Studies on the Reproductive Biology and Endangerment Mechanism of the Endangered Plant Manglietia aromatica

PAN Yue-Zhi, LIANG Han-Xing, GONG Xun*
(Kunning Institute of Botany, The Chinese Academy of Sciences, Kunning 650204, China)

Abstract: The embryogenesis, pollen germination, floral character and seed physiology of the endangered plant Manglietia aromatica Dandy were investigated. Based on this study, this species has very low seed set rate. The abortion rate of functional megaspores in all the ovules is 27.9%, the egg cell abortion rate of mature embryos is up to 80%, and the germination rate of pollen grains is as low as nearly 0.01%. In addition, the floral structure appears to be another limited factor for the effective pollination of this species. The endangerment mechanism of this species seems to be comprehensive. Human’s destroying actions are the direct factors that have made the population degenerate quickly: low reproductive ability and the destroyed environments are the main reasons that prevent the population from renovating and spreading. Therefore, the conservation measures suggested by this study are to research the breed technology, artificial population renovating, in situ conservation, and ex situ conservation.

Key words: endangered plant: Manglietia aromatica; reproductive biology; endangerment mechanisms

Manglietia aromatica, a species of the family Magnoliaceae, has a narrow distribution in the Southeast of Yunnan and the West of Guangxi, China, where only a few small populations sparsely occur in Guangan and Xichou of Yunnan and Longzhou, Nao and Baise of Guangxi (Liu et al., 1996). Some of the populations have disappeared today, such as those of Baise type locality of M. aromatica, and others are facing the increasing risk of extinction due to the change of environmental conditions, human’s destroying activities and especially low sexual reproductive capability. It was then listed in the China Red Data Book of Plants (1992). The individuals of M. aromatica usually produce a lot of flowers and fruits every year in the wild, but contain very few seeds. This appears to be the main cause of the low reproductive capability. For example, we collected more than 500 follicles in 1992, within which only 13 seeds were found. In this paper an attempt is made to find the possible factors affecting its sexual reproduction by researching its sexual reproductive biology. Moreover, conservation measures and management strategies are presented based on this study.

1 Materials and Methods

1.1 Materials and research sites
The materials of Manglietia aromatica Dandy were collected from a small population in Xichou County of Yunnan. Though the plants in this small population grow well, they were found to have very low seed set rate as mentioned above.

1.2 Research methods
1.2.1 Embryogenesis Buds and flowers at different stages of development were collected during the years of 1997 - 1999. Formalin-acetic-alcohol (FAA) solution was used as fixative. The customary methods of dehydration, inflation and embedding in paraffin were employed. Serial sections were cut in 6 - 8 μm thick for microspores and male gametophytes and 9 - 12 μm thick for macrospore and female gametophytes. The sections were stained in Heidenhain’s iron-alum haematoxylin and Orange-G. Observation and photographs were performed with Olympus PM-10AD.

1.2.2 Meiosis study of microspore mother cells The stamens were collected in March 1999 and fixed in Carnoy’s II fixative. Acetocarmine smears were used to study meiosis and observation and photographs were performed with Olympus PM-10AD.

1.2.3 Pollen in vitro culture The stamens were collected and brought to the temporary laboratory in Fadou in April 2000. Having been shedded from the locules naturally, the pollen grains were cultured by hanging drop culture technique. The medium consists of 1.0 × 10^{-4} mol/L boric acid with concentrations of sugar 5 and 10 percent. This kind of medium has been employed in our hybrid experiments of Magnoliaceae plants in recent years. The observation was made every two hours (Fan et al., 1992), and the three samples for statistic with microscope were 5 mm × 5 mm in size. The experiment was repeated three times.

1.2.4 Floral characters observation It is difficult and dangerous to observe the floral morphology on a big tree. So the branches with flowers not fully opening were collected and the lower parts were put in water for indoors observing.

1.2.5 The observation of seed structure and seed set rate In April 1999, a number of M. aromatica mature
follicles were collected and then opened artificially. The number of both fertile and aborted seeds per follicle was counted.

Seed set rate = developed seeds/developed seeds + undeveloped seeds × 100%

Well-developed mature seeds were selected, their exterior fleshy seed coats were washed off and the stony sarcocobs were peeled off. The embryo surrounded by endosperm was observed with a stereo and fixed in FAA. The next step of method was the same as that mentioned in 1.2.1.

2 Observation

2.1 Embryogenesis

2.1.1 Development of stamen The anther of *M. aromatica* is 4-sporangiate. The anther wall comprises epidermis, endothecium of 1–2 cells thick, middle layer of 1–2 cells thick and glandular tapetum with multinucleate cells (Fig. 1). No irregularities have been detected in the developmental process from the differentiation of archesporial cells to the formation of microspore mother cells. Modified simultaneous cytokinesis takes place by furrowing (Yoshiaki, 1960) during the meiotic division and tetrahedral- and isobilateral tetrads are produced. The tetrad cells are more or less irregular in shape (Figs. 5, 6). The newly formed microspore has dense cytoplasm with a nucleus situated near the center (Fig. 7). As the microspore increases in size, vacuoles appear in the cytoplasm and the nucleus moves to one side of the cell. Most of microspores are irregularly elliptical, semi-spherical and kidney-shaped (Fig. 8). Following this, the microspore is gradually filled up with cytoplasm till a spherical monocyte with a large vacuole is formed (Fig. 9). A similar process of male gametophyte development was observed in *M. insignis* as well (Pan et al. 2001). The microspore after mitotic division gives rise to a large vegetative cell and a small generative cell. The mature pollen grains are shed at the two-celled stage (Fig. 10), being (36.21–46.16) 40.08 × 56.61 (47.94–56.61) μm in size.

2.1.2 Development of pistil The flower of *M. aromatica* has 30–45 carpels, each with 8–13 anatropous ovules. No irregularities have been observed in the developing process from the initiation of ovule primordia to the formation of the megasporangium mother cell and the massive nucellus. When the ovule is at the stage of nearly anatropous, the megasporangium mother cell undergoes meiotic division and results in a linear type of tetrad of these megaspores. The chalazal one is functional and the remaining three degenerate and disappear soon or later (Fig. 15). The functional megaspore increases its volume. Then the nucleus divides and the daughter nuclei move apart to the two poles of the sac. Each of these two nuclei divides twice successively, resulting in the formation of eight nuclei, arranged in two quartets at the micropylar and chalazal ends of the embryo sac. As the macrogametophyte approaches the stage of maturity, three of the nuclei at the micropyllar pole constitute the egg apparatus embed-

ded in the thick protoplasm consisting of an egg cell and two synergids (Figs. 21, 22). At the chalazal end of the embryo sac, three nuclei differentiate as antipodal cells (Figs. 19, 20), and the remaining two polar nuclei migrating from the opposite ends of the embryo sac fuse to form a secondary nucleus before fertilization (Fig. 21). It is obvious that the embryo sac development is monosporous 8-nucleate polygonum type.

2.1.3 Abnormal development During the development of female gametophyte, abnormalities have been observed in two different stages: (i) Abnormal development occurs at the stage from the beginning of meiosis of the microspore mother cell to the formation of the functional microspore (Figs. 16, 17). Observations have been made on 462 ovules in total at this stage. 129 of them had no developed functional microspores but degenerated remaining trace. The degeneration rate is 27.99%. (ii) The egg apparatus of mature ovule degenerate before fertilization (Fig. 18). Of 55 mature ovules observed, 44 (80%) have degenerated egg apparatus, which occurs in *Manglietia glauca* var. *sumatrana* also (Liao et al. 2000).

2.2 Meiosis of microspore mother cell

The meiotic process of the microspore mother cell of *M. aromatica* is normal. The chromosome of microspore mother cell starts to condense and the homologous chromosomes pair after experiencing leptonema-amphiaphatena-pachynema-diplonema stage (Fig. 2). The chromosomes arrange on the metaphase I plate in different morpha like "rhombus", "E" and "rhaphodoid" (Fig. 3). The homologous chromosomes depart from each other at the stage of anaphase I and move to the either pole of the cell (Fig. 4). No cell plate is formed in telephase I. The chromosomes then arrange on the metaphase II plate once again shortly after condensing. Following this, the chromatids depart and move to the poles in metaphase II and condense in telephase II. Four microspores are produced after cytokinesis and formation of cell plates. Very few abnormal developments have been observed during the meiosis such as chromosome bridges in anaphase I and backward chromosomes.

2.3 Pollen in vitro culture

The pollen grains were observed when they had been cultured for two hours. The pollen grains were in the following three status: (i) most of them had not yet germinated; (ii) some of them had germinated, and the pollen tube spalled out from the tip of the pollen tube; (iii) pollen grains had germinated normally, while the pollen tubes were relatively short (Figs. 11–13). Four hours later, the number of the pollen grains in the state (ii) as mentioned above had increased. And six hours later, little change had occurred except for the number of the pollen grains with ruptured tubes continuing to increase.

Therefore, we concluded that the pollen germination capability of *M. aromatica* is very low. Among 1305 pollen grains we observed, a small part of them germinated abnormally with pollen tube spalling out of pollen tube, while most of the pollen grains did not germinate. Only one pollen grain germinated normally. The rate is less than 0.01%.
2.4 The floral characteristics

The flower of *M. aromatica* has 9 - 12 tepals attached on the gynoecium in whorls of three or four. Flowering begins from early April to late May. During the flower shortly before opening, the tepals still enclose the pistils and stamens. The flowers usually begin to open in the late afternoon from half past six to eight o'clock. During this time, the outer whorl of the tepals first opens a little widely, while the inner two or three whorls are just to be slack, not folding tightly over the gynoecium any more, but still keep closing. The inner two or three whorls of tepals close about half an hour later. From the hours before the flower begins to open to the time when the inner tepals close, the stigmas are receptive to the pollen grains. At about the same time of the following day, the flowers re-open and begin to shed pollen, but the stigmas are not receptive any more. At about 9 o'clock a.m. of the third day, the stamens start to detach and the tepals begin to fall away. Therefore *M. aromatica* is protogynous species and the insects must crawl into either the buds or unopened flowers in order to pollinate them effectively. This floral characteristics are very similar to those of *Magnolia delavayi* (Gong et al. 1998). Like other Magnoliaceae plants, the flowers of *M. aromatica* are highly specialized for exclusive pollination by insects such as beetles (Thien, 1974).

2.5 The structure of mature seed and seed-set rate

The seeds of *M. aromatica* open from later August to early October. The coats of plump seeds are three-layered. The outer layer is a slightly colored, oily, fleshy cover. Beneath the outer layer is a hard, bony, dark inner layer. The third layer is a thin membrane, which is difficult to identify when the seed is fresh. According to the study by Earle and Tiffney (Earle, 1938; Tiffney, 1977), the outer and the second layer seed coats are developed from the outer integument, and the third layer is...
developed from the inner integument. Inside the seed coats is the endosperm and morphologically well-developed embryo embedded within, which has a short radicle, a well-developed hypocotyl and two cotyledons. However, in the ripe follicles, seeds are mostly not filled with endosperm and embryos, even the bony second seed coats. In September 1999, we collected 18 ripe follicles from Fadou population, in which the total number of carpels is 663 containing 3,677 ovules (developed and undeveloped seeds). Only two of them developed into mature seeds.

3 The Endangered Mechanism of *M. aromatica*

3.1 Causes of low seed set rate

The reproductive system of the higher plants is more complicated than that of animals. Seeds will not be ripe and vital until the plant has experienced normal flower bud initiation and differentiation, regular microsporogenesis, macrogametogenesis, female and male gametophyte development, fertilization and normal development of embryo and endosperm. In our experiment an observation was made on 60 cross sections of 20 anthers. The anther is 10 mm long, and each slice section is 8 μm in thickness, on which there are 24 pollen grains found. Statistics show that each flower of *M. aromatica* has about 100 stamens, and may produce nearly 3 000 000 pollen grains.

If the germination rate of the pollen grains is not too low, they would be enough for the effective pollination.

The development of stamens in *M. aromatica* is normal morphologically, and no obvious abortions have been seen. However, the germination rate of the pollen grains is very low, less than 0.01% on vitro culture. In contrast, the same experiment was conducted on the other Magnoliaceous plants including *Magnolia* species, demonstrating that the germination rates were generally more than 50%, with the highest rate up to 90% and the lowest about 30%. Although the germination rate (0.01%) of the pollen grains is not the result of the experiment on the stigma, it may conclude that the germination rate of *M. aromatica* is very low. According to this rate, only less than 300 among the 3 000 000 pollen grains can germinate. This is obviously too poor to pollinate effectively for the pollination for 30-45 stigmas of every flower and, in addition, the pollen grains would be lost naturally during the pollination process.

In addition, the high abortion and degeneration rate of the ovules would also decrease the possibility of effective pollination and fertilization.

In conclusion, the high rate of ovule abortion and degeneration and low rate of pollen grain germination are the main factors affecting low seed set rates. The primary causes of endangerment of *M. aromatica* (Qi et al.)
There are two hypotheses about the sexual reproduction in plants. Resource mortality hypothesis predicts that less vigorous progeny are selectively aborted when resources available for seed production are limited, thus the ratios of fruit:flower and seed:ovule are less than 1:1. Genetic mortality (or genetic loads) hypothesis predicts that the accumulation of harmful genetic mutation resulted from secular environmental change and other biology invading such as pathogen and parasite increase the embryonic abnormality (Glenda, 1995). The flowers of *M. aromatica* open normally, and the follicles are ripe regularly even without producing any mature seeds. Accordingly, we predict that it is not the resource limitation but the genetic factors that caused the reproductive abortion. The development of molecular biology and developmental biology, we hope, will promote the research on the reproductive abortion in the near future.

### 3.2 Other causes of endangerment of *M. aromatica*

Knowledge of the reproductive biology about a species—especially the rare and endangered species—is essential for effective conservation programs (Schein, 1994; Bernardello et al., 1999). Seed is the only moving stage in the whole life cycle of plants and it is the embodiment of gene flow (Zhou et al., 1999). Producing enough seeds for plants to form the soil seed bank after the seed dispersal is very important for the population complement and growth (Xie and Li, 2000). In another word, normal seed development, seed dispersal, seed germination and the seedling establishment and growing are the direct determinants for the population complement and survival (Clampitt, 1987; Wang et al., 1998). The outer seed coats of *M. aromatica* are oily and fleshy, which are very easy to rot and cause the seeds to lose the germinating capability. In addition, the chemical materials in the outer seed coats will also inhibit the seed germination (Lu et al., 1999). The seeds, when the fruits rippen and split, are suspended from the fruit on funiculare threads with brightly red color. This makes the seeds be easily seen and preyed by the birds and small animals such as rats. The preying by the birds or small animals will benefit the dispersal of the seeds and the breaking of the outer coats, if the number of the seeds is large and then the plant can endure losing the seeds. However, the number of the seeds of *M. aromatica* is very small, and the preying is thus a big barrier for the formation of soil seed bank. In natural condition, the outer fleshy seed coats are broken by two ways: being decomposed naturally after falling into the soil; being digested by birds or other animals when passing by their digestive tracts. As a result, only the seeds surviving from the digestion and rot can germinate after the dormancy is broken by low temperature (Callaway, 1994). It seems that all the physiological and ecological characteristics of the seeds reduce the reproductive capability.

Because of the limitation of the seed quantity, we could not carry out the germination experiments systematically for *M. aromatica*. The experiments on a bit of seed propagation showed that the seeds with outer coats that become easily rotten in the soil fail to germinate at all, while the seeds without the outer coats have high germination rate (95%). In addition, the seed must be kept in moisture after the coat is removed. Otherwise the seed dries out, its vitality and viability would be reduced or completely lost. This physiological characteristic indicated that the seeds of *M. aromatica* need special environmental conditions to germinate in the field, and this is a factor of limitation affecting the population complement in time.

However the first cause of endangerment of *M. aromatica* that we discovered during the investigation comes directly from the human’s deforestation because of its fine, aromatic and anti-rotten timber. For example, the local people told us that there had once been a big population of *M. aromatica* on the mountain nearby village Chongjingzi in Fadou town. But only several individuals are left today due to the deforestation. At the same time, the ecosystem retrograded and the habitats of *M. aromatica* were destroyed badly, and as a result, the species has become fragmental there.

### 4 Suggestions for the Conservation

What we have learned about the reproductive biology suggest that features of the reproductive system do stand in the way of successful propagation. Thus, the first effort should be focused on the protection of the still living individuals in their native habitats from being destroyed any more in the future. Although the local people are willing to use the timber of *M. aromatica*, they do not know how to propagate this species. Therefore, the second effort should be to research and impart the propagation techniques to help them to produce the timber. The latter would be the basic measure for ex situ conservation and population renewal.

The main causes of low reproduction of *M. aromatica* are the physiological and genetic factors as well as the environmental effects. This species has only a few individuals in native habitats. As a result, poor gene exchange will occur among populations, causing the loss of genetic diversity. Thus another conservation strategy we suggested here is to plant some other Magnoliaceae plants with the same blooming period as *Manglietia aromatica* mixed with them. Thus they would attract more pollinators to visit the flowers of *M. aromatica* and increase the chance of successful pollination within or between populations, and eventually promote the outcrossing.

Acknowledgements: We thank Prof. GU Zhi-Jian and Prof. WEI Zhong-Xin for their assistance in micrography.

References:


香木莲有性生殖特性与其濒危机制的研究

潘跃芝 梁汉兴 龚涛*

（中国科学院昆明植物研究所，昆明 650204）

摘要：针对香木莲（Manglietia aromatica Lam.）生长率低以及野外实生苗稀少的现象，本文研究了香木莲雌配子体发育过程、花粉萌发力、开花生物学特性与种子结构的观察。结果表明，在雌配子体发育阶段存在以下退化现象：1）从大孢子母细胞开始减数分裂到功能大孢子形成阶段，在此过程中未完成的正常分裂，功能大孢子未能正形成，仅残留有退化痕迹，退化率为 27.9%；2）胚囊成熟时，受精卵细胞发育完全，退化率为 65%。花粉发育实验结果表明，在人工培养条件下具有正常萌发力的花粉不足 0.01%。这些是香木莲雌配子体发育的重要原因。同时，香木莲的开花生物学特性中受粉的成熟花粉通过调查和研究的结果显示，人为的采集花粉是造成香木莲种群数量减少的主要原因。有性生殖障碍和母本破败是制约香木莲种群更新的主要原因，因此提出了对香木莲的拯救和保护对策：增施保护；研究推广繁育技术，进行种群重建；进行迁地保护，保存尽可能多的种群资源。

关键词：濒危植物；香木莲；生殖生物学；濒危机制

中国分类号：Q945.53 文献标识码：A 论文编号：0577-7496/200303-0311-06

(责任编辑：彭丹（实习）)