Pollination Biology of *Hydrangea macrophylla*

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Abstract. Little information is available on the reproductive behavior of *Hydrangea macrophylla* (Thunb. Ex J.A. Murr.) Ser. The objectives of this study were to investigate time of stigma receptivity, viability of pollen from sterile flowers, and self-incompatibility in this popular ornamental shrub. Pollen germination and pollen tube growth in styles were examined using fluorescence microscopy. Stigma receptivity was examined in cross-pollinations made from 1 day before anthesis to 8 days after anthesis. Maximum stigma receptivity for the two cultivars examined occurred from anthesis to 4 days after anthesis. Viability of pollen from sterile flowers was evaluated through pollen staining and observations of pollen tube growth. No significant difference in percent stainable pollen between fertile and sterile flowers was observed in any of the six taxa examined. Pollen germination and pollen tube growth were studied in cross-pollinations made using pollen from fertile and sterile flowers of two cultivars. For both cultivars, pollen tubes from fertile and sterile flowers grew to the same length and had entered ovules by 72 hours after pollination. Self-incompatibility was evaluated by comparing pollen germination and pollen tube growth in cross- and self-pollinations. In the five taxa examined, self pollen tubes were significantly shorter than cross pollen tubes in flowers that were examined 72 hours after pollination. This finding indicates the presence of a gametophytic self-incompatibility system in *H. macrophylla*.

Hydrangea macrophylla is the most widely grown of the *Hydrangea* species (Dirr, 1998). Cultivated as both a garden and a florist's plant, it is highly valued for its large corymbs that range in color from white to pink to blue, depending on cultivar and soil pH. Like most *Hydrangea* species, the inflorescence of *H. macrophylla* is comprised of both small inconspicuous fertile flowers and large showy flowers that are referred to as sterile flowers (McClintock, 1957). Hortensia or mophead cultivars (*H. macrophylla* var. *macrophylla*) have rounded inflorescences containing numerous sterile flowers on the outside of the inflorescence and a few fertile flowers in the interior of the inflorescence. Lacecap cultivars (*H. macrophylla* var. *normalis*) have a flattened inflorescence with an outer ring of a few sterile flowers and an inner ring of many fertile flowers. *Hydrangea macrophylla* subsp. *serrata* cultivars may have either a lacecap or mophead type inflorescence. This subspecies, which is native to mountainous areas of Korea, is distinguished by its smaller leaves and flowers.

The increasing popularity of *H. macrophylla* in the landscape and the recent introduction of cultivars of this species with increased cold hardiness and ability to flower on current

year's growth (Adkins et al., 2002; Dirr, 2002; Lindstrom et al., 2003) have stimulated interest in breeding superior cultivars for garden use in the U.S. Even though a large number of *H. macrophylla* cultivars are available (Mallet, 1994; Mallet et al., 1992), little has been published concerning the reproductive behavior of this species. Based on evaluations of seed set and pollen tube growth, Kudo and Niimi (1999) reported that *H. macrophylla* was self-fertile. However, in another study, self-pollinations produced seed in only one of twelve *H. macrophylla* cultivars tested (Reed, 2000). While eight viable seed were obtained from self-pollinations of 'Alpengluhen', all but one of the seedlings died before developing true leaves. Dirr (2003) reported that *H. macrophylla* 'Dooley' plants grown in isolation produced 59 seedlings, which were presumed to be self progeny, but did not compare seed set in cross- and self-pollinated 'Dooley' plants.

Pollinations in mophead forms of *H. macrophylla* are often limited by the small number of fertile flowers present in a single inflorescence. According to McClintock (1957), the sterile flowers in *Hydrangea* usually consist of only an enlarged calyx. She noted that, in forms of *H. macrophylla* in which the inflorescences produce few fertile flowers, the sterile flowers may have petals, stamens, and pistils. We have observed anthers with abundant pollen in sterile flowers not only in mophead cultivars, but also in lacecap forms in which fertile flowers greatly outnumber sterile flowers. No information on the viability of the pollen produced by the sterile flowers is available. The only seed capsules we have observed in open-pollinated, field-grown

H. macrophylla plants have developed from fertile flowers, so presumably the pistil of the sterile flowers is not functional.

The overall objective of this study was to develop information on pollination biology of *H. macrophylla* that would assist in breeding efforts. Specific goals were to 1) determine time of stigma receptivity, 2) determine the viability of pollen produced by the so-called sterile flowers and, 3) reinvestigate self-incompatibility.

Materials and Methods

The following cultivars were utilized in this study: *H. macrophylla* var. *macrophylla* 'All Summer Beauty', 'Dooley', 'Balmer' (Endless Summer), 'Lemon Zest', 'Madame Emile Mouillere', 'Marechal Foch'; *H. macrophylla* var. *normalis* 'Blaumeise', 'Kardinal'; and *H. macrophylla* subsp. *serrata* 'Kiyosumi'. An unnamed seedling (PBB) with lacecap-type inflorescences that was derived from an *H. macrophylla* var. *macrophylla* 'Pia' × *H. macrophylla* subsp. *serrata* 'Blue Billow' hybridization was also used.

Plants were grown in 26.5-L containers under 60% shade and micro-irrigated using spray stakes. Growing media consisted of pine bark amended with 6.6 kg·m⁻³ 19N-2.1P-7.4K Osmocote Pro fertilizer (Scotts-Sierra Horticultural Products Co., Maryville, Ohio), 0.6 kg·m⁻³ Micromax (Scotts-Sierra Horticultural Products Co.), 0.6 kg·m⁻³ iron sulfate, and 0.2 kg·m⁻³ Epsom salts. Plants were brought into a greenhouse headhouse (22 to 25 °C) 1 to 2 d before flowers were emasculated.

Before pollination, open flowers were removed from inflorescences. Flowers that were to be used as sources of pollen were covered with a breathable plastic pollination bag (DelStar Technologies, Middletown, Del.). For the maternal parent, petals and anthers were removed from flowers that appeared, based on size and color, to be within 1 d of opening. Immature flowers were then removed, and the inflorescence covered with a pollination bag. For pollinations, anthers with newly dehiscent pollen were touched directly to the exposed stigmas of the emasculated flowers. For self-pollinations, pollen was obtained from a flower on the same plant as the emasculated flower. After pollination, inflorescences were covered with pollination bags which remained on the plants until the last flower was collected.

Stigma receptivity. Time of stigma receptivity was evaluated over a 10-day period. Ten 'Kardinal' × 'Marechal Foch' and five PBB × 'Lemon Zest' pollinations were made every day from the day of emasculatation (1 d before anthesis) to 8 d after anthesis. Flowers were collected 48 h after pollination.

Flowers were placed into FAA fixative (70% 18 ethanol : 1 formalin : 1 glacial acetic acid) (Johansen, 1940) immediately following collection. After 24 h at room temperature in fixative, flowers were transferred to 70% ethanol where they were stored for up to 2 months. Before staining, flowers were rinsed for 30 min in distilled water and then softened using 8 N NaOH for 3 h. Flowers were rinsed in distilled water for 30

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Table 1. Effect of time of pollination on pollen germination and pollen tube growth in *Hydrangea macrophylla*.

Time of pollination	Pollination			
	'Kardinal' × 'Marechal Foch'		('Pia' × 'Blue Billow') × 'Lemon Zest'	
	Flowers with germinated pollen (%)	Mean pollen tube length (mm) ^a	Flowers with germinated pollen (%)	Mean pollen tube length (mm)
Day before anthesis	70	1.4 c	0	---
Anthesis	100	2.4 ab	80	1.3 a
Days after anthesis				
1	90	2.3 ab	100	1.2 a
2	90	2.6 a	80	1.1 a
3	90	2.1 b	80	1.2 a
4	90	2.8 a	80	0.9 a
5	60	1.1 c	60	0.4 b
6	30	1.2 c	0	---
7	0	---	0	---
8	0	---	0	---

^aMean separation within a column based on Fisher's LSD test ($P \leq 0.05$).

Fig. 1. Percent stainable pollen from fertile and sterile flowers of six *Hydrangea macrophylla* taxa. Error bars = SE.

min before transfer to 0.1% (w/v) aniline blue in 0.1 N K_3PO_4 for 1 h (Martin, 1959).

After staining, the pistil was placed on a microscope slide in a drop of aniline blue stain, squashed under a cover slip, and observed using a microscope (BX-60; Olympus America, Inc., Melville, N.Y.) equipped with a 100-W high-pressure Hg lamp and an U-MNV near-ultraviolet (400 to 410 nm) filter. Callose was observed as a bright yellow-green fluorescence and was used to detect pollen germination and growth of the pollen tubes through the styles. Percentage of flowers in which germinated pollen was present on at least one of the three to four stigmas of the pistil was determined for each treatment. The longest pollen tube in each style was measured using an ocular micrometer and an average pollen tube length estimated for each flower. Mean pollen tube

length was calculated for each treatment using only those flowers in which pollen germination was observed. Pollen tube length data were analyzed by analysis of variance (ANOVA), with mean separation determined by Fisher's LSD at $P \leq 0.05$, using SigmaStat software ver. 3.0 (SPSS, Chicago).

Evaluation of sterile flowers. Three fertile

Table 2. Pollen germination and pollen tube length 72 h after pollination using fertile and sterile flowers of two *Hydrangea macrophylla* cultivars.

Pollination	Type of flower used as pollinizer	Flowers with germinated pollen (%)	Mean pollen tube length (mm)
(Pia × Blue Billow) × Bailmer	Fertile	80	2.8 a
	Sterile	80	2.2 a
(Pia × Blue Billow) × Dooley	Fertile	100	2.4 a
	Sterile	100	2.4 a

^aMean separation within a paternal parent based on paired t test ($P \leq 0.05$).

and three sterile flowers of 'All Summer Beauty', 'Balmer', 'Blaumeise', 'Dooley', 'Madame Emile Mouillere', and PBB that appeared to be within 1 d of opening were collected. Two anthers of each flower were placed on microscope slide and squashed in a drop of 1% acetocarmine stain. After removing debris and placing a cover slip over the stain, the slide was heated for 15 s over steam. Specimens were examined using a light microscope and pollen scored as stained (viable) or unstained (inviable). Five fields of 100 grains each were counted and a mean number of stained grains calculated for each slide. Student's t test was used to compare percent stainable pollen between fertile and sterile flowers within each taxa.

Ten PBB × 'Bailmer' and PBB × 'Dooley' pollinations were made. Half of each set of pollinations was made using pollen from sterile flowers, while the rest utilized pollen from fertile flowers. Flowers were collected 72 h after pollination and fixed and stained as described above. Pollen germination, mean pollen tube length and presence of pollen tubes inside ovules were recorded for each flower. Student's t test was used to compare mean pollen tube length between pollinations using fertile and sterile flowers as the pollen source.

Self-incompatibility. Thirty-five 'Kiyosumi' × PBB, 'Kiyosumi' selfed, 'Kardinal' × 'Marechal Foch', 'Kardinal' selfed, 'Marechal Foch' × 'Kardinal', 'Marechal Foch' selfed, PBB × 'Lemon Zest', and PBB selfed pollinations were made 2 d after emasculation, which corresponded to 1 d after anthesis. Five flowers from each set of cross- and self-pollinations were collected at 1, 2, 4, 8, 24, 48, and 72 h after pollination. Five 'Blaumeise' × PBB and five 'Blaumeise' selfed pollinations were also made 2 d after emasculation and collected 72 h after pollination. Flowers were fixed and pistils stained as described above. Percent flowers with germinated pollen, mean pollen tube length, and percent flowers in which pollen tubes had entered ovules were calculated for each treatment. Student's t test was used to compare pollen tube length between each set of cross- and self-pollinations at each collection time.

Results and Discussion

Stigma receptivity. Pollen germinated in over half of the 'Kardinal' × 'Marechal Foch' and PBB × 'Lemon Zest' pollinations made from anthesis to 5 d after anthesis (Table 1). Significant differences in pollen tube lengths among times of pollinations were noted for both crosses. Pollen tubes in 'Kardinal' × 'Marechal Foch' pollinations made the day

Table 3. Pollen germination and pollen tube length in cross- and self-pollinations of *Hydrangea macrophylla*.

Pollination	Hours after pollination				
	4	8	24	48	72
Flowers with germinated pollen (%)					
Kiyosumi × (Pia × Blue Billow)	100	100	100	100	100
Kiyosumi selfed	80	100	100	100	100
Kardinal × Marechal Foch	100	80	100	80	80
Kardinal selfed	0	100	100	100	100
Marechal Foch × Kardinal	20	0	100	100	100
Marechal Foch selfed	0	20	80	80	80
(Pia × Blue Billow) × Lemon Zest	0	20	80	60	60
(Pia × Blue Billow) selfed	20	60	100	80	60
Blaumeise × (Pia × Blue Billow)	---	---	---	---	80
Blaumeise selfed	---	---	---	---	80
Mean pollen tube length (mm) ^a					
Kiyosumi × (Pia × Blue Billow)	0.24 a	0.31 a	0.51 a	0.65 a	1.79 a
Kiyosumi selfed	0.37 a	0.20 a	0.37 a	0.40 a	0.35 b
Kardinal × Marechal Foch	0.11	0.20 a	1.32 a	2.00 a	3.77 a
Kardinal selfed	---	0.19 a	0.62 a	1.24 a	1.35 b
Marechal Foch × Kardinal	0.15	---	0.66 a	1.93 a	2.61 a
Marechal Foch selfed	---	0.13	0.51 a	0.43 b	0.59 b
(Pia × Blue Billow) × Lemon Zest	---	0.20 a	0.86 a	2.06 a	2.56 a
(Pia × Blue Billow) selfed	0.10	0.20 a	0.57 a	0.91 b	0.90 b
Blaumeise × (Pia × Blue Billow)	---	---	---	---	3.18 a
Blaumeise selfed	---	---	---	---	0.60 b

^aMean separation within maternal cultivar and time of collection based on paired *t* test ($P \leq 0.05$).

Fig. 2. Pollen tube growth 72 h after cross- and self-pollination of *H. macrophylla*. (A) 'Kardinal' × 'Marechal Foch'. (B) 'Kardinal' self-pollinated. Pistils stained with aniline blue and viewed using fluorescence microscopy.

before anthesis and 5 and 6 d after anthesis were significantly shorter than those from flowers pollinated between these times. PBB × 'Lemon Zest' pollinations made 5 d after pollination resulted in shorter pollen tubes than those made from the day of anthesis to 4 d after anthesis.

These results indicate that stigmas of 'Kardinal' and PBB are most receptive to pollen from the day of anthesis to 4 d after anthesis. Stigmas of two other *Hydrangea* species have also been found to be receptive to pollen for several days, with receptivity ranging from anthesis to 5 d after anthesis in *H. paniculata* and from 1 to 5 d after anthesis in *H. quercifolia* (Reed, 2004).

Evaluation of sterile flowers. No differences in percent stainable pollen were observed between sterile and fertile flowers of any of

the taxa examined (Fig. 1). While over half of the 'All Summer Beauty', 'Bailmer', 'Dooley', and PBB pollen appeared viable, <30% of the 'Blaumeise' and about 5% of the 'Madame Emile Mouillere' pollen was stainable. Both 'Blaumeise' and 'Madame Emile Mouillere' produced large quantities of small shrunken pollen grains.

Pollen collected from sterile flowers of 'Bailmer' and 'Dooley' germinated well and grew to the same length as pollen from fertile flowers in pollinations of PBB (Table 2). Pollen from both sterile and fertile 'Bailmer' flowers germinated in 80% of the pollinations; in all of these flowers, pollen tubes were observed entering ovules. With the exception of one flower in which sterile pollen was used, pollen tubes were observed entering ovules in all PBB × 'Dooley' pollinations.

Pollen from sterile flowers of *H. macrophylla* 'Bailmer' and 'Dooley' appeared to be as fully functional as that from fertile flowers of these cultivars. Use of pollen from sterile flowers in controlled pollinations would facilitate *H. macrophylla* pollinations, especially in mophead cultivars in which the number of fertile flowers is often extremely limited. While no differences in pollen viability were observed between sterile and fertile flowers of the six taxa examined, two cultivars with poor pollen viability were identified.

Self-incompatibility. No pollen had germinated in samples collected 1 and 2 h after pollination. Consistent germination in all self- and cross-pollinations did not occur until 24 h after pollination. Significant differences in pollen tube lengths between 'Kiyosumi' × PBB and 'Kiyosumi' selfed and between 'Kardinal' × 'Marechal Foch' and 'Kardinal' selfed pollinations were only observed in flowers collected 72 h after pollination (Table 3). For crosses involving 'Marechal Foch' and PBB as maternal parents, differences in pollen tube length between cross- and self-pollinations were observed in flowers collected 48 and 72 h after pollination. 'Blaumeise' × PBB and 'Blaumeise' selfed pollinations were collected only at 72 h after pollination, at which time cross pollen tubes were significantly longer than self pollen tubes. By 72 h after pollination, cross pollen tubes of the five taxa were about 3 to 5 times longer than corresponding self pollen tubes (Fig. 2).

Pollen tubes in 'Kiyosumi' × PBB crosses did not reach the bottom of the style until 72 h after pollination. In cross-pollinations involving 'Kardinal', 'Marechal Foch', and PBB as maternal parents, pollen tubes reached the bottom of the style by 48 h after pollination and had entered ovules by 72 h after pollination. Pollen tubes did not reach the bottom of the style or penetrate ovules in any of the self-pollinations.

Self-incompatibility has been defined as the inability of a plant with functional male and

female gametes to set seed when self-pollinated (Brewbaker, 1957). Gametophytic self-incompatibility is determined by the genotype of the pollen and is manifested by an inhibition of pollen tube growth in the style (de Nettancourt, 1977). In species with gametophytic self-incompatibility, pollen germinates freely on the stigma but self-pollen tubes usually reach only one-third to three-quarters the length of the style in the time required for compatible tubes to penetrate the entire style (Ascher, 1976). Gametophytic self-incompatibility is generally found in plants that, like *Hydrangea*, release pollen at the binucleate stage of development (Brewbaker, 1957, 1967).

In the five *H. macrophylla* taxa tested in this study, self-pollen germinated freely on the surface of the stigma, but growth of pollen tubes in the style was inhibited. This indicates the presence of a gametophytic self-incompatibility system in these taxa. Two other members of this genus, *H. paniculata* and *H. quercifolia*, have also been found to exhibit gametophytic self-incompatibility (Reed, 2004). Results of this study agree with previous observations regarding poor seed set following self-pollinations (Reed, 2000), but differ with results of a study conducted by Japanese researchers (Kudo and Niimi, 1999) in which pollen tubes of *H. macrophylla* 'Blue Diamond' grew to the bottom of the style by 48 h after self-pollination. It has been well documented that a single species may contain both self-compatible and self-incompatible individuals (Barrett, 1988). The cultivars in which good self-seed production was reported (Kudo and Niimi, 1999) were different from those in which we found poor seed set following self-pollination (Reed, 2000). A comparison of pollen tube growth following cross- and self-pollinations of a large number of *H. macrophylla* cultivars would help to determine how wide-spread self-incompatibility is in this species. Evidence of a strong and wide-spread self-incompatibility system in this species would eliminate the need

to emasculate flowers when making controlled pollinations.

Selfed progeny may be obtained from self-incompatible plants through pseudo-self-compatibility (PSC). PSC is expressed as limited self-pollination occurring on a regular basis or occasional full self-seed production (Ascher, 1976), and may be promoted by various factors. End of season PSC, in which plants that have failed to produce self seed throughout the growing season produce abundant self seed at the end of flowering, is one of the earliest documented forms of PSC (East and Park, 1917). Bud pollinations, which typically involving making self-pollinations two days before anthesis, encourage PSC in some species (East, 1923). High temperatures treatments have increased seed production from self-pollinations in several genera (Ascher, 1976). All pollinations used to evaluate self-incompatibility in this study were made one day after anthesis, involved plants early in their flowering cycle, and were made under moderate temperatures. None of these conditions are considered favorable for PSC. Evaluation of self-incompatibility under a range of environmental conditions would help to determine the strength of self-incompatibility in this species. In addition, since self-incompatibility may interfere with genetic analyses by reducing crossing success between siblings and other closely related plants, the development of pollination techniques that encourage PSC would be helpful for conducting breeding and genetic studies in *H. macrophylla*.

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