Postprint: Dynamic Morphological and Cellular Developmental Changes in Microsporangiate Strobili of Male-Sterile and Fertile Lines of Slash Pine

Authors: Zhou Yang, Yang Zhangqi, Huang Yongli

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Abstract

To investigate morphological variation during the development of microsporangiate strobili in slash pine 'Songtai', clarify its abortion process, pattern, and influencing factors, and provide a scientific basis for the utilization of male-sterile slash pine varieties and future related research, we used the 'Songtai' s10 sterile line and s9 fertile line as materials to observe morphological development changes of microsporangiate strobili and prepared paraffin sections for observation of microspore development under a light microscope. The results showed that the s10 sterile line and s9 fertile line exhibited no significant differences before microspore mother cell meiosis, with consistent growth trends of microsporangiate strobili. At the tetrad stage, s10 microspore cell development became abnormal, and microsporangiate strobilus morphological development also exhibited abnormalities, with synchronous abnormal development. The fertile line required approximately 5 d to develop from the tetrad to uninucleate microspore stage, whereas the sterile line continued development for approximately 20 d, lasting 4 times longer. During this period, abnormal tapetum cell development with slow degradation, disorganized microsporangial wall tissue arrangement, and delayed degradation were observed. Finally, s10 formed abnormal binucleate pollen without pollen release. Therefore, we infer that the main causes of s10 microspore abortion are: abnormal microsporangial wall cell development, abnormal microsporangiate strobilus morphology, correspondingly abnormal tapetum development at the tetrad stage, inability to timely secrete callase to degrade the callose wall surrounding the tetrad, inability to timely synthesize and transport energy substances required for pollen formation, and delayed degradation and accumulation of wall cells. This series of abnormal changes prevented normal tetrad formation, thereby causing pollen abortion.

Full Text

Dynamic Changes of Microstrobilus Morphology and Cell Development in Male Sterile and Fertile Lines of *Pinus elliottii*

ZHOU Yang[1], YANG Zhangqi[1,2,4,5]*, HUANG Yongli[3]
[1]College of Forestry, Guizhou University, Guiyang 550025, China
[2]Guangxi Forestry Research Institute, Nanning 530002, China
[3]Nanning Forestry Research Institute of Guangxi, Wuming 530107, Guangxi, China
[4]Masson Pine Engineering Technology Research Center of Guangxi, Nanning 530002, China
[5]Key Laboratory of Central South Fast-growing Timber Breeding, National Forestry and Grassland Administration, Nanning 530002, China

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Abstract

To understand morphological differences during microstrobilus development in *Pinus elliottii* 'Songtai', clarify its abortion process, mechanism, and influencing factors, and provide a scientific basis for utilizing male sterile varieties and future research, we examined morphological changes in microstrobili using the 'Songtai' s10 abortive line and s9 fertile line as materials. Paraffin sections of microstrobili were prepared and microspore development was observed under optical microscopy. The results showed no significant differences between the s10 abortive line and s9 fertile line before microspore mother cell meiosis, with both showing identical growth trends. During the tetrad stage, abnormal microspore cell development occurred in s10, accompanied by abnormal microstrobilus morphology, with synchronous abnormal development in both aspects. The fertile line required approximately 5 days to develop from tetrad to mononuclear microspore stage, whereas the abortive line continued developing for about 20 days—four times longer. During this period, abnormal tapetum cell development with slow degradation, disorganized microsporangial wall tissue arrangement, and delayed degradation were observed. Ultimately, s10 formed abnormal dinuclear pollen without pollen release. We conclude that the primary cause of microspore abortion in s10 is abnormal microsporangial wall cell development and abnormal microstrobilus morphology. Specifically, the tapetum develops abnormally during the tetrad stage, failing to secrete callase at the appropriate time to degrade the callose wall surrounding tetrads and unable to timely synthesize and transport energy substances required for pollen formation. Simultaneously, cyst wall cells show delayed degradation and lamination. These series of abnormalities prevent normal tetrad formation, resulting in pollen abortion.

Keywords: *Pinus elliottii*, microstrobilus, male sterility, cell development, morphological changes

Introduction

Pinus elliottii (slash pine), native to low-altitude regions of the southeastern United States with warm, humid, and rainy climates below 600 m elevation, is a large evergreen tree (Zheng & Fu, 1978; Zhu, 1993). China first introduced *P. elliottii* in the 1930s, establishing the first primary seed orchard in Taishan County, Guangdong Province in 1964 (Tang, 2015). Since large-scale promotion began in the 1970s, *P. elliottii* has been distributed across a wide geographic range in China, from Tunchang County in Hainan Island (110°26' E, 19°22' N, mean annual temperature 23.16°C) to Qingdao on the Shandong Peninsula (120°25' E, 36°09' N, mean annual temperature 11.9°C) and Yantai (122° E, 37°25' N, mean annual temperature 12°C), extending westward to Yunnan Province (Pan et al., 1994). Currently, the planted area of *P. elliottii* in China exceeds 3 million hectares, making it one of the ten dominant tree species in artificial forests (Zhang, 2017).

Male sterility (MS) is a common genetic phenomenon in higher plants, referring to the inability to produce functional pollen, anthers, or male gametes (Fan et al., 2016). First proposed by Coleman in 1876, male sterility has been documented in 320 species across 162 genera and 43 families (Kaul, 1988; Wise & Pring, 2002). Current research primarily focuses on economic crops with comprehensive and in-depth studies, while forest trees present greater complexity due to long breeding cycles, scarcity of sterile materials, extended growth periods, and difficulty in post-growth observation, resulting in relatively limited literature and shallow research depth. Studies on male sterility in gymnosperms such as Pinaceae are particularly rare.

The 'Songtai' variety originates from Florida *P. elliottii* provenances. In the 1980s, the National Exotic Pine Cooperative Group conducted provenance trials, and based on results from the Guangxi trial site, a first-generation improved *P. elliottii* clonal seed orchard was established at the Nanning Forestry Science Institute between 1992-1993. Through five consecutive years of phenological observations, the team found that 'Songtai' exhibited no pollen release, demonstrating male abortion characteristics (Tang, 2015). This study utilized the abortive line 'Songtai' s10 and fertile line s9 from this first-generation improved seed orchard to observe morphological development of microstrobili and structural development of microspores and their wall cells, tracking the abortion process, mechanism, and timing. The objectives were to provide a foundation for understanding the abortion mechanism of male sterility in *P. elliottii* and to enrich theoretical knowledge on sterility mechanisms in forest trees, thereby providing a basis for pine hybrid breeding and heterosis utilization.

1.1 Experimental Materials

All experimental materials were collected from 25-year-old trees of 'Songtai' s10 abortive line and s9 fertile line in the first-generation improved *P. elliottii* seed orchard at the Nanning Forestry Science Institute, Guangxi. The seed orchard is located at 108°00' E, 23°10' N within the southern margin of the south subtropical monsoon climate zone, with an average annual temperature of 21.5°C, annual precipitation of 1,246 mm, annual evaporation of 1,613.8 mm, and a frost-free period of 358 days. The site features distinct wet and dry seasons with average relative humidity of approximately 79%. Established on gentle hill terraces among limestone peak forests, the orchard sits at approximately 120 m elevation with deep lateritic red soil (pH 5.5-6.5).

1.2 Research Methods

Three sample trees each of 'Songtai' s10 abortive line and s9 fertile line were selected in the seed orchard. Sampling began on December 13, 2018 (when microstrobili emerged abundantly) and continued until abortive line microstrobili abscised (February 13) and normal line microstrobili completed pollen shedding (mid-February). Before January 23, 2019, samples were collected at 6-7 day intervals; from January 23 to February 7, sampling intervals were 3-4 days. At least 15 microstrobili were collected per tree, fixed in FAA fixative in 50 mL centrifuge tubes, labeled, and stored at 4°C for later use.

1) Microstrobilus Morphological Observation: Representative microstrobili were selected according to sampling dates to record external morphological changes. For each sampling, 30 microstrobili were randomly selected to measure length and width, with dynamic trends recorded and analyzed.

2) Microspore and Wall Tissue Development Observation: Conventional paraffin sectioning methods were employed, including Ehrlich's hematoxylin staining, dehydration, clearing, paraffin infiltration, embedding, sectioning, and mounting (Ye et al., 2011). Microsporophylls were cross-sectioned (5 m thickness), mounted with neutral balsam, and representative, clear sections were selected for observation using an automated optical microscope (Axio Imager Z2m) to document developmental changes at different stages.

2 Results and Analysis

2.1 Dynamic Changes in Microstrobilus Growth of 'Songtai' s10 and s9

Through two months of continuous sampling, growth trends of 'Songtai' s10 and s9 microstrobili are shown in [Figure 1: see original paper]. Before mid-January, both abortive and fertile lines showed slow individual growth with no significant differences in strobilus length and width between s10 and s9, indicating similar growth rates and trends. In late January, microstrobili entered a rapid growth phase with stem length increasing dramatically, and differences in stem length

growth between s10 and s9 became apparent, becoming more pronounced over time. We hypothesize that microspore abortion in s10 occurred before late January, which was confirmed through morphological and subsequent histological observations. After February, s9 microstrobilus growth slowed while s10 microstrobili ceased development by early February, becoming dry and shriveled with reduced size. The difference in stem length between s10 and s9 increased sharply, reaching maximum divergence by mid-February when microstrobili abscised. However, no significant differences in width were observed between s10 and s9 from initiation to abscission.

2.2 Morphological Development Comparison Between 'Songtai' s10 Abortive Line and s9 Fertile Line

Morphological observations revealed that 'Songtai' s10 abortive line began showing abnormal withering and loosening at the microstrobilus apex by mid-January, most pronounced by late January, without pollen release. Concurrently, s9 microstrobili developed normally. We therefore infer that s10 microstrobilus abortion began in mid-January, coinciding with the appearance of apical withering. Detailed temporal comparisons are as follows:

In mid-December, microstrobili clustered in leaf axils, wrapped in scales that were yellow-brown and oval-shaped ([Figure 2: see original paper]: a, s9), identical to s9 development with no abnormalities ([Figure 2: see original paper]: A, s10). By late December, apices emerged from scales with microsporophylls appearing purple-black ([Figure 2: see original paper]: b, s9). In early January, slow development produced conical shapes with purple-black microsporophylls ([Figure 2: see original paper]: C, s9), while s10 showed apical emergence with purple-black microsporophylls ([Figure 2: see original paper]: B, s10). By mid-January, slow development continued conically with purple-black microsporophylls showing loose, non-plump arrangement ([Figure 2: see original paper]: C, s10). In late January, s9 microstrobili grew rapidly beyond scales with microsporophylls arranged in rows or decussate pairs on the axis, tightly packed and light purple ([Figure 2: see original paper]: d, s9). In contrast, s10 microstrobili emerged slowly with similar microsporophyll arrangement but more pronounced apical withering, appearing light purple ([Figure 2: see original paper]: D, s10). In early February, s9 microsporophylls swelled plumply, the strobilus became soft and yellow, initiating pollen shedding ([Figure 2: see original paper]: e, s9), while s10 microstrobili appeared completely withered and shriveled, purple-red at the apex and pale yellow at the base, beginning to abscise without pollen release ([Figure 2: see original paper]: E, s10). By mid-February, s9 was in late pollen shedding stage with massive shedding ([Figure 2: see original paper]: f, G, s9), while s10 microstrobili were severely shriveled, significantly smaller than the fertile line, abscising in large numbers without pollen release ([Figure 2: see original paper]: F, G, s10).

2.3 Cross-Sectional Observation of 'Songtai' s10 Abortive Line and s9 Fertile Line Microstrobili

Gymnosperm pollen (male gametophyte) develops within microsporangia (Lin, 2013). Microspore development progresses through primary sporogenous tissue, sporogenous tissue, microspore mother cell, microspore mother cell meiosis, microspore tetrad, mononuclear, dinuclear, and pollen shedding stages (Lin, 2013; Sanchez, 2018). Cross-sectional observations ([Figure 3: see original paper], [Figure 4: see original paper]) revealed that *P. elliottii* microstrobilus development from sporogenous cell appearance to pollen shedding completion requires approximately 60 days. Compared with s9, s10 showed abnormal microspore development at the tetrad stage, with some microspores visibly shrunken and deformed, and asynchronous development within the same microstrobilus. Microsporangial wall tissues also showed clear differences at the tetrad stage, with abnormal tapetum cell arrangement and slow degradation in s10, while middle layer and endothecium showed lamination and delayed degradation during the mononuclear microspore stage. Detailed stage comparisons follow:

Primary sporogenous tissue stage: Initial microstrobilus development before mature microsporangia formation ([Figure 3: see original paper]: a, s9), identical to s9 development ([Figure 4: see original paper]: A, s10). **Sporogenous tissue stage:** Each microsporophyll contained two microsporangial cavities in a concentric symmetric structure. Microsporangial layers were distinct, from inner to outer: sporogenous cells, tapetum, middle layer, endothecium, and epidermis. Sporogenous cells and tapetum were tightly arranged with dense cytoplasm and full staining; middle layer, endothecium, and epidermis were orderly arranged around the tapetum with large, clearly stained nuclei ([Figure 3: see original paper]: b1, b2, s9), identical to s9 development with clear concentric structure ([Figure 4: see original paper]: B, s10). **Microspore mother cell stage:** Mother cells enlarged, rounded, with large, deeply stained nuclei and a non-staining vacuolated region around the nucleus. Tapetum cells underwent nuclear division, became binucleate, enlarged, with dense, deeply stained cytoplasm, arranged around microspore mother cells. Middle layer and endothecium cells elongated with deeply stained nuclei; epidermal nuclei began disappearing ([Figure 3: see original paper]: c, s9). s10 showed smaller cavity volume than s9 but otherwise identical cell development ([Figure 4: see original paper]: C, s10). **Microspore mother cell meiosis stage:** Mother cells were scattered in microsporangial cavities undergoing asynchronous meiosis, with tapetum tightly surrounding them ([Figure 3: see original paper]: d, s9). In s10, meiosis occurred with scattered microspores; tapetum surrounding microspores appeared stretched. Within the same microstrobilus, asynchronous developmental stages were observed from apex to base: tetrad stage, meiotic metaphase/anaphase, and interphase ([Figure 4: see original paper]: D, s10). **Microspore tetrad stage:** Mother cells formed tetrads surrounded by callose walls, scattered in cavities. Tapetum remained around tetrads with lighter staining. Microsporangial wall cells grew rapidly, cavity volume increased significantly, middle layer

and endothecium elongated and degraded, and epidermis became cutinized and thickened ([Figure 3: see original paper]: e1, e2, s9). In s10, tetrads formed with callose walls, but some microspores were visibly shrunken and deformed. Tapetum arrangement was irregular and stretched with deeply stained nuclei. Wall cells grew rapidly with increased cavity volume ([Figure 4: see original paper]: E1, E2, s10). **Mononuclear stage:** Callose released mononuclear microspores with clearly stained nuclei and two air sacs. Tapetum stained lightly and began degrading; middle layer and endothecium continued degrading ([Figure 3: see original paper]: f1, f2, s9). In s10, asynchronous development occurred within the same microstrobilus: (1) irregular mononuclear pollen formed with air sacs; tapetum degraded noticeably with disorganized arrangement; middle layer and endothecium showed lamination with delayed degradation and visible nuclear staining; (2) no mononuclear pollen formed, only bizarre, shriveled residues in empty cavities ([Figure 4: see original paper]: F1, F2, s10). **Dinuclear stage:** Dinuclear pollen formed with prominently inflated air sacs; tapetum degraded but retained residues; middle layer degraded completely and became vacuolated ([Figure 3: see original paper]: g1, g2, s9). In s10, asynchronous development occurred: (1) mononuclear microspores disintegrated and fragmented, undergoing abortion, though a few abnormal dinuclear pollen formed with air sacs; tapetum degraded with some residues; middle layer and endothecium cells swelled, vacuolated, and began massive degradation; (2) microsporangial cavities formed empty sacs with only residues ([Figure 4: see original paper]: G1, G2, s10). **Early and late pollen shedding stages:** Cavities filled with mature dinuclear pollen, tapetum, middle layer, and endothecium completely disappeared, epidermis cracked, and pollen shedding began ([Figure 3: see original paper]: h1, h2, s9). Late stage showed completed shedding with only residual sac walls ([Figure 3: see original paper]: i, s9). In s10, no normal pollen was released, with asynchronous development: (1) some abnormally mature pollen formed with shrunken, non-plump air sacs; tapetum, middle layer, and endothecium disappeared but epidermis did not crack; (2) no pollen formed in microsporangial cavities, only shriveled residues ([Figure 4: see original paper]: H1, H2, s10).

2.4 Comparison of Microstrobilus and Microspore Development Between s10 and s9

Combined morphological and histological observations revealed no significant differences between s10 and s9 before microspore mother cell meiosis, with identical growth trends. At the tetrad stage, s10 showed abnormal microspore cell development concurrent with abnormal microstrobilus morphology, indicating synchronized external and cellular abnormalities. The most significant differences occurred from tetrad to mononuclear microspore stage: s9 microstrobili grew rapidly and developed quickly until pollen shedding, while s10 grew slowly with delayed development, showing withering and shriveling until abscission. We therefore hypothesize that the tetrad to mononuclear microspore stage represents the critical abortion period for s10. The detailed developmental timeline

comparison is shown in .

TABLE:1 Comparison of microstrobilus and microspore development between s10 and s9

Sampling Date	s9 Fertile Line	s10 Abortive Line
13 Dec	Wrapped in scales	Wrapped in scales
19 Dec	Scales begin to show at top	Wrapped in scales
26 Dec	Scales begin to show at top	Scales begin to show at top
2 Jan	Microsporophylls slowly emerge from scales	Microsporophylls slowly emerge from scales
23 Jan	Microspore mother cells appear; microstrobili grow slowly	Microspore mother cells appear; microstrobili grow slowly
26 Jan	Meiosis; rapid growth	Meiosis; apical withering begins
2 Feb	Tetrad and mononuclear pollen; rapid growth and pollen shedding begins	Tetrad and abnormal mononuclear microspores; slow growth
7 Feb	Mononuclear and dinuclear pollen; epidermis cracks, pollen shedding	Abnormal mononuclear pollen, empty capsule formation; shriveling and abscission begins
13 Feb	Complete pollen shedding and massive abscission	No pollen release, massive abscission

3 Discussion and Conclusion

3.1 Discussion

Our results demonstrate that 'Songtai' microstrobili began showing apical withering and loosening abnormalities from mid-January. During this period, a single microstrobilus exhibited asynchronous development from apex to base (tetrad stage at apex, meiotic metaphase/anaphase in middle, meiotic interphase at base). Abnormalities during tetrad release of mononuclear microspores caused abortion. We conclude that 'Songtai' microstrobilus abortion initiates at the tetrad stage, consistent with Kaul (1988), who statistically analyzed multiple male sterility types and found that 70% of materials aborted after

the tetrad stage, though abortion can occur from sporogenous cell to dinuclear pollen stages.

During subsequent development, two abortion patterns coexisted within the same microstrobilus: (1) in upper portions, callose failed to release mononuclear microspores, which directly degraded and shriveled, forming flocculent material with the tapetum to create empty sacs that persisted until abscission without epidermal cracking; (2) in lower portions, tetrads released microspores after callose dissolution, forming abnormal mononuclear microspores that continued development. This dual abortion pattern within a single microstrobilus has also been reported in Chinese fir (*Cunninghamia lanceolata*) (Lin, 2013). Lü et al. (1999) identified three abortion types in Chinese fir: microsporangium absence, middle layer hyperplasia before meiosis I, and middle layer hyperplasia after meiosis I, concluding that abnormal middle layer and endothecium lamination and delayed degradation, along with non-cracking epidermis, affect subsequent microspore development and cause abortion.

The tapetum, the innermost anther wall layer surrounding microspores, is metabolically active in synthesis and secretion, rich in nutrients, and serves as the final transfer station for material transport, playing a critical role in normal pollen development (Fan, 2016; Tian et al., 2017). Our study showed that 'Songtai' microstrobili were normal before the tetrad stage compared with the fertile line. However, at the tetrad stage, abnormalities emerged with slow callose dissolution and delayed tapetum and middle layer degradation. The fertile line required approximately 5 days from tetrad to mononuclear microspore stage, while the abortive line needed about 20 days—four times longer. During this period, the abortive line's tapetum developed slowly with delayed degradation compared to the fertile line, preventing timely secretion and conversion of energy substances required for microspore development, thereby causing abortion. This aligns with Tang (2015) on *P. elliottii* microstrobilus abortion and Liu et al. (2019) on poplar male sterility, where delayed tapetum degradation caused microspore abortion. Similar abnormal tapetum and middle layer development has been reported in pear (Tan et al., 2013; Li, 2007), wheat (Zhao et al., 2015), cotton (Kong, 2017), and other plants. Additionally, slow callose degradation in the abortive line prevented or delayed mononuclear microspore release, directly affecting normal development. Since callose synthesis occurs during microspore mother cell meiosis and degradation happens during tetrad microspore release, abnormalities in any step—synthesis, accumulation, or degradation—may cause abortion (Yang & Zheng, 2013; Down et al., 2008; Wan et al., 2011).

3.2 Conclusion

Cytological observation of microspore development is crucial for understanding male abortion pathways in *P. elliottii* and forms the research foundation for utilizing male sterile families in hybrid breeding. Using 'Songtai' s10 abortive line and s9 fertile line, we observed dynamic morphological and cellular devel-

opment of microstrobili. No significant differences existed between s10 and s9 before microspore mother cell meiosis, with identical growth trends. However, at the tetrad stage, 'Songtai' showed abnormal microspore tissue development concurrent with visible microstrobilus withering, indicating synchronized external morphological and cellular abnormalities. Furthermore, the abortive line required significantly longer development from tetrad to mononuclear microspore stage compared to the fertile line, during which abnormal tapetum cell development with slow degradation and disorganized, delayed microsporangial wall tissue degradation occurred. Ultimately, s10 formed abnormal dinuclear pollen without pollen release. We conclude that the primary cause of s10 microspore abortion is abnormal microsporangial wall cell development and abnormal microstrobilus morphology. Specifically, the tapetum develops abnormally at the tetrad stage, failing to timely secrete callase to degrade the callose wall surrounding tetrads and unable to transport energy substances required for pollen formation, while cyst wall cells show delayed degradation and lamination. These abnormalities prevent normal tetrad formation, resulting in pollen abortion.

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