Assessing Reproductive Potential in a Federally Listed Species: Differential Staining for Pollen Viability in *Spiraea virginiana*

by

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Abstract

Spiraea virginiana Britton (*Rosaceae*) is a rare federally-listed, clonal shrub that inhabits riparian zones within the Ohio River drainage. The species often occurs in areas of high disturbance and predominantly uses asexual reproduction to propagate from upstream populations. Although the species' known range suggests that sexual reproduction played an important role in its evolutionary history, sexual reproduction is currently almost non-existent. Pollination studies have shown that the species can produce fertile seeds; however, this has not been witnessed in wild populations. Determining the role of pollen viability with regards to lack of fertile seed formation is an important step towards understanding the sexual reproductive barriers experienced in this species, possibly allowing for updated restoration efforts to be introduced. In this study, pollen viability was determined through *in vivo* studies on a Motic BA410E research microscope using brightfield technology and utilizing a modified Alexander's stain that differentiates viable and aborted pollen. Inflorescences were collected along the south fork of the New River before anthesis while pollen was mature, but anthers were non-dehiscent. This study aimed to assess pollen viability and determine the percentage of viable pollen produced among populations that were collected along this river drainage to help determine if pollen viability is a major contributing factor to sexual reproductive barriers within this species.

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Introduction

Spiraea virginiana Britton (*Rosaceae*) is a rare federally listed clonal shrub (USFWS, 1990) in the Rose family. Members within this family are primarily located in temperate regions of North America, Europe, and Eastern Asia (Zomlefer, 1994). Many of the species within the family have considerable economic value due to the edibility of their fruit (strawberries (*Fragaria*), raspberries (*Rubus*), and apples (*Malus*)) or showy flowers such as roses (genus *Rosa*) (Robertson & Sytsma, 2017). The agriculturally significant crops have been frequently studied, focusing on developing saturated linkage maps as a guide for fruit tree genetics and breeding (Dirlewanger et al., 2004), the understanding of self-incompatibility mechanisms (Sassa et al., 1996, Ashkani & Rees, 2016), and using genomic sequencing to study ripening patterns (Marti, Saski, Manganaris, Gasic, & Crisosto, 2018). *Spiraea virginiana* is 1 of more than 80 species of the genus *Spiraea* found in North America, Eastern Europe, and Asia (Rehder, 1940), belonging to the subfamily Amygdaloideae within the Rosaceae.

Spiraea virginiana is a rhizomatous, riparian shrub that is adapted to a high light riparian habitat along second and third order streams that are subject to predictable flooding events (Ogle, 1991b). *S. virginiana* has a restricted distribution and is isolated to the Ohio River drainages, including the sub-drainages of the Tennessee and Cumberland rivers (Ogle, 1991b, Pate, 2010). *The species* exploits a unique niche in riparian habitats, suggesting that it is a disturbance dependent species, requiring flooding and scouring events to facilitate its reproductive strategy and help eliminate competition for sunlight (Ogle, 1991b). Many anthropogenic activities, like

dam or bridge construction, and bank stabilization projects have reduced the specific habitat required by *S. virginiana.*

As a member of the genus *Spiraea*, *S. virginiana* is easily distinguished by several morphological characteristics. *S. virginiana* has a large and fibrous root system, growing from 0.6 to 3.05 meters in height, and displaying arching, upright stems. The leaves are simple and alternate with acute bases, dark green tops, a dull green or blue color on the underside, and margins that vary from entire to serrate. Serrations can range from course to fine, terminating abruptly at the apex with a sharp tip. Flowers can range from white to cream colored and occur as a flat-topped, compound corymb inflorescences that are 5 to 22cm wide, with stamens that are approximately twice the length of the sepals and extend past the floral cup (USFW, 1990, Ogle, 1991a). Plant phenology occurs from late May to late July depending on elevation and geographic location (Ogle, 1991a).

The United States Fish and Wildlife Service listed *S. virginiana* as federally endangered species in June of 1990, after originally being proposed for a threatened status in 1989 (USFWS, 1989 & 1990). The North Carolina Natural Heritage Program suggests the species is threatened across its range at both the state and federal levels. It is ranked as an S2G2 (state/global), meaning that it is considered imperiled. The species has between 6 and 20 extant populations remaining and is considered rare due to numerous factors such as habitat specifications (North Carolina Natural Heritage Program, 2017). *S. virginiana* persists in a narrowly defined riparian habitat, which has been subjected to extreme anthropogenic change due to infrastructure development. Increases in construction, especially dams and bridges, have created a scenario where population numbers are drastically decreasing to the point of, and up to, extinction for some populations (Wise, pers. obs.). *Spiraea virginiana* has a historically isolated and restricted

distribution that has decreased exponentially and is likely a result of the proximity of these populations to infrastructure projects (Ogle, 1991b, Bryzski & Culley, 2011). Additionally, it has been noted that *S. virginiana* does not compete well with other riparian species that occupy the same niche, such as invasive bamboo (Phyllostachys spp,) Japanese knotweed (*Fallopia japonica),* native Ninebark (*Physocarpus opulifolius*), or Elderberry (*Sambucus sp.*), further exacerbating the plight of this species.

During the Quaternary Period, spanning from 1.6 million (mya) to 11.7 thousand years ago (kya), the current northern range of *S. virginiana* was covered by glaciers that experienced various advances and retreat events (Delcourt & Delcourt, 1991). Plant communities were thought to expand and contract with glacial movements, and evidence from the analysis of pollen cores obtained from lakes and bogs in eastern North America suggests that this hypothesis is supported (Delcourt & Delcourt, 1991). Following the Quaternary Period, the Hypsithermal Period provided warmer and drier conditions across eastern North America. Based on the species present distribution, two hypotheses have been developed to explain the current distribution of the species; the species retreated and recolonized with glacial activity, or the species was able to persist in refugia by evading glaciation (Anders & Murrell, 2001, Williams, 2003).

Sexual reproduction within *S. virginiana* is extremely limited to non-existent in natural populations with no seedlings observed in the species natural habitat (Ogle, 1991a). Given the species current distribution it is possible that sexual reproduction (Ogle, 1991b) played an important role in its evolutionary history. The reproductive strategies of this species are poorly understood; it is believed that this species reproduces primarily through asexual propagation, by ramet formation, which break free of the rhizome and become lodged in rock crevices and around boulders downstream during flooding and scouring events (Ogle, 1991b). Subsequent

field observations have documented a lack of fertile seed production and demonstrated low germination rates (Emery, 2014), These observations suggest the possibility that the sexual reproductive barriers experienced in *S. virginiana* may be correlated with pollen viability, polyploidy, a self-incompatibility mechanism, or abnormal meiotic behavior that inhibits fertile seed formation.

Microsporogenesis is a relatively straight forward process involving multiple steps that regulate microspore (pollen) development. During this process, the pollen mother cell undergoes meiosis resulting in the formation of four haploid microspores enclosed in a callose (a plant polysaccharide comprised of glucose residues) sheath. After the sheath dissolves, the four microspores are released as single nucleated cells. Microspores continue to increase in size prior to microspore mitosis. Microspore mitosis results in the formation of two cells (vegetative and generative) with very different purposes. The vegetative cell does not undergo any additional mitotic functions and is responsible for the development of the pollen grain and the formation of the pollen tube. Alternatively, the generative cell undergoes another round of mitosis and forms 2 sperm cells (Sari-Gorla, Ferrario, Villa, & Pe, 1996). These limited reproductive options coupled with the premise that cross-pollination between river drainages is limited, suggests that there are likely multiple influences affecting sexual reproduction within *S. virginiana*. This study focuses on only one facet of these issues, pollen viability.

Previous studies conducted by Bryzski and Culley (2013) on pollen viability and germination analysis among a single robust population from Ohio (Scioto County) demonstrated a 90% ratio of viable pollen when observed using the Alexander's stain protocol (Alexander, 1980). Additionally, previous studies (Emery, 2014) were conducted to determine reproductive success under three hand-pollination treatments (selfing, inbreeding within populations, and

outbreeding among populations) from samples obtained in Western North Carolina drainages suggesting that pollen availability did not limit seed production. This study aims to assess and determine the percentage of viable pollen produced among populations that were collected along the New River drainage to help determine if pollen viability is a major contributing factor to sexual reproductive barriers within this species. We also examined the ratio of viable pollen to see if it is consistent with that found by Bryzski and Culley (2013). This study is important because species whose endangered or threatened status is combined with reproductive barriers must have their entire seed set liabilities evaluated to determine the proper conservation actions to take.

Methods

Field Collection Methods

Samples from nine populations using Elemental Occurrence (EO) data obtained from The NC Natural Heritage Program were visited, and samples collected along the South fork of the New River in Ashe County, North Carolina during the summer of 2016. Populations were accessed via kayak, or road when possible. Inflourescenceswere collected at varying developmental stages, fixed in Carnoy's II fixative (6:3:1 ratio of ethanol: chloroform: glacial acetic acid), placed in 50 mL polypropylene conical tubes, and stored temporarily in a portable cooler with ice packs until being placed in a refrigerator at 4^{0} C upon return to Appalachian State University.

Analysis Methods

Inflorescences were collected before anthesis while pollen was mature, but anthers were non-dehiscent. Inflorescences and anthers were dissected under a Wild Heerbrugg M-5 stereo scope. Slides were prepared for each sample by drawing 3 small black circles randomly on the

reverse side of the slides representing the areas being analyzed during pollen viability counts. The samples were stained and prepared for observation following the protocol set forth by Peterson et al. (2010), which utilized a modified Alexander's stain as the differential staining method. Briefly, this included dissecting anthers under a stereoscope, placing macerated anthers on a specimen slide, applying stain and allowing 10 minutes for the stain to be taken up, followed by placing a cover slip over the specimen. Pressure was applied using a chromosome squash technique to help spread the pollen out into a better field of view for observation. The slides were then observed using a Motic BA410E compound microscope fitted with an HD1500T Hi-Def camera from Meiji Techno America, under 10X and 100X magnification to ensure good resolution. Counting pollen under the microscope is a difficult and tedious process, so images were taken at 10x magnification (Figure 1A), placing the center of the black circle drawn on the slide at the center of the image. Three images per slide were taken, and each image was hand counted for viable and aborted pollen. When a discrepancy was noted, additional images were taken at 100x (Figure 2B) and analyzed by hand until all issues were resolved. A total of three slides were prepared from each population, with 100 pollen grains analyzed per slide, for a total of 300 pollen grains per population.

Results

The modified Alexander's stain produced easily distinguishable pollen viability (Fig 1). Viable pollen readily stained a pink to magenta color, while non-viable pollen appeared blue to blue-green. The nine populations collected along the South Fork of the New River exhibit viable pollen formation that ranges from 86.4 - 93%, with an average of 89.1% (Table 1 and Figure 2).

EO	#Viable	#Aborted	Total #	%Viable	%Aborted
45	268	32	300	89.3	10.7
48	271	29	300	90.3	9.7
23	262	38	300	87.3	12.7
16	266	34	300	88.7	11.3
44	269	31	300	89.7	10.3
15	279	21	300	93	7
17	254	46	300	84.6	15.4
63	267	33	300	89	11
64	270	30	300	90	10
Average	267.3	32.7	300	89.1	10.9

Table 1. Composite Pollen Viability Data

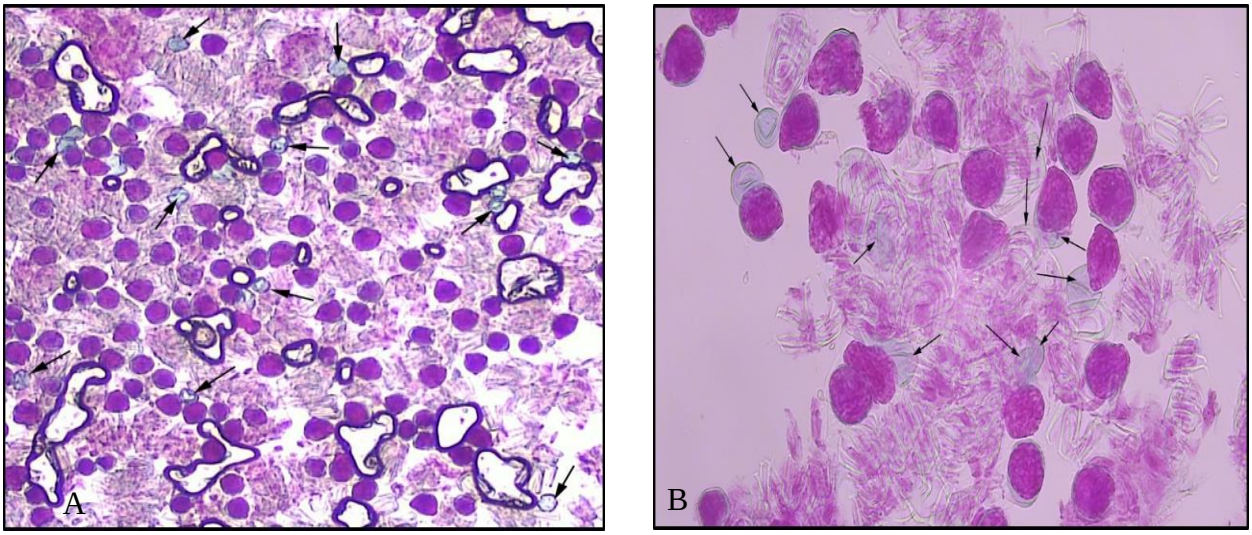

Figure 1. Pollen grains viewed at 10X (A) and 100X (B) using a Motic BA410E compound microscope, fitted with an HD1500T Hi-Def camera; Arrows indicate non-viable pollen grains.

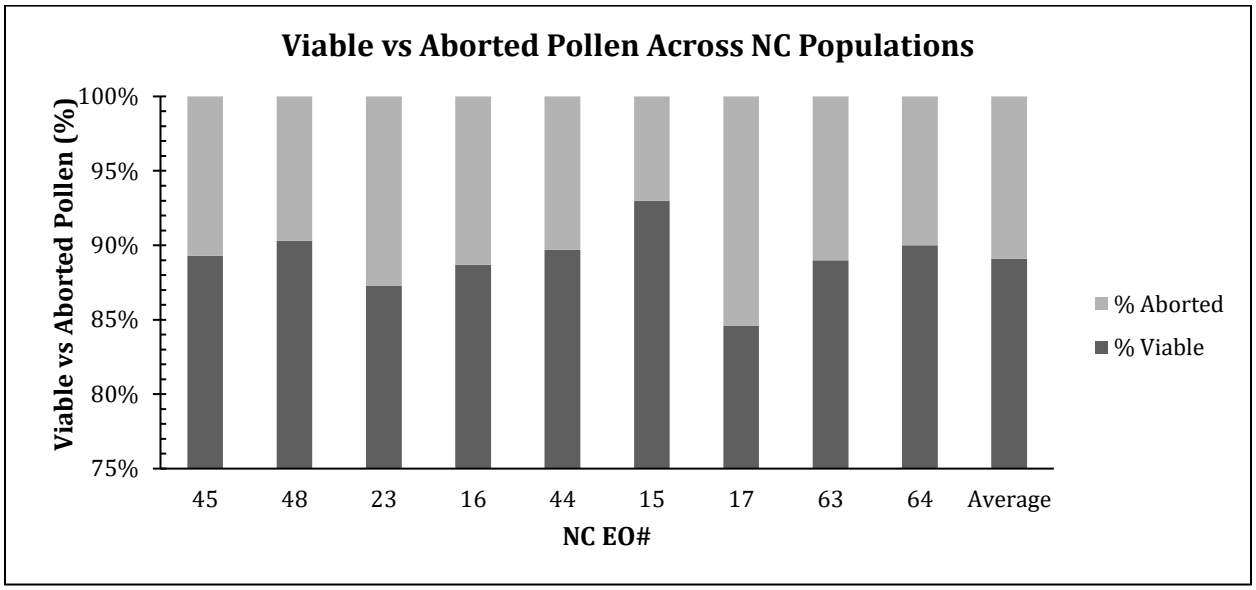

Figure 2. Percent of Viable and Aborted Pollen observed across the nine populations.

Discussion

Plant species that grow in restricted habitats, such as riparian zones, often struggle with seedling recruitment due to the nature of the flooding and scouring events that occurs within this extreme environment (Brzyski & Culley, 2013), and there are numerous factors that must be considered when determining reproductive success. Determining which factors are contributing to a lack of sexual reproduction in *S.virginiana* by examining pathways within the process of microsporogenesis is important. Analysis of pollen viability among populations of *Spiraea virginiana* suggests that the reproductive barriers faced by this species is not likely due to pollen viability, given the high degree (89.1%) of viable pollen formation in all populations examined. However, the lack of fertile seed formation in the wild suggests that other factors exist that are inhibiting sexual reproductive capabilities within this species.

In this study, the pollen examined across nine populations of *S. virginiana* along the North Fork of the New River in Ashe and Watauga Counties (North Carolina) exhibited consistently high viability ratios of nearly 90%. These numbers are consistent with those found by Bryzski and Culley (2013), where a high (90%) pollen viability was observed within a single

population in Ohio. This suggests that *S. virginiana* has no pollen viability issues and that viability among populations and across different watersheds and river drainages is consistent regardless of geographic location.

The differential staining of pollen during this study was accomplished using a modified Alexander's stain, which differs from that used by Bryzski and Culley (2013), where the original Alexander's stain formula was used and followed the staining protocol set forth by Alexander (1980). The modified Alexander's stain protocol and formula utilized in this study was obtained from Peterson, R., Slovin, J. P., & Chen, C. (2010). The modified stain employed was chosen for safety concerns, as well as a comparative analysis of the stainability of *S. virginiana* pollen grains. The widely used Alexander's (1980) protocol contains substances that are highly toxic and have known health hazards, including chloral hydrate, phenol, and mercuric chloride. In addition to safety concerns, chloral hydrate is heavily regulated. Chloral hydrate is considered a schedule IV-controlled substance due to its use as a mild sedative and hypnotic pharmaceutical drug that has been used to treat insomnia but also has been associated with use as a date rape drug according to a report by the US EPA (2000) entitled *Toxicological review on chloral hydrate*. The results of the comparative analysis of the modified stain versus the original formula demonstrated a consistent easily distinguishable differential staining of viable and aborted pollen, having nearly identical results with regards to percent viability within this species. This suggests that the safer, modified staining methodachieved results that were as consistent as the original stain formula. Additionally, it should be noted that the sample preparation time and staining of the pollen grains using the modified Alexander's stain protocol can be accomplished in a shorter time frame than the original version and can be used in the field under lower magnification field compound microscopes (Peterson, R., Slovin, J. P., & Chen, C., 2010).

The results of this study support and confirm the findings in previous studies (Bryzski and Culley, 2013) which found a high degree of viable pollen in the population sampled among a single Ohio population. However, a secondary purpose for this study allowed for the analysis of populations (EO-15) that have provided natural seed germination in a controlled setting and determine if the viability of this population was significantly different from any of the others. Emory (2015) suggested that pollen availability did not limit seed production, and demonstrated viable, fertile seed production among a single population (EO 15) collected along the New River. Although this was the only EO that demonstrated fertile seed production, pollen viability analysis suggests there is likely no significant difference in the viability of pollen (93%, Table 1) among this population even though it was 3% higher than the average among all populations analyzed.

Numerous issues can arise when genes that control the process of microsporogenesis experience mutations. One such example of this is the gene gaMS-1 (a maize mutant). In their studies, Sari-Gorla et al. (1996) demonstrated that expression of this gametophytically acting gene during or just after the first microspore division in maize leads to the production of immature, sterile pollen grains. This suggest that issues arising in the process of microsporogenesis are likely to affect sexual reproductive capabilities within the species. However, fertile seed set, and germination in *S. virginiana* have been successfully demonstrated in greenhouse and common garden experiments (Ogle, 1991b) where individuals from different drainages were crossed. These observations suggest the possibility of a self-incompatibility mechanism hindering sexual reproduction in *S. virginiana*, preventing inbreeding (Murrell & Anders, 2001; Brzyski & Culley, 2013; Emory, 2014). Members of the Rosaceae family have demonstrated an RNase based self-incompatibility system, resulting in the failure to produce

viable zygotes during self-pollination (Ashkani et al., 2016). This mechanism prevents the occurrence of an inbreeding depression within species of the family but appears to limit the reproductive potential of rare species.

Continued efforts to study the sexual reproductive pathways, or lack thereof, in *S. virginiana* must be continued to meet the guidelines set forth by the United States Fish and Wildlife Service (USFWS, 1992) in their recovery plan. The primary objective stated in this plan is the delisting of *S. virginiana* from the federal and state registers. Douglas Ogle, who prepared this recovery plan for the USFWS, states that one of the actions necessary to achieve the delisting is to re-establish populations within the historic range of the species (USFWS, 1992). One of the methods that has been suggested to accomplish this monumental task is by augmenting existing, current populations. The data in this study suggests that the reproductive barriers in this species is unlikely due to any of the numerous processes associated with microsporogenesis, including the formation of viable pollen, and that other biotic factors exist that are hindering sexual reproduction. Although the data does not significantly contribute to the recovery of this species, it does suggest that research can and must now focus on addressing the low genetic diversity among populations and drainages, habitat preservation and maintenance, and landowner awareness. Fertilization was observed among several populations of *S. virginiana* in North Carolina and Virginia during the summer of 2017 suggesting that further research should focus on *in-situ* pollination and germination studies within and among populations to determine if seedling recruitment in natural habitats is possible.

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