Identification of Sterile, Noninvasive Cultivars of Japanese Spirea

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Abstract. Japanese spirea (*Spiraea japonica* L. f.), a popular landscape shrub, has shown the potential to become an invasive weed in both North America and Europe. Twenty commonly available *S. japonica* cultivars were evaluated for fertility using pollen and seed germination. Clones were grown in a randomized, replicated field plot, and additional seed samples were obtained from commercial nurseries and hand-pollinations in the greenhouse. Three sterile cultivars were identified: ‘Crispa’, ‘Dart’s Red’, and ‘Neon Flash’. These cultivars demonstrated poor anther dehiscence and very low mean pollen germination, 2.7%, 3.0%, and 1.3%, respectively, which often produced abnormal pollen tubes. None of these three cultivars produced viable seed in the field plot, at commercial nurseries, or when hand-pollinated in the greenhouse, whereas seed germination from fertile clones ranged from 91.5% to 100%. The other 17 cultivars tested, which should be treated as entirely fertile for the purposes of invasive plant management, were ‘Albiflora’, ‘Anthony Waterer’, ‘Candlelight’, ‘Dakota Goldcharm’, var. *alpina* ‘Daphne’, ‘Flaming Mound’, ‘Flowering Choice’, ‘Froebelii’, ‘Golden Princess’, ‘Goldflame’, ‘Goldmound’, ‘Gumball’, ‘Lemon Princess’, ‘Little Princess’, ‘Magic Carpet’, ‘Norman’, and ‘Shibori’. Measurements of DNA content indicated that all tested clones are diploid; therefore, the observed sterility was not related to polyploidy. The identification of these three sterile cultivars can help reduce the use of fertile varieties in areas where Japanese spirea has shown the potential to become invasive.

The escape and establishment of exotic plants from ornamental landscapes is causing significant ecosystem and environmental damage throughout much of the United States. These nonnative plants have affected natural ecosystems in a variety of ways, including a reduction in diversity through the displacement of native species, reduced soil stability and water quality, interference with plant succession dynamics and wildlife habitat and foraging, and altered fire regimes and nutrient cycling (Dukes and Mooney, 2004; Mack et al., 2000; Raizada et al., 2008). Economic costs associated with invasive plants total more than \$120 billion annually (Pimentel et al., 2005). Many of these exotic species, particularly among woody plants, originated as ornamental introductions. Reichard (1997) determined that 85% of invasive woody plants were originally introduced for landscape purposes.

Japanese spirea, *Spiraea japonica* L. f. (synonym *S. ×bumalda*) has become an extremely popular landscape plant as a result of

its adaptability, ease of culture, and desirable ornamental characteristics (Dirr, 1998). Sales of *Spiraea* species and cultivars, the majority of which are *S. japonica*, rank second only to roses in total sales of deciduous ornamental plants in the United States [U.S. Department of Agriculture (USDA), 1998]. Unfortunately, Japanese spirea has also shown the potential to become an invasive weed, naturalizing in much of the eastern United States (USDA, 2009).

A number of state governments have banned, or are in the process of banning, the import and sale of invasive ornamentals (Mehrhoft et al., 2003). An alternative to banning the sale of Japanese spirea would be the use of sterile, noninvasive clones. The availability of sterile cultivars would precipitate the elimination of fertile varieties from commerce in areas where it has shown the potential to become invasive.

Invasions of exotic species are often marked by a lag time between initial colonization and the onset of rapid population growth and range expansion (Sakai et al., 2001). There is compelling evidence that many, if not most, successful invasions are preceded by multiple introduction events that provide allelic variation that drives adaptive evolution (Culley and Hardiman, 2007; Durka et al., 2005; Lavergne and Molofsky, 2007; Pérez de la Vega et al., 1991; Prentis et al., 2008; Sakai et al., 2001). Available evidence suggests that many populations of Japanese spirea are in the initial establishment and/or lag phases of invasion both in

North America (Bowen et al., 2002; Garrett, 2007; Jog and Delong, 2005; USDA Forest Service, 2007) and Europe (Essl, 2005). Eliminating fertile Japanese spirea cultivars, at least in areas where this species has demonstrated the potential to naturalize, may be of particular importance early in the invasion process when multiple introductions, genetic recombination, and local selection are taking place, potentially contributing to the evolution of highly invasive populations.

In this study, 20 commonly available cultivars of *S. japonica* were evaluated for fertility by measuring the germination of pollen and open-pollinated seed from plants grown in a replicated field plot. In addition, seed collected from commercial nurseries were tested, and plants demonstrating sterility in the test plot were pollinated under controlled conditions in the greenhouse. The DNA content of all clones was determined using flow cytometry.

Materials and Methods

Plant material. Eighteen cultivars of *S. japonica* were maintained at the Montana State University Horticulture Farm, Bozeman, MT, in a randomized complete block design with three replicated blocks. All plants in the field plot were obtained from Bailey Nurseries (St. Paul, MN). Two clones that were not available when the field plot was planted, ‘Candlelight’ and ‘Lemon Princess’, were acquired from Forest Farm (Williams, OR) and three replicates of each were maintained in the greenhouse for analysis of pollen and seed germination and DNA content. Three cultivars that demonstrated sterility in our test plot, ‘Crispa’, ‘Dart’s Red’, and ‘Neon Flash’, were further tested through hand-pollinations in the greenhouse. For these pollinations, container-grown plants of ‘Dart’s Red’ were obtained from Means Nursery (Scappoose, OR) and ‘Crispa’ and ‘Neon Flash’ from Bailey Nurseries (St. Paul, MN). All pollen and seed samples were collected during 2008.

Pollen germination. Pollen germination was measured for each of the three replicate plants for all cultivars in the field test plot between 15 July and 8 Aug. 2008. Pollen from ‘Candlelight’ and ‘Lemon Princess’ was obtained from each of three replicates in the greenhouse. At anthesis, pollen from one inflorescence was sprinkled onto pollen germination media by gently tapping the inflorescence directly above the surface of the media. Pollen germination media consisted of 1.6 mM H₃BO₃, 1.3 mM Ca(NO₃)₂, 0.8 mM MgSO₄·7H₂O, 0.6 mM MnSO₄·1H₂O, 1 mM KNO₃, 15% sucrose, and 1% agar. After 8 h of incubation at 23 °C, the percentage of germinated pollen grains was determined from 100 total grains on randomly selected sections of the petri dish observed at 100× magnification using an Olympus microscope (Olympus America Inc., Center Valley, PA). A pollen grain was considered to have germinated if a pollen tube was visible and had a length that was equal to or

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greater than the diameter of the pollen grain (Rajora and Zsuffa, 1986).

Greenhouse pollinations. Three cultivars that demonstrated sterility in our test plot, 'Crispa', 'Dart's Red', and 'Neon Flash', were further evaluated through hand-pollinations in the greenhouse. Because self-incompatibility of *Spiraea* has not been characterized, and our work from a previous study indicates that *S. japonica* is primarily an outcrossing species (W. Hoch, unpublished data), each hand-pollination was performed using pollen from two different clones. One inflorescence on each of three replicates of the three cultivars was pollinated with a mixture of pollen from 'Albiflora' and 'Gumball' plants maintained in the greenhouse. These two clones were also reciprocally crossed as a positive control. Pollen was checked on germination media before pollination to assure a high percentage of viable pollen, and florets were pollinated at least twice over a period of several days to increase the probability of stigmatic receptivity during pollinations. Seed heads were collected 2 to 3 months after pollination, dried, and the contents removed under an Olympus dissecting scope (Olympus America Inc.).

Seed germination. Mature seed heads from each of the three replicate plants per clone in the test plot were collected and dried at room temperature to release the seed. Seed samples of several cultivars were obtained from commercial nurseries either because additional samples were desired to verify our findings of sterility in the test plot ('Crispa', 'Dart's Red', and 'Neon Flash') or because the length of the growing season at our test plot in 2008 precluded the maturation of enough seed for a full analysis ('Albiflora', 'Anthony Waterer', 'Dakota Goldcharm', var. *alpina* 'Daphne', 'Flaming Mound', 'Flowering Choice', 'Goldflame', 'Goldmound', 'Little Princess', 'Magic Carpet', 'Norman', and 'Shibori'). Nursery samples, which consisted of open-pollinated seed heads from many plants per clone pooled into a common container, were dried on arrival. Samples were obtained from the following nurseries: Bailey Nurseries (Yamhill, OR), Cross Nurseries Inc. (Lakeville, MN), Johnson's Nursery Inc. (Menomonee Falls, WI), Lawyer Nursery Inc. (Plains, MT), McKay Nursery (Waterloo, WI), and Sampson Nursery Inc. (Godwin, NC). A total of three replicates of 100 seeds from the field plot and nurseries were tested for each cultivar. When adequate seed were available from the field plot, 100 seeds from each of three replicates of each cultivar were tested. When adequate seed were not available from the field plot, seed from commercial nurseries was tested to bring the total number of replicates for each clone to three. In that case, when possible, tests were performed on seed from more than one nursery. For plants demonstrating sterility in the field plot, three replicates of 100 seeds from commercial nurseries were tested in addition to the three replicates from the field plot. For each germination test, 100 filled seed were placed onto moistened #42 filter paper (Whatman Inc., Florham Park, NJ)

inside 100 × 15-mm petri dishes and wrapped with parafilm (Pechiney Plastic Packaging, Menasha, WI). Petri dishes were placed on a laboratory bench 1.2 m beneath a 400-W high-pressure sodium vapor lamp (average photon flux of 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) operating on an 18-h photoperiod. For all tests, seed germination was recorded every 3 d until germination was complete (up to 8 weeks).

Flow cytometry. To release nuclei, fresh, newly expanded leaves of spirea samples [≈ 30 mg fresh weight (FW)] and the DNA standard (≈ 50 mg FW) were chopped together with a double-edged razor blade in 1 mL ice-cold chopping buffer for ≈ 60 s. Care was taken not to include spirea leaves with noticeable levels of anthocyanins, because the presence of anthocyanins has been found to inhibit propidium iodide DNA fluorescence (Bennett et al., 2008). The chopping buffer consisted of 10 mM MgSO_4 , 50 mM KCl, 5 mM Hepes, 6.5 mM dl-dithiothreitol, 2% polyvinylpyrrolidone 40, and 0.7% Tween 20. *Sorghum bicolor* Pioneer 8695 (Pioneer Hi-Bred International, Johnston, IA), which has a DNA content of 1.74 pg/2C (Johnston et al., 1999), was used as the standard in all samples. The suspension was filtered through a 30- μm nylon mesh filter (Spectrum Laboratories, Rancho Dominguez, CA) and stored on ice until staining. After nuclei extraction, propidium iodide and RNase (RNase One™ Ribonuclease; Promega Corp., Madison, WI) were added to each sample to a final concentration of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ and 20 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively, and briefly vortexed. Samples were kept on ice in the dark and analyzed by flow cytometry within 1 to 2 h. The ploidy level of *S. japonica* cultivars was based on the known tetraploid ($2n = 4x = 36$) *S. japonica* var. *fortunei* (Zhao-Yang et al., 2002).

The mean fluorescence of nuclei was quantified using a BD FACSCalibur Flow Cytometer (Becton, Dickinson and Company, San Jose, CA.) equipped with an air-cooled argon ion laser tuned at 488 nm and 15 mW. Fluorescence was collected by a photomultiplier screened by a long-pass filter. The results were acquired using the BD CellQuest Pro software, Version 5.2 (Becton, Dickinson and Company, San Jose, CA).

Statistical analysis. Pollen and seed germination data were analyzed using the Tukey-Kramer multiple comparisons test with SAS for Windows Version 9.1 (SAS Institute Inc., Cary, NC) using an experimentwise error rate of $\alpha = 0.05$.

Results

Cultivars with the highest and lowest mean percent pollen germination were in two distinct groups (67.0% to 69.3% and 1.3% to 4.0%, respectively) (Table 1), whereas most clones fell into overlapping groups with pollen germination between 36.3% and 57.3%. Among the four cultivars with the lowest germination percentage ('Crispa', 'Dart's Red', 'Flaming Mound', and 'Neon Flash'), germinating pollen frequently developed tubes with abnormal morphology similar to

those observed in pollen from triploid plants such as stunted growth, twisting, and swelling (Park et al., 2002). Anther dehiscence was also abnormal in these clones with the majority of anthers opening only partially or not at all. This indicates that the pollen of these four cultivars is mostly, if not entirely, nonviable. Pollen from all other cultivars germinated at significantly higher rates and produced normal pollen tubes. Pollen collected from the two greenhouse-grown cultivars, Candle Light and Lemon Princess, displayed significantly higher germination percentages (69.3% and 67.0%, respectively) than the other clones.

Seed germination of the 20 cultivars revealed two markedly different groups: those with a high mean percentage of germination (greater than 91%) and those that did not yield any viable seed (Table 1). As a result of the length of the growing season at our test plot in 2008 (118 d; U.S. Department of Interior, 2009), 12 clones did not produce enough mature seed for a full analysis of germination. For these cultivars, available seed from the field plot were tested for germination, and additional seed samples were obtained from commercial nurseries to complete the analysis. Three cultivars, Crispa, Dart's Red, and Neon Flash, did not produce viable seed in the field plot, at commercial nurseries, or when hand-pollinated in the greenhouse. In all instances, the dried dehiscent follicles of these three clones contained small, shriveled seed, sometimes with small amounts of abnormal endosperm. For these three cultivars, the entire sample from each source was processed through the seed germination protocol, but in all instances, no germination occurred.

Pollinations in the greenhouse produced viable seed in the fertile cultivars Candle Light and Lemon Princess (Table 1) as well as in the reciprocal crosses between the two cultivars, Albiflora and Gumball, used as male parents for all hand-pollinations (data not shown). For all cultivars, the majority of seed germination occurred during the first 14 d of each test, whereas sporadic germination continued for up to 8 weeks for some clones. Prolonged germination times were particularly evident in seed from the field plot and greenhouse.

Flow cytometric analysis found the DNA content of the 20 cultivars ranged from 0.52 to 0.61 pg/2C (Table 2) with a mean of 0.56 pg/2C. Zhao-Yang et al. (2002) karyotyped the eight varieties comprising the *S. japonica* complex and found seven to be diploid with the one tetraploid being *S. japonica* var. *fortunei* ($2n = 4x = 36$). In our study, *S. japonica* var. *fortunei* was found to have a DNA content of 1.14 pg/2C, which is very close to double the mean DNA content of the tested cultivars. This indicates that all tested clones are diploid.

Discussion

This study identified three sterile cultivars of *S. japonica* that can be used as landscape

Table 1. Mean pollen and seed germination for twenty *Spiraea japonica* cultivars in 2008.

Cultivar	Mean pollen germination ^z (%)	Mean seed germination (%)			
		Field plot ^y	Nursery ^x	Greenhouse ^w	Clone total
Albiflora	44.3 bc ^v	NS ^u	99.7 (B, M)	—	99.7 a
Anthony Waterer	36.7 c	NS	97.7 (J,S)	—	97.7 ab
Candlelight	69.3 a	—	—	91.5	91.5 b
Crispa	2.7 d	0.0	0.0 (L)	0.0	0.0 c
Dakota Goldcharm	47.7 bc	96.0 (1)	100.0 (B, M)	—	98.7 a
var. <i>alpina</i> Daphne	41.3 bc	99.0 (1)	96.5 (B, L)	—	97.3 ab
Darts Red	3.0 d	0.0	0.0 (C)	0.0	0.0 c
Flaming Mound	4.0 d	NS	100.0 (M)	—	100.0 a
Flowering Choice	43.7 bc	NS	100.0 (B)	—	100.0 a
Froebelii	49.3 bc	95.3	—	—	95.3 ab
Golden Princess	57.3 ab	94.0	—	—	94.0 ab
Goldflame	41.7 bc	NS	100.0 (B)	—	100.0 a
Goldmound	48.7 bc	100.0 (2)	100.0 (B)	—	100.0 a
Gumball	40.7 c	95.7	—	—	95.7 ab
Lemon Princess	67.0 a	—	—	98.0	98.0 ab
Little Princess	36.3 c	100.0 (1)	100.0 (B, M)	—	100.0 a
Magic Carpet	50.3 bc	NS	100.0 (B, M)	—	100.0 a
Neon Flash	1.3 d	0.0	0.0 (L, S)	0.0	0.0 c
Norman	50.0 bc	96.0 (2)	100.0 (M)	—	97.3 ab
Shibori	40.7 c	NS	100.0 (M, S)	—	100.0 a

^zData are from three replicates of 100. Pollen from 'Candlelight' and 'Lemon Princess' are from greenhouse plants; all other pollen collected were from the field plot in Bozeman, MT between 15 July and 8 Aug. 2008.

^yData are from three replicates of 100 unless number of replicates is indicated in parentheses as a result of inadequate availability of mature seed from the field plot in Bozeman, MT.

^xFor clones that produced inadequate amounts of mature seed before the end of the growing season or demonstrated sterility in the field plot, additional samples were obtained from commercial nurseries that are indicated in parentheses: B = Bailey Nursery; C = Cross Nurseries; J = Johnson's Nursery; L = Lawyer Nursery; M = McKay Nursery; S = Sampson Nursery. Data are from three replicates of 100 for clones that demonstrated sterility or did not produce mature seed in the field plot and for the remainder tests of 100 were performed to bring the total number of replicates from the field plot and nurseries to three.

^wData are from three replicates of 100. Clones that demonstrated sterility in the field plot along with 'Candlelight' and 'Lemon Princess', which were not in the field plot, were hand-pollinated in the greenhouse.

^vMeans within columns followed by the same letter are not significantly different at $\alpha = 0.05$ by the Tukey-Kramer multiple comparisons test.

^uNS = No seed matured before the end of the growing season.

Table 2. Mean 2C DNA content and ploidy of 20 *Spiraea japonica* cultivars and *S. japonica* var. *fortunei*.

Cultivar	2C DNA content ^z (pg)	Ploidy ^y
Albiflora	0.55 ± <0.01	2x
Anthony Waterer	0.55 ± 0.01	2x
Candlelight	0.56 ± 0.01	2x
Crispa	0.61 ± <0.01	2x
Dakota Goldcharm	0.55 ± 0.02	2x
var. <i>alpina</i> Daphne	0.53 ± <0.01	2x
Darts Red	0.53 ± <0.01	2x
Flaming Mound	0.58 ± <0.01	2x
Flowering Choice	0.57 ± <0.01	2x
Froebelii	0.58 ± 0.01	2x
Golden Princess	0.54 ± 0.02	2x
Goldflame	0.52 ± <0.01	2x
Goldmound	0.55 ± <0.01	2x
Gumball	0.61 ± <0.01	2x
Lemon Princess	0.58 ± 0.01	2x
Little Princess	0.53 ± 0.01	2x
Magic Carpet	0.54 ± 0.01	2x
Neon Flash	0.54 ± <0.01	2x
Norman	0.55 ± 0.01	2x
Shibori	0.54 ± 0.02	2x
<i>S.j.</i> var. <i>fortunei</i>	1.14 ± 0.02	4x

^zData are from three replicates ± SE. All samples are from the field plot in Bozeman MT in 2008, except for 'Candlelight' and 'Lemon Princess', which were from greenhouse plants.

^yPloidy based on known tetraploid (2n = 4x = 36) *Spiraea japonica* var. *fortunei*.

plants with minimal risk of escape into natural areas. These three cultivars produced little, if any, viable pollen and no viable seed from any of the sources. The other 17 clones demonstrated considerable pollen germination and female fertility with the exception of 'Flaming Mound', which exhibited very low pollen viability despite having a high percentage (100%) of seed germination. The high degree of seed viability of the fertile cultivars from both the field plot and commercial nurseries indicates that adequate cross-pollination existed at all locations. These results strongly support the conclusion that 'Crispa', 'Dart's Red', and 'Neon Flash' are sterile cultivars.

The significantly higher percentage of pollen germination from the two cultivars grown in the greenhouse, 'Candle Light' and 'Lemon Princess', is most likely the result of more stable conditions in the greenhouse relative to the field plot. High temperatures, ultraviolet-B radiation, and large changes in relative humidity can shorten pollen viability (Bots and Mariani, 2005) and are conditions that would be more pronounced in the field. Although we could not find data concerning the effect of relative humidity on pollen viability for any rosaceous species, Stone et al. (1995) found that low humidity greatly shortened pollen viability in a number of plant families. During the summer of 2008 at our field plot, daytime relative humidity

commonly fell below 20% (U.S. Department of Interior, 2009) and was below 12% on 6 d during the period of pollen sampling, which may have affected the duration of pollen viability during this study.

The long period of seed germination (up to 8 weeks) for some samples could be the result of this seed becoming excessively dry. Although references indicate that no stratification is required for *S. japonica* seed (Dirr and Heuser, 1987; USDA Forest Service, 2008), Dirr and Heuser (1987) suggest that if *Spiraea* seed are allowed to dry, germination may be improved by 1- to 2-month cold stratification. Calmes and Zasada (1982) found 30-d cold stratification improved the germination of *Spiraea stevenii*. Thus, it is possible that a short period of cold stratification may have resulted in more uniform seed germination among these samples.

The variation in DNA content among the cultivars is possibly the result of differences in chromosome morphology within the *S. japonica* complex. Zhao-Yang et al. (2002) found considerable karyomorphological variation in chromosome size and organization among the eight taxa comprising the *S. japonica* complex. With a wide diversity of origins, the cultivars in this study (Dirr, 1998) likely include at least some of the chromosomal variation observed among the *S. japonica* taxa.

Polyploidy, in particular triploidy, is a common cause of sterility in ornamental selections (Datta, 1960; Godo et al., 2004; Kondo et al., 1993; Saito et al., 2003; Tanaka et al., 2005; Wybe and Peterse, 1999). These sterile plants probably result from chance unreduced gametes contributed by one or both parents (Otto and Whitton, 2000) and are then preferentially selected as a result of pleiotropic effects of polyploidy, e.g., larger flowers and fruit, longer bloom time, compact growth habit, and reduced fruiting (Anurita and Girjesh, 2007; Liu et al., 2007). However, polyploidy is not a factor in the sterility of the Japanese spirea cultivars identified in this study, because all clones were determined to be diploid based on DNA content. Aneuploidy is one possible explanation for the sterility of these cultivars, particularly if it involves one or only a few chromosomes, because karyotypes were not performed, and the inherent variation in DNA content among the cultivars prohibited detection of small differences in chromosome number using flow cytometry.

The variation in seed and pollen germination data among the fertile clones of this study should not be interpreted as relative differences in invasive potential. As described in the beginning of this article, the establishment and lag phases of successful plant invasions are often marked by multiple introduction events that contribute to the evolution of invasive genotypes. It is entirely possible that, relative to more prolific clones, a cultivar with lower seed and/or pollen germination may actually produce offspring that are better locally adapted and/or supply alleles that contribute more to the adaptive evolution of established *S. japonica* populations. Thus,

in the absence of extensive study, there is no way of knowing how the offspring of any particular cultivar would perform in the wild and/or interact genetically with local, naturalized populations. Therefore, we recommend the three cultivars that produced no offspring in our study ('Crispa', 'Dart's Red', and 'Neon Flash') be considered to have extremely low invasive potential, whereas the other 17 clones be treated as entirely fertile for the purposes of invasive plant management.

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