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ANATOMICAL AND HISTOCHEMICAL OBSERVATION OF MICROSPORE ABORTION AND TAPETUM DEGENERATION IN MALE-STERILE *ROSA STERILISER* S. D. SHI (ROSACEAE)

XING-YIN CHEN, PING GUAN*, JIAN-MING SHI, PENG YANG AND KAI-KAI ZHANG

Department of Life Science, Guizhou University, 550025, Guiyang, P.R. China
**Corresponding author's email:guanp508@163.com*

Abstract

A high frequency of pollen grain abortion causes male sterility in *Rosa sterilis* S. D. Shi. To study the cytological mechanism of male sterility in *R. sterilis*, we compared microspore development and histochemical distribution of nutritive materials at different stages of anther development in *R. sterilis* and its fertile close relative *Rosa roxburghii* Tratt by light microscopy. The pollen mother cells of *R. sterilis* and *R. roxburghii* develop consistently, and undergo normal meiosis. At the tetrad stage, the tapetum cells of *R. sterilis* showed binuclearte and trinucleate augmentation and no signs of degeneration, whereas *R. roxburghii* showed evidence for initiation of tapetum degeneration. At the vacuolate microspore (VMP) and mature pollen stages, the nucleus degenerated in *R. sterilis* microspores, resulting in empty pollen grains. Non-nucleate microspores comprised 76.70% of total microspores in late-VMP anther of *R. sterilis*, but only 2.4% in *R. roxburghii* anthers. The distribution of nutritive materials in *R. sterilis* and *R. roxburghii* anthers showed no notable differenc at the meiosis stage, except for that of starch grains. At the mature pollen stage, nutritive materials (protein, polysaccharides, starch grains) accumulated in *R. roxburghii* pollen grains, whereas nutrients failed to accumulate in *R. sterilis* pollen grains. The delayed disintegration of the tapetum and lack of accumulation of nutritive material may be cause of pollen abortion in *R. sterilis*. late VMP stage is a critical period for *R. sterilis* pollen abortion. Nuclear matter melted was the key factor resulting in pollen abortion of *R. sterilis*.

Key words: *Rosa sterilis*, Male sterility, Pollen development, Tapetum, Cytology.

Introduction

Male sterility plays an important role in the utilization of heterosis in crop breeding and production. Male sterility includes genic male sterility (GMS) and cytoplasmic male sterility (CMS). The former exhibits Mendelian inheritance, whereas the latter shows non-Mendelian inheritance patterns (Mohan & Kaul, 1988). Production of fertile pollen involves physiological, biochemical, and morphological processes that are controlled by a large number of genes. Thus, mutations that impact on any stage of stamen development, such as microsporangium differentiation, meiosis, microspore development, microspore mitosis, and pollen differentiation, and on flowering related genes may lead to male sterility in plants (Glover *et al*., 1998). In maize, rice, tomato, and barley, numerous male sterility genes have been identified (Jinguo & Rutger, 1992; Okamuro *et al*., 1993).

The anther wall consists of four layers: the epidermis, endothecium, middle layer, and the tapetum (Bedinger, 1992). As the innermost cell layer of the anther wall, the tapetum provides nutrients for pollen development and plays a crucial role in the normal development of pollen mother cells (PMCs) and microspores (Pacini *et al*., 1985). Tapetum abnormality mainly results from programmed cell death (PCD) of the tapetum cells, which may lead to pollen microspore abortion. Thus, the abnormal expression of PCD genes controlling tapetum can lead to premature or delay PCD of the tapetum, affecting the normal development of pollen microspores, and ultimately results in male sterility (Varnier *et al*., 2005). Male sterility resulting from PCD has been detected in Arabidopsis (*Arabidopsis thaliana*; Vizcay-Barrena & Wilson, 2006), rice (*Oryza sativa*; Li *et al*., 2006), kiwifruit (*Actinidia deliciosa*; Coimbra *et al*., 2004), pepper (*Capsicum annuum*; Luo *et al*., 2006), and Chinese cabbage-pak-choi (*Brassica rapa* subsp. *Chinensis*; Xie *et al*., 2005).

R. sterilis is a climbing shrub that was discovered during an investigation at Guizhou Agricultural College of *R. roxburghii* genetic resources in Guizhou province in 1981. Subsequently, *R. sterilis* was described as a species by Shi (1984). The fruits of *R. sterilis* have a crisp, non-astringent, sweet taste, are rich in sugars, superoxide dismutase and vitamin C, and shows development potential on account if its medicinal and nutritional value (Fu *et al*., 2012). In the 1980s and 1990s, Guizhou College initiated a domestication, cultivation and breeding program for *R. sterilis* (Liu & Zhao, 2014). The Anshun Forestry Science Research Institute from 2000 continued research on the introduction and cultivation of wild *R. sterilis* germplasm (Wei *et al*., 2007). As of 2011, in Anshun city districts and counties an area of 333.3 hm^2 was under cultivation of *R. sterilis* (Wei *et al*., 2012). *R. sterilis* is not only of high economic and social value, but also is of ecological importance in desertification control and ecological restoration for soil and water conservation and is attracting increasing attention from academia and commercial enterprises (Yang *et al*., 2016).

In mature fruit of *R. sterilis* the seeds are withered and non-viable (Fig. 1a). In contrast, the seeds of *R. roxburghii* are fertile (Fig. 1b). *R. sterilis* reproduces asexually and shows stable sterility. No variability in the male sterility and no fertile lines of *R. sterilis* are known. Analysis of random amplified polymorphic DNA markers revealed that *R. sterilis* shows an extremely close genetic relationship to *R. roxburghii* (Wen & Deng, 2004). Deng *et al*., (2015) concluded that *R. roxburghii* was the paternal parent of *R. sterilis* as indicated by DNA barcod. Shi (1984) believed that *R. sterilis* and *R. roxburghii* are closely related based on morphological similarities. Thus, comparative studies that include *R. roxburghii* as a fertile control may be informative to elucidate the mechanism and developmental timing of male sterility in *R. sterilis*.

Fig. 1. Morphology of *R. sterilis* (a) and *R. roxburghii* (b) fruits.

At present, research on *R. sterilis* has mostly focused on morphology, propagation from cuttings and tissue culture, analysis of aroma components and pharmacological properties, resistance to powdery mildew, and analysis of other biological and chemical components (Yang *et al*., 2016). Little information on the sterility of *R. sterilis* is available. Shi *et al*., (1994) examined pollen morphology of *R. sterilis* by light microscopy and scanning electron microscopy, and observed that *R. sterilis* pollen grains were malformed, empty, and abortive. The cause and timing of pollen grain abortion in *R. sterilis* has not been reported previously. The objective of the present study was to investigate the cytological mechanism of pollen sterility in *R. sterilis*, and examine the causes and timing of pollen grain abortion. In this paper, we report on *R. sterilis* microspore development, anatomical changes in the anther, and the histochemical distribution of polysaccharides, starch grains and protein in paraffin-embedded sections at different stages of anther development. The results indicate that Nuclear matter melted, resulting in pollen abortion of *R. sterilis*, and infers that delayed tapetum degeneration and inadequate nutrient and energy supply to sustain microspore mitosis, result in degradation of the microspore nucleus and pollen abortion.

Materials and Methods

Plant material: *R. sterilis* and *R. roxburghii* were used in this work. The male sterility of *R. sterilis* is natural and stable. Thus, the reproductive system of *R. sterilis* is asexual. *R. roxburghii* was selected as a fertile control because it is a close relative of *R. sterilis* and produces fertile pollen. Plants of *R. sterilis* and *R. -roxburghii* were grown in the grounds of the Guizhou Botanical Garden, Guiyang City, China.

Light microscopy: Stamens of different sizes were collected carefully from plants of *R. sterilis* and *R. roxburghii*. The samples were classified into groups according to stamen length. The anthers were excised and immediately fixed in a solution of 50% alcohol (90ml), glacial acetic acid (5ml), and 30% formaldehyde (5ml) at room temperature. After 3–4 days in the fixative solution, the samples were dehydrated in an alcohol graded series (50%, 70%, 75%, 80%, 85%, 95%, and 100%), cleared in xylene, and embedded in paraffin. Thin sections (8–10 μm) were cut from blocks with a microtome. For anatomical examination, sections were stained with 1% safranin O and fast green (Zhang *et al*., 2011). For detection of polysaccharides, starch and protein, sections were stained in Schiffs reagent, iodine-potassium iodide (1% I-KI_2), udan black B, and Coomassie brilliant blue R250 solutions (Li, 2012). As an indicator of pollen grain viability, fresh pollen of *R. sterilis* and *R. roxburghii* was stained with 1% I-KI_2 and acetocarmine solutions. The stained sections and pollen grains were observed with Olympus SZ 61 photomicroscope under bright field illumination and photographed using Olympus SZ 61 digital camera. Pollen of *R. roxburghii* and *R. sterilis* was stained with 1% I_2-KI and viewed at 20× magnification under a light microscope. Three repeated trials were performed each consisting of 10 fields of view. Thus, the pollen grains within a total of 30 fields of view were counted, each not less than 100 pollen grains. The type and proportion of stained pollen grains were counted.

Results

Development of *R. sterilis* and *R. roxburghii* anthers: Microscopic observation of sections cut from randomly selected blocks permitted the relation between anther size and pollen developmental stage to be established. phases of sporogenous cells (SC) and PMC development in *R. sterilis* were observed in stamens less than 5 mm in length, which contained gametophytic and sporophytic tissues, including (SCs) and PMCs, and the layers of the anther wall (epidermis, endothecium, middle layer and tapetum). The tetrad stage in *R. sterilis* was observed in stamens between 4.5 and 5.3 mm in length. Stamens 5.3–7.0 mm in length showed subsequent stages of microspore development up to themed-microspore and vacuolate microspore (VMP) stages. Mature pollen grains in *R. sterilis* were observed in stamens greater than 7.0 mm in length. Anther development proceeded normally in *R. roxburghii*. At the sporogenous cell stage, differentiation of the four locules in *R. roxburghii* anthers resulted in formation of large archesporial cells containing dense cytoplasm, and in cross section the anthers were butterfly like in shape (Fig. 2a). Periclinal division of the archesporial cells resulted in formation of the outer parietal cells and the (SCs) to the inner (Fig. 2b). The development of *R. sterilis* and *R. roxburghii* anthers at these stages were almost identical (Fig. 2c, d).

Subsequently, in *R. roxburghii* anthers, SCs gave rise to PMCs after several cell divisions. At meiosis, the PMCs were located in the center of the pollen sac. The PMCs were large in volume, deeply stained, with a relatively large nucleus. The PMCs were closely packed. The tapetum was clearly identifiable; the tapetum cells were larger with dense cytoplasm, dark staining, and were binucleate. The middle layer cells were flat and lightly stained. The PMC stage was identical in *R. roxburghii* and *R. sterilis* anthers, with normal meiosis characterized by simultaneous cytokinesis observed. The tapetum cells were not separated from the endothecium, but were retained in situ. The tapetum was of the glandular type (Fig. 2e-g). In meiosis, the PMCs of *R. sterilis* passed through leptotene (Fig. 2h), zygotene (Fig. 2i), pachytene

(Fig. 2j), diplotene (Fig. 2k), and diakinesis (Fig. 2l), with normal chromosome pairing and gradual condensation of the chromosomes, which were shorter and thicker at diakinesis. After spindle formation, the chromosomes were arranged on the equatorial plate in metaphase I (Fig. 2m), late metaphase I (Fig. 2n) and then entered prophase II (Fig. 2o) and anaphase II (Fig. 2p). Finally, the PMCs entered telophase II (Fig. 2q) to complete meiosis. These observations showed that in *R. sterilis* the PMCs undergo normal meiosis.

At the completion of PMCs meiotic division in *R. roxburghii*, tetrad cells (TC) were formed. The arrangement of the tetrad of microspores was tetrahedral. The four microspores were surrounded by callose. Each microspore in a tetrad was densely cytoplasmic with a centrally located, darkly stained, large nucleus. At the tetrad stage, the tapetum cells were characterized by large volume, dark staining, dense cytoplasm, and the karyoplasm was not distinguishable. Subsequently, the tapetum began to degrade (Fig. 2r). The TCs of *R. sterilis* were also tetrahedral, but the tapetum cells of the *R. sterilis* anther were binucleate or trinucleate, and the cells were larger in volume compared with PMCs. Thus, at this stage the tapetum showed no signs of degeneration in *R. sterilis* and, compared with *R.-roxburghii*, disintegration of the tapetum cells was delayed in *R. sterilis* (Fig. 2s).

Subsequently, the microspores were released from the TCs, the mid-microspore stage the microspores were characterized by small cytoplasmic vacuoles and a central nucleus. As the microspore continuously absorbs nutrients from the tapetum cells, the cell volume gradually increased and a large central vacuole gradually formed, forcing the nucleus from the center to the cell periphery; when entering the VMP stage. At the VMP stage, the tapetum of the *R. roxburghii* anther had almost disintegrated (Fig. 2t). In contrast, the tapetum cells of the *R. sterilis* anther were darkly stained with dense cytoplasm, and mononucleate; the tapetum of the *R. sterilis* anther showed the onset of degeneration (Fig. 2u). Thus, disintegration of the tapetum of the *R. sterilis* anther was indicated to occur in the late VMP and mature pollen stages (Fig. 2v).

To identify the peak period of pollen grain abortion, the types of microspores at the VMP and late VMP stages were counted in 30 locules of *R. roxburghii* and *R. sterilis* anthers. At the VMP stage of *R. -sterilis* anther development, mononucleate microspores accounted for 54.2%, non-nucleate microspores 42.5%, and binucleate microspores 3.21% of the total number of microspores. At the late VMP stage of *R. sterilis* anther development, mononucleate microspores accounted for 25.70%, non-nucleate microspores 76.70%, and binucleate microspores 2.57% of the total number of microspores (Table 1, Fig. 3). These data showed that non-nucleate microspores were vastly more frequent than nucleate microspores at the VMP stage of *R. sterilis* pollen development. Thus, it can be inferred that the peak phase of pollen bortion was at the VMP and late VMP stages, and in the mononucleate to binucleate transition period, binucleate microspores were not formed. The mature pollen grain of *R. roxburghii* showed three distinct germinal pores in the pollen wall (Fig. 2t). At pollen maturity the exine was formed, and coincided with complete disintegration of the tapetum. Mature pollen grains contained a vegetative cell and a germ cell (Fig. 2w). The vegetative cell was large in volume, had a larger nucleus and was lightly stained. The germ cells were small in volume, and the nucleus was dense and darkly stained. Almost all mature pollen grains of *R. sterilis* observed were empty, non-nucleate and abortive. Only a small percentage of pollen grains containing nuclei were observed (Fig. 2w, x). At the VMP stage of *R. sterilis* anther development, the chamber was found to contain a vegetative cell and a germ cell of a few pollen grains (Fig. 2y).

Histochemistry of anther development in *R. sterilis* and *R. roxburghii*: During anther development, the distribution of nutritive material (protein, polysaccharides and starch grains) in the anther correlated with different stages of male gametophyte and microspore development. In the *R. roxburghii* anther, starch grains accumulate in the cells of the epidermis, endothecium, and middle layer of the anther wall (Fig. 4a, i, o). However, in the *R. sterilis* anther, very few starch grains accumulated in cells of the anther wall (Fig. 4b, l, r). The distribution of protein and polysaccharides showed no notable differences between the anthers of *R. roxburghii* and *R. sterilis* during meiosis (Fig. 4c-f). During early microspore development of *R. roxburghii* and *R. sterilis*, irregular microspores were formed At this stage the tapetum cells of the *R. roxburghii* anther had become vacuolated and were lightly stained, implying that disintegration the of tapetum had begun, and staining indicated that amounts of protein, polysaccharides, and starch grains in the tapetum cells were decreased (Fig. 4g-i). The tapetum cells of the *R. sterilis* anther were not vacuolated and were darkly stained, implying that the tapetum was not degraded and contained ample amounts of protein, polysaccharides, and starch grains (Fig. 4j-l). At an advanced stage of microspore development the microspore forms vacuoles, and the microspore gradually becomes spherical. At this stage the tapetum of the *R. roxburghii* anther had almost completely disintegrated, and protein, polysaccharides and starch grains accumulated in the microspores (Fig. 4m-o). In contrast, the tapetum of the *R. sterilis* anther showed no obvious changes, and protein, polysaccharides and starch grains did not accumulate in the microspores (Fig. 5p-r). At the mature pollen stage, the tapetum of the *R. roxburghii* anther had degenerated completely, and large amounts of protein, polysaccharides, and starch grains had accumulated in the mature pollen grains (Fig. 4s-u), which indicated that *R. roxburghii* pollen grain development was normal. However, the *R. -sterilis* pollen grains showed almost no accumulation of protein, polysaccharides and starch grains (Fig. 4v-y). These results indicated that the lack of accumulation of nutritive material in *R. sterilis* pollen grains may be one factor leading to pollen abortion, and may be associated with the delayed disintegration and metabolic disturbance of the tapetum.

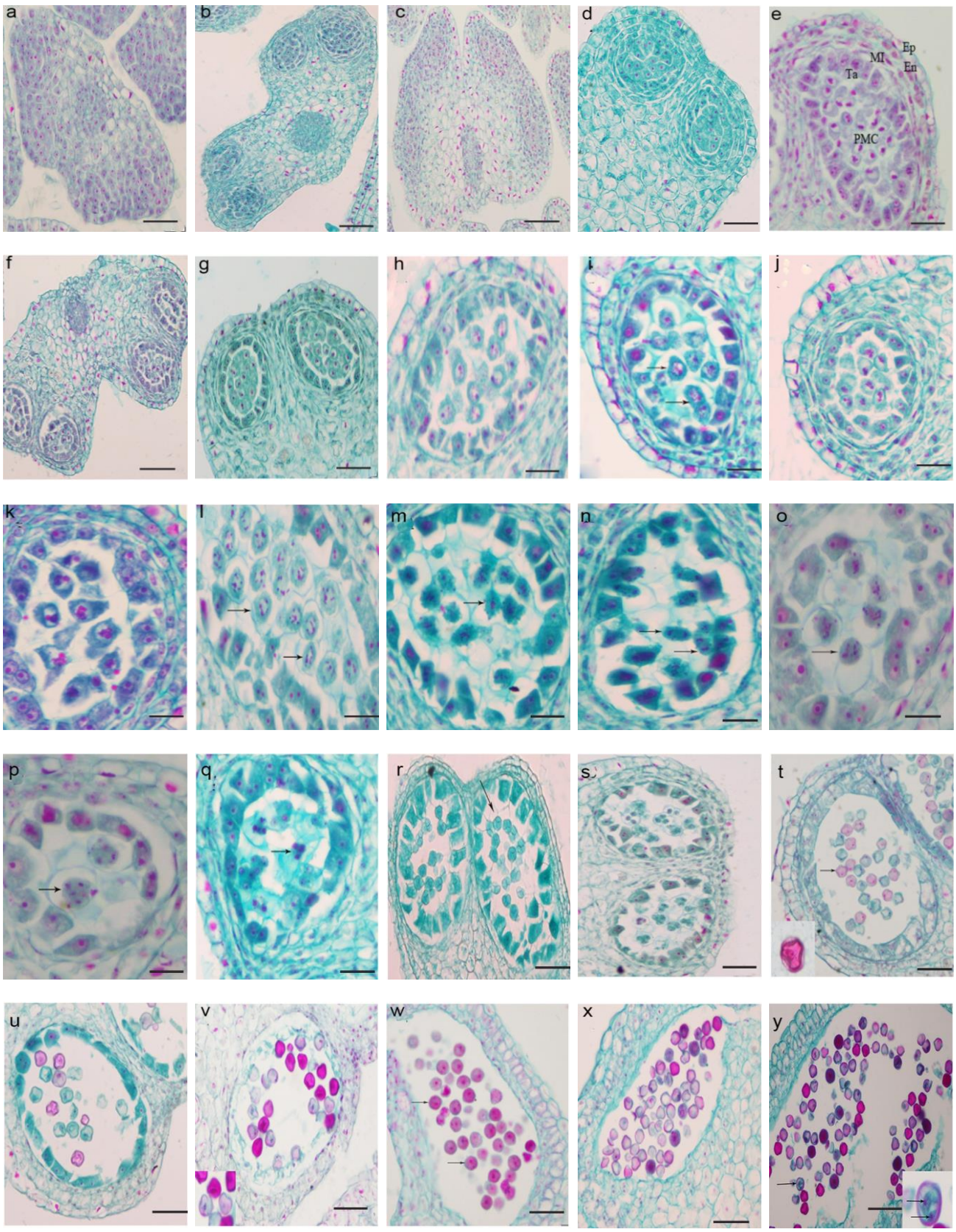

Fig. 2. Stages of microspore development in *R. roxburghii* and *R. sterilis*. Paraffin-embedded sections stained with safranin O and fast green were prepared from anthers of the *R. roxburghii* (a, b, e, r, t, w) and the *R. sterilis* (c, d, f-q, s, u, v). The following developmental stages are shown: (a-d) sporogenous cells, (e-q) pollen mother cells, (r, s) tetrad cells, (t-v) vacuolated microspores (VMP), (y) late vacuolated microspores, (w, x) mature pollen grain. At the tetrad stage, the tapetum of the *R. roxburghii* anther began to disintegrate (r), whereas the tapetum of the *R. sterilis* anther showed no sign of disintegration (s). This phenomenon extended to the VMP stage (t-v). A pollen maturity, the *R. sterilis* anther contained obviously non-nucleate pollen (w, x). At late vacuolated microspores stage, the *R. sterilis* anther contained a pollen grains that has a vegetative cell and a germ cell (y). bar = 10 μm.

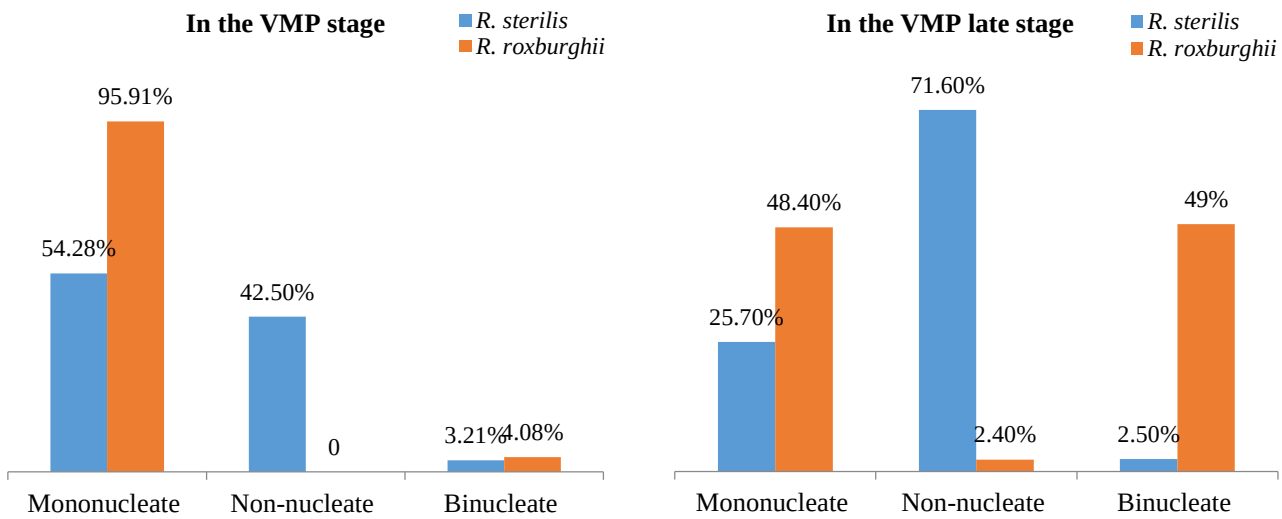

Fig. 3. Microspore cell type ratio in *R. roxburghii* and *R. sterilis* anthers at the vaculoate microspore (VMP) stage of pollen development.

Table 1. Frequency of microspore type in *R. roxburghii* and *R. sterilis* anthers at the vaculoate Microspores (VMP) and late VMP stages of pollen development.

Material	VMP				Late VMP			
Name	Mononucleate	Non-nucleate	Binucleate	total	Mononucleate	Non-nucleate	Binucleate	total
R. sterilis	304	238	18	560	240	668	24	932
R. roxburghii	892	0	38	930	280	14	284	578

Stainability of *R. sterilis* and *R. roxburghii* pollen: *R.roxburghii* pollen grains stained purple with 1% I_2-KI solution and 98% of the pollen grains were stained and of normal appearance (Fig. 5a). In contrast, 95.5% of *R. sterilis* pollen grains, were not stained (Fig. 5b). Pollen grains of *R. roxburghii* were strongly stained with acetocarmine (Fig. 5c), whereas *R. sterilis* pollen grains were not stained with acetocarmine, indicating that the grains were non-nucleate and abortive (Fig. 5d).

Discussion

We used light microscopy to examine the anatomical development of the anther of *R. sterilis* with the aim of gaining insights into the mechanism and timing of pollen abortion during anther development. The cause and timing of pollen grain abortion in *R. sterilis* has not been reported previously. The present report gives a complete and detailed analysis of *R. sterilis*.

In the process of microspore development, any gene or gene system abnormality may lead to male sterility. Laser and Lersten 1972 reported that the forms and timing of pollen abortion in angiosperm male-sterile lines are diverse. Male-sterility may be expressed during microspore formation before abnormal phenomena are detectable, such as premature disintegration of the tapetum or premature disintegration of the callose wall of PMCs. The meiotic process in PMCs may be abnormal, and pollen abortion may occur prior to tetrad formation. In some male sterile plants pollen abortion occurs during microspore development after TC release, or during the binucleate and trinucleate pollen stages (Yang & Li, 1984; Liu *et al*., 1995). In environmentally induced genetic male-sterile rice (Ku *et al*., 2003) and PET1 CMS in sunflower (Balk & Leaver, 2001). There is a lot of research on wheat, Hussain research shows that soil that K application may increase Zn accumulation in wheat grown on calcareous saline-sodic soils (Hussain *et al*., 2001), and Including studies on wheat male sterility, pollen development aborts after meiosis, and pollen abortion in the thermo-sensitive male -sterile wheat line BNS366 (He *et al*., 2014) occurs at the late mononucleate stage. The onset of pollen abortion is critical to understanding mechanisms of control for male sterility in higher plants. In the present study, *R. sterilis* anthers undergo normal meiosis and the microspores are released after the tetrad phase. However, most of the microspores at the VMP and late VMP stage do not form binucleate microspores, but instead undergo nuclear degradation, resulting in a proportion of 76.7% non-nucleate microspores. Staining of mature pollen with 1% I_2-KI, solution showed that the proportion of abnormal pollen grains was as high as 95.5%. Staining of mature pollen with acetocarmine revealed the presence of non-nucleate showed microspores. The present results suggested that binucleate microspore formation failed before nuclear degradation at the VMP and late VMP phases leading to non-nucleate microspore generation, which thus may be the main reason for male sterility in *R. sterilis*.

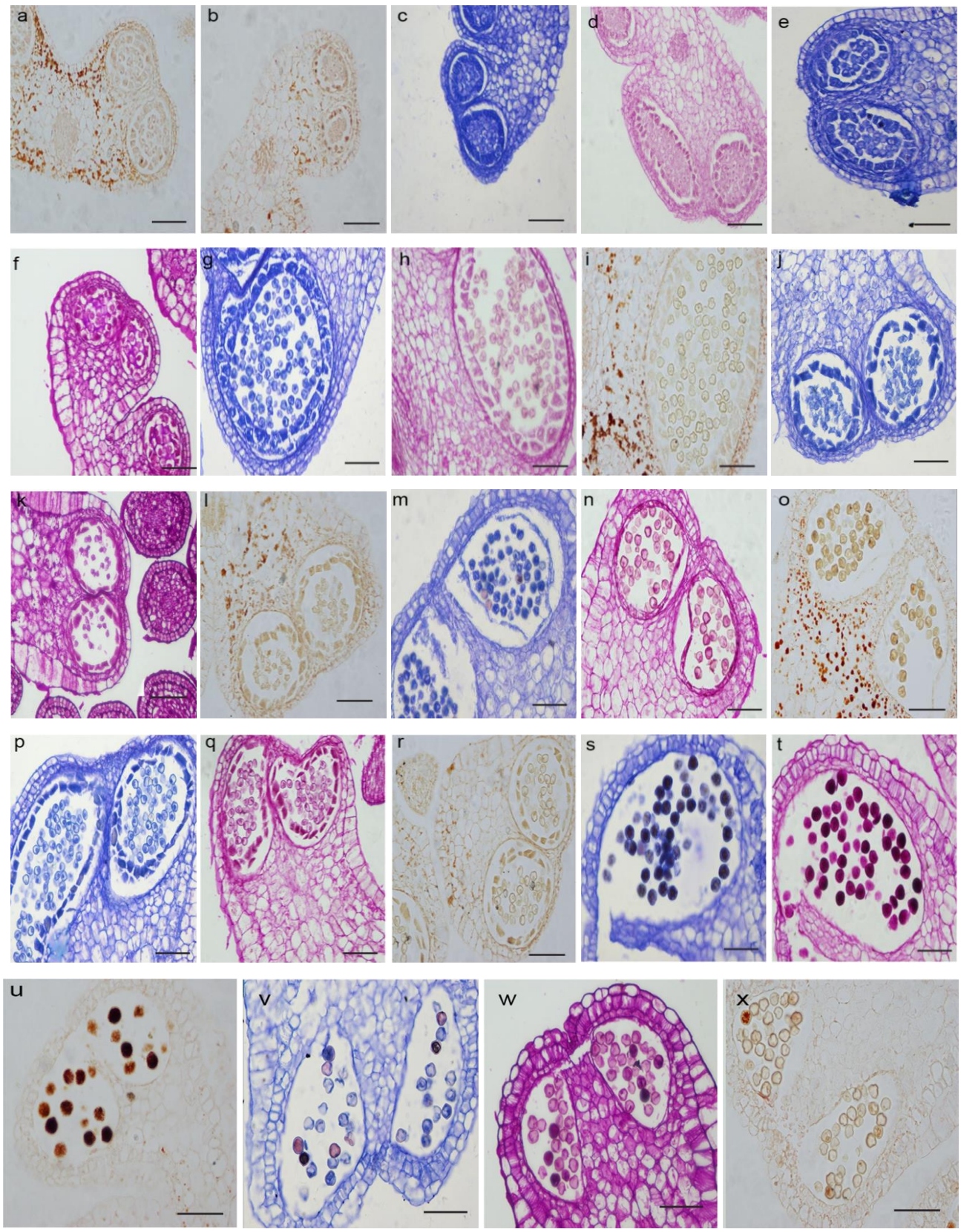

Fig. 4. Distribution of nutritive materials during anther development in *R. roxburghii* and *R. sterilis*. Paraffin-embedded sections were stained with Schiffs reagent for *R. roxburghii* (d, h, n, t) and *R. sterilis* (f, k, q, w), I-KI_2 solution for *R. roxburghii* (a, i, o, u) and *R. sterilis* (b, l, r, y). Coomassie brilliant blue R250 for *R. roxburghii* (c, g, m, s) and *R. sterilis* (e, j, p, v). The following developmental stages are shown pollen mother cells (PMC) stage (a-f), early microspore stage (g-l), late microspore stage (m-r) and mature pollen stage (s-x,). At the PMC stage, starch grain accumulation in the endothecium of *R. sterilis* was notably less than that in *R. roxburghii* (a, b). At the early microspore stage, tapetum disintegration was delayed in *R. sterilis* compared with *R. roxburghii* (g-l). This phenomenon extended to the late microspore stage (m-r). At the mature pollen stage, *R. sterilis* pollen lacked accumulation of protein (v), polysaccharides (w), starch grains (x). bar = 10 μm.

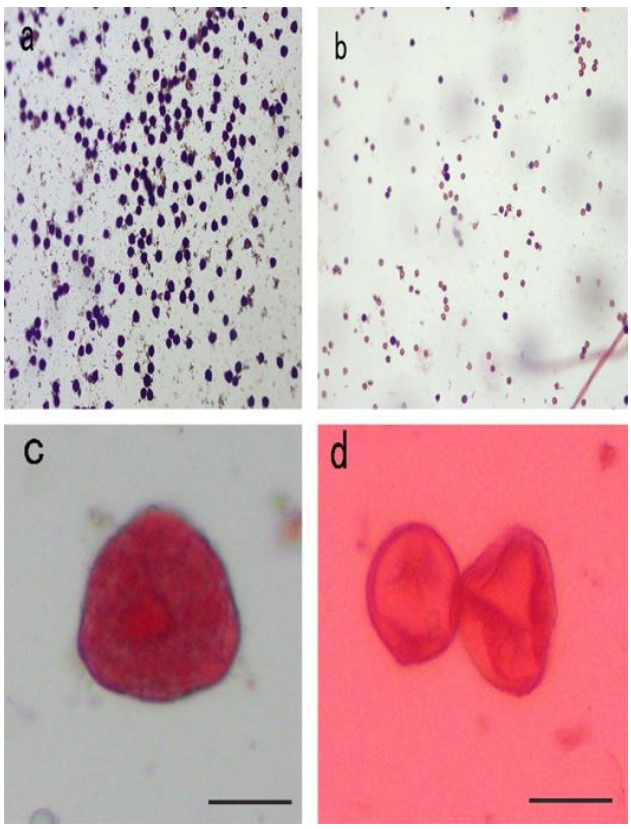

Fig. 5. Stainability of *R. roxburghii* and *R. sterilis* pollen grains. Pollen grains of *R. roxburghii* pollen stained with 1% I_2-KI solution (a) and acetocarmine (c). Pollen grains of *R. sterilis* stained with 1% I_2-KI solution (b) and acetocarmine (d). bar = 2 μm.

In the process of microspore development, at the VMP phases, the mononuclear of microspore was moved to the edge of the pollen wall, its late VMP phases, mononuclear microspore is further divided into mitosis to form mature pollen. In the late VMP phase, RNA synthesis and chromosome doubling occur, and the nucleus undergoes a mitotic division. A series of important life activities within the cell must have strong physiological activity as the foundation, after pollen mature phases. Pollen grains accumulate a large amount of nutritive materials, of which the most prominent are starch and fatty acids, indicating that pollen grains show high basal nutrient metabolism. In this phase, decreased protein synthesis causes a lack of protein accumulation, and leads to decline in the number and type of enzymatic activities. Ultimately, it may cause failure of DNA synthesis and chromosome doubling, and cessation of mitosis. The starch content is an important manifestation of pollen vitality. A low rate of starch synthesis and obstruction of carbohydrate transport can lead to severe deficiency in the nutritive materials and energy needed to maintain pollen vitality and promote pollen germination. Thus, inadequate nutrient supply will disrupt the normal development of pollen, which will be unable to enter the gametophytic development stage and remain at the late VMP phase. Finally, pollen abortion is induced (Liu, 1992). In the late VMP phase of anther development in *R. sterilis* predominantly non-nucleate pollen was formed. However, in this period extremely rare microspores containing a vegetative cell and a germ cell were observed (Fig. 2y), and protein, polysaccharides, and starch grains failed to accumulate in *R. sterilis* pollen grains as indicated by histochemical observations. Thus, we speculate that *R. sterilis* pollen abortion may be due to inadequate supply of nutrient materials for normal mitosis, resulting in degradation of the nucleus and pollen abortion.

The tapetum is the innermost cell layer of the anther wall, and is responsible for the transpfer of nutritive substances to PMC, thereby playing an important role in the normal development of PMCs and microspores (Zhang *et al*., 1996; Pacini, 1997). One important function of tapetal cells was to provide nutrients substance for the development of microspore (Wu *et al*., 1997). Many researchers have attributed male sterility to Tapetum abnormality (Laser & Lersten, 1972). In the present study, at the meiosis stage, the tapetum of *R. roxburghii* and *R. sterilis* anthers showed no obvious difference, but at this stage considerably fewer starch grains accumulated in the endothecium of the *R. sterilis* anther compared with that of the *R. roxburghii* anther. At the tetrad stage, the tapetum of the two species begun to show differences, with the *R. sterilis* anther showing delayed disintegration of the tapetum. At the mature pollen stage, nutritive materials (protein and polysaccharides) failed to accumulate in *R. sterilis* pollen grains. One cause of pollen abortion might be delayed disintegration of the tapetum, which lead to assimilates translocation protein and polysaccharides. Compared with *R. roxburghii*, in the *R. sterilis* anther very few starch grains accumulated in cells of the anther wall. Starch grains content of *R. sterilis* was low because of its low synthesis (Fig. 5b, l, r). The microspore may enter the binucleate stage from the mononucleate stage because of delayed degradation of the tapetum and low starch grains synthesis, which fails to provide nutrients to the microspore and leads to nuclear degradation.

According to the concept of classical genetics, the occurrence of male sterility in plants is the result of interaction between gene and environment. However, its male sterility was stable, and Unaffected by the environment. Thus, In the study of the male sterility mechanism of *R. sterilis*, we can exclude its specific expression of gene regulation because of its environmental impact, and can accurately locate the function gene that causes male sterility and cause male sterility. Thus, Additional research is required to test this hypothesis and to elucidate the origin and molecular mechanism of male sterility in *R. sterilis*.

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