Self-incompatibility and Time of Stigma Receptivity in Two Species of *Hydrangea*

Sandra M. Reed¹

Floral and Nursery Plants Research Unit, U.S. National Arboretum, Agricultural Research Service, U.S. Department of Agriculture, Tennessee State University Nursery Crop Research Station, 472 Cadillac Lane, McMinnville, TN 37110

Additional index words. breeding, pollen tube growth, *Hydrangea paniculata*, *Hydrangea quercifolia*, oakleaf hydrangea, panicle hydrangea

Abstract. The objectives of this study were to evaluate self-incompatibility in *Hydrangea paniculata* Sieb. and *H. quercifolia* Bartr. and to determine optimum time for pollination of these two species. Flowers from three genotypes of each species were collected 1, 2, 4, 8, 24, 48, and 72 hours after cross- and self-pollination, stained with aniline blue and observed using a fluorescence microscope. In both species, pollen germination was observed on stigmas of over half of the flowers collected 4 to 72 hours after cross- or self-pollination. Differences in pollen tube length between cross- and self-pollinated flowers were noted from 8 to 72 hours after pollination in *H. paniculata* and from 24 to 72 hours after pollination in *H. quercifolia*. By 72 hours after pollination, most self-pollen tubes had only penetrated the top third of the style but cross-pollen tubes had grown to the base of the style and entered 40% to 60% of the ovules. Stigmas of *H. paniculata* were receptive to pollen from anthesis to 5 days after anthesis, while stigmas of *H. quercifolia* were receptive from 1 to 5 days after anthesis. This study provides evidence of a gametophytic self-incompatibility system in *H. paniculata* and *H. quercifolia*. Occasional self-seed set previously observed in these species was theorized to have been due to pseudo-self compatibility.

The genus *Hydrangea* consists of ≈23 species, several of which are cultivated as ornamentals (McClintock, 1957; Dirr, 1998). *Hydrangea macrophylla* (Thunb. ex J.A. Murr.) Ser., which is grown both as a garden and a florist's plant, has long been the most widely grown member of the genus; however, the popularity of several other *Hydrangea* species has greatly increased in recent years. In particular, consumer interest in panicle (*H. paniculata*) and oakleaf (*H. quercifolia*) hydrangeas has grown during the past decade (Dirr, 1998).

Hydrangea paniculata, which is native to China and Japan, grows 3 to 6 m in height and spread. Inflorescences are produced in mid-summer and consist of 15- to 20-cm long panicles of white to pale pink flowers. Hardy to U.S. Dept. of Agriculture (USDA) hardiness zone 4, *H. paniculata* is the most cold-hardy member of the genus. *Hydrangea quercifolia* is native to the southeastern United States and is hardy to USDA hardiness zone 5. Most plants grow 2 m or taller in height with an equal to wider spread; however, a few compact cultivars are available. Cream-colored conical inflorescences up to 30 cm in length are produced in early summer and often turn an

attractive rose-color as they age. Mahogany red autumn foliage and exfoliating bark provide autumn and winter interest in the landscape (Dirr, 1998). Inflorescences of both species consist of a combination of showy sterile and inconspicuous fertile flowers. Fertile flowers are small (<10 mm in length) and have two to four styles (McClintock, 1957). Both species have the potential to produce a tremendous number of seeds as a single inflorescence may have 2000–4000 fertile flowers with 30–100 ovules each (Reed, unpublished data).

Cultivar development efforts with *H. paniculata* and *H. quercifolia* have primarily relied on the selection of superior plants from open-pollinated seedling populations (Dirr, 1998; Lawson-Hall and Rothera, 1995; Mallet, 1994; Mallet et al., 1992). Continued improvement of these species, especially for traits such as growth habit and pest resistance, may require making controlled pollinations between selected individuals; however, little has been published concerning the genetics and breeding behavior of *Hydrangea*. The presence of a self-incompatibility system is of particular interest as emasculations are very difficult in *Hydrangea* due to the small size and close proximity of individual flowers.

Conflicting information concerning self-incompatibility in this genus has been reported. In one study, only a very few viable seeds were obtained from self-pollinations of *H. macrophylla*, *H. paniculata*, and *H. quercifolia*, and no self-pollinated *H. arborescens* seeds were obtained (Reed, 2000). In contrast, Kudo and Niimi (1999) obtained large numbers of viable

seed from self-pollinations of *H. macrophylla* and *H. arborescens*. They reported that pollen tubes reached the bottom of the style 48 h after self-pollinations of these two species. A Tennessee nursery producer has obtained good seed yields from *H. quercifolia* 'Snow Queen' plants planted in a block physically isolated from other *H. quercifolia* plants (personal communication; Don Shadow, Shadow Nursery, Winchester, Tenn.). The objective of this study was to evaluate self-incompatibility in *H. paniculata* and *H. quercifolia* by comparing pollen germination and pollen tube growth in cross- and self-pollinated flowers. Stigma receptivity was also evaluated over a 7-d period to identify optimum times for controlled pollinations in these two species.

Materials and Methods

Plant materials and growing conditions. A single plant of each of the following was used in this study: *H. paniculata* 'Tardiva', 'Unique', and 'Pink Diamond'; *H. quercifolia* 'Pee Wee'; and, two unnamed *H. quercifolia* seedlings (seedling 1 and seedling 2). *Hydrangea paniculata* plants were grown in 56.8-L containers in a pot-in-pot growing system in full sun. *Hydrangea quercifolia* plants were grown in 26.5-L containers under 60% shade. Both sets of plants were 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.), and 0.2 kg·m⁻³ Epsom salts. Plants were brought into a greenhouse 2 to 3 d prior to making pollinations. All pollinations were performed between 31 July and 17 Aug. 2002. Mean daily temperature in the greenhouse was 26.3 °C, while outdoor temperature averaged 25.6 °C during this period.

Controlled pollinations. Prior to 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, color and position in the inflorescence, to be within 1 d of opening. Immature flowers were then removed, and the inflorescence covered with a pollination bag.

For pollinations, flowers with newly dehiscent pollen were removed and used immediately. The bag was removed from the inflorescence of the maternal parent, and newly dehiscent anthers were touched directly to the exposed stigmas of the emasculated flowers. The bag was placed back over the female inflorescence. For self-pollinations, pollen was obtained from a flower on the same plant as the emasculated flower.

Pollen tube staining and observations. 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%

Received for publication 23 Dec. 2002. Accepted for publication 17 Apr. 2003. Mention of trade names of commercial products in the publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Dept. of Agriculture.

¹Research Geneticist.

ethanol where they were stored for up to 2 months. Prior to staining, flowers were rinsed in distilled water and then softened using 8 N NaOH for 3 h. Flowers were rinsed in distilled water for 30 min prior to transfer to 0.1% (w/v) aniline blue in 0.1 N K₃PO₄ for 1 h (Martin, 1959).

After staining, flowers were sliced vertically to separate the pistils. All material from an individual flower was placed on the same microscope slide in a drop of aniline blue stain, squashed under a cover slip, and observed using an Olympus BX-60 microscope (Olympus America, Melville, N.Y.) equipped with a 100-W high-pressure Hg lamp and an U-MNV near-ultraviolet (400–410 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 one or more of the stigmas 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. Ovules were examined for evidence of pollen tube penetration.

Evaluation of self-incompatibility. Thirty-five cross- and self-pollinations were made on each of the six plants in this study 2 d after emasculation, which corresponded to 1 d after anthesis. All cross- and self-pollinations were made on an individual plant within a 15-min. time period. Five flowers from each set of cross- and self-pollinations were collected at 1, 2, 4, 8, 24, 48, and 72 h after pollination. An additional five flowers of each cultivar and seedling were emasculated but not pollinated; these were collected 48 h after emasculation. Percent flowers with germinated pollen, mean pollen tube length, percent flowers in which pollen tubes had entered ovules, and percent ovules that had been penetrated by pollen tubes were calculated for each treatment.

Time of stigma receptivity. Time of stigma receptivity was evaluated over a 7 d period. Ten *H. paniculata* 'Unique' × 'Tardiva', 'Tardiva', × 'Pink Diamond', 'Pink Diamond' × 'Tardiva' and five *H. quercifolia* 'Pee Wee' × seedling 1, seedling 1 × 'Pee Wee', and seedling 2 × seedling 1 pollinations were made on the day of emasculation (1 d prior to anthesis), on the day of anthesis, and 1, 2, 3, 4, and 5 d after anthesis. Flowers were collected 48 h after pollination. Percent flowers with germinated pollen and mean pollen tube length were calculated for each pollination time.

Statistical analyses. For the evaluation of self-incompatibility, Student's *t* test was used to compare pollen tube growth between self- and cross-pollinations at each collection time. Stigma receptivity data were analyzed by analysis of variance (ANOVA), with mean separation determined by Fisher's least significant difference (LSD) at *P* ≤ 0.05. All statistical analyses were performed using SigmaStat software ver. 2.03 (SPSS, Chicago). For both species, no significant differences among

genotypes were observed; therefore, data from genotypes were combined.

Results

Evaluation of self-incompatibility in *H. paniculata*. Germinated pollen was observed on a few stigmas collected 1 to 2 h after either cross- or self-pollination of *H. paniculata* (Table 1; Fig. 1A). From 4 to 72 h pollination, germinated pollen was observed on stigmas of over half of the flowers in both sets of pollinations. Large numbers of germinating pollen grains were observed on most stigmas, making it impossible to quantify numbers of germinating grains (Fig. 1B). No pollen germination was observed on stigmas of any of the emasculated, unpollinated flowers.

Hydrangea paniculata pollen tubes were easily detected in styles, due to their bright fluorescence and minimal background fluorescence. Callose plugs were visible when the pollen tubes were viewed at ×200 magnification (Fig. 1C). By 24 h after pollination, pollen tubes from *H. paniculata* cross-pollinations had grown to the base of the styles, but most had not yet reached the ovules. A pollen tube was observed in only one of the 364 ovules examined 24 h after cross-pollination. Pollen tubes from cross-pollinations had reached their maximum length by 48 h after pollination (Fig. 2), and were observed entering ovules in all of the flowers in which germinated pollen was observed (Fig. 1D; Table 1). Pollen tubes were observed in 60% of the ovules examined 48 and 72 h after cross-pollination; mean number of ovules examined per flower for both collection periods was 31.

Differences in pollen tube length between *H. paniculata* cross- and self-pollinations

became apparent by 8 h after pollination (Fig. 2). From 24 to 72 h after pollination, *H. paniculata* self-pollen tubes were only 20% to 25% the length of pollen tubes from cross-pollinations collected at the same time. Pollen tubes from self-pollinations reached their maximum length by 24 h after pollination. A pollen tube was observed inside one ovule in an *H. paniculata* self-pollinated specimen collected 72 h after pollination, but no other pollen tubes in this or other self-pollinated specimens grew to the base of the style.

Evaluation of self-incompatibility in *H. quercifolia*. No germinated pollen was observed in *H. quercifolia* cross- and self-pollinations collected 1 h after pollination (Table 1). From 4 to 72 h after pollination, germinated pollen was observed on stigmas of over half of the flowers in both types of pollinations. As with *H. paniculata*, large numbers of germinating pollen grains were observed on most stigmas, but no pollen was present on any of the emasculated, unpollinated flowers. Stained *H. quercifolia* stigmas were covered with a thick mucilaginous exudate that was not observed on *H. paniculata* stigmas.

Pollen tubes and callose plugs in *H. quercifolia* styles were similar in appearance to those of *H. paniculata* (Fig. 1E). *Hydrangea quercifolia* pollen tubes reached the bottom of the style 48 h after cross-pollination. By 72 h after cross-pollination, pollen tubes had entered ovules in all of the flowers in which pollen germination was observed (Fig. 1F; Table 1). Pollen tubes were observed in ≈40% of the ovules in flowers collected 72 h after cross-pollination, but were not present in ovules from any other of the *H. quercifolia* cross-pollinations. Mean number of ovules examined per *H. quercifolia* flower was 85.

Table 1. Pollen germination and penetration of pollen tubes into ovules following cross- and self-pollinations of *H. paniculata* and *H. quercifolia* (n = 15)

Species	Type of pollination	Hours after pollination	Flowers with germinated pollen (%)	Flowers in which pollen tubes entered ovules (%)
<i>H. paniculata</i>	cross	1	20	0
		2	27	0
		4	60	0
		8	73	0
		24	87	7
		48	93	93
		72	87	87
	self	1	20	0
		2	40	0
		4	80	0
		8	80	0
		24	93	0
		48	100	0
<i>H. quercifolia</i>	cross	72	87	7
		1	0	---
		2	47	0
		4	60	0
		8	73	0
		24	87	0
		48	93	0
		72	87	87
	self	1	0	---
		2	53	0
		4	53	0
		8	80	0
		24	93	0
		48	93	0
		72	80	0

Fig. 1. Pollen germination and pollen tube growth in *Hydrangea* styles stained with aniline blue and viewed using fluorescence microscopy. (A–D) *H. paniculata*. (A) Germinating pollen grains on surface of stigma, 1 h after cross-pollination. (B) Pollen tube elongation, 24 h after self-pollination. (C) Pollen tubes with callose plugs growing in styles, 48 h after cross-pollination. (D) Pollen tubes entering ovules, 72 h after cross-pollination. (E–F) *H. quercifolia*. (E) Pollen tube elongation, 8 h after self-pollination. (F) Pollen tubes entering ovules, 72 h after cross-pollination. Scales represent 0.1 mm.

Pollen tube length differed between cross- and self-pollinated *H. quercifolia* flowers collected from 24 to 72 h after pollination (Fig. 3). During this time period, pollen tubes in self-pollinated flowers were one-third to one-fourth the length of cross-pollen tubes.

Self-pollen tubes reached their maximum length 48 h after pollination. No pollen tubes were observed entering ovules in the *H. quercifolia* self-pollinated flowers.

Time of stigma receptivity. Pollen germinated on stigmas of over 90% of the *H.*

Fig. 2. Pollen tube growth following cross- and self-pollination of *Hydrangea paniculata*. Values represent mean $\pm$ SE; $n = 30$.

paniculata flowers pollinated from 1 d before to 5 d after anthesis (Table 2). No differences in pollen tube length were observed among flowers pollinated from anthesis to 5 d after anthesis, but mean pollen tube length in flowers pollinated 1 d before anthesis was significantly less than in the other six pollination times.

No germinated pollen was present on *H. quercifolia* stigmas when pollinations were made on the day of or 1 d prior to anthesis. Pollen germination occurred in over 70% of the *H. quercifolia* pollinations made from 1 to 5 d after anthesis. No differences in pollen tube length among flowers collected from 1 to 5 d after anthesis was observed.

Discussion

Self-incompatibility is the inability of a plant with functional male and female gametes to set seed when self-pollinated (Brewbaker, 1957). This phenomenon, which is under genetic control, is estimated to be present in almost half of all angiosperm species (Brewbaker, 1959). Two homomorphic self-incompatibility systems are recognized (de Nettancourt, 1977). In the sporophytic system, incompatibility is determined by the genotype of the pollen parent and germination of incompatible pollen is inhibited at the stigmatic surface. Gametophytic self-incompatibility is determined by the genotype of the pollen, and is manifested by an inhibition of pollen tube growth in the style. In species with gametophytic self-incompatibility, self-pollen tubes generally reach one-third to three-quarters the length of the style in the time required for compatible tubes to penetrate the entire style (Ascher, 1976).

This study provides evidence of a self-incompatibility system, which appears to be gametophytic in nature, in *H. paniculata* and *H. quercifolia*. Pollen germinated freely after self-pollination in both species, but self-pollen tubes grew much slower than cross-pollen tubes. By the time pollen tubes had reached the bottom of the style in cross-pollinations, self-pollen tubes had grown only one-fourth to one-third of the way down the style. One *H. paniculata* self-pollen tube had penetrated an ovule; however, no pollen tubes grew to the base of the style in any *H. quercifolia* self-pollinated flowers. While only three genotypes of each species were examined in this study, low or no seed set following self-pollination has been observed in an additional two *H. paniculata* and six *H. quercifolia* cultivars (Reed, 2000); therefore, self-incompatibility may be widespread in these two species.

Self-incompatible species may sometimes produce self-seed. Pseudo-self-compatibility (PSC) is defined as the limited or occasionally full seed set following incompatible pollination of a plant known to possess self-incompatibility (Ascher, 1976). Progeny resulting from PSC retain a fully functional self-incompatibility system. The most common form of PSC is the regular production of a few seeds from a small percentage of self-pollinations. The frequency of PSC may be affected by environmental conditions, particularly high

Table 2. Pollen germination and pollen tube growth in *H. paniculata* and *H. quercifolia* cross-pollinations made over a 7-d period (n = 15) and examined 48 h after pollination.

Species	Day of pollination	Flowers with germinated pollen (%)	Mean pollen tube length (mm) ^z
<i>H. paniculata</i>	1 d before anthesis	96	2.8 b
	anthesis	96	3.2 a
	1 d after anthesis	100	3.5 a
	2 d after anthesis	100	3.3 a
	3 d after anthesis	100	3.3 a
	4 d after anthesis	100	3.4 a
	5 d after anthesis	92	3.3 a
<i>H. quercifolia</i>	1 d before anthesis	0	---
	anthesis	0	---
	1 d after anthesis	87	1.3 a
	2 d after anthesis	73	1.3 a
	3 d after anthesis	87	1.3 a
	4 d after anthesis	100	1.4 a
	5 d after anthesis	73	1.4 a

^zValues within a column followed by the same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$).

temperatures. In radish (*Raphanus sativa*), seed yields from self-pollinations increased when flowering plants were incubated at 26 °C rather than 15 °C (El Murabaa, 1957). High-temperature induced PSC has been confirmed in several additional species (Ascher, 1976). PSC has also been observed at the end of the flowering season, where plants that had previously failed to produce seed upon self-pollination suddenly began to set self-seed (East and Park, 1917). End-of-season PSC may be an expression of heat inactivation of the self-incompatibility system (East, 1923).

Low seed set that was previously observed in *H. paniculata* and *H. quercifolia* controlled self-pollinations (Reed, 2000), along with the single pollen tube that penetrated an ovule following self-pollination of *H. paniculata* in this study, may be evidence of PSC. Because

a single *H. paniculata* or *H. quercifolia* plant has the potential to produce several million seed, even a low level of PSC would produce a "good" seed set in plants isolated physically or temporally from a source of compatible pollen. Studying the effects of high temperatures and end-of-season pollinations on PSC may help explain differences in self-incompatibility assessments in other members of *Hydrangea* (Kudo and Niimi, 1999; Reed, 2000), and may also more fully explain seed set in isolated *H. quercifolia* plants. The possibility that there may be self-compatible *H. paniculata* and *H. quercifolia* cultivars should also be considered and investigated.

A secondary objective of this study was to determine optimum time for cross-pollinations. Stigmas of *H. quercifolia* were not receptive to pollen until the day after anthesis.

Fig. 3. Pollen tube growth following cross- and self-pollination of *Hydrangea quercifolia*. Values represent mean $\pm$ SE; n = 15.

While pollen germinated on *H. paniculata* stigmas pollinated 1 d prior to anthesis, pollen tubes were shorter than when pollen was applied at anthesis or later. In *H. paniculata* flowers that were pollinated prior to anthesis, pollen germination may have been delayed until stigmas became receptive. Stigmas of both species remained receptive to pollen for at least 5 d after anthesis; therefore, it appears that cross-pollinations should be effective if made from anthesis to 5 d after anthesis in *H. paniculata* and from 1 to 5 d after anthesis in *H. quercifolia*.

In summary, a gametophytic self-incompatibility system appears to be present in at least some *H. paniculata* and *H. quercifolia* genotypes. The decision on whether to emasculate flowers before making controlled pollinations depends on the tolerance in the experimental design to a few self-pollinated offspring and/or the ability to identify and eliminate self-progeny. Further investigations into the frequency and causes of pseudo-self compatibility are needed for developing better guidelines on eliminating or producing self-progeny in *Hydrangea*.

Literature Cited

- Ascher, P.D. 1976. Self-incompatibility systems in floriculture crops. *Acta Hort.* 63:205–15.
- Brewbaker, J.L. 1957. Pollen cytology and self-incompatibility systems in plants. *J. Hered.* 48:271–177.
- Brewbaker, J.L. 1959. Biology of the angiosperm pollen grain. *Ind. J. Genet. Plant Breed.* 19:121–133.
- de Nettancourt, D. 1977. Incompatibility in angiosperms. Springer-Verlag, Berlin.
- Dirr, M.A. 1998. Manual of woody landscape plants. Stipes Publishing, Champaign, Ill.
- East, E.M. 1923. Genetical aspects of self- and cross-sterility. *Amer. J. Bot.* 10:468–473.
- East, E.M. and J.B. Park. 1917. Studies on self-sterility. I. The behavior of self-sterile plants. *Genetics* 2:505–609.
- El Murabaa, A.I.M. 1957. Effect of high temperature on incompatibility in radish. *Euphytica* 6:268–270.
- Johansen, D.A. 1940. Plant microtechnique. McGraw-Hill, New York.
- Kudo, N. and Y. Niimi. 1999. Production of interspecific hybrids between *Hydrangea macrophylla* f. *hortensia* (Lam.) Rehd. and *H. arborescens* L. *Japan. Soc. Hort. Sci.* 68:428–439.
- Lawson-Hall, T. and B. Rothera. 1995. *Hydrangeas. A gardeners' guide*. Timber Press, Portland, Ore.
- Mallet, C., R. Mallet, and H. van Trier. 1992. *Hydrangeas. species and cultivars*. Centre d'Art Floral, Varengeville, France.
- Mallet, C. 1994. *Hydrangeas. species and cultivars*. Vol. 2. Centre d'Art Floral, Varengeville, France.
- Martin, F.W. 1959. Staining and observing pollen tubes in the style by means of fluorescence microscopy. *Stain Technol.* 34:125–128.
- McClintock, E. 1957. A monograph of the genus *Hydrangea*. *Proc. Calif. Acad. Sci.* 24:147–256.
- Reed, S. M. 2000. Compatibility studies in *Hydrangea*. *J. Environ. Hort.* 18:29–33.