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Pollination Biology and Seed Traits of *Spiraea virginiana* L. (Virginia spiraea) Populations in Western North Carolina

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Abstract

Spiraea virginiana L. (Virginia spiraea) is a federally threatened riparian rose native to the Appalachians. Due to its predominantly asexual reproduction, *S. virginiana* relies on regular flooding to send broken stems downstream and establish new populations. Since the introduction of dams, however, opportunities for population expansion have greatly decreased. Sexual reproduction is uncommon in *S. virginiana*; however, the limiting factor(s) have yet to be identified. Reproductive constraints for this species could include pollen and ovule production or ratios; visitor type, frequency, and effectiveness; seed production; or seed viability. This study examined pollination behavior and seed traits in *S. virginiana* from two western North Carolina sites. Pollen counts made in 2017 from Ashe County were compared to ovule counts of 2018 to determine pollen:ovule ratios. In summer 2018, open-pollinated flowers were collected from one population made up of many subpopulations in Graham County. Seeds from each sample were counted and compared among subpopulations. Seeds were tested for viability using a 1% triphenyl tetrazolium chloride solution (TTC), and 2018 viable seed counts were also compared to those from 2010 and 2013. Flower visitors of *S. virginiana* were collected from a neighboring population in Macon County to identify pollination characteristics of this species. Pollen and ovule counts were significantly different among flowers (pollen) and corymbs (ovules) in Ashe County ($P<0.0001$ and $P=0.0119$, respectively). Pollen:ovule ratio was low (60.3) but consistent with biotic pollination. Seed count and seed viability did not differ among subpopulations in Graham County ($P= 0.9353$ and $P=0.6410$, respectively). Most visitors of Macon County subpopulations are classified as generalists, suggesting that *S. virginiana* may have a generalist pollination syndrome. These results suggest interannual variation in seed traits for *S. virginiana* due to either resource allocation in the form of light availability or pollination effectiveness based on pollination syndrome.

1. Introduction

Riparian plants have adapted to withstand the habitual disturbances of river flow, and in doing so, have become reliant on frequent flooding for seed or vegetative fragment dispersal[1,2]. The establishment of dams, however, disrupts larger rivers, creating smaller river flows and reservoirs downstream. The river fragmentation caused by damming can negatively affect species success by either restricting population dispersal or enabling other riparian plants to compete for resources such as light availability.

One such plant that has been impacted by damming is *Spiraea virginiana* (Virginia spiraea)[3]. *S. virginiana* a is a riparian shrub in the Rosaceae family that is endemic to the Appalachian Mountains of the eastern United States[4]. Historically, this species ranged from Alabama up to Pennsylvania; isolated populations are currently located in Georgia, North Carolina, Virginia, Tennessee, Kentucky, West Virginia, and Ohio[5]. In 1990, *S. virginiana* was classified as a federally threatened species[5].

Although *S. virginiana* is capable of both sexual and asexual reproduction, the species favors vegetative fragmentation of rhizomes or stems[6]. Sexual reproduction is uncommon in current populations, potentially due to limitations on resource allocation to reproductive structures. Tradeoffs are often made between sexual and asexual reproduction in plants when resources are limited[5,6,7]. High rates of asexual reproduction are a consequence of the high clonality of this species and the absence of seedlings in the field[8,6]. Although seedlings have yet to be reported, seed production does occur in small quantities[9,10]. Conspecific pollen grains have been discovered on stigma loads with low levels of foreign grains[10]. In the same study, low seed set was not related to stigma loads, suggesting that pollen receipt is not a driving force behind low seed production[10]. However, the few seeds that are produced tend to be inviable. Viability of seeds from Graham County, for instance, was less than 1% in both 2010 and 2013[11,10]. Such low levels of reproductive output may be due to the high levels of clonality within populations and the potential presence of a mechanism that prevents self-fertilization[11]. In fact, manual outbreeding has yielded higher levels of viable seeds[12].

Disruptions to population expansion and gene flow, in conjunction with increased interspecific competition for resources, contribute to the population decline of *S. virginiana*[5]. Populations have been proven to be genetically isolated from each other, as indicated by the low number of alleles within a population, the rarity of alleles per population, and the high genetic variation among populations[3,13,8]. Although low genetic variability within a population may contribute to reduced seed recruitment, some studies reveal consistent seed quantity regardless of self-pollination or outbreeding[14,10].

Plant reproductive success can be correlated with reproductive effort, interactions with pollinators, reproductive output, or a combination of these factors. Reproductive effort is the absolute and relative production of pollen and ovules, often measured through pollen:ovule ratios[15]. Another potential area of interest is the impact of pollen transfer on reproductive success. Visitation frequency is positively associated with pollen load, which in turn is correlated with seed set[16]. Floral morphology, including color, fragrance, and flower shape, determine the pollination syndrome, or ability of a plant to attract specific types of visitors[17]. Seed traits such as quantity and quality might also limit reproductive success in *S. virginiana.*

Neither pollen:ovule ratio nor pollination syndrome has yet to be determined for *S. virginiana*. Seed quantity, on the other hand, has been determined to be typically low for this species[6]. Perhaps more critical is the low production of viable seeds as exhibited in previous studies[11,10]. Through analysis of pollen and ovule counts and ratios, visitor observation and identification, and seed count and viability across three populations in western North Carolina, this study serves to determine which of the previously mentioned factors contribute to low rates of sexual reproduction in *S. virginiana.*

2. Methods

2.1 Study Sites

S. virginiana populations have been located across western North Carolina in Graham, Ashe, and Macon counties[20,3]. A total of 16 subpopulations have been located along the Cheoah River, below the Santeetlah Dam in Graham County. In 2013, 10 of the 16 subpopulations were studied, 8 of which are along Highway 129, an area with biennial mowing by the North Carolina Department of Transportation[10,21]. Elemental occurrences of *S. virginiana* in Ashe and Macon counties were identified in 1988[3]. An additional 2 new subpopulations were found in Macon County alongside the Little Tennessee River Greenway in 2011, and 6 additional subpopulations were found in Ashe County along the South Fork of the New River[10]. This study used 6 subpopulations along the Little Tennessee River Greenway in Franklin (Macon County), 4 subpopulations along US 129 highway within the Cheoah River corridor in Graham County, and 4 subpopulations along the New River in West Jefferson (Ashe County).

2.2 Pollen Counts

A preliminary pollen analysis was done on samples to determine the total number of pollen grains per flower. A corymb from Ashe County was collected in June 2017 and preserved in 70% ethanol. A total of 12 flowers were randomly selected from this corymb for pollen analysis. Each flower was ground using a micro pestle in a 5% sodium dodecyl sulfate (SDS):95% ddH_2O solution for a total volume of 120 μL[18]. Twelve replicate depression microscope slides were prepared to capture the complete variation within a sample. Each slide contained a 10 μL drop of floral solution, accounting for the entire pollen volume per sample. Slides were viewed at 10X magnification under a Nikon ECLIPSE TE2000-S inverted confocal microscope with a 12v 100 W halogen bulb. Images were captured for each sample using a SPOT RT KE/SE™ camera and imaging software, focusing on the center of the depression slide. Photos were uploaded to ImageJ software and total pollen count per spot was determined using an auto-count feature[19]. Pollen counts from the 12 spots were averaged to calculate mean pollen per flower. Pollen count differences among flowers were determined using one-way ANOVA in RStudio[22].

2.3 Ovule Counts

In the summer of 2018, unopened flowers were collected from 1-5 plants at each of three sites (Ashe County, Graham County, and Macon County) and stored in 70% ethanol. Initial ovule counts were completed for 2 Ashe County corymbs collected in 2017. A total of nine flowers per corymb were dissected using a Leica ZOOM 2000™ dissecting scope at 20X magnification to reveal ovules, and all ovules were counted. One-way ANOVA in RStudio was used to compare ovule counts across corymbs[22].

2.4 Corymb Collection

Open-pollinated plants in Graham County subpopulations were used in this study. Open-pollinated plants were undisturbed, enabling normal pollination behaviors to occur. These plants were marked with a single yellow bead threaded through clear fishing line. Corymbs with flowers already exhibiting pollinated stigmas were bagged with tulle to prevent any seed loss or visitation from additional insects. Corymbs were collected 3 months later in mid-September 2018 after maturation and seed development. Stalks containing 1-3 corymbs were cut and placed in labeled, brown paper bags. Flowers air dried in the open bags for a total of 10 days prior to seed trait analyses.

2.5 Visitor Collection

Kill jars were prepared prior to collection with a cotton ball soaked in ethyl acetate and placed in a 50 mL plastic tubes[18]. Visitors located on (or had recently been on) *S. virginiana* flowers located in Macon County were captured and stored in sealed kill jars until visual identification.

2.6 Seed Counts

Five corymbs were collected from three subpopulations in Graham County and dried at room temperature to prevent fungal growth. After drying, sepals and petals were removed to reveal the follicle (with seed coat intact). Counts were made by assessing the number of seeds per cluster under a Leica ZOOM 2000™ dissecting scope at 20X magnification. A subset of 50 seeds per sample were used for viability testing, and the remaining seeds were retained for future germination studies. One-way ANOVA in RStudio was used to evaluate differences in seed counts among subpopulations[22].

2.7 Seed Viability

Seeds soaked in ddH_2O for approximately 48 hours to soften the seed coats. After the 48-hour period, the seed coats were broken with forceps. With the seed coat broken, the seeds were placed in a 1% triphenyl tetrazolium chloride (TTC) solution for ~24 h in the dark. Afterwards, seeds were dissected, and color was noted; presence of a red color indicated cellular respiration and viability of a seed. Endosperm and embryos of inviable seeds would remain white, with no red color. Viability counts were run through a one-way ANOVA test to determine any variation between counts and subpopulation. A regression was also performed to elucidate relationships between seed counts and viability. Both statistical tests were done in RStudio[22].

3. Results

3.1 Pollen and Ovule Production and Ratio

The mean number of pollen grains per flower in Ashe County ranged from 27-155 grains (Fig. 1). Pollen production varied significantly among flowers (df=11/130, n=12, F=22.53, P<0.0001). Ovule counts were also significantly different between the 2 corymbs in Ashe County (df=1/20, n= 2, F=7.663, P=0.0119; Fig. 2).

Because pollen and ovule production were not sampled from the same corymb, it is difficult to determine the true pollen:ovule ratios of Ashe County flowers. However, a ratio (60.3) was determined using the ovule production data from 2018 (mean=16.4) and the pollen production data from 2017 (mean=989.7) from the same parent plant.

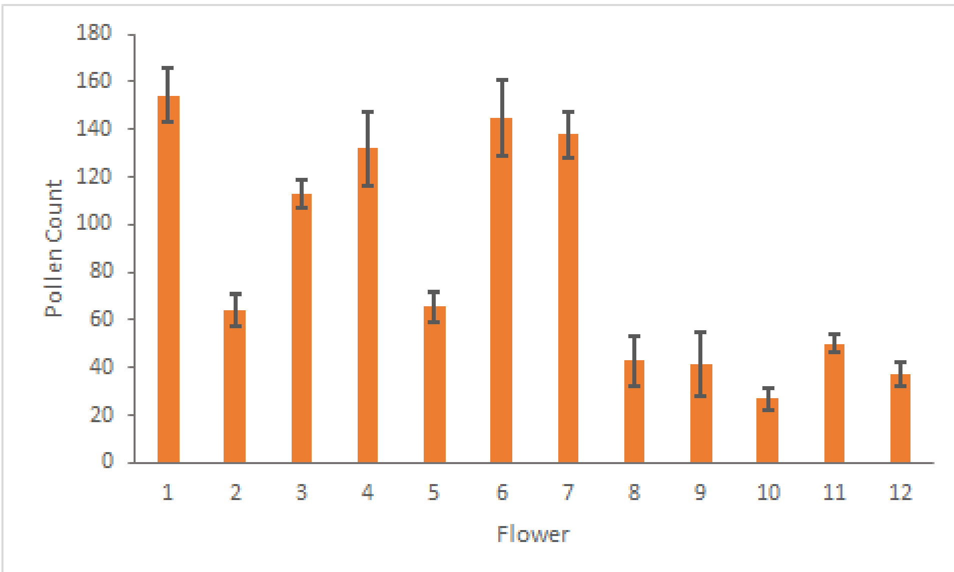

Figure 1. Mean (± SE) pollen grains per flower in one corymb from Ashe County

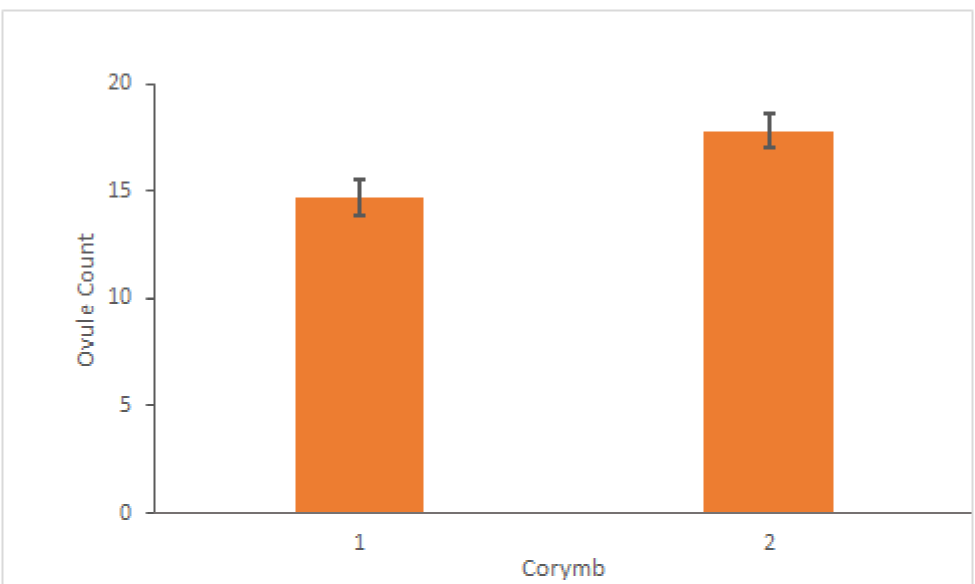

Figure 2. Mean (± SE) number of ovules per corymb from Ashe County.

3.3 Flower Visitors

Flower visitors consisted of moths, bees, flies, wasps, and beetles (Table 4). The most common pollination syndrome among visitors is Cantharophily, frequently associated with beetles. Most of the visitors are classified as pollen or nectar-seekers; however, Erebidae, Scarabaeidae, and Curculionidae are known to feed off solely floral structures or nectar. Many of these families visit a wide range of plant families, with Asteraceae being the most common.

Table 4. Pollination traits of visitors near Macon County subpopulations[23, 17, 24,25,26, 27]

Family	Pollination Syndrome	Visits/Pollinates	Reward
Apidae	Melittophily	Asteraceae Plantaginaceae Ranunculaceae Rosaceae Violaceae	Nectar Pollen
Cantharidae	Cantharophily	Apiaceae Asteraceae	Pollen
Cerambycidae	Cantharophily	Ranunculaceae Rosaceae	Nectar Pollen
Crabronidae	-	Orchidaceae	Prey on Pollinators
Curculionidae	Cantharophily	Annonaceae	Flowers
Erebidae	Phalaenophily	Apocynaceae Asteraceae	Nectar
Miridae	-	Asteraceae	Nectar Pollen
Mordellidae	Cantharophily	Asteraceae	Pollen Egg-laying
Scarabaeidae	Cantharophily	Araceae	Flowers
Syrphidae	Myophily	Asteraceae Primula Lamiaceae	Nectar Pollen

3.4 Seed Set

Mean numbers of seeds ranged from 581-807 for subpopulations in Graham County (Fig. 3). Total seed set did not vary significantly among subpopulations (df=2/6, n=3, F=0.0676, P=0.9353).

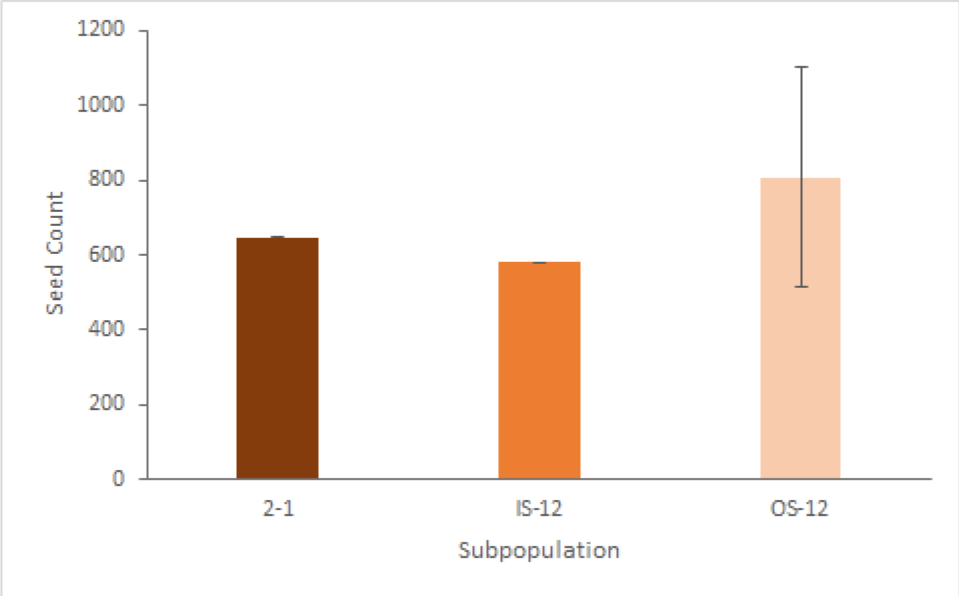

Figure 3. Mean seed counts for subpopulations in Graham County.

3.5 Seed Viability

Percent viable seeds did not differ significantly among subpopulations in Graham County (df=2/5, n=3, F=0.4387, P=0.6675; Figure 4). There was no relationship between number of seeds and the percentage of viable seeds (df=1/6, F=1.932, P=0.214).

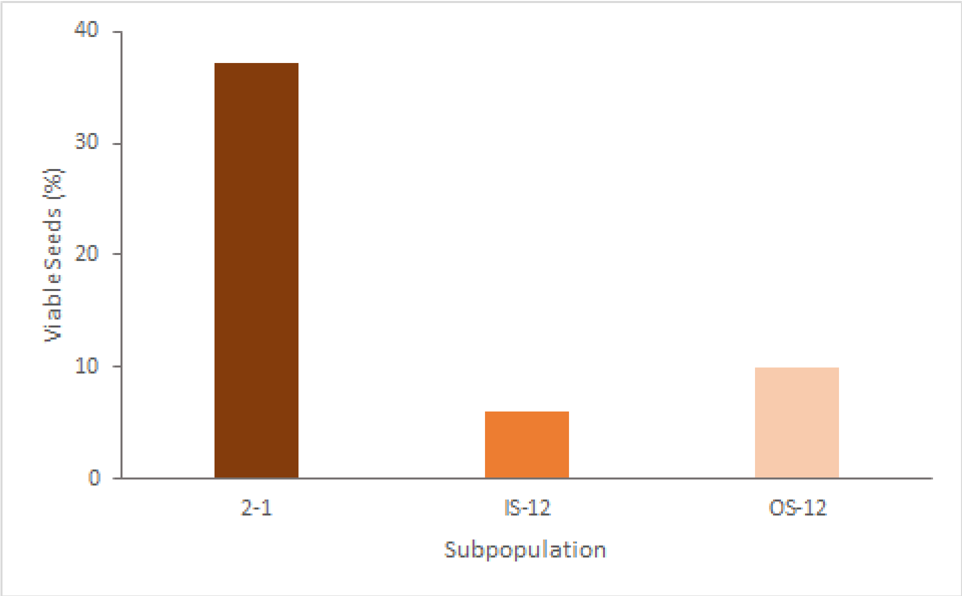

Figure 4. Viable seeds (%) for subpopulations in Graham County.

4. Discussion

Pollen counts differed among flowers in Ashe County. Pollen production is related to environmental conditions and resource availability such as soil quality, and mycorrhizal interactions[29]. This might suggest that the differences in pollen production per flower in Ashe County may be due to allocation of resources. Reproductive allocation considers the scope of resources and how they are utilized in reproduction, while reproductive effort is the actual production of reproductive structures using those resources[28]. The differences in ovule counts among corymbs may also be a result of reproductive allocation. Ovule production in *S. virginiana* has not been analyzed prior to this study. This may be due to the historically low seed counts in the field and the suggestion that seed inviability is caused by ovule termination when resources are scarce[6,10]. Although resource allocation is usually studied between populations or subpopulations, significant differences in pollen and ovule production among corymbs appear to be caused by resource allocation in individual plants. Pollen and ovule counts not only suggest differences in resource allocation of *S. virginiana*, but also suggest that reproductive effort is not contributing to the low reproductive success of the species. The ratio of pollen to ovules has been associated with high stigma loads, which in turn, measures pollination success[15,30]. Pollen:ovule ratios are influenced by morphological and ecological features of specific plant taxa[17]. For example, wind-pollinated plants tend to exhibit much higher ratios compared to plants that rely on animal pollination[17]. The 60.3 ratio for *S. virginiana* falls within the range of other animal-pollinated plant species. Thus, pollen and ovule production are most likely not causing depressed sexual reproduction in this species.

Another factor that seems to not contribute to low reproductive success in *S. virginiana* is its pollination syndrome, which can be predicted from the visitors identified in this study. Literature suggests that plants visited by generalist families might have a generalist pollination syndrome. Almost half of the visitors seen are part of generalist pollinator families (Apidae, Cerambycidae, Cantharidae, and Syrphidae)[17]. These families typically visit a variety of plants in the Asteraceae (daisy), Ranunculaceae (buttercup), and Rosaceae (rose) families[17]. The morphological characteristics under this syndrome enable a variety of pollinators, thus encouraging pollination success for the plant. Plants classified under this syndrome share many of the same morphological features of *S. virginiana* flowers: open and radial, often bowl-shaped, cream in color, accessible pollen, and clustered in inflorescences[17]. Plants with a generalist syndrome also have low volumes of nectar in high concentration[17]. It is not known whether *S. virginiana* produces nectar; however, other *Spiraea* species make nectar rewards for their pollinators[31,32]. At least one nectar robber was found on flowers of the Macon County subpopulations (Erebidae), as well as many beetles and flies, most of which collect both pollen and nectar. Should nectar production be confirmed in *S. virginiana*, its classification as having a generalist pollination syndrome suggests that pollination is not limiting sexual success. Another area of examination that may confirm the generalist syndrome for this species is to compare visitor types of other Rosaceae plants with that of *S. virginiana.*

Previous studies have related low seed count to low genetic variation in this species[6,10]. Seed count was relatively high among all three subpopulations collected in 2018. Viable seeds were also present within these subpopulations, even though no viable seeds were observed in 2013[10]. The higher number of seeds produced in conjunction with the presence of viable seeds suggests the possibility of interannual variation in seed traits for *S. virginiana.* Variation in seed set could be caused by allocation of resources[33]. Although there were no significant differences in either seed count or viability among Graham County subpopulations, seed production and viability could vary over time. Perhaps the biennial long-arm mowing established in 2011 has contributed to seed success for the subpopulations in Graham County by creating more light resources that can lead to increased carbon availability for seed production[24,28]. The most recent study that examined seed viability for this population was in 2013, only 2 years after the determination that mowing is beneficial. Now that it has been seven years since the 2011 study, it may be possible that the long-term effects of mowing may contribute to changes in resource allocation over time, thus explaining the change in seed viability from 2014 to 2018. Further physiological measurements should be done to determine if light availability is limiting reproductive success for *S. virginiana.*

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