Studies on new cultivar breeding and the aging of tree peony

（ボタンの新品種育成ならびに樹体老化に関する研究）

Qing Hao

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The United Graduate School of Agricultural Sciences,

Tottori University

Attachment: Shimane University

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Chapter 1

Introduction

Tree peony (*Paeonia suffruticosa* Andr.), a woody shrub, belongs to the section *Moutan* DC. of the genus *Paeonia* L. (Paeoniaceae). It is known as 'the king of flowers' because of its excellent ornamental traits, i.e., large, showy, fragrant, and beautiful flowers, as well its deep cultural importance and pharmaceutical value as a widely-used component in Chinese traditional medicine.

Tree peony has been widely cultivated in China as a traditional ornamental plant and has been introduced to Japan, America, and Europe, and formed peony tour resorts. They are popular and loved by lots of people. Tree peony is an especially important tour resource. Amounts of tourists visit tree peony thematic gardens to see the beautiful flowers at anthesis, and enjoy the sight-seeing, especially in China and Japan (Photograph 1-1). The introduction of new cultivars with high ornamental value which different from existing ones in flower color and flower form was requested for maintaining and developing the tour resource. On the other hand, as plants in peony gardens age, the plants grow weak, caused the phenomenon of no flowering or bad flowering which was a big problem in tree peony gardens, reducing their ornamental value (Photograph 1-2). Therefore, the new cultivar breeding and clarifying the causes of aging are necessary for maintaining and developing tree peony tour resource.

1.1 Genetic resources of tree peony

1.1.1 Wild species

The wild species of tree peony are distributed in China, except for *P. delavayi* France., which occurs in Bhutan (Liu, 2003). Nine wild species are currently known (Hong and Pan, 1999a, b, 2005a, b, 2007; Li, 1999): (1) *P. suffruticosa* Andrews; (2) *P. jishanensis* T. Hong et W. Z. Zhao; (3) *P. qiui* Y. L. Pei et D. Y. Hong; (4) *P. ostii* T. Hong et J. X. Zhang; (5) *P. rockii* (S. G. Haw et L. A. Lauener) T. Hong et J. J. Li; (6) *P. decomposita* Hand.-Mazz.; (7) *P. delavayi* France. (including three infra-species, *P. delavayi* (Photograph 1-3), *P. potaninii* and *P. lutea* (Photograph 1-4)); (8) *P. ludlowii* (Stern et Taylor) D. Y. Hong; and (9) *P. cathayana* D. Y. Hong & K. Y. Pan.

Based on the nature of the flower discs, tree peonies are classified into two subsections: subsect. *Delavayanae* F. C. Stern includes *P. delavayi* and *P. ludlowii*, while the remaining species belong to subsect. *Vaginatae* F. C. Stern.

1.1.2 Cultivars

Tree peony was famous among the earliest cultivated horticultural plants in the world. It was introduced to Japan from its native China in the 8th century and brought to Europe and America in the 18th and 19th centuries, respectively (Wister, 1928). Unique cultivars have been developed throughout its modern distribution. These tree peony cultivars are classified based on morphology and geographic location into four groups: Chinese, Japanese, French, and American (Liu, 2003).

1.1.2.1 Chinese tree peony cultivar group

In China, tree peonies have been cultivated as ornamental plants since the Dongjin Dynasty

1,600 years ago (Wang, 1998). Five wild species — *P. suffruticosa*, *P. rockii, P. ostii, P. jishanensis*, and *P. qiui* wchich belong to subsect. *Vaginatae*— were involved in the differentiation and development of cultivars belonging to Chinese group (Photograph 1-5) (Li, 1992, 1998, 1999; Zhou et al., 2003).

The many cultivars of the Chinese tree peony group can be divided into four regional subgroups that differ in climate preferences, and flower appearance: Zhongyuan, Xibei, Xinan, and Jiangnan. The Zhongyuan tree peony cultivar group has the richest colors, with white, pink, red, purple, green, or light yellow flowers as well as secondary colors; some cultivars in this group are also shy-flowering. The Xibei cultivar group includes large plants with violet-purple or purple-red blotches at the petal bases and high flowering ratios. Cultivars in the Jiangnan group have excellent in heat-wet resistance. Finally, the Xinan cultivar group comprises large plants with purple blotches or red blushes at the petal bases and low flowering ratios. However, cultivars in different groups may be similar because the four groups interbred long ago (Li, 1998; Wang, 1998).

1.1.2.2 Japanese tree peony cultivar group

Since the tree peony was introduced to Japan, many Japanese cultivars have been hybridized or selected from open-pollinated seedlings or bud sports, especially during the Edo period (1603–1867) (Hashida, 1990), to form the Japanese cultivar group. In the Meiji era (1867–1912), cultivars were propagated in Ikeda City, Osaka Prefecture, and introduced into Yatsuka-cho (Daikon-jima Island), Shimane Pref., and Gosen City, Niigata Pref. Since then, two prefectures have become major production areas of tree peonies in Japan (Hosoki et al., 1997). The Japanese

group is characterized by large flowers, graceful and stiff stems that hold the flowers upright (Photograph 1-6), and bright, pure flower colors of white, red, or purple (Photograph 1-7) (Li et al., 2011). ‘Ten I’ and ‘Yatsuka Jishi’ are two representative cultivars of the Japanese group because of their large flowers with diameters of about 30–40 cm.

1.1.2.3 French tree peony cultivar group

In France during the early 20th century, Henry and Lemoine hybridized *P. suffruticosa* cultivars with *P. lutea* Delav. Ex Franch which belong to subsect. *Delavayanae* and obtained yellow cultivars (Photograph 1-8, the above three ones) (Wister and Wolfe, 1962). Five yellow cultivars were introduced to Japan and China; they are ‘Kinkaku’, ‘Kinkou’, ‘Kinshi’, ‘Kinti’, and ‘Kinyou’. French cultivars inherited from *P. lutea* yellow flowers with reddish-brown blotches at the petal bases, small flowers with drooping stalks, and some flowers that bloom laterally or hide in the leaves (Li et al., 2011).

1.1.2.4 American tree peony cultivar group

In the United States, Saunders and Daphnis crossed Japanese cultivars with *P. lutea* and *P. delavayi* which belong to Subsect. *Delavayanae* created many American cultivars. They are popular for their rare flower colors of yellow, orange, and purple-black (Photograph 1-8, the bottom three ones). The yellow pigment in American and French cultivars was inherited from the wild *P. delavayi* and *P. lutea* (Hosoki et al., 1991). However, these cultivars have small flowers, the stems are too weak to hold the flowers upright, and some flowers bloom laterally, reducing their ornamental value (Li et al., 2011) (Photograph 1-9). The cultivar ‘High Noon’ is an American-group hybrid (*P. lutea* × *P. suffruticosa*) bred by Saunders in 1952 (Kessenich and APS

Nomenclature Committee, 1976). It bears cup-shaped and semi-double flowers that are sunshine yellow with crimson blotches, tends to re-bloom in August, and has much stronger stems subtending the flowers than of other American and French yellow-flowered cultivars (Photograph 1-10).

1.2 Recent research developments in breeding tree peonies

1.2.1 The ancient and the conventional breeding-Introduction and domestication, selective breeding and close-relative crosses

Introduction and domestication was the earliest method of cultivating tree peony cultivars. It provided the base material for other breeding methods and expanded the geographic distribution of tree peony. Selective breeding, such as selecting bud sports, grafting chimaera, and seedling selection, played an important role in conventional breeding of tree peony (Dai, 1987; Yu and Yang, 1962; Zhou et al., 2003). The famous Japanese cultivar 'Shima Nishiki' and Chinese cultivar 'Er Qiao' were bred by the bud sport method. A large number of cultivars have also been bred by cross-breeding close relatives, especially, in China and Japan (Cheng and Chen, 1998; Hashida, 1990; Zhuang, 1995; Yu, 1982) and by crossing more distant relatives in Europe and America (Wister, 1995).

There are about 2,500 tree peony cultivars world-wide, among them, 1,700 in the Chinese group, more than 300 in the Japanese group, nearly 400 in the American group, and more than 100 in the Europe group (Cheng et al., 1998; Hashida, 1990; Li, 1999). Most of these cultivars were bred from closely-related parents via intra-subsection, intra-specific, or intra-group hybridization.

These crosses improved seed set but yielded few good variants and hybrids low in heterosis, limiting the breeding new tree peony cultivars. Few tree peony cultivars were bred from crossing distantly-related parents.

1.2.2 Crosses between distantly-related tree peonies

1.2.2.1 Inter-subsection crosses

In the 19th century, French breeders Victor Lemoine and Emile Lemoine used *P. delavayi* and *P. lutea* crossed with Chinese group tree peony to obtain the earliest inter-subsection cultivars. In the early 20th century, American breeder A. P. Saunders began to breed cultivars by crossing *P. delavayi* and *P. lutea* with Japanese-group tree peony (Wister, 1995). Since then, more gardeners and breeders have engaged in this breeding work to form the American French and American cultivar groups. Several of these cultivars were introduced to China and Japan, where they were quite popular for their rare flower colors.

1.2.2.2 Crosses between different cultivar groups

American and French tree peonies are inter-subsection cultivars**,** they have desirable flower colors, but small flowers, the stems are too weak to hold the flowers upright, and some flowers bloom laterally or hide in the leaves, which were inherited from the wild *P. delavayi* and *P. lutea*, reducing their ornamental value. It is hopeful to improve the American (and French) cultivars by crossing with Japanese and Chinese tree peonies, especially Japanese ones which have larger flowers with better presentation. Superior cultivars that combine the advantage of Japanese and American (and French) cultivars are highly desirable. Due to their different genetic backgrounds, hybrids between American (and French) and Japanese cultivars are difficult to obtain. Several

crosses with Japanese cultivars have been attempted using ‘High Noon’ as the crossing parent, but no seeds were obtained (He, 2006).

Cross-breeding of distant tree peony relatives is most promising means of cultivar improvement at present and in the future. However, the long period is necessary for breeding and cross abortion are the two main obstacles to obtaining viable new cultivars.

1.2.3.1 Cross-incompatibility between distant relatives

1.2.3.1.1 Fertilization barriers

Hybridization barriers occur frequently when distant crosses are attempted. Breeding barriers can happen at different stages during the process of hybridization. The phenomena underlying crossing barriers can be divided into pre- and post-fertilization barriers. Pre-fertilization breeding barriers mainly include the failure of pollen grains to germinate on the stigma and the arrest of pollen tube growth and ovule penetration, whereas, post-fertilization barriers mainly include failures in embryo development, seed germination, and seedling survival (Marta et al., 2004; Okamoto and Suto, 2004). Failure of pollen to germinate and anomalous pollen-tube growth occur commonly in interspecific crosses (Ali and Fujieda, 1990; Pimienta et al., 1983). One possible reason for pollen tube arrest is an inability to use stylar nutrients, perhaps due to a lack of suitable nutrients in the transmitting tract or suitable enzymes in the pollen tubes (Shivanna, 1996).

1.2.3.1.2 Methods for overcoming crossing barriers

Crossability is determined by both genetic and environmental factors. Therefore, one must test different accessions of both parents in hybridization programs. Sometimes, a cross will be successful in one direction, whereas the reciprocal cross fails. In some plants, pre-fertilization

barriers can be overcome by using mixed or mentor pollen, the cut-style method, the grafted-style method, or the in *vitro* isolated ovule pollination technique (Brown and Adiwilaga, 1991; Nikiforova and Khromaa, 1987; Van Tuyl et al., 1988). To overcome pre-fertilization barriers, environmental conditions or chemical treatments, such as high temperature (Ascher and Peloquin, 1968; Okazaki and Murakami, 1992), floral aging (Ascher and Peloquin, 1966), hormone treatment (Sankin, 1976; Ascher, 1973), specific proteins and exudates (Martin, 1970), 6-mercaptopurine (Kravtson et al., 1975), or lipids (Wolter-Arts et al., 1998), can be applied.

A range of in *vitro* rescue methods, such as embryo (Buitendijk et al., 1992; Kursakov, 1978), ovary (Kerlan et al., 1992), ovary-slice (Van Tuyl et al., 1991), and ovule culture (Bridgen et al., 1989; Gray et al., 1990; Neal and Topoleski, 1983) have been developed to overcome post-fertilization barriers in a number of ornamental plants. Fertilization can be divided into embryo and endosperm formation. Embryo development is highly affected by changes in the endosperm; if the endosperm fails to develop, the embryo can rarely survive. In such cases, very early embryo rescue, such as via ovary slice culture, may help.

1.2.4 Polyploidy breeding

1.2.4.1 The method of polyploidy breeding

Polyploidy is widespread in the plant kingdom. Among angiosperms, 30% are polyploids (Bretagnolle and Thompson, 1995). Ploidy manipulation is a valuable tool for plant breeders, particularly in crops grown for their vegetative parts, because polyploidy can result in larger, more succulent vegetative organs and greater vegetative vigor. Polyploids may arise either by sporadic chromosome doubling or by the union of unreduced gametes with other unreduced gametes or

with normal reduced gametes. The latter case is considered more likely to occur in nature (Harlan and De Wet, 1975). Most polyploidy breeding has focused on unreduced pollen grains, which are easier to isolate.

1.2.4.2 Detection of 2n pollen

Unreduced (2n) pollen can be detected in four ways (Bretagnolle and Thompson, 1995): pollen size measurements, flow cytometric detection of pollen DNA content, analysis of the microsporogenesis, and ploidy analysis of the progeny. Pollen diameter is an easy and commonly-used method to screen for 2n pollen. Large pollen has frequently been attributed to 2n pollen in many genera, because of the positive correlation between DNA content and cell volume, which in turn influences pollen diameter. In crops such as Japanese persimmon, banana, rose, and sweet potato, the diameter of 2n pollen was approximately 30% larger than that of haploid pollen (Becerra Lopez-Lavalle and Orjeda, 2002; Crespel et al., 2006; Ortiz, 1997; Sugiura et al., 2000; Zhuang, 1990).

1.2.4.3 Polyploidy breeding in tree peony

All of the wild species of tree peony are diploid (2n = 2x = 10), as are most of the cultivars, with the exception of the triploid (2n = 3x = 15) '*P. suffruticosa Shou An Hong*' (Photograph 1-11) with many virtues such as bigger flower, numerous petals, and high disease resistance (Li and Zhang, 1982; Li and Zhang, 1996). The American tree peony breeder D. Reath reported that tree peony was more sensitive than herbaceous peony to colchicine and suggested using 0.5-1.0% colchicine to treat Japanese tree peonies for polyploidy breeding (Reath, 1972). Cheng et al. (2009) used colchicine to induce mixed buds and *in vitro* embryos of 'Feng Dan' and obtained tetraploid

test-tube plantlets, but no polyploid cultivar has resulted from either study.

1.3 Plant senescence

As plants age, their survival and reproductive abilities gradually decrease. Older plants, exhibit slower growth, declining vigor, chloroplasts disintegration, degradation of CO_2 carboxylase and other proteins, decreased antioxidant capacity, more sensitivity to environmental changes, and less resistance to diseases and insects, all of which ultimately lead to the death of the plant. Under natural conditions, plant death and turnover have important positive consequences for natural selection and ecological adaptation of populations, but the declines caused by aging greatly limit potential crop yields and impact ornamental value.

A decrease of protein content typically occurs with plant aging (Li et al., 2004). In addition, malondialdehyde (MDA) is a product of membrane lipid peroxidation, and an increase in MDA levels is an inherent characteristic of aging cells (Li et al., 2004). The accumulation of free radicals can speed the aging process. Superoxide dismutase (SOD) plays a important role in clearing free radicals, so a decrease in SOD activity could accelerate aging (Del Rio, 1998; Leshem, 1988). In flowers of the herbaceous plants *Chrysanthemum morifolium* Ramat (Strickland, 1972), *Digitalis purpurea* L. (Stead and Moore, 1977), *Hemerocallis* hybrid (Lay-Yee et al., 1992), and *Sandersonia aurantiaca* (Eason and Webster, 1995), protein content decreased as petals aged. As *Chrysanthemum morifolium* (Bartoli et al., 1995) and *Gladiolus* (Yamane et al., 1999) petals grew older, SOD activity decreased, while SOD activity increased in *Hemerocallis* hybrid

(Panavas and Rubinstein, 1998). In addition, MDA content increased with petal age in *Hemerocallis* (Chakrabarty et al., 2009) and *Freesia* (Shu et al., 2010). In the woody *Pinus taiwanensis*, protein content in leaves decreased with aging (Li et al., 1998), and SOD activity in leaves of ancients tree was lower than in young trees of *Pinus tabulaeformis* (Guo et al., 2011).

Heat, cold damage, water stress, salt stress, and nutritional deficiency can cause plant senescence (Nooden et al., 1997), and the hormone ethylene can accelerate within-plant leaf senescence (Leopold and Nooden, 1984; Mattoo and Aharoni, 1988).

1.4 Hot topics in tree peony recent research

In recent decades, many tree peony researchers have focused attention on five key aspects: (1) the origination and classification of wild species (Hong and Pan, 1999a, b, 2005a, b, 2007; Hong et al., 1992; Hong and Osti, 1994; Zhou et al., 2003); (2) flower bud differentiation and flower type evolution of cultivars (Aoki, 1992b; Aoki et al., 2000; Aoki and Yoshino, 1989; Liu et al., 2002; Wang, 1986); (3) forcing and retarding culture (Aoki, 1992a; Aoki et al., 2001; Aoki and Yoshino, 1984a, b; Cheng et al., 2001; Hosoki et al., 1983, 1984, 1992; Hosoki and Kimura, 1996; Liu et al., 2003a, b; Jiang et al., 2007; Zhang, 2004); (4) molecular biology (Guo and Luo, 2006; Guo et al., 2009; Han et al., 2008a, b; Hao et al., 2008a; Hou et al., 2006; Huang et al.,2008a,b; Wang et al., 2009; Zhou and Dong, 2008; Shi et al., 2009; Shu et al., 2009; Su et al., 2006; Suo et al., 2005; Zhang et al., 2012); and (5) flower color and pigment in tree peony petals (Hosoki et al., 1991; Li et al., 2009; Sakata et al., 1995; Wang et al., 2001a, b; Zhang et al., 2007). In addition,

there were also many researchers turned to the researches of physiology (Cui et al., 2009; Li et al., 2006; Wei, 2009; Zhang et al., 2009) and chemical analysis (Xu et al., 2006; Han et al., 2008c; Wu et al., 2010).

The basic theoretical and applied research related to cross-breeding tree peonies is lacking, especially for crosses among cultivar groups and polyploids. The aging process of tree peony is also rarely studied.

1.5 Objectives of this study

The overall goal of this work was to improve tree peony cultivars and determining the causes of aging for maintaining and developing tree peony tour resource. This study had two main objectives, one was to breed new cultivars, for this purpose, two parts of experiment were performed, 1) to hybridize cultivars of two different subgroups to produce superior cultivars; 2) to confirm the existence and generation rate of giant pollen grains for the use in polyploidy breeding; the other was to discuss the reason of aging, in this part of experiment, 3) to combine measures of tree growth status with three physiological indexes to study tree peony aging. These objectives were addressed in the following chapters:

(1) Crossability of American tree peony ‘High Noon’ as seed parent with Japanese cultivars to breed superior cultivars

These experiments evaluated Japanese cultivars as pollen parents crossed with the American cultivar ‘High Noon’ to obtain superior cultivars with the combined advantages of both parents in

terms of flower color and presentation. Moreover, better cross combinations were identified based on seed set and seedling survival, and pre- and post-fertilization barriers were analyzed to assist future breeding programs. Finally, the floral morphology and DNA of the hybrids that have already flowered and their parents were also investigated.

(2) Occurrence of giant pollen grains in tree peony cultivars

To obtain unreduced pollen for polyploidy breeding of new cultivars, we surveyed the occurrence of giant pollen grains in several cultivars of Japanese tree peonies. Pollen diameter was measured, and pollen size distribution was determined.

(3) Changes in tree body, soluble protein content, SOD activity and MDA content in aging tree peony

The tree body, protein content, SOD activity, and MDA content in leaves of ‘Luo Yang Hong’ trees of different ages planted in the same resource garden were measured. These data were used to establish a baseline for tree peony aging and to understand the changes that take place as plants age. Correlations between plant age after division and protein content, SOD activity, and MDA content in leaves were evaluated.

Chapter 2

Crossability of American tree peony 'High Noon' as seed parent with Japanese cultivars to breed superior cultivars

2.1 Introduction

The Japanese group is characterized by large flowers, graceful and stiff stems that hold the flower upright, and bright, pure flower color of white, red, or purple (Li et al., 2011). American cultivars are popular for their rare flower colors of yellow, orange, and purple black. However, they have small flowers with drooping stalks, because their stems are too weak to hold the flowers upright, and some flowers bloom laterally or hiding in the leaves, reducing their ornamental value (Li et al., 2011). 'High Noon (HN)' is one cultivar of American tree peony. It has much stronger stems subtending the flowers than that of other American ones. Superior cultivars that combine the advantage of Japanese and American cultivar 'HN' are highly desirable. However, 'HN' is closely related to *P. lutea* and distantly related to Japanese cultivars (Zhang et al., 2012). Due to their different genetic backgrounds, hybrids between 'HN' and Japanese cultivars are difficult to obtain.

The cross-compatibility and causes of fertilization barriers between American and Japanese cultivars of tree peonies remains unknown till now. This study evaluated Japanese cultivars as pollen parents crossed with 'HN' to obtain superior cultivars with the combined advantages of both parents in terms of flower color and presentation. Moreover, better cross combinations were

identified, and fertilization barriers were analyzed to assist future breeding programs.

2.2 Materials and methods

2.2.1 Plant materials

From 2004–2006 and 2009–2011, one American cultivar 'HN' (*P. lutea* × *P. suffruticosa*) (yellow; seed parent) and 57 Japanese cultivars (*P. suffruticosa*) (3 black, 16 pink, 7 purple, 19 red, and 12 white; pollen parents; Table 2-1) were crossed to form 57 cross combinations. All plants were obtained from the private collections of flower growers on Daikonjima Island (Matsue city, Shimane Prefecture, Japan).

2.2.2 Emasculation, pollination, and cross compatibility

To avoid self-pollination and unwanted crosses with nearby plants, flowers of the seed parent were emasculated and covered with waxed paper bags one day before they opened in late April to mid- May. Meanwhile, unopened flowers of the pollen parents were taken and dried at room temperature. Dried pollen was applied to the seed-parent stigma two days later. The number of pollinated flowers in each combination was recorded in Table 2-2. All of the pollinated flowers were re-bagged to avoid contamination with other pollen (Photograph 2-1).

Seeds were collected from August to September. The numbers of mature (plump, fully developed) and immature (hollow, poorly developed) seeds were counted. All of the seeds were then sown and evaluated for germination; the number of seedlings for each cross was considered a measure of crossability. Cross compatibility was calculated as the percentage of pollinated flowers

that yielded seedlings.

2.2.3 Floral morphology of parents and offspring

The floral morphology of those hybrids and their parents was investigated in the spring when the plants blossom. The color of fresh petals at their middles was measured with a spectrophotometer CR-300 (Nippon Minolta Co., Ltd., Osaka, Japan). If the petal was blotched, the blotch color was also measured. Lightness (L^*) and two chromatic components a^* and b^* on the CIELAB color coordinate were measured, and chroma (C^*) was calculated from the equation $(a^{*2}+b^{*2})^{1/2}$. Hue angle (h°) was calculated from the equation ATAN $(b^*/a^*)/6.2832 \times 360$ (McGuire, 1992). Flower diameter and petal number of hybrids were recorded for all flowers to blossom and subsequently averaged.

2.2.4 DNA isolation and amplification

Total genomic DNA of those hybrids and their parents was extracted from leaf tissues by a modified CTAB method (Hao et al. 2008a). DNA was amplified via sequence related amplified polymorphism (SRAP) markers. Primer sequence were 5'- TGA GTC CAA ACC GGA AG-3' (Me5) and 5'-GAC TGCGTA CGA ATT AAT-3' (Em1) which reported by Li and Quiros (2001). The PCR reaction mixtures (20 μL total volume) consisted of 25 ng genomic DNA, 0.2 μM of each primer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 10×Taq buffer and 1 unit of *Taq* DNA polymerase (KAPA Biosystems). The amplification was carried out in an Program Temp Control System PC-320 (ASTEC) using the following program: 3 min at 94 °C, eight cycles of 30 s at 94 °C, 30 s at 37 °C and 90 s at 72 °C, followed by 35 cycles, 30 s at 94 °C, 30 s at 50 °C, 90 s at 72 °C, with a final elongation at 72 °C for 7 min. Each PCR product (10 μL) was displayed by electrophoresis

on 2% agarose gels at 100 V for 1 h and stained with ehidium bromide for 30 min. The gel was photographed by Printgraph (DT-20MP).

2.2.5 Pollen tube growth behavior

Two cross combinations ('HN' ×'KKJ' and 'SDJ' × 'KKJ') were used to observe pollen germination on the stigma and pollen tube growth in the style. Styles were collected at intervals of 6 h, 12 h, 1 d, 2 d, and 10 d after pollination. Three pistils were collected from each seed parent, fixed in FAA (1:13:1, formalin : 70% ethanol : acetic acid (v/v/v)) solution for 24 h, then preserved in 70% ethanol. The fixed pistils were soaked in 1 N NaOH for 24 h, washed three times in distilled water, and stained with 0.1% aniline blue dye dissolved in 0.1 N K_3PO_4 for 3 h (Martin, 1959). The stigma lobes from each flower were carefully divided into two or three parts on a slide and the stigmas and styles were squashed. The preparations were examined under a Nikon Optiphot XF-21 microscope with a high-pressure mercury vapor lamp and a combined filter (EX330-380, BA520, DM400; Nikon, Tokyo, Japan). Pollen tube growth was evaluated by counting the pollen tubes in the styles and that penetrated the ovules.

2.3 Results

2.3.1 Crosses and cross compatibility

During the six years of this study, we generated a total of 1,927 crosses involving 57 parental combinations of the American cultivar 'HN' with Japanese cultivars. A total of 9,184 pistils in 1,927 flowers were pollinated. Of those crosses, 135 flowers (157 pistils) from 38 cross

combinations set 181 seeds, 86 (47.5 %) mature and 95 (52.5 %) immature (Table 2-2).

Both fruit and seed set in all cross combinations were very low (Table 2-2). The fruit-set rate ranged from 0 % (19 crosses, including 'ASK', 'BG', and 'BM' as pollen parents) to 9.51 % ('SR' as pollen parent), with an average of 1.68 %. The seed-set rate ranged from 0 % (29 crosses including 'ASK', 'BG', and 'BM' as pollen parents) to 7.22 % ('SR' as pollen parent), with an average of 0.97 %. Japanese accessions differed compatibility, but all were low; values ranged from 0 % (44 crosses, including 'ASK', 'BG', and 'BM' as pollen parents) to 6.67 % ('HRUM' as pollen parent) with an average of 0.68 %. Thirteen cultivars ('CTFJ', 'FM', 'HJM' 'HRUM', 'KJD', 'KY', 'MK', 'MKHR', 'SPZ', 'SR', 'SRK', 'SYD' and 'TI') had higher cross compatibility with 'HN' and fathered a total of 22 seedlings. Five of the hybrids since 2004 (from pollen parents 'SPZ', 'SR' and 'SYD') have already flowered. The overall seed germination rate was 12.2 % (mature seeds germinated 25.6 %). One seedling (from pollen parent 'KY') crossed in 2006 was weak and died. In 2011, two seedlings from pollen parent 'TI' were also in bad growth. Some cross combinations, e.g., with pollen parents 'TI', 'DKK' and 'HJM', produced many seeds, but most were immature.

The 57 cross combinations could be divided into five groups based on the seedlings (Table 2-3). In the first group, no seeds were set in 19 crosses (involving 2 black, 4 pink, 11 red, and 2 white Japanese cultivars). In the second group, seeds were set, but did not mature in 10 crosses (3 pink, 4 purple, and 3 white). In this third group, seeds matured but did not germinate in 15 crosses (1 black, 3 pink, 6 red, and 5 white). In the fourth group, seeds germinated, but all of the seedlings were weak or died in two crosses (1 pink and 1 red). In the fifth group, 11 crosses (5 pink, 3 purple,

1 red, and 2 white) produced seedlings and survived.

2.3.2 Floral morphology of parents and hybrids

Five hybrids from three combinations crossed in 2004 are growing vigorously and have already flowered. All bear yellow flowers with blotches, and the flowers were larger in diameter and had more petals than 'HN'. The hybrids with 'SYD' and 'SR' as pollen parents had similar color, with light yellow petals with red flushes and purple-black blotches. The hybrids with 'SPZ' as pollen parents had pure yellow petals and purple-red blotches similar to 'HN' (Fig. 2-1). Stalks of the hybrids with 'SPZ' and 'SR' as pollen parents were stronger than 'HN' and held the flowers upright, and 'SYD' as pollen parents, the stalks were stronger than 'HN' and held the flowers upward, but not fully upright (Table 2-4).

2.3.3 Analysis of hybridity by SRAP markers

The hybridity was confirmed by SRAP (sequence-related amplified polymorphism) markers. The results indicated that the five hybrids had the genetic information inherited from their parents (Fig. 2-2). For the cross combination 'HN' × 'SPZ', a total of seven amplicons were produced in the hybrid, among which, three fragments of 220 bp, 500 bp and 700 bp in length respectively detected in the hybrid and their parents, one fragment about 580 bp was also observed in the hybrid exhibited the same genotypes as the pollen parents, one fragment about 1400 bp was observed in the hybrid and seed parents. Two hybrids were obtained when 'SR' as the pollen parent. In the hybrid of 'HN' × 'SR'-1, two fragments were detected, one with 500 bp long was also detected in the parents, one polymorphic fragment about 450 bp was only detected in the hybrid while parents lacked. In the other hybrid 'HN' × 'SR'-2, two fragments 500 bp and 1400 bp

in length detected in the hybrid and their parents. When ‘SYD’ as the pollen parent, two hybrids were obtained, one band with 500 bp long was detected in both of them and their parents. One band about 1400 bp long detected in both of the two hybrids and seed parents. Two bands about 900 bp and 1000 bp long were detected in ‘HN’ × ‘SYD’-1 and the pollen parent, one band about 950 bp long was detected in ‘HN’ × ‘SYD’-2 and the pollen parent. One band about 2000 bp in ‘HN’ × ‘SYD’-1 and about 700 bp in ‘HN’ × ‘SYD’-2 was detected while parents lacked. All the above demonstrated that the hybrids obtained the genetic information from their parents, meanwhile they also present genetic variation as compared with their parents, which were the genetic base for their phenotype difference especially with ideal flower morphology.

2.3.4 Pollen tube growth behavior

The ‘SDJ’ × ‘KKJ’ cross between two Japanese cultivars had high cross compatibility, while, ‘HN’ × ‘KKJ’, between American and Japanese cultivars, had low cross compatibility. Pollen germination took place in both the high and low cross-compatibility combinations tested, but the pollen tubes grew differently. In the ‘SDJ’ × ‘KKJ’ cross, pollen tubes grew normally to the base of the style and penetrated the ovule (Fig. 2-3a-d). In the ‘HN’ × ‘KKJ’ cross, pollen tubes grew abnormally (Fig. 2-3e-h); the tubes twisted anomalously in the middle of the style (Fig. 2-3f) and could not penetrate the ovule because it bent when it reached the ovule (Fig. 2-3h). There were more pollen tubes in ‘SDJ’ × ‘KKJ’ than in ‘HN’ × ‘KKJ’ at the top, middle, and base of the style, and they reached the base of the style earlier. The pollen tube of ‘SDJ’ × ‘KKJ’ reached to the base of the style at 12 h after pollination, and the pollen tube of ‘HN’ × ‘KKJ’ was at 1 d after pollination reached to the base of the style. There were 19.0 and 15.0 pollen tubes, respectively, at

the bases of the styles in ‘SDJ’ × ‘KKJ’ and ‘HN’ × ‘KKJ’ after pollination for 2 d (Table 2-5). There were an average of 7.64 ovules per ovary in ‘SDJ’ and 12.73 in ‘HN’ (data not shown). Pollen tubes/ovule (P/O) was 2.49 in ‘SDJ’ × ‘KKJ’ and 1.18 in ‘HN’ × ‘KKJ’, respectively.

2.4 Discussion

In this study, 1,927 ‘HN’ flowers were crossed with Japanese cultivars, but 1,792 did not set seed, indicating that pre-fertilization barriers were significant, so we examined the performance of pollen grains in high (‘SDJ’ × ‘KKJ’) and low (‘HN’ × ‘KKJ’) compatibility crosses. Pollen grains of the Japanese cultivar ‘KKJ’ germinated on ‘HN’ stigmas, so pollen germination was not the fertilization barrier for this cross. He and Cheng (2006) also found that pollen grains of two Chinese cultivars, ‘Shuo Sheng Peng Mo’ and ‘Feng Dan Bai’, germinated well on ‘HN’ stigmas. More pollen tubes reached the base of the style where the ovules are available for fertilization in both crosses, but the ratio in ‘SDJ’ × ‘KKJ’ was higher than that of in ‘HN’ × ‘KKJ’ combination. Thus, the probability of fertilization was higher in the former cross, but the number of pollen tubes was not a major pre-fertilization barrier. Pollen tubes in the ‘HN’ × ‘KKJ’ cross grew abnormally in the middle of the style and bent at the ovule, so they could not penetrate it. This suggested that anomalous pollen tube growth was a significant pre-fertilization barrier. Abnormal pollen tube growth was observed in a lot of plants. One possible reason for pollen tube arrest is an inability to use stylar nutrients, perhaps due to a lack of suitable nutrients in the transmitting tract or suitable enzymes in the pollen tubes (Shivanna, 1996). The pollen tube of ‘HN’ × ‘KKJ’ cross reached to

the base of the style was later than the one in ‘SDJ’ × ‘KKJ’ cross maybe due to the pollen grew abnormally and twisted.

Of the 135 pollinated flowers that set seeds, 61 did not set mature seed; overall we obtained 47.5 % mature seeds and 52.5 % immature seeds. This suggested that the failure of fertilized ovules development was a significant post-fertilization barrier. Of the 74 pollinated flowers that set mature seed, the seeds of 53 could not germinate (nor could any of the immature seeds). The mature seed germination rate was 25.6 %, so seed germination was another important post-fertilization barrier. Of the 22 seedlings, two were weak and one died, suggesting that seedling health was only a minor post-fertilization barrier.

Although black-flowered pollen parents did not produce seedlings, seeds from all other flower colors germinated. This indicated that cross compatibility had no correlation with flower color. Only three black-flowered cultivars were studied, which may not completely explain the poor germination results.

Among ornamental plants, tree peonies have relatively short bloom durance. American and French tree peonies flower later than that of Japanese cultivars, which can elongate the flowering time of tree peony. In our studies, we observed that the five hybrids bloomed later than that of the Japanese cultivars did and had better presentation than that of ‘HN’. They could enhance the ornamental value of later-blooming tree peonies. Five of the hybrids crossed in 2004 have already flowered and are growing vigorously. They inherited the ‘HN’ flower color and had improved ornamental characteristics, increasing the aesthetic value of the whole plants. These results

suggested that we can breed superior cultivars by introducing genes of Japanese cultivars into French and American ones.

The morphological characteristics and flower color have maternal inheritance in the cross breeding of camellia (Nishimoto et al., 2003). Pollen germination rate of 'High Noon' in *vitro* was low, and most of Japanese cultivars was about 100% (data was not shown). To obtain yellow-flowered cultivars was one of the objectives in this study, so we used 'High Noon' as seed parents. Five of the hybrids have already flowered and have the flower-color and leaf shape near to seed parent, the tree performance inherited the characteristics of pollen parents, the hybrids had the stranger stalks which could hold the flower upright.

All the five hybrids and their parents were analyzed by SRAP markers. SRAP analysis has been used for identification of intersectional hybrid between section *Moutan* and section *Paeonia* and analysis of tree peony bud sports (Hao et al., 2008a, 2008b; Han et al., 2008a). In this study, the results of DNA analysis indicated that genetic information was changed among the hybrids. New special fragments were detected in the hybrids as well as the typical ones that same as the parents. When 'SR' and 'SYD' as the pollen parent, there were two hybrids were obtained, respectively, but the genetic differences between them were also observed. The two hybrids which obtained from same cross combination have different genotype. This showed that kinds of genetic restructuring were generated in the process of distant cross-breeding.

The American cultivar 'HN' is the offspring of *P. lutea* and a cultivar that may have come from Japan (Jane 1999). There is no evidence that *P. lutea* was involved in the differentiation of Japanese and Chinese cultivars (Li 1998; Zhou et al. 2003). Although 'HN' has Japanese cultivar

ancestry, it is only distantly related to Japanese cultivars at the molecular (Zhang et al., 2012) and morphological levels. ‘HN’ has yellow flowers like *P. lutea* and a similar growth habit, suggesting cross incompatibility between the American ‘HN’ and Japanese cultivars. However, the incompatibility was not complete. In this study, pollen grains from 57 Japanese cultivars were used to pollinate ‘HN’. The pollen parents differed in fruit-set rate, seed-set rate, and cross compatibility, although all were low. Pollination with pollen grains of thirteen cultivars (‘CTFJ’, ‘FM’, ‘HJM’ ‘HRUM’, ‘KJD’, ‘KY’, ‘MK’, ‘MKHR’, ‘SPZ’, ‘SR’, ‘SRK’, ‘SYD’, and ‘TI’) had higher cross compatibility with ‘HN’ than those of other cultivars and yielded hybrids. Evaluating the crossability between American ‘HN’ and Japanese cultivars may increase the frequency of superior cultivars through traditional breeding. Our results will enhance future tree peony breeding and can assist breeders in parent selection.

In summary, the three most important barriers to crosses between ‘HN’ and Japanese cultivars were abnormal pollen tube growth, the failure of fertilized ovules to develop, and poor seed germination. To overcome the failure of fertilized ovules to develop and seeds to germinate, *in vitro* culture of ovules or immature embryos may be appropriate. Abnormal pollen tube growth might be mitigated by ovary pollination, *in vitro* fertilization, or cutting the styles prior to pollination. The five hybrids that have flowered will be backcrossed in the future. More backcross generations will improve the inherited characteristics of tree peony offspring, producing better ornamental traits.

2.5 Abstract

The tree peony (*Paeonia suffruticosa* Andr.) is a valuable ornamental plant. American and French cultivars have desirable flower colors, while, Japanese cultivars have larger flowers with better presentation. We hybridized an American tree peony cultivar 'High Noon (HN)' (the seed parent) with 57 different Japanese cultivars (pollen parents) to investigate cross compatibility, with the ultimate goal of obtaining improved hybrids. Of the 1,927 crosses performed, 135 (38 cross combinations) yielded a total of 181 seeds (86 mature) and 22 seedlings. Five of the hybrids have already flowered and exhibited their parent character with large yellow flowers. To investigate the causes of cross incompatibility, we examined pollen tube growth in a cross between 'HN' and a Japanese cultivar. The result indicated that there are three most important causes of incompatibility, namely, abnormal pollen tube growth, the failure of fertilized ovules, and poor seed germination. Although cross compatibility in each combination was low, crosses between 'HN' and Japanese cultivars might be successful with the right paternal plants. In this study, 13 Japanese cultivars had higher cross compatibility with 'HN' than the others. Our results will enhance tree peony breeding and guide the selection of parents for hybridization.

Table 2-1. Fifty-seven Japanese cultivars used as pollen parents in this study.

Cultivar	Abbreviation	Flower color group[z]
Fuya Jou	FYJ	Black
Karasu ga San	KGS	Black
Yo Garasu	YG	Black
Asuka	ASK	Pink
Fuku Mukae	FM	Pink
Hana Kisoi	HNK	Pink
Himiko	HMK	Pink
Hare Umi	HRUM	Pink
Kai Hou	KHO	Pink
Mai Ka	MK	Pink
Muko Hara	MKHR	Pink
Shima Bijin	SBJ	Pink
Shimane Ji	SNJ	Pink
Sakara Jou	SJ	Pink
Silver River	SR	Pink
Ten I	TI	Pink
Tou Ka Sen	TKS	Pink
Yatsuka no Kaori	YTK	Pink
Yatsuka Jishi	YTKJ	Pink
Chitose Fuji	CTFJ	Purple
Ki Jou Den	KJD	Purple
Shima Daijin	SDJ	Purple
Shima no Hokori	SNH	Purple
Shi Ou Den	SOD	Purple
Sharaku	SRK	Purple
Yatsuka no Fuji	YTF	Purple
Beni Guruma	BG	Red
Beni Musume	BM	Red
Dai Ki Kou	DKK	Red
Daichi no Asa	DNA	Red
Heisei Ko	HSK	Red
Hi Sen	HSN	Red
Hou Tou	HT	Red
Koki Jishi	KKJ	Red
Ko Ka Den	KKD	Red
Kosai	KS	Red
Kou You	KY	Red

Table 2-1. Continued.

Cultivar	Abbreviation	Flower color group[z]
Ki Se Beni	KSB	Red
Ko You Den	KYD	Red
Shuku En	SE	Red
Shin Ka Jishi	SKJ	Red
Shima Zu Beni	SMZB	Red
Shima Nishiki	SNK	Red
Shin Shima no Kagayaki	SSK	Red
Shou You Den	SYD	Red
Hakuju Mon	HJM	White
Hakuba	HKB	White
Hakkoden	HKZ	White
Hakuraku Den	HRS	White
Hakutei Jou	HTJ	White
Hakuunzan	HUN	White
Kikkou Shiro	KKH	White
Shin Fusou	SFS	White
Shokaku	SK	White
Seppakuzan	SPZ	White
Shirasagi	SS	White
Shiro Hokkyokusei	WP	White

[z]Tree peony cultivars are classified into nine flower-color groups: red, pink, purple, white, yellow, black, green, blue, and double color.

Table 2-2. Fruit-set rate, seed-set rate, and cross compatibility of Japanese tree peony cultivars used as pollen parents with the American cultivar 'HN'.

Pollen parent	Number of pollinated flowers	Number of pollinated pistils	Number of flowers that set seed	Number of pistils that set seed	Number of seeds set			Number of seedlings	Fruit-set rate[z] (%)	Seed-set rate[y] (%)	Cross compatibility[x] (%)
					Total	Mature	Immature				
Seed germinated and seedling survived											
CTFJ	47	239	1	1	1	1	0	1	0.42	0.42	2.13
FM	30	156	1	1	1	1	0	1	0.64	0.64	3.33
HJM	80	371	16	21	21	1	20	1	5.66	0.27	1.25
HRUM	15	76	2	2	2	1	1	1	2.63	1.32	6.67
KJD	88	405	8	9	9	3	6	3	2.22	0.74	3.41
MK	60	272	10	11	15	8	7	2	4.04	2.94	3.33
MKHR	42	203	3	4	4	2	2	1	1.97	0.99	2.38
SPZ	73	326	8	9	10	5	5	2	2.76	1.53	2.74
SR	56	263	14	25	29	19	10	3	9.51	7.22	5.36
SRK	269	1258	19	19	22	15	7	1	1.51	1.19	0.37
SYD	77	369	3	4	7	5	2	3	1.08	1.36	3.90
Seed germinated but seedling grew weak or died											
KY	68	311	7	7	10	3	7	1	2.25	0.96	1.47
TI	80	425	4	5	10	3	7	2	1.18	0.71	2.50
Mature seed set but didn't germinate											
DKK	32	159	4	4	5	1	4	0	2.52	0.63	0.00
HSK	7	33	1	1	1	1	0	0	3.03	3.03	0.00
KKD	6	31	1	1	1	1	0	0	3.23	3.23	0.00
KKJ	10	48	1	1	1	1	0	0	2.08	2.08	0.00
HKZ	21	100	1	1	1	1	0	0	1.00	1.00	0.00
HNK	26	133	3	3	3	3	0	0	2.26	2.26	0.00
HRD	6	24	1	1	1	1	0	0	4.17	4.17	0.00
HUZ	15	71	2	2	2	1	1	0	2.82	1.41	0.00
KYD	10	38	2	2	2	2	0	0	5.26	5.26	0.00
SBJ	121	642	3	3	3	2	1	0	0.47	0.31	0.00
SK	16	67	1	1	1	1	0	0	1.49	1.49	0.00
SMZB	59	272	2	2	2	1	1	0	0.74	0.37	0.00
SNJ	5	22	1	1	1	1	0	0	4.55	4.55	0.00
SS	8	35	1	1	1	1	0	0	2.86	2.86	0.00
YG	10	46	1	1	1	1	0	0	2.17	2.17	0.00
No mature seed set											
HKB	40	180	2	2	2	0	2	0	1.11	0.00	0.00
KHO	53	249	1	1	1	0	1	0	0.40	0.00	0.00
KKH	15	69	2	2	2	0	2	0	2.90	0.00	0.00
SDJ	20	94	1	1	1	0	1	0	1.06	0.00	0
SNH	10	43	1	1	1	0	1	0	2.33	0.00	0

Table 2-1. Fifty-seven Japanese cultivars used as pollen parents in this study.

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Hana Kisoi	HNK	Pink
Himiko	HMK	Pink
Hare Umi	HRUM	Pink
Kai Hou	KHO	Pink
Mai Ka	MK	Pink
Muko Hara	MKHR	Pink
Shima Bijin	SBJ	Pink
Shimane Ji	SNJ	Pink
Sakara Jou	SJ	Pink
Silver River	SR	Pink
Ten I	TI	Pink
Tou Ka Sen	TKS	Pink
Yatsuka no Kaori	YTK	Pink
Yatsuka Jishi	YTKJ	Pink
Chitose Fuji	CTFJ	Purple
Ki Jou Den	KJD	Purple
Shima Daijin	SDJ	Purple
Shima no Hokori	SNH	Purple
Shi Ou Den	SOD	Purple
Sharaku	SRK	Purple
Yatsuka no Fuji	YTF	Purple
Beni Guruma	BG	Red
Beni Musume	BM	Red
Dai Ki Kou	DKK	Red
Daichi no Asa	DNA	Red
Heisei Ko	HSK	Red
Hi Sen	HSN	Red
Hou Tou	HT	Red
Koki Jishi	KKJ	Red
Ko Ka Den	KKD	Red
Kosai	KS	Red
Kou You	KY	Red

Table 2-3. Summary of hybridization results by pollen-parent flower color.

Results of hybridization	Number of cultivars					Total
	Red	White	Purple	Pink	Black	
No seeds set	11	2	0	4	2	19
Only immature seeds set	0	3	4	3	0	10
Mature seeds set but did not germinate	6	5	0	3	1	15
Seeds germinated, but seedlings were weak or died	1	0	0	1	0	2
Seeds germinated and seedlings survived	1	2	3	5	0	11
Total	19	12	7	16	3	57

Table 2-4. Floral morphology of parents and their hybrid offspring.

Cultivar	Flower color						Flower diameter (cm)	Number of petals	Flower stalk
	Petal			Blotch					
	L*	C*	h°	L*	C*	h°			
HN	77.7	41.3	108.8	51.3	28.0	49.4	13.0	26.4	Sideward
SPZ	77.1	3.3	120.1		_[z]		20.5	28.4	Upright
SR	56.8	17.8	9.2	16.5	14.2	3.5	17.2	45.0	Upward
SYD	30.3	38.9	17.5		_		19.5	63.2	Upright
HN × SPZ	77.3	29.9	112.0	42.6	35.8	10.7	15.9	57.3	Upright
HN × SR-1	73.0	31.5	108.7	25.0	32.7	12.7	18.2	38.3	Upright
HN × SR-2	73.4	33.6	102.6	20.7	28.5	8.7	15.9	34.3	Upright
HN × SYD-1	68.6	22.9	101.3	23.4	34.1	10.7	17.2	58.8	Upward
HN × SYD-2	65.6	17.3	98.6	26.9	37.7	9.2	13.8	55.5	Upward

[z] The flower petals of SPZ and SYD don't have blotch.

Table 2-5. Number of pollen tubes at different points in the style at different times after pollination in two crosses with high (‘SDJ’ × ‘KKJ’) and low (‘HN’ × ‘KKJ’) compatibility.

Cross combination	Time after pollination	Number of pollen tubes		
		Top of the style	Middle of the style	Base of the style
‘SDJ’×‘KKJ’	6 h	43.6±5.8	34.2±8.2	0.0±0.0
	12 h	40.4±9.4	21.2±8.1	2.0±4.5
	1 d	40.8±2.9	28.4±5.4	3.6±5.7
	2 d	50.0±13.2	27.0±8.3	19.0±2.5
	10 d	42.0±7.3	31.8±4.7	16.6±2.6
‘HN’×‘KKJ’	6 h	28.2±2.9	24.2±3.4	0.0±0.0
	12 h	27.2±5.6	23.8±5.8	0.0±0.0
	1 d	25.4±2.7	24.0±3.8	3.6±5.4
	2 d	26.8±4.0	20.6±1.5	15.0±1.6
	10 d	28.4±7.1	23.8±2.9	15.0±1.6

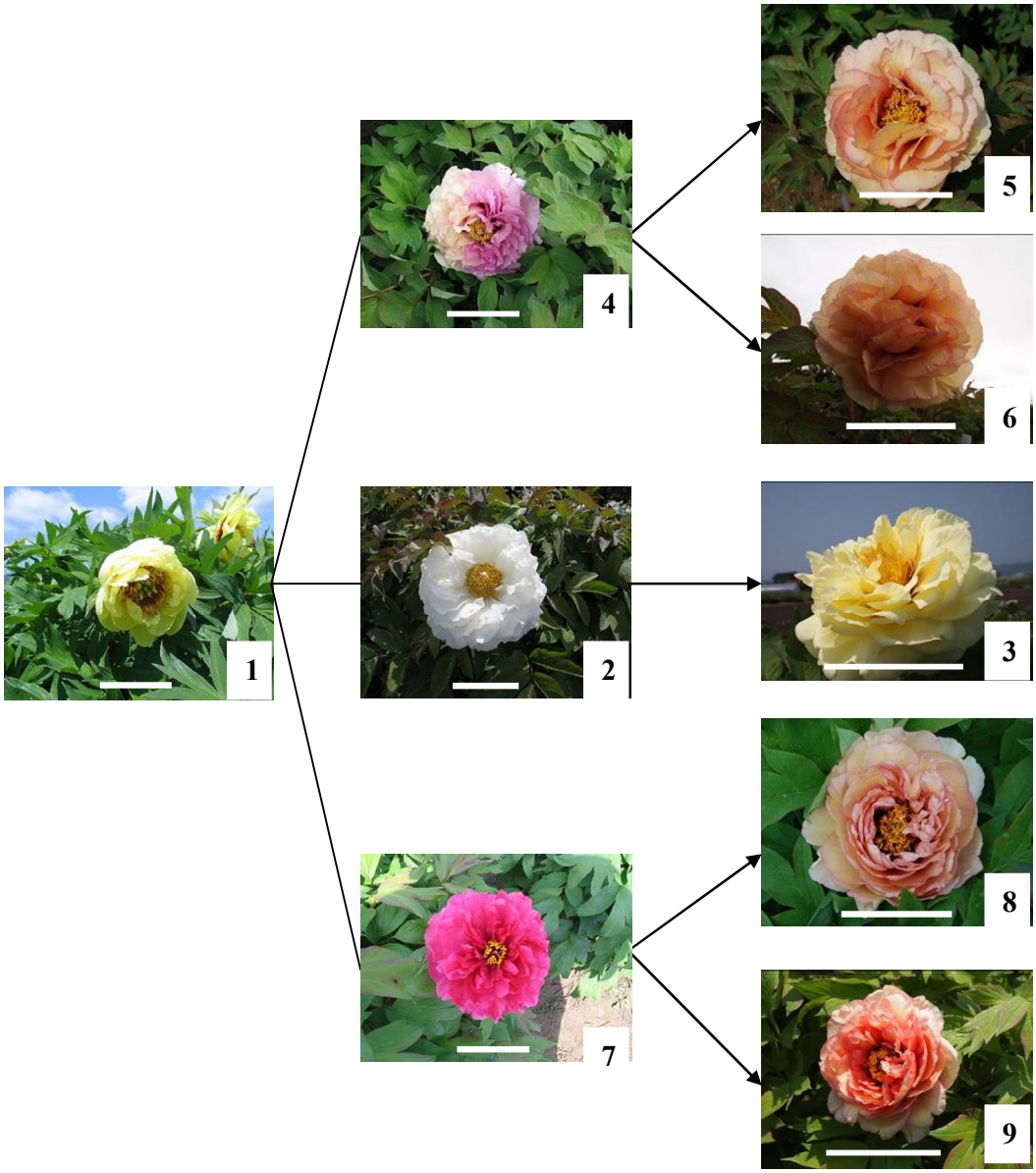

Fig. 2-1. Photographs of hybrids which have flowers and their parents used in this study.

1 ‘HN’: seed parent, 2 ‘SPZ’, 4 ‘SR’, 7 ‘SYD’: pollen parent, 3 ‘HN’ × ‘SPZ’: the hybrids of ‘HN’ and ‘SPZ’, 5 ‘HN’ × ‘SR’-1, 6 ‘HN’ × ‘SR’-2: two hybrids of ‘HN’ and ‘SR’, 8 ‘HN’ × ‘SYD’-1, 9 ‘HN’ × ‘SYD’-2: two hybrids of ‘HN’ and ‘SYD’. Scale bars = 10 cm.

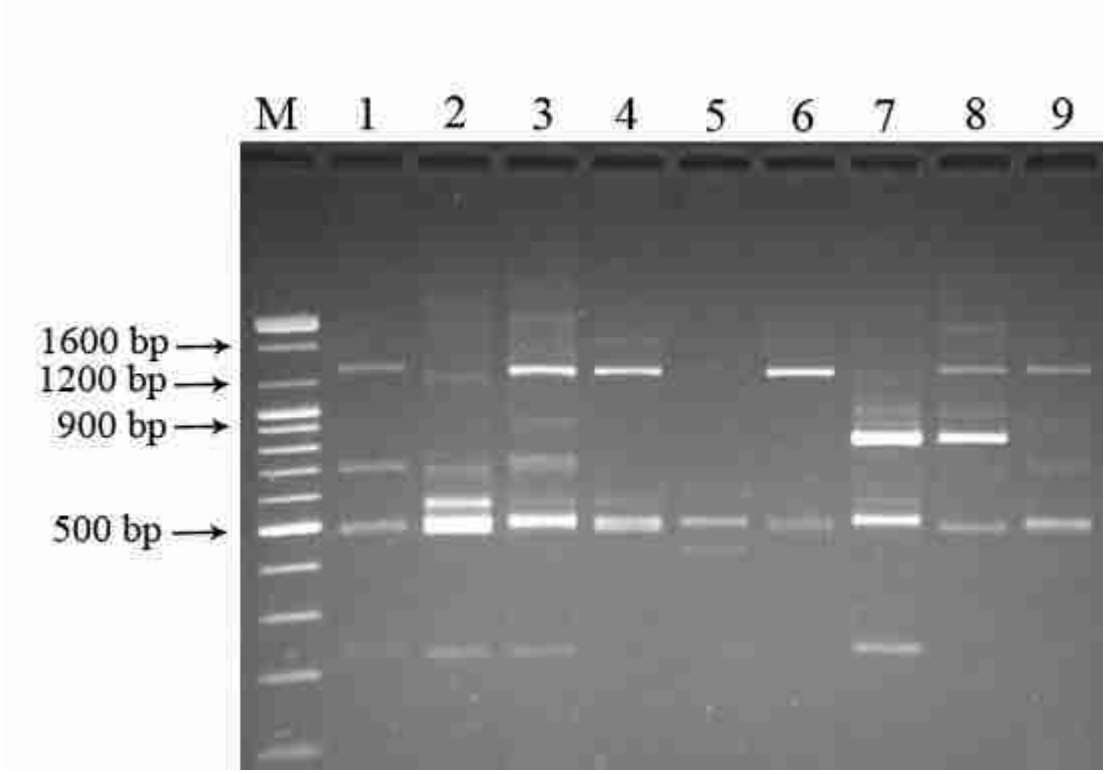

Fig. 2-2. A profile of PCR products of the hybrids and their parents using primer pair of Me5/Em1.

Lane M: 100 bp DNA ladder marker. Lanes: 1 ‘HN’, 2 ‘SPZ’, 3 ‘HN’ × ‘SPZ’, 4 ‘SR’, 5 ‘HN’ × ‘SR’-1, 6 ‘HN’ × ‘SR’-2, 7 ‘SYD’, 8 ‘HN’ × ‘SYD’-1, 9 ‘HN’ × ‘SYD’-2.

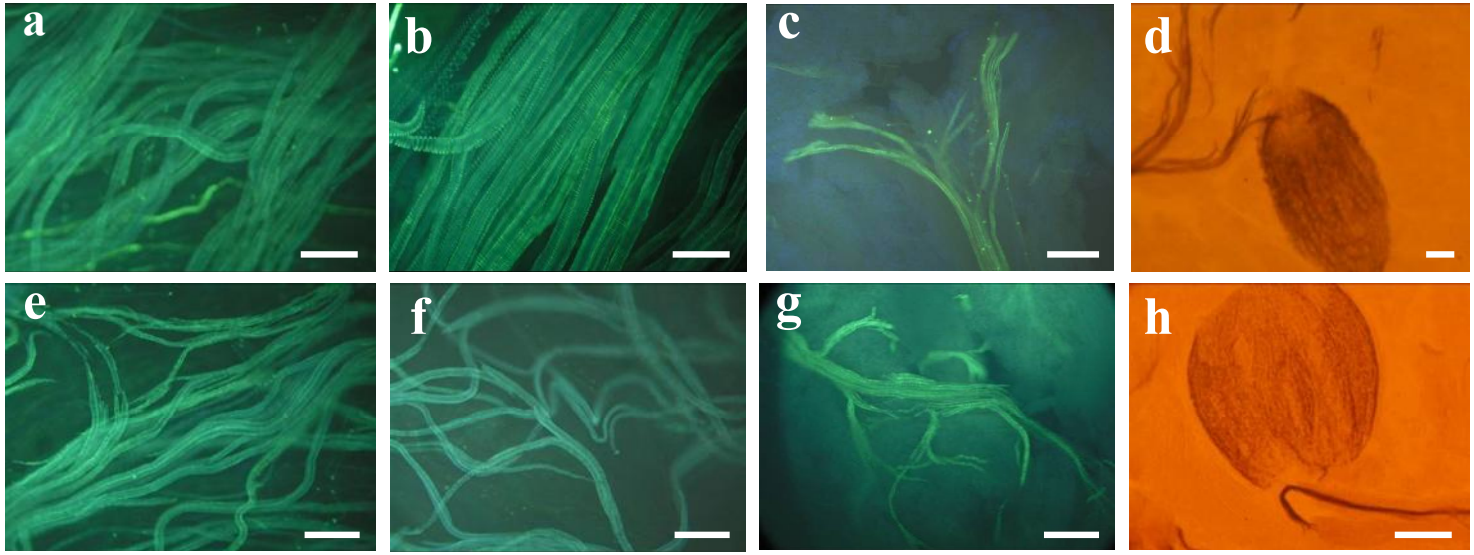

Fig. 2-3. Pollen tube growth in the styles of ‘SDJ’×‘KKJ’ and ‘HN’×‘KKJ’.

Pollen tubes in the top (a), middle (b), and base (c) of the style and penetrating the ovule (d) in ‘SDJ’×‘KKJ’.

Pollen tubes in the top (e), middle (f), and base (g) of the style and penetrating the ovule (h) in ‘HN’×‘KKJ’.

Scale bars =50 μm.

Chapter 3

Occurrence of giant pollen grains in tree peony cultivars

3.1 Introduction

Most tree peony cultivars are diploid, with the exception of the Chinese cultivar 'Shou An Hong', which with many virtues include larger flowers with numerous petals and high disease resistance. Polyploids arise either by sporadic chromosome doubling or by the union of unreduced gametes with other unreduced gametes or with normal reduced gametes. The latter case is considered more likely to occur in nature (Sugiura et al., 2000). It is possible to obtain polyploids by using the unreduced pollen grains which generating by abnormal meiotic of pollen mother cell to pollination. It was reported that the polyploids were bred by using the unreduced pollen grains in persimmon and citrus, etc. Breeders hope to exploit unreduced pollen to breed polyploid tree peony cultivars with desirable ornamental characteristics. According to previous studies of 2n pollen grains, there is a positive correlation between pollen size and number of chromosomes, so we can determine whether the pollen is diploid by measuring its diameter. In this study, the occurrence of giant pollen in several Japanese tree peony cultivars was surveyed, and pollen diameter was measured and the size distribution determined for each cultivar. Moreover, pollen germination was also observed.

3.2 **Materials and methods**

3.2.1 Plant materials

Seven Japanese tree peony cultivars: ‘Kai Hou’, ‘Ki Jou Den’, ‘Kou You’, ‘Sharaku’, ‘Shi Ou Den’, ‘Shou You Den’ and ‘Yachiyo Tsubaki’ were used as materials. All plants were obtained from the private collections of flower growers on Daikonjima Island (Matsue, Shimane Prefecture, Japan).

3.2.2 Collection of pollen grains

Flowers of the seven cultivars were collected 2 days before anthesis. The corollas were removed with forceps, and the flowers were dried over 2 days at room temperature (≈18 °C). The released pollen grains were collected and the germination was observed immediately. Other pollen grains were stored with silica gel and used to survey size.

3.2.3 Pollen size

The dried pollen grains were put in the slide glass, distilled water was added to make the pollen scattered, and the pollen diameter was measured under a microscope, the rate of giant pollen was counted.

3.2.4 Pollen germination

The pollen grains were scattered in liquid culture medium (0.01% (*w*/*v*) $CaCl_2$, 0.01% (*w*/*v*) H_3BO_3, 0.0007% (*w*/*v*) KH_2PO_4, 10% (*w*/*v*) sucrose, and 0.01% (*w*/*v*) yeast extract, pH 5.8) in a culture dish. After 10 min to 1 h, pollen germination was observed under a microscope (Hirano and Hoshino, 2009).

3.3 Results

The pollen grains of tree peonies existed in three forms: abortive, normal, and giant pollen (Fig. 3-1, 3-2). The normal pollen grain of 'Kai Hou', 'Ki Jou Den', 'Kou You', 'Shi Ou Den', 'Sharaku', 'Shou You Den' and 'Yachiyo Tsubaki' showed a major peak in size distribution about 44 μm, 40 μm, 42 μm, 44 μm, 42 μm, 39 μm and 42 μm, respectively. According to the diameter of 2n pollen was approximately 30% larger than that of haploid pollen in other plants was reported, and combining the pollen diameter distribution in this study, the diameter of giant pollen grains in tree peony cultivars distributed in about 53-63 μm were got (Fig. 3-3). The pollen grain which pollen diameter $\geqslant$ 53 μm was defined as giant pollen and the giant pollen rate was counted. The percentage of giant pollen differed among the seven cultivars was low, from approximately 0-1.16%. 'Kou You' lacked giant pollen, while giant pollen occurred at rates of 0.12% in 'Shou You Den', 0.23% in 'Shi Ou Den', 0.42% in 'Ki Jou Den', 0.58% in 'Sharaku', 0.80% in 'Kai Hou', and 1.16% in 'Yachiyo Tsubaki' (Table 3-1). Giant pollen could germinate but did so later than normal pollen which showed that the germination of giant pollen was difficult (Fig. 3-4).

3.4 Discussion

These data confirmed that giant pollen exists in tree peony cultivars but at very low rates, and the germination of giant pollen was later than normal pollen that consistent with the conclusion of

the study in persimmon reported by Gu and Luo (2002). Making their direct use in cross-fertilization is difficult. In practice, physical methods of separation or chemical methods to induce 2n pollen will be needed. Sugiura et al. (2000) used nylon mesh to efficiently separate giant and normal pollen. Tree peony pollen grains will be sorted with nylon mesh to isolate giant grains from normal ones in the future. The nuclear DNA content of sorted pollen grains will also be determined by flow cytometry to verify the ploidy level of giant pollen.

3.5 Abstract

Pollen diameter and germination of seven Japanese tree peony cultivars were studied in order to confirm the existing of giant pollen and the rate in tree peony. The pollen grains of Japanese tree peony cultivars existed in three forms: abortive pollen, normal pollen and giant pollen. The natural pollen diameter mainly focused on 39 to 44 m and giant pollen diameter distributed in 53-63 m. The percentage of giant pollen was different among the seven cultivars, about 0-1.16%. The giant pollen could germinate but it was later than natural pollen.

Fig. 3-1. The three pollen forms of ‘Kai Hou’.

1. Giant pollen 2. Normal pollen 3. Small pollen

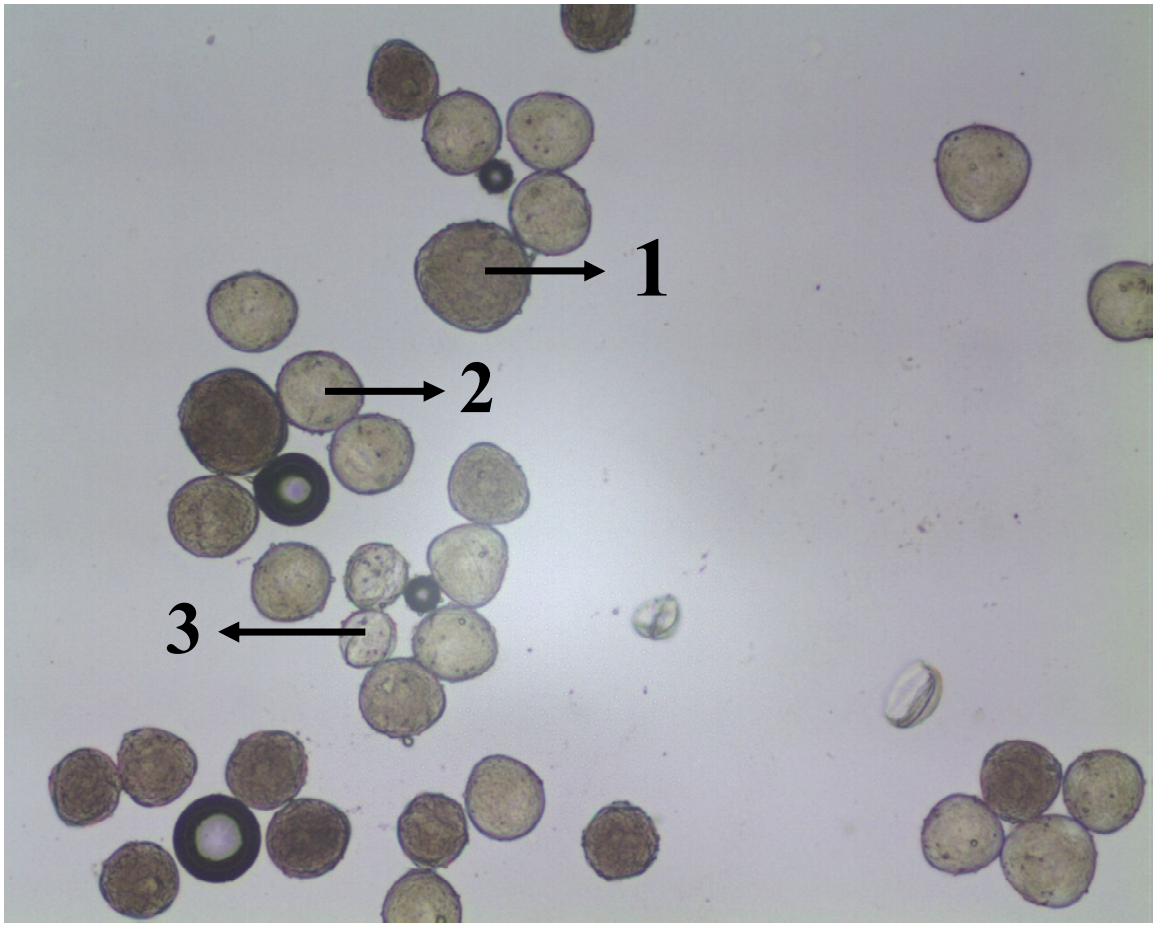

Fig. 3-2. The three pollen forms of ‘Yachiyo Tsubaki’.

1. Giant pollen; 2. Normal pollen; 3. Small pollen.

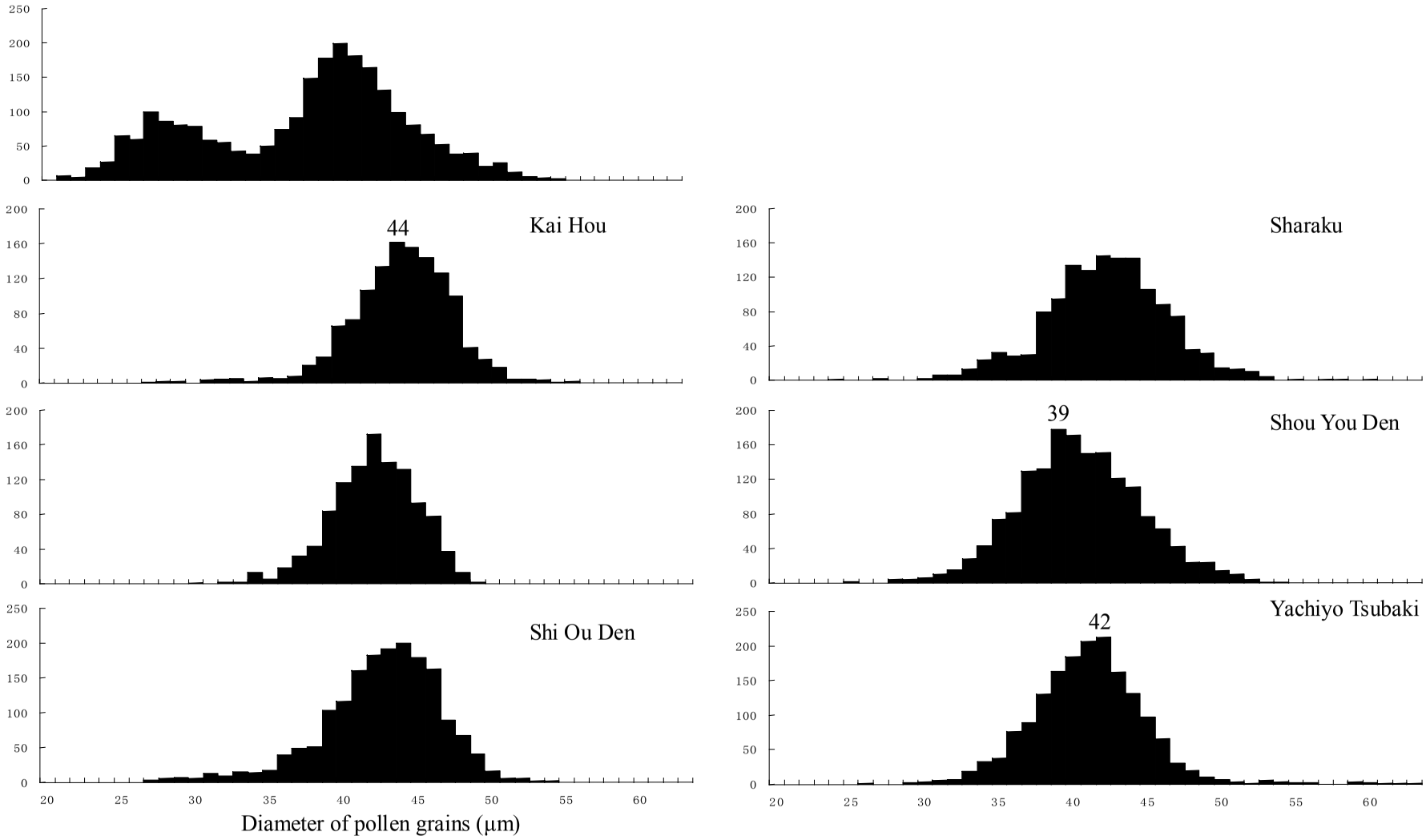

Fig. 3-3. The distribution pattern of pollen size in 7 cultivars.

Table 3-1. Number of pollen grains were measured and the frequency of giant pollen.

Cultivar	Number of pollen grains		Giant pollen (%)
	Total	Giant (≥53 μm)	
Kai Hou	1255	10	0.80
Ki Jou Den	2374	10	0.42
Kou You	1118	0	0.00
Sharaku	1391	8	0.58
Shi Ou Den	1746	4	0.23
Shou You Den	1671	2	0.12
Yachiyo Tsubaki	1718	20	1.16

Fig. 3-4. The pollen germination of giant pollen (arrow) and normal pollen.

Chapter 4

Changes in tree body, soluble protein content, SOD activity, and MDA content in aging tree peony

4.1 Introduction

The cultivation status of tree peony in China and Japan is shown in Table 4-1. Heze, Shandong Province, and Luoyang, Henan Province are famous in China for their tree peonies. In Japan, tree peonies are widely cultivated in temples and parks in Matsue, Shimane Prefecture, in Sukagawa, Fukushima Pref., and in Sakurai, Nara Prefecture.

Tree peony is an especially important tour resource. However, as plants in peony gardens age, their branches wither and dry and the plants grow weak and eventually die, reducing their ornamental value. Therefore, determining the causes of and inhibiting the progress of aging to extend ornamental lifetimes are very important and significant goals in horticulture.

Protein content, SOD activity and MDA content are three indexes that used for investigating postharvest aging of leaf and flower. A decrease of protein content typically occurs with plant aging, in addition, MDA is a product of membrane lipid peroxidation, and an increase in MDA levels is an inherent characteristic of aging cells (Li et al., 2004). The accumulation of free radicals can speed the aging process. SOD plays an important role in clearing free radicals, so a decrease in SOD activity could accelerate aging (Del Rio, 1998; Leshem, 1988). In the woody

Pinus taiwanensis, protein content in leaves decreased with aging (Li et al., 1998), and SOD activity in leaves of ancients tree was lower than in young trees of *Pinus tabulaeformis* (Guo et al., 2011).

'Luo Yang Hong', with purple-red petals, is one of the most representative Chinese tree peony cultivars. It is mainly cultivated in parks and temples in China as an ornamental plant and is currently one of the most commonly-used cultivars in forcing culture. It also had been introduced to Yatsuka-cho, Matsue, in Japan. In this study, the tree body and leaf protein content, SOD activity, and MDA content of different-age 'Luo Yang Hong' plants in the same resource garden were measured to establish baselines and to examine changes with aging. Correlations between plant age after division and these leaf parameters were also studied.

4.2 Materials and methods

4.2.1 Plant materials

Three trees in each of six age brackets (5, 10, 15, 20, 25, and 30 years after division) of tree peony 'Luo Yang Hong' growing in the same resources garden of Baihua Park, Heze, Shandong Province, China were examined.

4.2.2 Measurements of tree body

In April 2006 (the flowering season), plant height and width and numbers of flower buds, flowers, and blasting flower buds per tree were investigated. Five new flowering branches of each tree were evaluated for branch length, leaf expansion width, largest leaf size (length and width),

leaf color, flower diameter, and flower color. Color was measured with an NF-333 spectrophotometer (Nippon Minolta, Osaka, Japan). Lightness (L^*) and two chromatic components a^* and b^* on the CIELAB color scale were measured, and chroma (C^*) was calculated from the equation $(a^{*2}+b^{*2})^{1/2}$. Hue angle (h°) was calculated from the equation ATAN $(b^*/a^*)/6.2832 \times 360$ (McGuire, 1992).

4.2.3 Protein content, SOD activity and MDA content

In early August, three branches per tree were selected, and three compound leaves per branch (near the apex, middle, and base) were picked, wrapped immediately in 5 × 10 cm paper, frozen in liquid nitrogen, and finally stored in a freezer at -70 °C until needed.

Leaf samples (0.5 g) were triturated in 2.5 mL of cold solution (50 mM phosphate buffer, pH 7.8, 2% *w/v* polyvinyl pyrrolidone) and centrifuged at 1,000 rpm for 20 min at 4 °C. The supernatant was used to estimate protein content, SOD activity, and MDA content according to the method of Li et al. (2000).

4.2.4 Statistical analyses

All statistical treatments were performed using the SPSS for Windows package 19.0.

4.3 Results

4.3.1 Growth vigor at different plant ages

Plant growth vigor increased in 5- to 15-year-old ones and decreased in 15-to 30-year-old (Fig. 4-1). Plant height increased with age. Plant width was smallest in 5-year-old plants and largest in

25 year olds. New branch length increased in plants from 5–15 years after division, peaked at the age of 15, and decreased in older plants. The leaf expansion width 30-year-old plants was significantly smaller than in 5- to 15-year-old ones. Leaf length was the shortest in the oldest plants and longest in 15-year-old trees, which had significantly longer leaves than 25- and 30-year-old plants. There were no significant differences in leaf width among ages (Table 4-2).

4.3.2 Leaf and flower color at different ages

Leaf and flower color in plants of 'Luo Yang Hong' of different ages are summarized in Table 4-3. For leaf color, there were no significant difference in L^* or C^* among ages, although h° was smallest in 10-year-old plants and largest in 15-year-old ones; these values were significantly different. There were no significant differences in flower L^*, C^*, or h° among plant ages.

4.3.3 Flowering at different ages

The number of flower buds tended to increase between 5 and 15 years after division, however, it tended to decrease after 15 years. Flower number also increased with age; 5-year-old plants had significantly fewer flowers than older plants, which did not differ significantly from one another, although flower number was highest in 15- and 25-year-old plants. Plants 5 and 10 years of age had few blasting flower buds and high flowering rates (62% and 81%, respectively). However, the older plants with many blasting flower buds had low flowering rates (46%); the 30-year-old plants had the lowest flowering rate (18%). There were no significant differences in flower diameter among plants of different ages (Table 4-4).

4.3.4 Protein content, SOD activity, and MDA content in the leaves

Protein content and plant age were positively correlated with age from 5-15 years ($r=0.992$, P

≤ 0.01; Fig. 4-2A), and negatively correlated with age from 15–30 years (r＝-0.982, $P \leq 0.01$; Fig. 4-2A). SOD activity tended to decrease with plant age (r＝-0.888, $P < 0.05$, Fig. 4-2B), while MDA content increased (r＝0.940, $P < 0.01$; Fig. 4-2C).

4.4 Discussion

In ‘Luo Yang Hong’ tree peonies, measures of plant bodies (new branch length, leaf length, and number of flower buds) increased until 15 years after division, after which they tended to decrease. Therefore, these results showed that shorter branches and leaves and fewer flowers were characteristics of aging. In particular, new branch length declined drastically in plants older than 20 years, so this characteristic of aging accelerated. In contrast, leaf and flower color were not correlated with plant age, indicating no direct relationship between these traits and aging.

Protein content and plant age were positively correlated until 15 years post-division and negatively correlated afterwards. In addition, SOD activity was inversely correlated and MDA content was directly correlated with plant age. Therefore, these changes were characteristic of aging in ‘Luo Yang Hong’. In cut flowers of tree peony during low-temperature storage (Liu et al., 2005) and in freesia after full bloom (Shu et al., 2010), protein content and SOD activity decreased and MDA content increased over time. Although these two previous studies examined petals and our study analyzed leaves, the results were consistent. These data support the view that changes in protein content, SOD activity, and MDA content are useful indices of plant aging.

Various environmental stresses cause active oxygen to accumulate in cells, and changes in

antioxidant enzyme activity and lipid peroxidation are causes of plant aging (Mittler and Zilinskas, 1994; Shalata and Tal, 1998; Tsang et al., 1991; Wang et al., 2007). In the resources garden where this study was conducted, organic (soybean meal) and chemical fertilizers were applied in the autumn of each year. Chemical fertilizers are rarely applied to tree peony in Japan, and can promote long-term accumulation of salts in the soil. We believe that these salts were a major cause of tree peony aging in this study.

Polyamine, a plant hormone, can improve stress tolerance, delay protein degradation, and slow MDA accumulation due to membrane lipid peroxidation (Tiburcio et al., 1994; Galston, 1990). Moreover, the immersion in polyamine solution prolonged the life of cut flowers of carnation (Lee et, 1997). Furthermore, sunflowers grown in sand that were sprayed with salicylic acid, another plant hormone, had increased SOD activity even under salt stress (Noreen et al., 2009). Therefore, the effectiveness of these two substances in delaying aging in tree peony should be investigated.

Tree peonies are propagated in Japan by grafting onto herbaceous peony roots and in China by division and grafting onto tree peony ‘Feng Dan’ roots. There are no differences between the two types of stock, because these roots can provide nutrition even after 5–10 years. Therefore, we expect no direct relationship between the type of propagation and aging in tree peony.

To examine the effects of foliar application of salicylic acid and polyamine on delaying aging in tree peony, plants of different ages must be studied in the future.

4.5 Abstract

Tree peony ‘Luo Yang Hong’ with different ages (5, 10, 15, 20, 25, 30-year-old) in the same resources garden were studied, based on the investigation on tree body and soluble protein content, superoxide dismutase (SOD) activity, malondialdehyde (MDA) content in the leaves, the senescence phenomenon of tree peony were clarified. The results indicated that new branch length, leaf length and the number of flower buds increased till to 15 years after division and tended to decrease from the ones of 20-year-old. Especially, the new branch length extremely became shorter in the plants after 20-year-old, the aging was the characteristic of the aging was accelerated. There was positive correlation between protein content and plant age in the plants of 5 to 15-year-old, and was negative correlation in the ones from 15- to 30- year-old. In addition, the negative correlation between SOD activity and plant age, and positive correlation between MDA content and plant age were demonstrated. New branch length, leaf length, the number of flower buds, protein content, SOD activity and MDA content could be used as indices to diagnose the senescence and weakness of ancient tree peony.

Table 4-1. The cultivation status of tree peony in the main producing areas and tour resorts.

Peony-producing area		Growing area (ha)	Number of cultivars	Number of plants sold/year	Number of plants cultivated	Planting purpose
Country	Place					
Main producing area						
China	Heze City (荷澤市), Shandong Province	16,700	1,240	2,500,000	750,000,000	Sale, sightseeing, medicine and seed oil
	Luoyang City (洛陽市) , Henan Province	6,700	1,260	1,500,000	300,000,000	Sale, sightseeing, medicine and seed oil
	Tongling City (銅陵市), Anhui Province	600	20	10,000,000	300,000,000	Medicine
Janpan	Matsue City, Shimane Prefecture	70	300	1,500,000	3,000,000	Sale and sightseeing
	Gosen City, Niigata Prefecture	20	210	161,000	400,000	Sale and sightseeing
Tour resort						
Japan	Sukagawa peony park, Fukushima Prefecture	10 [z]	290	2,000	7,000	Sale and sightseeing
	Hase Temple, Nara Prefecture	83 [z]	150	—[y]	7,000	Sightseeing

[z]This is the area of the whole park not growing area.

[y]Tree peonies cultivated in Hase Temple are just for sightseeing, and not for sale.

Table 4-2. Comparison of growth vigor and leaf size among plants of different ages.

Plant age	Plant vigor (cm)				Leaf size (cm)	
	Plant height	Plant width	New branch length	Leaf expansion width/cut flower	Length	Width
5 Y	77.3 d[z]	108.7 c	16.9 c	51.7 a	30.2 abc	18.9 a
10 Y	100.3 cd	123.0 c	22.8 b	52.4 a	33.0 ab	20.7 a
15 Y	112.7 bc	126.7 c	29.2 a	53.4 a	33.7 a	21.6 a
20 Y	123.3 bc	169.0 b	25.5 ab	49.6 ab	30.6 abc	20.8 a
25 Y	135.3 ab	201.7 a	16.0 c	48.8 ab	26.6 bc	18.8 a
30 Y	147.7 a	182.7 ab	12.9 c	42.7 b	25.8 c	18.5 a

[z]The numbers followed by the same letter within a column are not significantly different according to Tukey-HSD test at the 5% level.

Table 4-3. Comparison of leaf and flower color among plants of different ages.

Plant age	Leaf color			Flower color		
	L^*	C^*	h°	L^*	C^*	h°
5 Y	43.4 a[z]	33.8 a	119.1 ab	37.5 a	43.5 a	344.7 a
10 Y	45.8 a	35.1 a	117.7 b	35.9 a	44.9 a	345.8 a
15 Y	41.8 a	33.0 a	123.5 a	36.5 a	45.7 a	345.7 a
20 Y	44.0 a	34.5 a	118.8 ab	36.4 a	47.5 a	347.2 a
25 Y	43.3 a	32.5 a	121.1 ab	37.0 a	43.7 a	343.4 a
30 Y	43.2 a	33.0 a	121.2 ab	36.5 a	46.3 a	346.5 a

[z]The numbers followed by the same letter within a column are not significantly different according to Tukey-HSD test at the 5% level.

Table 4-4. Comparison of flowering behavior among plants of different ages.

Plant age	Number of flower buds	Number of flowers	Number of blasting flower buds	Flowering rate (%)	Flower diameter (cm)
5 Y	8.6 c[z]	5.3 b	3.3 b	61.6 ab	14.3 a
10 Y	19.4 c	15.7 ab	3.7 b	80.9 a	14.0 a
15 Y	96.4 a	37.7 a	58.7 a	39.1 abc	12.5 a
20 Y	83.3 ab	25.3 ab	58.0 a	30.4 bc	13.7 a
25 Y	79.3 ab	36.3 a	43.0 a	45.8 abc	14.3 a
30 Y	66.0 b	11.7 ab	54.3 a	17.7 c	13.2 a

[z]The numbers followed by the same letter within a column are not significantly different according to Tukey-HSD test at the 5% level.

Fig. 4-1. The growth status of 'Luo Yang Hong' at different plant ages.
[z] Number in each photograph is the plant age.

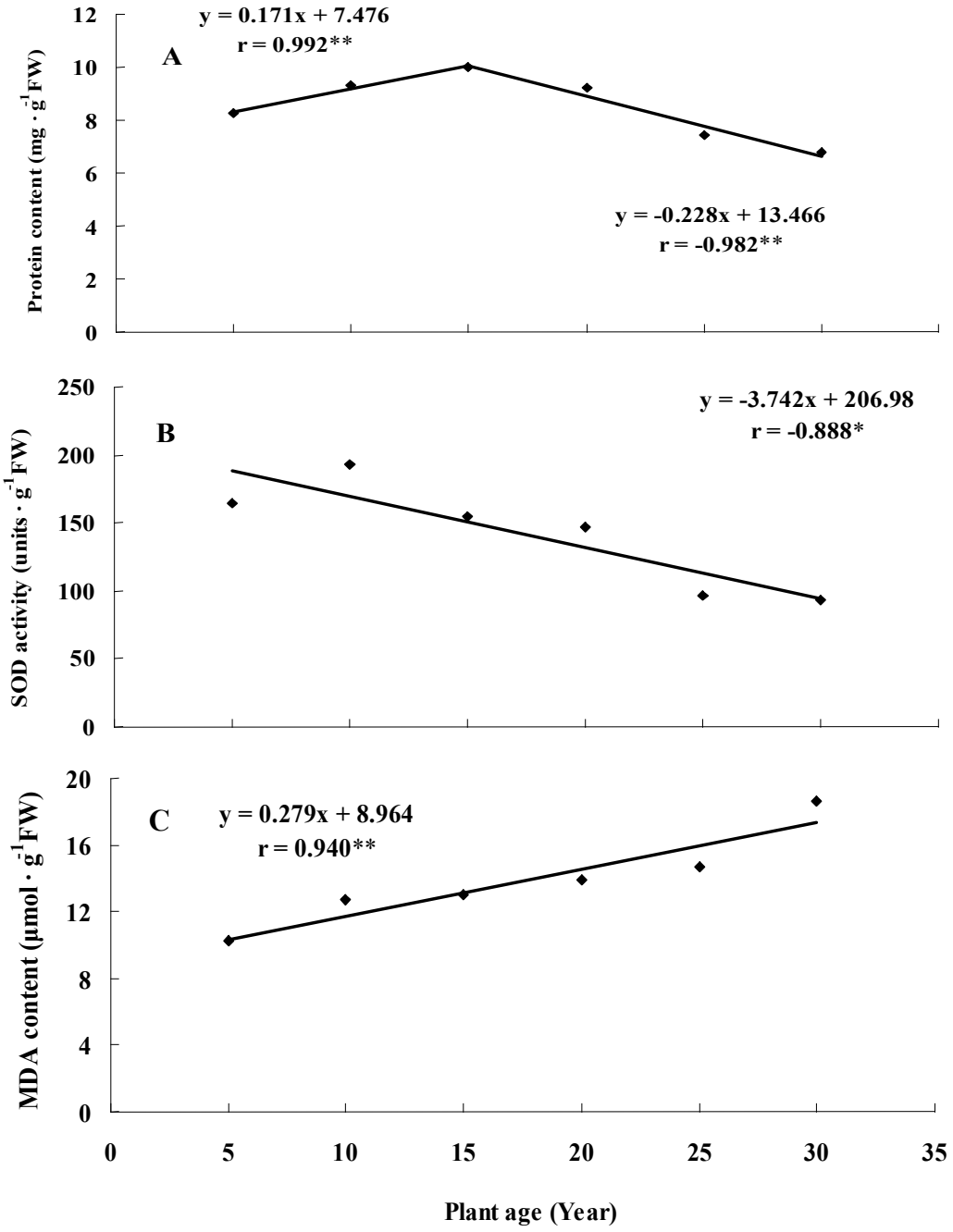

Fig. 4-2. Relationship between plant age and protein content (A), SOD activity (B), and MDA content (C), respectively. * and ** indicate significance at 5 and 1% levels, respectively.

Chapter 5

Conclusion and future work

Tree peonies are widely cultivated in Shandong, Henan, Gansu, and Anhui Provinces of China as an ornamental plant due to the large flowers and ornamental foliage. They were introduced to Japan, America and Europe, and have been developed (Wister 1928, Li, 1999). Yatsuka-cho (Daikon-jima Island), Shimane Prefecture and Gosen City, Niigata Prefecture have became two major production areas of tree peonies in Japan and many cultivars were hybridized or selected (Hosoki et al., 1991, 1997). So far, there are more than 2000 tree peony cultivars around the world were cultivated and nine wild species were known. How should we use these resources to breed more superior cultivars to improve tree peony ornamental values is an important point in tree peony breeding research.

Tree peony cultivars are classified into four groups: Chinese, Japanese, American and French (Liu, 2003). There is no evidence that *P. delavayi* and *P. lutea* which participated in the forming of American and French were involved in the differentiation of Japanese and Chinese cultivars (Li 1998, 1999; Zhou et al., 2003). There is different genetic background among American and French groups from Japanese and Chinese ones. Japanese cultivar group is characterized by large flowers, graceful and stiff stems that hold the flower upright, and bright, pure flower color, while, American group has rare flower color and its cultivars flower later than that of Japanese cultivars, it is hopefully to breed superior cultivars by the crosses among them although it is difficult.

On the other hand, most of tree peony cultivars are diploid except triploid of Chinese cultivar ‘Shou An Hong’ with many virtues such as bigger flower, numerous petals, high resistance, etc. Polyploid breeding is another way that can increase the diversity of tree peony. The superior polyploidy tree peony cultivars may be bred by exploiting of unreduced pollen (giant pollen) grains.

Tree peony is an especially important tour resource. However, as plants in peony gardens age, the plants grow weak and appear the status of no flowering or bad flowering, affecting their ornamental value. Therefore, determining the causes of and inhibiting the progress of aging to extend ornamental lifetimes are very important and significant goals for tree peony management.

New cultivar breeding and clarifying the causes of aging are necessary for maintaining and developing tree peony tour resource. For these above purposes, from 2004 to 2012, the experiments were performed in Education and Research Center for Biological Resources, Faculty Life and Environmental science, Shimane University, Yatsuka-cho, Institute of Botany, Chinese Academy of Sciences and Baihua Park, Heze city, Shandong Province, China. The main results are as follows.

1. A total of 1,927 flowers in 57 cross combinations were pollinated, of those crosses, 38 cross combinations of 135 flowers set 181 seeds, 86 mature and 95 immature, and obtained 22 seedlings. Although seed-set rate was low in all of cross combinations, 13 Japanese cultivars had higher cross compatibility with ‘High Noon’ compared to the other 44 ones. Crosses between ‘High Noon’ and Japanese cultivars might be successful when the right paternal plants were selected.

These 13 cultivars will play an important role in tree peony breeding. The more superior cultivars may be bred from these 13 cultivars cross with ‘High Noon’.

2. There are three most important causes of incompatibility, abnormal pollen tube growth, the failure of fertilized ovules, and poor seed germination. To overcome these barriers, ovary pollination, *in vitro* fertilization, or cutting the styles prior to pollination, *in vitro* culture of ovules or immature embryos may be appropriate. The study of this aspect will be considered in the future.

3. Five of the hybrids have already flowered and inherited the yellow flower color from seed parents and big flower diameter and stronger stalks from pollen parents. They will be backcrossed in the future. More backcross generations will improve the inherited characteristics of tree peony offspring, producing better ornamental traits. These results suggested that we can breed superior cultivars by introducing genes of Japanese cultivars into French and American ones. It is hopefully to breed better cultivars by using these resources as cross materials.

4. The giant pollen grains were observed in six cultivars among seven cultivars used in this study. The giant pollen ratio was low, the highest only 1.16%. Giant pollen could germinate but did later than normal pollen. This study provided the basic data for tree peony polyploidy breeding. In subsequent study, giant pollen grains and normal ones will be isolated by using nylon mesh, and nuclear DNA content of sorted pollen grains also will be investigated to verify the ploidy level of giant pollen in order to perform the confirmed giant pollen to pollination.

5. Shorter branches and leaves, and fewer flowers were characteristics of aging in tree peony. Especially, new branch length declined drastically in plants older than 20 years, so this characteristic of aging accelerated. Therefore, strengthening cultivation management after the plants grow 15 to 20 years after division or grating is beneficial to tree peonies in tree peony thematic garden.

6. Protein content and plant age were positively correlated until 15 years post-division and negatively correlated afterwards. In addition, SOD activity was inversely correlated and MDA content was directly correlated with plant age. Therefore, these changes were characteristic of aging in ‘Luo Yang Hong’. Protein content, SOD activity and MDA content could be used as indices to diagnose the senescence and weakness of ancient tree peony. To investigate the effectiveness of foliar application of salicylic acid and polyamine on delaying aging in tree peony, plants of different ages would be studied in the future.

Summary

Tree peony, which originates in China, is an important ornamental plant. Various cultivars have been developed in Europe and America as well as in China and Japanese. The breeding of new cultivars with high ornamental value, which different from existing ones in flower color and flower form was requested for tree peony cultivar improvement, moreover, as the tour resource, tree peony aging caused the phenomenon of no flowering or bad flowering was a big problem in peony gardens.

This study aims to evaluate the crossability of American tree peony ‘High Noon’ as seed parents crossed with Japanese tree peonies, analyze fertilization barriers and select better cross combinations to breed the superior cultivars with high ornamental values. The existing and the occurrence rate of unreduced pollen grains (giant pollen grains) among different cultivars were also investigated in order to explore the possibility of tree peony polyploidy breeding. Moreover, with the plant age increasing, the tree body, protein content, soluble protein content, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) content were measured to collect the basic data of preventing tree peony aging process was another purpose. From 2004 to 2012, the experiments were performed in Education and Research Center for Biological Resources, Faculty Life and Environmental science, Shimane University, Yatsuka-cho, Institute of Botany, Chinese Academy of Sciences and Baihua Park, Heze city, Shandong Province, China. The main results are as follows.

1. The investigation in the causes of cross incompatibility and the selection of better cross combinations when American tree peony 'High Noon' as seed parent crossed with Japanese cultivars

To investigate the cross incompatibility and select better cross combinations, 1,927 flowers were crossed that American tree peony cultivar 'High Noon' as the seed parent with 57 different Japanese cultivars. The results indicated that 38 cross combinations set 181 seeds, and obtained 22 seedlings. There are three most important causes of incompatibility in the crosses of 'High Noon' as the seed parent with Japanese cultivars, namely, abnormal pollen tube growth, the failure of fertilized ovules, and poor seed germination. Although cross compatibility in each combination was low, crosses between 'High Noon' and Japanese cultivars might be successful with the right paternal plants. In this study, 13 Japanese cultivars had higher cross compatibility with 'High Noon' than the other 44 ones. Our results will enhance tree peony breeding and guide the selection of parents for hybridization. Five of the hybrids have already flowered and inherited the flower color of seed parent that bear yellow flowers, and the larger flowers, stronger stalks from pollen parents. Moreover, the results of genetic identification showed that the bands from the parents were detected in the hybrids.

2. Occurrence of giant pollen grains

The pollen diameter and germination of seven cultivars were measured in order to confirm the

existing and the rate of giant pollen grains. The pollen grains of tree peonies existed in three forms, namely, abortive pollen, normal pollen and giant pollen. For the seven cultivars used in this study, the giant pollen grains were observed in six cultivars, the rate of giant pollen was low, from approximately 0-1.16%. 'Kou You' lacked giant pollen, while giant pollen occurred at rates of 0.12% in 'Shou You Den', 0.23% in 'Shi Ou Den', 0.42% in 'Ki Jou Den', 0.58% in 'Sharaku', 0.80% in 'Kai Hou', and 1.16% in 'Yachiyo Tsubaki'. Giant pollen could germinate but did later than normal pollen which showed that the germination of giant pollen was difficult.

3. Changes in tree body, soluble protein content, SOD activity, and MDA content in aging tree peony

For the problem of no flowering or bad flowering caused by the aging tree peonies, tree body and soluble protein content, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) content in the leaves on different plant age (6 age brackets: 5, 10, 15, 20, 25 and 30-year-old plants after division) of tree peony 'Luo Yang Hong' which planted in the same resources garden were investigated to clarify the senescence phenomenon of tree peony. The results indicated that new branch length, leaf length and the number of flower buds increased in the plants which grew 5 to 15 years after division, however, tended to decrease after 20 years. Especially, the results showed that new branch length became shorter with plant age in 'Luo Yang Hong'. Protein content increased till 15 years after division, since then decreased; the negative correlation between SOD and plant age, and positive correlation between MDA and plant age were demonstrated.

ボタンの新品種育成ならびに樹体老化に関する研究

要約

中国原産のボタンは，中国や日本をはじめとして欧米でも多様な品種が発達している重要な花木である．このボタンの品種改良においては従来の花色や花形と異なる観賞価値の高い新品種の育成が求められており，また，観光資源として活用したボタン園などにおいては，樹体老化による着花不良等が問題となっている．

そこで，本研究は観賞価値の高い新品種育成のために，アメリカボタン品種‘ハイヌーン’を種子親として，日本ボタン品種との交雑能力を検定し，不和合性の原因を究明し，有効な交雑組み合わせを選択すること，また，倍数体育種の可能性を探るため，非還元花粉である巨大花粉の存在と比率を調査すること，さらに，ボタン樹体の老化進行を抑制するための基礎資料を得るために，樹年生の進行による樹体変化および葉における可溶性タンパク質含量，スーパーオキシドジスムターゼ（SOD）活性ならびにマロンジアルデヒド（MDA）含量を測定することを目的とした．なお，これらの研究は 2004 年から 2012 年にかけて，島根大学生物資源科学部附属生物資源教育研究センター，島根県松江市八束町，中国科学院植物研究所ならびに中国山東省荷澤市百花園において実験を行ったものであり，これらの結果は以下のとおりである．

1. アメリカボタン品種‘ハイヌーン’を種子親として日本ボタン品種を交雑した場合の

交雑不和合性要因の調査と交雑和合性組み合せの選択

アメリカボタンと日本ボタンとの交雑和合性を調査するために，アメリカボタン品種‘ハイヌーン’を種子親として日本ボタン 57 品種との間で 1,927 花の交雑を行った．その結果，38 組み合わせから合計 181 粒の種子が得られ，22 株の苗を得た．続いて，不和合性の原因を明らかにするために，‘ハイヌーン’と日本ボタンならびに日本ボタン間の交雑における花粉管伸長状況を調査した．‘ハイヌーン’と日本ボタンの交雑では，①花粉管が歪曲に伸長し，胚珠への貫入に失敗する，②胚珠が発育停止する，③種子発芽が不能である，という三つが交雑不和合性の要因と考えられる．さらに，ほとんどの交雑組み合わせは和合性が低いが，適切な品種の花粉を選択した場合，‘ハイヌーン’と日本ボタンとの交雑種子が獲得できる可能性が高かった．本研究では，使用した日本ボタン 57 品種中，13 品種がそれ以外の 44 品種より，‘ハイヌーン’との交雑和合性が高いことが明らかとなった．現在までに開花した雑種 5 個体の花は，種子親から受け継いだ黄色の花色を有し，花粉親からは大きい花径と，剛直な花梗を受け継ぎ，両親には見られない新規な花色と花形を有した．また，開花した交雑個体の遺伝子検定の結果，両親由来のバンドが検出された．

2. 巨大花粉の出現比率

巨大花粉の存在と比率を調査するため，7 品種の花粉直径と発芽率を測定した．ボタン花粉は，①発育不全の小花粉，②正常花粉，③巨大花粉の三種類が混在した．使用した 7

品種中，6 品種において巨大花粉の存在が確認されたが，その出現比率は 0～1.16%の範囲で低率であった．‘向陽’は 0%，‘照陽殿’は 0.12%，‘紫王殿’は 0.23%，‘貴城殿’は 0.42%，‘写楽’は 0.58%，‘海峰’は 0.80%，‘八千代椿’は 1.16%であった．なお，巨大花粉の発芽は正常花粉によりやや遅く，発芽に時間を要することが明らかとなった.

3. ボタンの加齢に伴う樹体変化およびタンパク質含量，SOD活性ならびにMDA含量の消長

樹体老化の進行によって着花不良が問題になっていることから，中国山東省荷澤市百花園に植栽されている株分け後の年生が異なるボタン樹（5，10，15，20，25，30 年生）‘洛陽紅（Luo Yang Hong）’を供試し，樹体変化および葉における可溶性タンパク質含量，スーパーオキシドジスムターゼ（SOD）活性ならびにマロンジアルデヒド（MDA）含量を測定し，樹体の老化現象を明らかにした．新梢の長さ，葉の長さおよび着蕾数は，5～15 年生樹までは増加し，20 年生以後の樹からは減少傾向を示した．特に，新梢の長さは加齢とともに著しく短くなった．また，タンパク質含量は 15 年目までは増加し，その後は年生とともに減少した．SOD 活性は年生とともに減少する負の相関を，さらに MDA 含量は年生とともに増加する正の相関を示した.

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