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Effects of Open Pollination, Selfing, Inbreeding, and Outbreeding on Seed Set and Viability in *Spiraea virginiana* Britton (Rosaceae)

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Abstract

Spiraea virginiana Britton (Virginia spiraea), an endangered riparian shrub found in the Appalachian and Blue Ridge Mountains, reproduces primarily via asexual reproduction. Sexual reproduction is rare under field conditions, but it is unclear whether pollen limitation or genetic incompatibilities are the underlying cause. Open, selfed, inbred, and outbred pollination treatments were applied to populations of *S. virginiana* from three Western North Carolina counties. Stigmas from open pollinated treatments were collected to quantify pollen loads. Flowers from pollination treatments were collected 90-120 days later, and a subset of seeds were stratified for three months before monitoring germination rates. In addition, 100 seeds from each replicate were tested for viability using TTC. Both foreign and conspecific pollen loads on Graham County stigmas were significantly lower (P=0.0001) than pollen loads on stigmas from other populations. Seed set from Ashe County plants was significantly lower than other populations (P=0.0001), but treatment had no significant effect on seed set (P=0.18). Only the Ashe County population produced viable seeds (0.44%). Because seed set was not correlated with pollen load, variation in reproductive success could be due to other factors. Genetic variation within and among populations is currently being investigated to see if it could help explain differential seed production.

1. Introduction

Genetic diversity allows plant populations to adapt to selective pressures in their environments[1]. Populations with more genetic diversity are better able to evolve in response to external pressures and less likely to be extirpated if environmental conditions change[2-5]. Declining population genetic diversity can also be harmful to the wider plant community and might negatively affect other species[6]. For these reasons, genetic studies are often incorporated into conservation plans for rare plant species[7]. Results of these studies can be used to supplement limited populations or to create genetically representative reserves of seeds for the species' reestablishment[1,7].

A population's primary mode of reproduction can play a role in shaping its genetic diversity[2,3]. Asexual reproduction happens without the recombination of genetic material, and, unless somatic mutations occur, all resulting offspring are genetically identical to the parent[2,8]. Populations that reproduce primarily via asexual reproduction typically contain a limited number of genetic lineages and have lower genetic diversity than those which reproduce sexually[2,3]. In stable environments, asexual reproduction might result in populations that can utilize available resources most effectively[2,5,8]. Asexual reproduction is also advantageous because producing vegetative propagules is less energetically expensive than making flowers and seeds, and pollinators are never required [2,9,8].

Some clonal species display high levels of phenotypic plasticity, but in highly disturbed habitats asexual reproduction can still be a handicap[5]. Sexual reproduction is typically required to adapt to changing habitat conditions, because the offspring have more genetic variability and can better withstand fluctuating conditions or disasters[2,3,8,10,11]. Seeds created by sexual reproduction can also be dispersed to colonize new habitats or to introduce new genotypes

into disconnected populations[1,2,5,8]. Many plant species utilize both sexual and asexual reproduction, favoring one or the other depending on environmental cues[2,5].

The mating system of a population can also affect its genetic diversity[12]. The introduction of pollen from a different population can increase genetic variation[3,5]. Alternatively, self-fertilization (selfing) or frequent pollination among closely related individuals can lower genetic diversity and sometimes result in inbreeding depression, which can reduce seed production, viability, and germination[13,14,15].

Self-pollination can also lead to a loss of genetic diversity within a population[3,5,15]. To increase the chance of outbreeding and prevent self-pollination, many plant species have protandrous flowers, with male reproductive parts that mature before female reproductive parts[16,17]. Protogynous flowers, with female reproductive parts that mature first, are also utilized by plants to avoid self-pollination, but they are less common than protandrous flowers[16]. Plants can also be genetically self-incompatible, using chemical inhibition to stop pollen germination[1]. Some species are capable of producing viable seeds through self-pollination, which can be advantageous in small or isolated populations, where pollen import is unlikely[3]. Reproducing solely via self-pollination can lower a population's genetic diversity, however[3,18,13].

Rare species are characterized by narrow ranges (endemism), small population sizes, or both[12]. Rare species frequently have lower levels of genetic variation than widespread species in the same family or genus, often due to low levels of outcrossing among small or distant populations[19,20]. Species with small or isolated population can have high levels of selfing and inbreeding, resulting in reduced and small seed set[12,15,16] These traits are associated with populations whose genetic diversity is reduced; such populations more likely to be negatively influenced by human activities such as development and water diversion[12,15,16].

This study focused on *Spiraea virginiana* Britton (Virginia spiraea), a rare riparian shrub found in the Appalachian and Blue Ridge Mountains[10,21-23]. *Spiraea virginiana* plants reach up to 4 m in height and grow in clumps along river and stream banks[10,22,24]. Large clusters grow in wet, marshy riverbanks, and smaller clusters develop on disturbed, rocky riverbanks, where moisture is limited[22]. Although the shrub can transfer photosynthate along its rhizomes, individual ramets depend on floods to remove competing tree and shrub species, allowing for better access to sunlight[10,25].

The flowers of *S. virginiana* are white, perfect (containing both male and female reproductive parts), and 4-8 mm broad[21,22]. These flowers grow as compound corymbs, flat-topped clusters of flowers that grow at the ends of the branches[9,20,21,22]. Inflorescences are generally 3-8 cm wide but can be up to 14 cm wide [9,21,22]. Flowers bloom from mid-May to August and are typically pollinated by beetles [10,22,26]. Flowers are also self-compatible, and seeds can result from both self-pollination and open pollination[27]. However, seed set is limited under field conditions, and only some corymbs produce seeds[9]. Brzyski and Culley tested the pollen viability of *S. virginiana* pollen from a population in Ohio, and found that 90% of the pollen grains were viable, so pollen does not limit seed production[26].

Asexual reproduction is the normal mode of reproduction in *S. virginiana*, and sexual reproduction is limited under field conditions[10]. *Spiraea virginiana* takes advantage of regular disturbances such as flooding to reproduce asexually by fragmentation, where woody fragments are carried downstream to establish a clone of the parent, within a new or existing colony[9,28]. When flowers are self-pollinated or pollinated with pollen from plants in the same population, seeds are produced infrequently[9,10]. Sexual reproduction is thought to be more successful under field conditions when individuals from upstream subpopulations are washed downstream to cross-breed with different subpopulations[10].

Natural pollination of *S. virginiana* produces few viable seeds, as shown in a preliminary study along the Cheoah River (Graham County, NC,) in which 1 out of 1300 ovules developed into a viable seed[29]. Only 10.3% of the seeds collected from open-pollination treatments of *S. virginiana* in Ohio germinated successfully[26]. *Spiraea virginiana* individuals from common gardens have produced viable seeds when crossbred with pollen from individuals in different river drainages, however[30]. Individuals in three subpopulations along the New River in North Carolina that were outbred with pollen from Tennessee populations in a hand-pollination study produced a similar percentage of seeds as individuals in the same population that were self-pollinated, (22.6% and 29.7% respectively)[27].

Spiraea virginiana is federally designated as threatened in North Carolina due to its narrow range of habitats, limited colonization opportunities, and rare sexual reproduction[31]. Populations in Ohio, Kentucky, and Tennessee also have low levels of genetic variation, probably due to small population sizes and the species' reliance on asexual propagation[10,11,32]. Reduced genetic variability and sexual reproduction in populations of *S. virginiana* is partially due to the construction of dams, which prevent regular flooding events and restrict exchange of genetically-distant fragments among *S. virginiana* populations[10]. The lack of frequent floods also endangers *S. virginiana* populations due to increased competition for light by other woody species that would normally be swept away in a flood[9,10]. Finally, the species is threatened by competition for light with invasive species like the congener *Spiraea japonica* (Japanese spiraea) and insect pests such as aphids[10,31].

Because information on the genetic variation of North Carolina *S. virginiana* populations is limited, sequences of microsatellite markers were used to examine the genetic relationships within and among seven populations of *S. virginiana* occurring in the Western North Carolina. It was hypothesized that there would be more genetic diversity among rather than within populations. This could be due to limited exchange of pollen or vegetative propagules among populations and high levels of asexual reproduction within populations**.**

Open, self, inbred, and outbred pollination treatments were established in three populations of *S. virginiana* in Western North Carolina. Seeds were collected from each treatment and tested for viability and germination. If outbred or inbred pollination treatments yielded high rates of viable seeds, this approach could be adopted with populations of *S. virginiana* in other states, and incorporated into a conservation strategy for the species as a whole. It was hypothesized that a higher percentage of seeds from the outbred treatments would be viable than those from the open or self-pollinated treatments. Unmanipulated stigmas were also collected and examine for pollen load deposition, to determine if the flowers were receiving pollen under field conditions. It was hypothesized that stigma pollen loads would vary among study sites due to differences in habitat that promote or deter flower visitation, and that stigma pollen loads would be positively correlated with the seed set of plants in the open pollination treatment.

2. Methods

2.1 Genetics

2.1.1 study sites

Populations of *S. virginiana* were identified in seven different river corridors in Western North Carolina[23]. Populations were defined as all plants a single river corridor. Subpopulations were defined as groups of plants at least 100 m[29].

2.1.2 DNA extraction

Forty *S. virginiana* leaf samples from seven different populations in Western North Carolina were collected during the summer of 2011 (Table 1). A modified CTAB procedure was used to extract DNA from the samples[33]. Extracted DNA was quantified using a spectrophotometer (ND-1000, Nanodrop Products, Wilmington, DE)**.** If the concentration of the DNA was below 10 ng/µl, the DNA extraction procedure was repeated.

Table 1. Number of subpopulations and samples within the seven populations of *S. virginiana* studied.

Population	Number of subpopulations	Number of samples
Cane River	1	1
Cheoah River	16	63
French Broad	1	1
Little Tennessee	3	7
New River (South Fork)	18	18
Nolichucky	2	4
North Toe	3	3
South Toe	3	6

2.1.3 polymerase chain reaction

A multiplex polymerase chain reaction was performed on all samples with primers VS 2, VS 5, VS 8, and VS 10. Each reaction had a volume of 20 µL, containing 10 µL QIAGEN Multiplex PCR Master Mix, 2 µL of a mix of primers VS 2, VS 5, VS 8, and VS 10, 7.6 µL ddH_2O, and 0.4 µL template DNA[34]. The samples were run in a thermocycler (T100, BIO-RAD®, Hercules, CA) following the Brzyski protocol of 15 minutes at 95^{0}C, 30 cycles of 30 s at 94^{0}C, 45 s at 57^{0}C, and 45 s at 72^{0}C, finished by ten minutes at 72^{0}C[32].

2.1.4. sequencing

The PCR products were purified using a QIAQuick® PCR Purification Kit™ and quantified using a spectrophotometer (ND-1000, Nanodrop Products, Wilmington, DE)[35]. The PCR products were diluted to a concentration of 1 ng/ µL and each of the four microsatellite primers were diluted to a concentration of 5 µmol, using ddH2O. Ten µL of each diluted PCR product were mixed with 5 µL of each diluted primer and sent to Genewiz (Genewiz, South Plainfield, NJ) for sequencing.

2.2 Pollination

2.2.1 study sites

The Santeetlah Dam on the Cheoah River, a tributary of the Little Tennessee River in Graham County, was built in 1928, disrupting natural *S. virginiana* dispersal patterns and creating subpopulations of special concern[10,36]. Sixteen subpopulations of *S. virginiana* were found below the dam, 10 of which flowered in the summer of 2013. Eight of these subpopulations were adjacent to Highway 129, where the North Carolina Department of Transportation used long-arm mowing until 2010 (subpopulations 2-1, 3-1, 3-2, 10-2, 11-3, 12-1, 12-4, and 12-7)[37]. No-mow zones were created in 2011 to protect the subpopulations of *S. virginiana* found along Highway 129[37]. The other two flowering subpopulations were located on the other bank of the river, not adjacent to the highway (subpopulations 13-1 and 16-1). Many of these subpopulations were under substantial tree cover.

Two flowering subpopulations of *S. virginiana*, 7-1 and 7-2, were found on the Little Tennessee River in Macon County, North Carolina in 2011. These subpopulations were located alongside the Little Tennessee Greenway, inside the city limits of Franklin, with sparse tree cover[36].

The South Fork of the New River winds through eastern Ashe County, North Carolina. In the summer of 2013, NC Natural Heritage Program data[28] were used to locate 6 flowering subpopulations (2-1, 6-1, 16-1, 17-1, 19-1, and 46-1) along the river. Sparse tree cover was found at these subpopulations, all of which were adjacent to roads.

2.2.2 hand pollination

Hand pollination can be used to supplement populations of rare species [37,38]. The ability of a plant to produce seeds via self-pollination is tested by pollinating flowers with other inflorescences from the same plant[13]. Flowers are emasculated and pollinated with pollen from a different plant in the same population to test the effectiveness of inbred pollination[13]. Emasculated flowers can also be pollinated with pollen from a distant population to test outbred pollination[13]. To assess natural levels of pollination, flowers are left open to all visitors[13].

In summer 2013, pollination treatments were established in *S. virginiana* populations from Ashe, Graham, and Macon Counties in North Carolina. Four different pollination treatments were set up at each population. Flowers were examined under a dissecting microscope to determine the stage at which the anthers were dehiscent, which was after they exited the hypanthium but before they turned brown. Corymbs in the open-pollination treatment were marked while in full bloom and bagged after the stigmas had turned brown and became unreceptive to pollen. Self-pollinated corymbs were fertilized with pollen from other flowers on the same plant. Corymbs in the inbred pollination treatments had all their anthers removed, to ensure that no self-pollination occurred, before pollen from another subpopulation was applied to the stigmas. To transport pollen for short distances (inbred treatments), flowers with recently dehisced anthers were collected in microcentrifuge tubes and kept on ice. To ensure pollen viability, flowers with dehisced anthers were used within 24 h. Corymbs in the outbred pollination treatment had all their anthers removed before pollen from subpopulations at another site was applied to the stigmas. To transport pollen over long distances (outbred treatments), flowers with un-dehisced anthers were collected in microcentrifuge tubes and kept on ice. The pollen was used within 48 h to ensure that it remained viable. The collected flowers were pinched at the back of their calices and rubbed over the flowers of the recipient plant, allowing the pollen of the transported plant to contact the stigmas.

All corymbs used in the self, inbred, and outbred pollination treatments were bagged with a fine mesh after pollination, to exclude insect visitors and prevent the loss of seeds. The inflorescences were allowed to mature until late September, when seeds were collected. Seeds were dried for one week at room temperature to prevent fungal infection.

2.3 Pollen Load

Pollination success can be measured via pollen deposition, the number of pollen grains that adhere to the stigma[13, 38,39]. Large amounts of pollen deposited on the stigma can help ensure successful fertilization of the ovules, and low rates of deposition or ineffective pollinators could limit seed production[38-41]. Stains such as malachite green can be used to stain stigmatic surfaces so that conspecific and foreign pollen grains can be counted[13,16].

Stigmas were collected from ten flowers per subpopulation for a total of 30-50 stigmas per population to analyze pollen loads. The stigmas were stored in microcentrifuge tubes filled with a solution of 0.1% malachite green in 95% ethanol. The stained styles were mounted onto slides and visualized under an inverted brightfield microscope (Eclipse TE2000-S, Nikon, Melville, NY) at 100x magnification and the number of *S. virginiana* pollen grains and foreign pollen grains on each style were counted to determine if natural pollen transfer was sufficient to ensure seed set[13,16].

2.4 Seed Tests

Flowers were dissected under a dissecting scope (EZ4, Leica, Buffalo Grove, IL) and seed counts were recorded for individual flowers. For each treatment and subpopulation combined, ten flowers, or enough flowers to yield 100 seeds, were dissected. Average seed set of each pollination treatment and population were compared.

The viability of 100 seeds from each pollination replicate were tested by soaking seeds for 48 hours in ddH_2O, stabbing the seeds to disrupt the seed coat, and soaking for 24 h in a 1.0% solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC). Embryos of viable seeds turned red or pink, while inviable seeds remained white[13,15,42]. The number of viable seeds from each pollination treatment was counted, and ratios of viable to inviable seeds were calculated.

To test for germination, seeds were planted in a sterilized, moistened mixture of 2:1:1 peat, perlite, and vermiculite. After cold stratification in the dark for 90 days at 4 ^{0}C, the seeds were placed in a growth chamber (E8 growth chamber, Conviron, Winnipeg, Canada**)** set to a light level of 500 µmoles m^{-2} s^{-1} and a temperature of 22 ^{0}C[30,33].The seeds were monitored and counted to calculate germination percentages of each pollination treatment.

3. Results

3.1 Pollen Load

Both foreign and conspecific pollen loads on Graham County stigmas were significantly lower (P = 0.0001) than pollen loads on stigmas from other populations. Within populations, foreign pollen loads were significantly different among subpopulations (P=0.0052). Conspecific pollen loads were also significantly different among subpopulations (P<0.0001) (Figure 1).

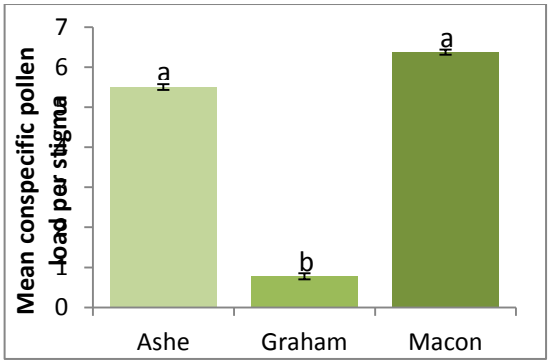

Figure 1. Mean conspecific pollen load by population.

Figure 1. Mean number of conspecific foreign grains per stigma by population.

3.2 Seed Set

Mean seed set per flower from Ashe County was significantly lower than mean seed set per flower from Graham County (P=0.0022) and Franklin County (P<0.0001) (Figure 2A). Mean seed set per flower was not significantly different between the Graham and Franklin County populations (P=0.2432). Pollination treatment had no significant effect on seed set (P=018) (Figure 2B).

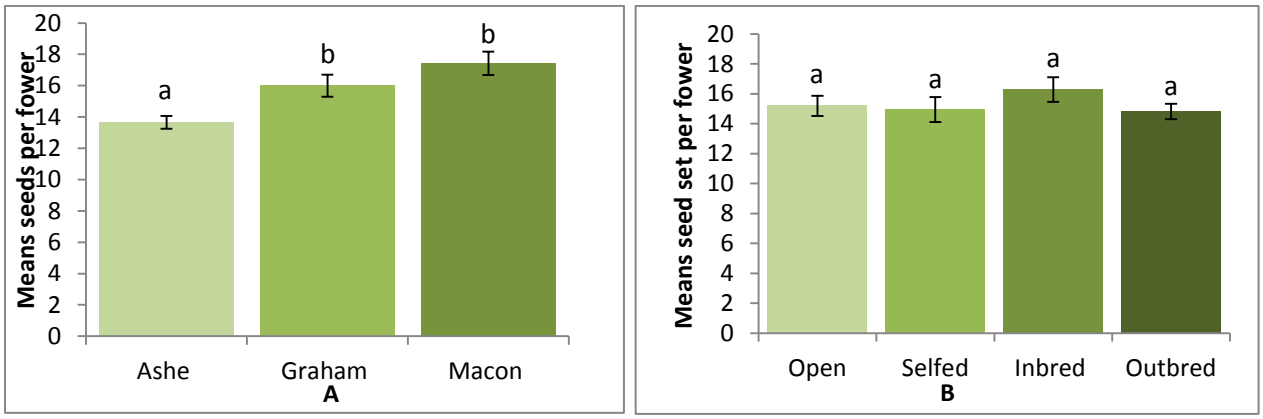

Figure 2. Mean seed set per flower by population and treatment.

Figure 2. Mean (±SE) seed set per flower in (A) County populations and Treatment (B).

3.3 Seed Viability And Germination

None of the seeds tested for viability from the Graham and Macon County populations were viable (Figure 3). Out of 6,314 seeds tested from the Ashe County population, 28 seeds were viable (Figure 3). Of the 28 viable seeds, thirteen were from flowers in the open pollination treatment, six were from the selfed treatment, six were from the inbred treatment, and three were from the outbred treatment (Table 2). None of the planted seeds germinated after four weeks.

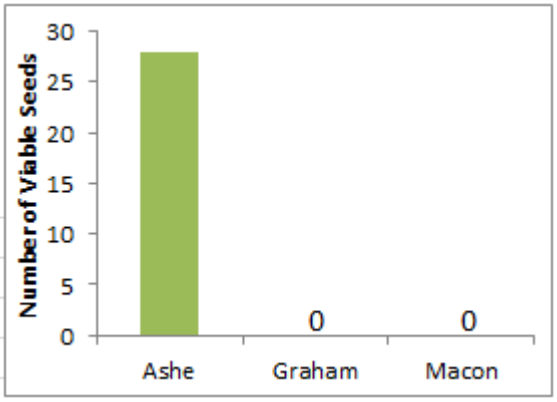

Figure 3. Number of viable seeds by population.

Table 2. Seed viability in Ashe County population by pollination treatment.

Treatment	Number of seeds tested	Viable seeds	Inviable seeds
Open	1614	13	1601
Selfed	1300	6	1294
Inbred	1500	6	1494
Outbred	2000	3	1997

4. Discussion

4.1 Pollen Load

The differences in pollen load among the three populations of *S. virginiana* could be caused by differences among the three populations in habitat or pollinator community[16,43,44]. Pollinator activity can also be affected by habitat differences such as shade[44]. Plants in the shade receive fewer visits from pollinators than those in the sun[44]. The plants in the Graham County population, which had the lowest pollen loads, are in shadier environments than the plants in the other two populations, which could contribute to the low pollen loads from that population.

Insects from the following genera were observed visiting *S. virginiana* inflorescences over the summer: *Crabro* (hornets), *Leptura* (soldier beetles), *Mordella* (tumblingflower beetles), *Popillia* (scarab beetles), and *Phymata* (ambush bugs). The flowers of *S. virginiana* white and radially symmetrical, providing a broad platform for the insect to walk on, as are many generalist flowers that rely on beetles for pollination[43]. Beetles can also carry pollen for distances of up to 20 m, which would be advantageous for the scattered subpopulations of *S. virginiana*[43]. For instance, larger beetle species have been found to be more efficient at cross-pollinating than small beetles and bees in populations of *Magnolia obovata*[43].

Few studies have been done on the effectiveness of beetles as pollinators[43]. Although numerous beetle visitors were observed on *S. virginiana* inflorescences, floral visitation does not guarantee that pollination is actually occurring[13,43]. Even if beetles are pollinators of *S. virginiana*, they might not be good pollinators. Beetles do not disperse as easily from flowers as other pollinators (e.g., bees or flies), which means that they might not transfer pollen among plants as effectively[43]. Differences in effectiveness of local pollinators and pollinator community might influence difference in pollen load among populations of *S. virginiana*.

Because of the low numbers of foreign pollen grains counted on stigmas from any of the three study sites, it is unlikely that pollen competition occurred. In addition, there were few other plants in flower near the study plants at the time that stigmas were collected, meaning that there were not many potential sources of foreign pollen. However, foreign pollen load might have been underestimated, due to the difficulty of distinguishing foreign pollen grains from fungal bodies and other forms of debris deposited on the stigmas.

4.2 Seed Set

Seed set is a good indicator of pollen viability and pollen limitation[13,45]. Because seed set was not correlated with pollen load of *S. virginiana*, lack of natural pollination is probably not the cause of low reproductive success in *S. virginiana*[46]. Differences in seed set among different populations of *S. virginiana* could be due to other factors such as population size, soil resource availability, and lack of genetic variation among individuals[47].

Seed mass and seed number can be negatively correlated[47]. Large seeds have an advantage over small seeds in establishment, meaning that if the Ashe County population has fewer but larger seeds, that population might experience more reproductive success[46]. Future studies could weigh seeds from populations of *S. virginiana* to see if seed set and seed weight are negatively correlated, and if seed weight is positively correlated with viability and germination.

Seed set was not affected by pollination treatment. Other hand pollination studies done on *S. virginiana* have also shown no difference in seed set among pollination treatments[31]. These subpopulations and populations of *S. virginiana* might be so close genetically that it would not make any difference whether the pollen came from the same plant or a different one in another population.

4.3 Seed Viability

Plant populations with more biomass have more resources to allocate to reproduction, and are often more successful[48]. As seed filling is a more resource-expensive act than floral production, this might explain why the Ashe County population, which was larger than the Graham or Macon County subpopulations, was the only population to produce viable seeds[48]. Seed filling can also be determined by sink strength (activity and number of sinks)[49]. Flowers act as sinks, and sink strength can be combined across inflorescences[49]. The corymbs of the plants in the Ashe County population were noticeably larger than those in the other two populations, creating larger sinks, which could also contribute to the ability of these plants to make viable seeds. Sources also influence seed filling; the larger and more efficient the source is, the more photosynthate can be sent to the sinks for seed filling[49]. The plants in the Graham and Macon County populations were more heavily shaded than those in the Ashe County population, meaning that their sources might be less strong. Future studies could compare light environment and physiological responses among different populations of *S. virginiana* to seed viability and germination rates.

Resource limitations can negatively affect reproductive output[43,48,50]. If soils are shallow and nutrient depleted, plants might be resource limited even in summer[43]. Many of the plants in the Graham County population are lodged between rocks in thin layers of soil, which might play a role in the lack of viability found in seeds from those plants. Resource limitations are also caused by competition with other plants[48,50]. Plants in both the Macon County populations were frequently surrounded and even crowded by plants of other species, possibly lowering their reproductive output. The lack of viable seeds from the Graham and Macon County populations despite large seed set might be caused by excess ovule termination in the face of insufficient resources[45]. Future studies could analyze soil depth and nutrient availability among populations of *S. virginiana* to see if soil depth and nutrient levels correlate with reproductive output.

Seed banks can be used to supplement existing populations of rare plants, or to re-introduce species in areas where they have been extirpated[7]. Such a plan would only work for *S. virginiana* if the collected seeds were viable. This study shows that lack of seed viability is a serious concern in populations of *S. virginiana* in North Carolina. Future studies on the environmental and genetic factors that influence the reproductive success of *S. virginiana* are needed to create a strategy for its preservation.

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