle of May	Late May–early June

mitochondria, plastids, dictyosomes, and vacuoles (Fig. 1e, f). Undifferentiated microsporocytes at later stage of development became microspore mother cells and have roundish form. Meiotic stage occurs at the end of July in *R. ledebourii* and at the end of August-early September in *R. catawbiense* and *R. luteum* resulting in tetrad formation (Table 1).

Tetrad stage The four daughter cells from a meiosis form an aggregation called a pollen tetrad. Those four cells are originally interconnected and surrounded by common callose wall (Fig. 2a, b); tetrads of microspores in genus *Rhododendron* do not dissociate. At this stage of pollen development, the exine formation is initiated through the primexine deposition as a

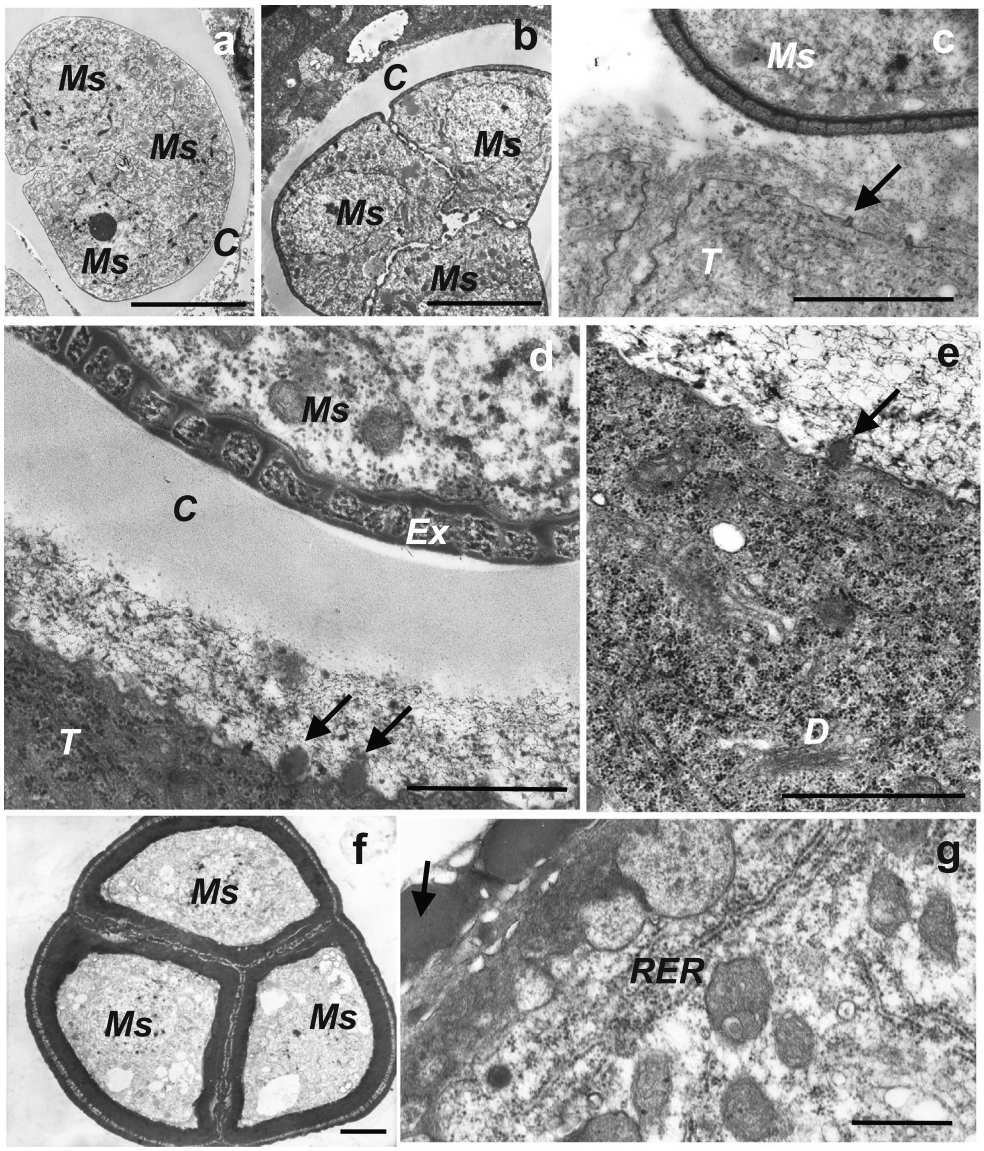

Fig. 2 Tapetum and microspore ultrastructure at the stage of tetrad and early vacuolization in *R. luteum* (**a**, **c**, **f**, **g**) in September (**a**, **c**) and October (**f**, **g**) and in *R. ledebourii* (**b**, **d**, **e**) in July (**b**) and August (**d**, **e**). **a**, **b** Microspores at the early tetrad stage surrounded by common callose wall at the early (**a**) and more pronounced (**b**) stages of primexine development. **c**, **d** Exine formation is evident between the callose wall and the microspore plasma membrane at the middle (**c**) and late (**d**) tetrad stage with initial and pronounced pro-tectum, pro-columellae, and foot-layer precursors formation. **e** Secretory tapetum at the stage of tetrads. **f** Tetrads of microspores at the stage of early vacuolization with all exine layers formed. **g** Ultrastructure of tapetum at the stage of microspores vacuolization showing decrease of activity. *Arrows* show orbicules secreted from tapetum. *C*, callose; *D*, dictyosomes; *Ex*, exine; *Ms*, microspore; *RER*, rough endoplasmic reticulum; *T*, tapetum. *Scale bars*: **a**–**c**, **f**—5 μm; **d**, **e**, **g**—1 μm

thin dark layer between the callose wall and plasma membrane in early tetrads (see Fig. 2a, b), followed by development of pro-tectum, pro-columellae, and foot-layer precursors in mid-tetrads (Fig. 2c) with increasing in thickness at the late tetrad (Fig. 2d). During microspore meiosis and the exine formation, tapetal cells show hypersecretory ultrastructure having numerous dictyosomes and cisterns of RER near the plasma membrane, abundant smooth endoplasmic reticulum (SER) in the center of the cell, and polysomes through the cytosol (Fig. 2e). Tapetum of *R. ledebourii* sometimes contains amyloplasts with starch granules. Tapetum cell wall towards the anther loculus is loose, and orbicules are secreted from the tapetum cells to loculus (Fig. 2c–e).

Stage of vacuolization This stage starts in early August in *R. ledebourii* and in early September in *R. catawbiense* and *R. luteum* (Table 1). During early vacuolization stage, the nucleus of microspore is located in the center of cell, and only a few vacuoles are in cytoplasm (Fig. 2f). The exine development continues with the endexine formation. Exine now contains ectexine with three layers (tectum, columellae, and foot layer) and endexine (Fig. 2f). Later, the microspore forms an intine which is the inner layer of its wall with very pronounced thickening near the aperture. Tapetal cells contain numerous mitochondria, ribosomes, and vacuoles, and stacking of RER cisterns to parallel arrangement is characteristic for this stage. Density of cytosol in tapetal cell is decreased (Fig. 2g). During the late vacuolization stage, the nucleus and cytoplasm of the microspore are displaced towards to periphery of the cell with large vacuole situated in the center (Fig. 3d, e).

Stage of bicellular pollen grains and the phenomenon of fall bloom in R. ledebourii This species shows asynchronous microgametogenesis among the developing flowers resulting in flowers at two different stages of development by fall. They differ in the timing of mitosis and subsequent formation of

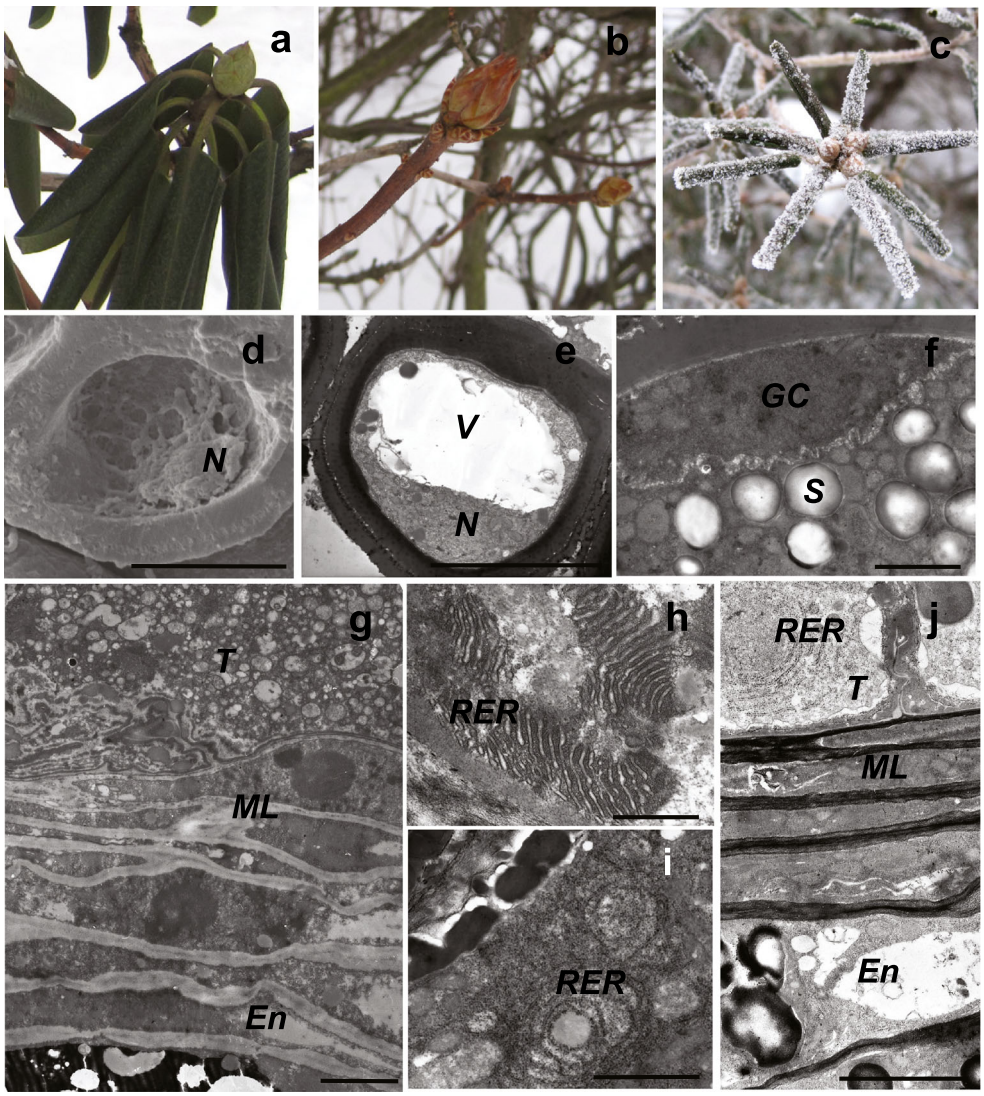

Fig. 3 Floral buds and anther structural features at the stage of winter dormancy in three *Rhododendron* species, *R. catawbiense* (**a**, **h**), *R. luteum* (**b**, **d**, **e**, **g**, **i**) and *R. ledebourii* (**c**, **f**, **j**). **a**–**c** General view of branches in December showing leaf thermonasty reaction in *R. catawbiense* (**a**) and *R. ledebourii* (**c**) and no foliage in *R. luteum* (**b**). **d**, **e** Microspores on the stage of late vacuolization at the end of November. **f** Bicellular pollen grain with starch accumulation in vegetative cell at January. **g**, **j** Cross section of anther wall at December showing vesiculation of tapetal cells in *R. luteum* (**g**) and «stacks» of RER in tapetal cells of *R. ledebourii* (**j**). **h**, **i** «Stacks» of RER in tapetal cell at February (**h**) and January (**i**). *En*, endothecium; *GC*, generative cell; *ML*, middle layers; *N*, nucleus; *RER*, rough endoplasmic reticulum; *S*, starch granules; *T*, tapetum; *V*, vacuole. *Scale bars*: **d**, **e**—10 μm; **f**–**i**—1 μm; **j**—5 μm

bicellular pollen grains containing generative and vegetative cells (Table 1). In flower buds which are more apically positioned at branch axis (apical buds), mitosis occurs at the end of August. In lower positioned flower buds (subapical buds), microspores remain in the late vacuolization stage until November, with the same structural features in microspores and tapetum cells as *R. catawbiense* and *R. luteum* have. In addition, these flowers are smaller sized and have more tightly closed bud scales than the apical buds. In subapical flower buds, mitosis occurs throughout November leading to bicellular pollen grain formation.

R. ledebourii is the only species among the three being studied which is prone to autumn flowering during unusually warm fall temperatures (September–November). In St. Petersburg (Russia), these warm conditions occurred during the 3 years the current study was conducted (Supplementary Fig. 1). Our observations of flowers indicate that the apical flower buds containing bicellular pollen grains are more likely to bloom in the fall than flower buds at the lower position on the stem. Subapical flower buds usually develop bicellular pollen grains later or occasionally pass winter at vacuolated stage and do not flower in fall under favorable temperatures.

Observation of pollen germination during the fall and spring bloom shows the similar percentage of germinated pollens, ~95 %.

Winter resting stage (October–April) R. catawbiense and *R. ledebourii* are evergreen with leaves exhibiting thermonasty (curling and drooping) under freezing conditions (Fig. 3a, c) while *R. luteum* as a deciduous species loses foliage in October (Fig. 3b). Microspores of *R. catawbiense* and *R. luteum* overwinter at the stage of late vacuolization until the May (Table 1, Fig. 3d, e; Fig. 4a). Tapetum cells appear to be metabolically inactive in autumn and winter. The vesiculation of tapetal cell cytosol (Fig. 3g), a decrease in number of organelles, and the formation of RER "stacks" are characteristic for this period (Fig. 3h, i), and these are specific features of the cell dormancy. A significant reorganization of tapetal cell ultrastructure is observed in April showing recovery of secretory activity. The main ultrastructural changes are multiple nuclei, disappearance of vesicles, disassembling of RER "stacks," abundance of Golgi bodies, and accumulation of electron-dense substances with sporopollenin-like material secreted towards the anther loculus (Fig. 4b).

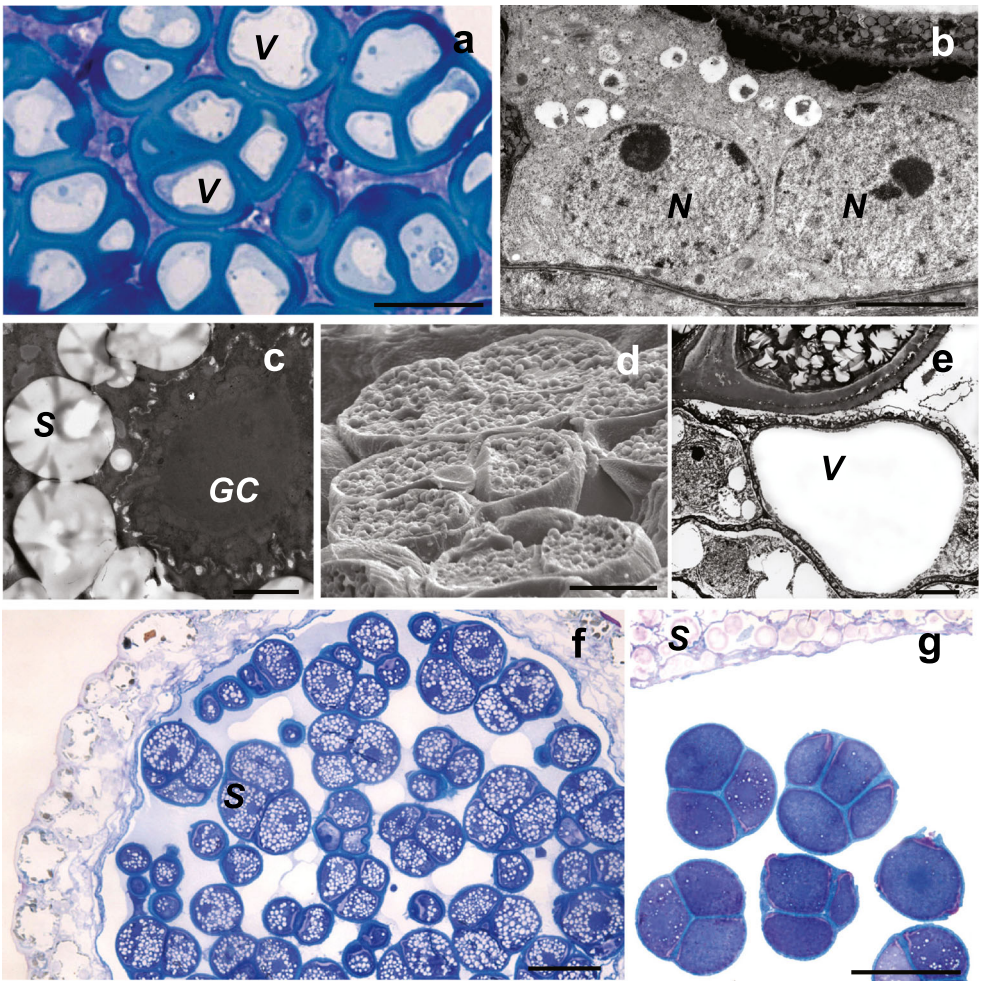

Fig. 4 Last stages of pollen development in *Rhododendron luteum* (**a**–**f**) and *R. catawbiense* (**g**) after winter dormancy release at spring, April (**a**, **b**) and middle of May (**c**–**g**). **a** Microspores on the stage of late vacuolization. **b** Reorganized tapetum with restored secretory activity. **c**, **d** Bicellular pollen grains with starch accumulation in amyloplasts of vegetative cell. **e** Early events in the destruction of tapetal cells with cytoplasm vacuolization. **f** Anther cross section showing bicellular pollen grains in tetrads and destroyed tapetum. **g** Anther cross section with mature pollen grains in tetrads with little starch in pollen and evident starch granules in anther wall. *GC*, generative cell; *N*, nucleus; *S*, starch grain; *V*, vacuole. *Scale bars*: **a**—20 μm; **b**, **e**—5 μm; **c**, **d**—10 μm; **f**, **g**—40 μm

R. ledebourii shows different strategy of pollen development and overwinters with microspores at the pronounced stage of bicellular pollen grains (Fig. 3f). During winter dormancy, the generative cell tends to be located at the periphery of the pollen grain and has smooth plasma membrane towards the inner layer of pollen wall, intine, but has very irregular plasma membrane shape where it joins to the plasma membrane of the vegetative cell. The dense cytosol of both cells and starch granules in amyloplasts of the vegetative cell are characteristic of ultrastructure at this stage (Fig. 3f). Specific position and ultrastructural features remain mainly unchanged during the winter until middle of May with only progressive increasing of sizes of starch granules in vegetative cell in spring. Unlike *R. catawbiense* and *R. luteum*, tapetal cells in *R. ledebourii* during the cold season are not vesiculated and characterized by special concentric arrangement ("whorls") of RER surrounding the lipid bodies (Fig. 3j). Starch granules are accumulated in the plastids of endothecium of the anther wall (Fig. 3j).

Stage of bicellular pollen grains in R. catawbiense and R. luteum Pollen grains in *R. catawbiense* and *R. luteum* complete development shortly before flowering, which is observed in June (Table 1). Bicellular pollen grains with generative and vegetative cells are distinguished at the second part of May (Fig. 4c, d). The generative cell is located at the center of the pollen grain. Numerous amyloplasts of vegetative cell contain starch grains (Fig. 4c, d). Pollen grain completes cell wall formation after intine deposition. At this time, the tapetum collapses with destruction starting from large vacuole formation and followed by total cytoplasm shrinkage; finally only remains of orbicules are observed (Fig. 4f).

Mature pollen grains Complete maturation of pollen grains in all three species occurs shortly before flowering. Flower buds which are in process of budburst (Supplementary Fig. 2a, b, c) contain fully mature pollen grains. The pollen grains are shed as tetrads (Supplementary Fig. 2g, h, i) that are typical for Ericaceae. Dehiscent anthers have two pollen sacs which originate from four pollen loculi of the tetrasporangiate anthers during the last stages of pollen maturation. By the time of flowering (May for *R. ledebourii* (Supplementary Fig. 2d) and June for *R. luteum* (Supplementary Fig. 2e) and *R. catawbiense* (Supplementary Fig. 2f)), starch grains in amyloplasts of vegetative cells become very small and rare (compare Fig. 4f, g); in *R. ledebourii*, the generative cell moves away from the wall to a more central position. Mature pollen grains are bicellular; it was shown for *R. luteum* that division of generative cell undergoes in the pollen tube (Jakobson 1969). Mature pollen grains in tetrads are 3-colporate with viscin threads at the surface which are easily recognized under the SEM (Supplementary Fig. 2g, h, i) and which hold the pollen grains together when leaving the anther. At maturity, the tapetum is completely destroyed and the remains of orbicules stick to the exine layer of the pollen grain. Plastids of cells in the remaining anther layers contain large grains of starch (Fig. 4g).

Discussion

Winter resting stage of pollen development

The results showed that in the three *Rhododendron* species studied, pollen development takes about 11 months and includes six stages: I—sporogenous tissue, II—meiosis and tetrads of microsporocytes, III—early vacuolization, IV—late vacuolization, V—mitosis and bicellular pollen grains, VI—mature pollen grains. These stages are common for the most studied plants irrespective of origin, distribution, and features of ontogenesis indicating highly conserved evolutionary character of pollen development among angiosperms (Blackmore et al. 2007; Borg et al. 2009; Ma 2005; McCormick 2004; Wilson and Zhang 2009). In many woody perennial plants from temperate climate, male gametophyte development continues about 10–11 months; however, the time scale of each stage and their durations and winter resting stage vary from species to species (Bell and Burchill 1955; El-Ghazaly and Grafström 1995; Jacobs and Lersten 1994; Jansson and Douglas 2007; Julian et al. 2011).

In this study, the timing of flower initiation and flowering as well as the time scale of stages of pollen development differed in *R. ledebourii* versus *R. catawbiense* and *R. luteum* (Fig. 5). Transition from vegetative to reproductive structures occurred earlier in *R. ledebourii* (June) than in *R. catawbiense* and *R. luteum* (July). Early initiation allows to *R. ledebourii* to complete pollen differentiation by November, resulting in microspores that overwinter as bicellular pollen grains. Earlier differentiation of pollen in *R. ledebourii* is associated with earlier spring bloom, at least 1 month earlier than flowering in *R. catawbiense* and *R. luteum*. In contrast, the delay in pollen development in *R. catawbiense* and *R. luteum* results in prolonged stage of microspore vacuolization occurring during the period of lower temperatures in winter. Maturation of pollen in these two species completes just before blooming in middle of May when the probability of microspore damage from spring frost is lower (Fig. 5).

Among the plant studied, only herbaceous early-spring ephemeral *Scilla sibirica* (Miroslavov et al. 2008) and woody perennials *Epigaea repens* and *Chamaedaphne calyculata* (Bell and Burchill 1955) and *Corylus avellana* (Frenguelli et al. 1997) have microspores as bicellular pollen grains during the cold season similar to *R. ledebourii*. Besides *R. catawbiense* and *R. luteum*, overwintering at the stage of vacuolization was also shown for herbaceous early-spring ephemeral *Corydalis bracteata* (Khodorova et al. 2010) and

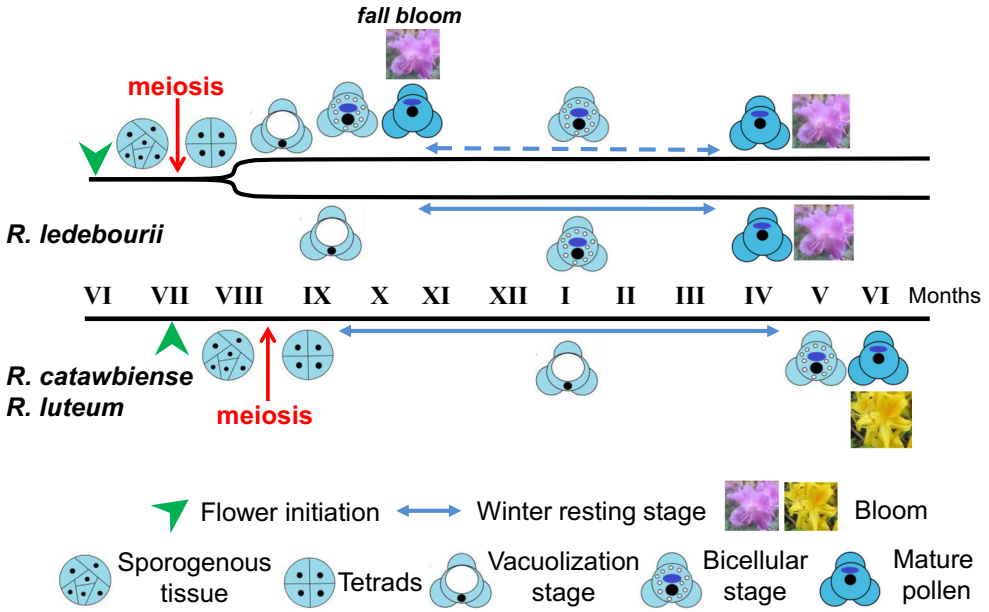

Fig. 5 Schematic illustration of microsporogenesis and microgametogenesis in three *Rhododendron* species showing time scale of developmental events with similar schedule for *R. catawbiense* and *R. luteum* and two different types of flowers in *R. ledebourii.* Five stages of pollen development are represented by diagrams: (1) sporogenous tissue, (2) tetrads of microspores in common callose wall, (3) vacuolization stage with microspores containing one nucleus (*black circle*) and vacuole, (4) bicellular stage with pollen grains containing vegetative cell with starch in amyloplasts and one nucleus (*black circle*) and generative cell (*blue oval*), and (5) stage of mature pollen grains which are still in tetrad and contain vegetative cell (*nucleus as a black circle*) and generative cell (*blue oval*)

Ericaceae species, *Kalmia polifolia* and *R. canadense* (Bell and Burchill 1955), *R. schlippenbachii* (Shamrov and Babro 2008), and Leach hybrid rhododendrons (Stowe et al. 1989).

Among seed plants, several additional variants were shown which are differing in winter resting stage of pollen development. Microspores of some conifers overwinter at the stage of meiosis (Ekberg et al. 1968; Owens and Molder 1974; Rowley and Walles 1985a; Rowley and Walles 1985b; Walles and Rowley 1982; Zhang et al. 2008) or at the stage of sporogenous tissue (Cecich 1984; Kupila-Ahvenniemi et al. 1978). The latter strategy is also found for angiosperms, *Prunus cerasus* (Felker et al. 1983), *Acer saccharum* (Jacobs and Lersten 1994), *Cerasus vulgaris* and *Cerasus tomentosa* (Shamrov and Yandovka 2006), *Prunus armeniaca* (Julian et al. 2011), four Ericaceae species (Bell and Burchill 1955), and *Ribes nigrum* (Koteyeva et al. 2015). Thus, the most plants studied overwinter at the sporogenous stage, and no one plant found resting at the stage of early vacuolization or completely mature pollen grains. Additionally, only among conifers the prolonged stage of meiosis was found occurring during period of low temperatures. The most *Rhododendron* species and cultivars studied overwinter at the stage of late vacuolization, with only *R. ledebourii* having more pronounced stage of pollen development during the winter. The strategy of pollen development is not depending on leaf drop during winter but appear to be associated with the time of spring flowering as *R. ledebourii* flowers first at the conditions under the current study.

Each species has a fairly definite resting stage of microspore development regardless of year and locality with some rare exceptions (Bell and Burchill 1955). Variations in pollen development reflect different methods that temperate zone plants use to adapt to seasonal climate. It seems that temperature-dependent and/or temperature-sensitive stages of pollen development are different among these strategies. For example, for species overwintering at the stage of sporogenous tissue, the meiosis depends on low temperature applying (Julian et al. 2011; Koteyeva et al. 2015) and does not occur if to avoid chilling during winter dormancy (Koteyeva et al. 2015). In contrast, in the *Rhododendron* being studied, microspore meiosis does not require low temperatures since meiosis occurs before the first frost. The limited data on pollen development strategies to survive low winter temperatures do not permit us to suggest the most successive between them; more experimental data is needed taking in consideration the pollen viability. It has been shown that overwintering at the meiosis stage of microspores has the largest percent of abnormality, while pre- or postmeiotic stages appear to be more stable (Bazhina et al. 2011; Eriksson 1968; Hak and Russell 2004; Owens and Molder 1974). The sporogenous stage also appears to be freeze tolerant due to its meristematic nature (Julian et al. 2011; Koteyeva et al. 2015). For several annual crops was shown that during anther development the most sensitive stages for chilling injury are meiosis, tetrads, and uni-nucleate microspore (Barton et al. 2014; Ohnishi et al. 2010; Oliver et al. 2005).

Phenomenon of fall bloom in *R. ledebourii*

R. ledebourii develops initially two different types of flower buds. One type of flower is characterized by early microspore meiosis and mitosis leading to development of bicellular pollen grains by the end of August; they are typically larger and apically positioned on shoot axis. Microspores of the second type have a more prolonged vacuolization stage with mitosis and subsequent bicellular pollen grains occurring in November. All three individuals of *R. ledebourii* studied were characterized by expanded sparse bloom from end of August to end of October under the warm conditions. Only apical flower buds that have formed bicellular pollen grains very early in August were capable to bloom at fall supposing very short chilling period required for break of dormancy or developmental regulation of this flower blooming independently from dormancy status. To understand the regulating factor provoking the fall bloom, the more plants resting at pronounced stage of microspore development need to be studied, and experiments using controlled conditions should be applied. However, there is very limited number of plants known to have microspores as bicellular pollen grains during the cold season (Bell and Burchill 1955; Frenguelli et al. 1997; Miroslavov et al. 2008) with no data available on their unexpected autumn bloom. According to our observation, *Daphne mezereum* L. which is natural to St. Petersburg region and overwinters at the stage of bicellular pollen grains is prone to fall and even to early winter bloom with most flower buds enabled resulting often in the whole plant death (Olga Mirgorodskaya, observations during 2010–2012, Botanical Garden of Komarov Botanical Institute, unpublished). In case of *R. ledebourii*, only part of flowers enables to bloom during the autumn.

The physiological and ecological role of production of two separate populations of flowers in *R. ledebourii* is not clear. Viability of pollens and percent of germination are not different in autumn and spring blooming flowers; however, no one event of successful pollination was recorded in autumn under conditions studied. Area of origin and recent natural distribution of this species are characterized by seasonal climate with cold winters, and the fall bloom is frequently reported for natural areas from August to November depending on temperature conditions (Miroslava Sakhnevich, annual personal observations in Altaisky State Nature Biosphere Reserve for the more than 14 years). Nevertheless, the existence of floral buds with asynchronous male gametophyte development in *R. ledebourii* reduces the fraction of flower buds which incorrectly break dormancy due to temperature fluctuations.

Tapetum function during prolonged pollen development

The tapetum, an inner parietal layer of the anther wall adjacent to sporogenous tissue, consists of secretory cells which nourish and regulate the pollen grains during development (Ariizumi and Toriyama 2011; Liu and Fan 2013; Pacini 2010), and which collapse during the last stages of pollen development. Our study shows two different ways of tapetum functioning in flowers associated with two overwintering strategies in *R. ledebourii* versus *R. catawbiense* and *R. luteum.*

Flowers which overwinter with vacuolated microspores (*R. catawbiense* and *R. luteum*) have two peaks of secretory activity in tapetal cells interrupted by a winter dormancy period. The first peak of tapetal activity is observed during meiosis and tetrad formation; it is characteristic for this stage of development in all plants studied and was described by Rowley (1993) as a period of metabolic hyperactivity in tapetal cells. The tapetum remains active during late tetrad formation assisting in exine formation, indicated by exocytosis of sporopollenin-like-containing orbicules to the anther loculus and pronounced cytoplasm development. Tapetal cell participation in exine formation has been studied in numerous taxa (Ariizumi and Toriyama 2011; Dickinson and Bell 1976; Gabarayeva et al. 2009; Galati et al. 2007; Liu and Fan 2013; Quilichini et al. 2014; Vinckier and Smets 2005). During the winter period, vesiculation of cytosol, arrangement of RER to "stacks", and a decrease in mitochondria and Golgi content are indicative of tapetal cell inactivation. It has been shown that "stacks" of RER produced in dormant tissue are a prerequisite configuration for future protein biosynthesis in these organelles (Cecich 1984; Kupila-Ahvenniemi et al. 1978; Muravnik 2008). Reactivation of tapetal cell metabolism and function as secretory structure occurs during further development of pollen grains in the spring, in May–June for *R. catawbiense* and *R. luteum* followed by disintegration of the tapetum shortly before flowering.

In contrast, the tapetum of *R. ledebourii* anthers with bicellular pollen grain formation in autumn actively functions as a secretory structure during the meiosis (July) followed by assistance in exine formation at the tetrad stage (August). During the winter period, the ultrastructure of tapetum cells indicates a dormant state for this tissue, and it finally collapses shortly before flowering in May without reactivation.

In summary, two different strategies of pollen development were shown for three *Rhododendron* species in this study. The observed differences in the seasonal timing of floral differentiation were not dependent on the foliage behavior (loss/preservation) during the winter but associated with timing of spring bloom. There was a close association between the early pollen development and the probability of fall bloom; floral buds that delayed mitosis and formation of bicellular pollen grains until late fall avoided flowering during warm fall temperatures. Pollen development in more *Rhododendron* species needs to be studied to understand the advantages of each strategy and their evolution in relation with species origin/distribution, and to predict the species reaction to introduction as ornamental plants or to global climate changes.

Acknowledgments This material is based upon work supported by the Research Foundation of American Rhododendron Society, grant # 133 and by the Russian Foundation of Basic Research, grant # 13-04-00876. We thank Irina Descharmes for reviewing the English. We appreciate the Core Center "Cell and Molecular Technology in the Plant Science" at the Komarov Botanical Institute (St. Petersburg) for use of its facilities.

Conflict of interest The authors declare that they have no conflict of interest.

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