

Outcrossing and fecundity in the woodland sedge, *Carex pensylvanica*: implications for ecological restoration

by

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**Abstract:**

The growing field of ecological restoration has prompted the horticulture industry to include a specialty niche of growers who breed native plants for restoration projects. The goals of native plant production are different than those of traditional horticulture, and native plant growers face a host of economic and technical challenges. Such challenges can limit their ability to produce adequate quantities and diversity of plants, and many ecologically important species are often underrepresented in restoration plantings. An example of this is the woodland sedge *Carex pensylvanica* -- an herbaceous-layer dominant in dry eastern forests -- which is marked by poor seed yield and germination rates, and is, as such, difficult to produce from seed. *C. pensylvanica* is self-compatible, and because of its expansive, clonal growth form, it is possible that long-term, self-pollination in many wild populations has resulted in inbreeding depression and reduced seed production and fitness. I tested this hypothesis in a greenhouse experiment where I controlled the breeding system of *C. pensylvanica* through hand-pollination to compare the reproductive output between outcrossed and self-pollinated manipulations. Results showed no effects of the breeding system manipulation on seed weight, but seed set in outcrossed plants was significantly higher (1.6x) than seed set in self-pollinated subjects. Based on this data, I developed models that predicted outcrossing seed set at 4.692 seeds/flower while selfing seed set predicted at 2.835 seeds/flower, supporting the hypothesis that long-term selfing is a significant contributor to the low seed production in this species. This study demonstrates that manipulating the breeding system of *C. pensylvanica* to achieve increased outcrossing is an effective way to increase seed production.

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**Introduction:**

Alterations to the environment as a result of anthropogenic global change have already had pronounced effects on abiotic and biotic ecosystem processes and function (Walther et al 2002, Lewis and Maslin 2015). Ecological restoration has become an important tool for countering the detrimental effects of global change (Young 2000), and as the discipline has matured over the past several decades, a specialty niche for plant growers providing native propagules for restoration projects has emerged within the plant nursery industry (Booth and Jones 2001). While native plant production has its roots in traditional horticultural knowledge, the goals for restoration plant materials are very different from those destined for traditional horticultural settings. As such, native plant production for restoration projects requires a large corpus of supplemental skills and knowledge, and growers must consider novel decision-making factors. In addition to considering the economic tradeoffs always involved in plant production, restoration growers must consider ecological tradeoffs as well. For example, growers must balance the use of locally adapted genotypes, which would thrive under current environmental conditions, with expanded regional and genetic diversity to allow for evolutionary potential as climate changes (Herman et al 2014, Rice and Emery 2003).

Supplying sufficient native plant material for restorations is often hampered by knowledge gaps on both ends of the production line. Restoration clients tend to favor iconic and showy native species (Bill Schneider personal communication) and project managers cite limited and inconsistent client demand and interest as major obstacles preventing them from expanding their use of native plants (Hooper et al 2008). Additionally, plants appropriate for restoration tend to have more complex life histories and growth requirements than the developed cultivars favored in traditional horticulture, and production is often restricted due to a lack of production knowledge among growers (Peppin et al 2010). For researchers and professionals alike, these challenges present frontiers for plant research and identify gaps in our knowledge that stand as barriers to supplying adequate plant materials for diverse restorations.

To restore appropriate levels of biodiversity to degraded sites, native plant producers must expand the diversity of their plant offerings to include ecologically important species that have often been overlooked. A primary step in the process is to continue to expand native plant research through studies targeting their biology with an applied focus on production procedures (Boyer 2008). Sedges of the genus *Carex* are excellent candidates with which to begin such studies. Comprised of over 2,000 species with nearly global distribution, *Carex* is one of the most morphologically and ecologically diverse plant genera (Ball and Reznicek 2003). Carices are herbaceous layer dominants across a diversity of North American ecosystems, but are often underrepresented in ecological restoration planting (Handel 2015).

A body of literature has developed about the reproductive ecology of sedges that is relevant to native plant production, including the physiological dormancy and germination requirements for seeds of *Carex* species (Schütz and Rave 1999, Brandel and Schütz 2005, Kettering and Galatowisch 2007). There is a general consensus that most *Carex* species require a period of cold stratification followed by exposure to light and warm or varying temperatures for significant germination. The exact requirements vary between species.

There has also been progress in understanding the correlation between certain life history traits and seed production. Generally speaking, cespitose *Carex* species tend to produce larger quantities of seed, while in rhizomatous species viable seed production is minimal even for species that produce many flowers (Schütz 1997). Estimates of seed set at the species level are valuable not only for nursery professionals in planning their seed collection, but also for

restoration practitioners when evaluating the potential for restored populations to persist or expand over multiple generations (Montalvo et al 1997).

Other studies have attempted to characterize the breeding system and genetic diversity of *Carex* populations. A study by Friedman and Barrett (2009) examined the breeding systems of seven woodland *Carex* species from temperate North America and found indications of high rates of self-pollination in all species. Such breeding behavior has potential to have a strong impact on seed production and fitness, a topic that will be treated in greater detail below.

One thing that emerges from the literature on *Carex* regeneration is that while there are generalizable trends, the high species diversity of the genus brings with it high diversity in reproductive niches, and for every genus-level trend, there are species-level exceptions. For example, for the 32 European *Carex* species examined by Schütz and Rave (1999), germination rates were improved with cold stratification in 28 of them, but four species showed no change or diminished germination rates with stratification. Plant production is a precise field, and growers need predictable processes for generating viable seed. With such variation in the genus, it becomes critical to identify regeneration niches on a species-specific basis to suggest protocols for seed production by growers.

The sedge *Carex pensylvanica*, known commonly as Pennsylvania or Penn Sedge, Early Sedge, Oak Sedge, and Early Oak Sedge, is a widespread species and ground layer dominant in a number of ecosystems in the Eastern United States including dry mesic forest, oak savannas and pine and oak barrens (Voss and Reznicek 2012). This species grows vigorously after disturbance, and has been noted for importance in ecological restoration for its ability to compete with exotic species and buffer against invasion (Mottl et al 2007). There is also horticultural interest in *C. pensylvanica* as a lawn alternative for its grass-like form and shade tolerance (Meyer 2004). While *C. pensylvanica* reproduces readily vegetatively, it is notoriously difficult to obtain adequate quantities of seed due to low seed set (Friedman and Barrett 2009) and low rates of germination (Farrer and Goldberg 2011).

The potential for this sedge to be used in horticulture and restoration has prompted research to improve seed production. A study by McGinnis and Meyer (2011) attributed the difficulty in germinating this species to complex dormancy requirements. Through after-ripening, cold stratification and temperature and light treatments, the authors achieved substantially improved germination rates, but their results varied significantly between the two years of their trials (57-96% germination in year one and 31-67% in year two) in which two different seed stocks were used. The authors suggest that the observed variation between trials may be a result of underlying population or genetic differences between the seed stocks, indicating that to understand the regeneration characteristics of *Carex pensylvanica* as pertaining to native plant production, other aspects of its reproductive life cycle must also be examined.

In plants, seeds can be produced through autogamy (self-fertilization), allogamy (outcrossed fertilization), apomixis (seed production without fertilization) or a combination (Shivanna and Tandon 2014). In all organisms, any factors that determine the patterns of gene inheritance between generations are collectively known as the breeding or mating system (Kearns and Inouye 1993) and for plants, the breeding system is essentially a measure of the relative dependency on outcrossing or selfing for reproductive success. Flowers of *C. pensylvanica* are monoecious, with the staminate spike superposed above one or several pistillate spikes (Hipp 2008). Friedman and Barrett (2009) found *C. pensylvanica* to be self-compatible, and at least in their study system, to demonstrate high rates of self-pollination.

Additional evidence that *C. pensylvanica* may tend towards selfing under natural conditions can be determined through its growth habit. *Carex pensylvanica* is a long-lived perennial plant with vigorous vegetative reproduction that produces large mats of genetically identical ramets that can be acres in size (Bernard 1990). As clonal plants increase in size, each flower is increasingly surrounded by more genetically identical ramets. This leads to a tight relationship between clonal spread and incidences of self-pollination that has been demonstrated in other species (Handel 1985).

The potential effects of the breeding system on the long-term reproductive output of plants are discussed at length in the literature. Long-term self-pollination can lead to reduced fitness from inbreeding depression (Charlesworth and Charlesworth 1987) and several studies have found the negative effects of inbreeding to be expressed in seed characteristics such as seed set and germination (Carta et al 2015, Joschinski et al 2015, Rymer et al 2015, Keller and Waller 2002, Kalisz 1989). However, other studies posit that highly selfing plants may, over time, purge the deleterious alleles causing inbreeding depression (Barrett and Harder 1996). In such cases, outcrossing with genetically distinct individuals may disrupt these stable genetics, resulting in a reproductive diminishment (Schierup and Christiansen 1996).

Low seed set and germination rates, and poor seed fitness characterize sexual reproduction in *Carex pensylvanica*. Evidence in the literature and observations of the physiology of *C. pensylvanica* suggest that it is highly selfing. From this, I hypothesize that 1) *Carex pensylvanica*'s typical poor seed quality is a result of long-term inbreeding through self-pollination and that 2) outcrossing should produce seeds with improved fitness characteristics. To test this hypothesis, I performed a hand pollination experiment, in which I artificially manipulated the breeding system of *C. pensylvanica* clones to produce crops of outcrossed and self-pollinated seeds for comparison. This study has important implications for better understanding the reproductive ecology and evolutionary trajectory of the species as a whole. It also has practical applications for aiding native plant growers in their ability to produce *C. pensylvanica* stocks for ecological restoration.

## **Methods:**

### *Sampling and Experimental Setup:*

Plant material for the study was obtained from ten wild *C. pensylvanica* clones at the University of Michigan's Matthaei Botanical Gardens and Radrick Forest properties in Ann Arbor, Michigan (42.2994° N, 83.6622° W) in July of 2015. Clones were identified on a ground survey through the study area. All clones used were a minimum distance of 100 meters apart from each other and separated by natural breaks such as rivers, wetlands, or steep cliffs to maximize the likelihood that the samples were from genetically distinct clones. All sampling took place in closed canopy Oak-Hickory woodlands on glacially deposited till. Clones ranged in size from 5-50 meters in diameter. At each sampling location, fifty 6" diameter plant masses were dug from each clone (n=500 total) to a depth of 8 inches in order to leave roots intact, and transported back to the greenhouses of the Matthaei Botanical Gardens where they were potted into 6" square pots. The potted sedges were grown under ambient outdoor conditions from July-September, and fertilized weekly, until they were transferred to outdoor, subterranean cold frames for pre-vernalization hardening off. In mid-September, pots were placed in a refrigerator room maintained at 5°C for 16 weeks of vernalization treatment.

In January of 2016, all plants were transferred from the cold room to the greenhouses of the Matthaei Botanical Gardens where they were placed under 8 hour-day length grow lights

with average daytime temperature ranging 18-24°C to simulate early spring conditions and induce flowering. With 30-40% mortality occurring during vernalization, the remaining samples were randomly assigned to pollination treatments, with 15 sedges per clone assigned outcrossing treatment, 15 sedges per clone assigned selfing treatment, and any remaining plant designated as a pollen source.

### *Pollination Manipulation*

Treatments were applied at the initiation of flower development, which began ~4 weeks after release from vernalization cold treatment. For all outcrossing treatments, the developing staminate spike was removed from the floral culm with small surgical scissors. The culm was then bagged with a 4"x6" pollen-proof bag (Carolina Biological Supply, Burlington, North Carolina), to prevent contamination through geitonogamy, another form of self-pollination where pollen is transferred between two genetically identical ramets within a single genet. For the self-pollination treatments, floral culms remained intact, and the full culm was bagged to prevent contamination from outside pollen. Treatments were separated into adjacent greenhouses to prevent cross-contamination.

Visual assessment of pistil receptivity and pollen dehiscence was performed daily. When outcrossing treatment pistils were receptive, pollen was collected from flowers of multiple other genets and combined on the rim of 16 x100 mm glass test tubes (Carolina Biological Supply, Burlington, North Carolina) to randomize pollen donation and control for the effect of male fitness differences on the seed development. The pollen was then transferred directly from the rim of the tube to the receptive pistils. For the selfing treatment, pollen was gathered using the same technique from the staminate spike of the focal flower and applied to the receptive female flowers. Because Penn sedge is protogynous, pollen availability and pistil receptivity was often offset temporally on the same culm, and in such cases, pollen was gathered from other attached culms and transferred to the receptive pistils, maintaining the selfing treatment through geitonogamy.

Observations and pollination manipulations continued for six weeks from the first flowering episode and concluded in March of 2016. Seeds were allowed to mature on the culm for six weeks after the pollen transfer and then collected for analysis. The reproductive outputs of the trials were evaluated using two proxies for reproductive fitness: seed set and seed weight.

Seed set is defined as the number of viable seeds (with a developed embryo) per flower. Seed development was evaluated visually under a Coddington 10x magnifying lens, and deemed developed if a rounded embryo was visible inside the seed coat. Seed weight values were obtained as an average weight per flower, by weighing each flower's crop of viable seeds on an XS104 microbalance (Mettler-Toledo, Columbus, OH), and dividing this total weight by the number of seeds per flower.

### *Statistical Analysis*

Seed set was analyzed with generalized linear mixed models (GLMM) (R Core Team 2014, Bates et al 2014) to account for the fact that multiple flowers per clone were sampled. The final model was fit to accommodate a poisson distribution typical of count data. Average seed weight was also initially analyzed with a GLMM (R Core Team 2014, Bates et al 2014). Analysis of both models considered pollination treatment as the fixed-effect variable and clonal origin as the random-effect variable and utilized restricted maximum likelihood (REML) to characterize the covariance between responses of individual plants.

For the sake of model parsimony, the random effect was ultimately excluded from the final seed weight model as it failed to explain a significant proportion (using  $\alpha=0.05$ ) of the variation in the dependent variable. Additionally, because the seed weight data fit a normal distribution, the average seed weight was ultimately analyzed with a simple linear regression.

In both models, the significance of the fixed effect on the dependent variable was determined through Type II Wald Chi-square tests (Fox and Weisberg 2011), and predicted values for both models were determined (R Core Team 2014).

It should be noted that only flowers that set one or more seeds were included in statistical analysis because it was not possible to evaluate whether the failure of pollinated flowers to produce seed was a result of a biological mechanism, which would have been fit for inclusion in analysis, or a result of experimental error, for example mistimed pollen transfers, which would have been unfit for inclusion. Pollen transfers were attempted on 210 floral culms, and based on the aforementioned seed development criteria, 121 culms and 176 individual flowers (92 outcrossed and 84 selfed) were included in the analysis.

## Results:

### *Seed Set:*

Seed set in outcrossed flowers was significantly higher than in self-pollinated flowers (outcrossed mean= 4.692 SE= 0.302, selfed mean= 3.393 SE=0.360,  $P<0.001$ , see Figure 1). Based on the generalized linear mixed models, the predicted value for seed set for the outcrossing treatment was 4.639 seeds per flower and the value predicted for the selfing treatment was 2.835 seeds per flower. The range of seed set in this experiment was between 1 and 19 seeds per flower. These results support the hypothesis that the breeding system influences seed production in *C. pennsylvanica* and that outcrossed individuals demonstrate higher reproductive fitness.

### *Seed Weight:*

There was no significant difference between the average seed weight of outcrossed and self-pollinated progeny (mean outcrossed=1.127 mg, SE= 0.031, mean selfed= 1.145 mg SE=0.037,  $P=0.719$ , see Figure 2). The range for average seed weight was between 0.2 and 2.33 mg. This result failed to support the hypothesis that seed weight, a proxy for reproductive fitness, was influenced by the breeding system of *C. pennsylvanica*.

## Discussion:

The higher seed set derived from outcrossed flowers of *C. pennsylvanica* when compared to the self-pollination treatment suggests inbreeding depression from long-term selfing as the likely cause of low seed set. While few other studies examine the breeding system of this species directly, the results of this experiment are consistent with Friedman and Barrett's (2009) study, which found that *C. pennsylvanica* culms bagged for selfing set significantly less seed than open-pollinated stems.

These results indicate that manipulating the breeding systems of *C. pennsylvanica* to favor outcrossing may be a useful approach to increasing seed production, despite observed seed set being relatively low in both treatments. As is consistent with expectations for plants that have been vernalized to accelerate the annual reproductive cycle, the sedges studied in this experiment displayed several irregular floral morphologies including pistillate spikes intermingled or superposed above staminate spikes, or staminate spikes lacking entirely (Vonk Noordegraaf and



Welles 1995, Anton Reznicek personal communication). While it was confirmed that both male and female flowers were viable and receptive even in these arrangements, the full effect of these anomalies on seed development is unknown. Because these altered morphologies were equally pervasive across both pollination treatments, the comparative results between treatments are sound, but caution should be used when considering the absolute values for seed set obtained in this experiment in comparison to wild populations with more characteristic floral morphologies.

In this experiment, no significant effect of breeding system on seed weight was found, which is consistent with observations in the literature that mean seed size is relatively invariant across a given species, especially when seeds are produced under similar environmental conditions (Silvertown 1989). Though not observed in this study, it is generally understood that there is a trade-off between seed size and seed set, and there is selective pressure to produce more seeds (Westoby et al 1992). At the same time, larger seeds increase the likelihood of seedling establishment and are associated with increased tolerance in stressful conditions (Leishman et al 2000). Smaller seeds are also generally associated with persistence in seed banks (Leck and Shutz 2005). These tradeoffs have resulted in stabilizing selection for seed weight over time, resulting in relatively consistent patterns in mean seed size despite high variability in seed set for a given species (Sandras 2007).

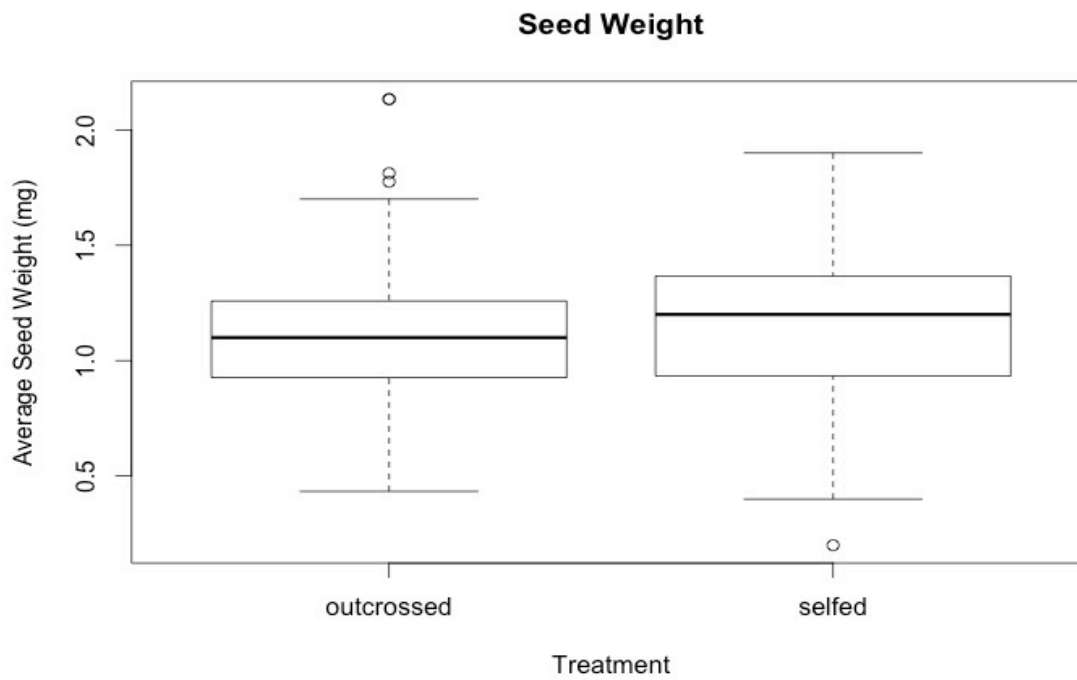
While the results of this study indicated the breeding system was an important determinant of seed set and should be considered in *C. pensylvanica* nursery production, several other biological and ecological factors have been shown to affect seed production in other species and warrant further study. While the clones used in this experiment were selected from sites with coarse homogeneity, there was also certainly latent, micro-variation between sites. It is possible that the influence of original site factors, such as nutrient availability, soil moisture or exposure to light may have persisted through the experiment and affected flower and seed production (Fenner and Thompson 2005), though precautions were taken to minimize this possibility by growing all sedges under standardized conditions for several months after initial collection. Other factors not evaluated in this study that have been shown to influence seed production include maternal environment (Gutterman 1991), age of maternal plant (Lembicz et al 2011) and paternal contributions (de Jong et al 2011). These factors were beyond the scope of this study, but should be evaluated in future work to provide native plant growers with a more complete picture of the regeneration requirements of *C. pensylvanica* in the context of restoration production.

It also important to note that the relevant ecological theories surrounding inbreeding depression all deal with the effects of long-term selfing; repeated self-pollination over multiple generations. This study examined only one reproduction event and did not include background observations or analysis about differences in the genetic history or population structures for the genets in the study. With only one season's worth of observations and unknown genetic backgrounds, it is difficult to evaluate the full implications of the results obtained in the study towards the long-term productivity of these focal plants as seed sources. Because reliable production is an important factor for native plant growers, further research should evaluate the effect of outcrossing in *C. pensylvanica* in multi-year studies to help determine if the fitness benefits demonstrated in this study persist in the long term.

To return to the applied question of this study, can growers use these results to better produce *C. pensylvanica* propagules for restoration? The model developed through this study projected a 1.6x increase in seed set when outcrossing was compared to selfing. On a production scale, this difference could significantly affect a grower's ability to economically produce *C.*

*pensylvanica* propagules. While there is a general preference towards wild collected restoration propagules to prevent the inadvertent but inevitable breeder's selection on cultivated materials (Schröder and Prasse 2013), the high selfing rates for *C. pensylvanica* makes wild collection of seed nearly impossible. Instead, growers might consider constructing seed gardens assembled spatially to maximize the interactions between plants of different genotypes as an option for increasing *C. pensylvanica* seed yield. Such gardens would not only increase the likelihood of outcrossing, but if regularly supplemented, could serve the dual purpose of maintaining a large genetic pool to preserve the adaptive capacity of the plant stock as local conditions change.

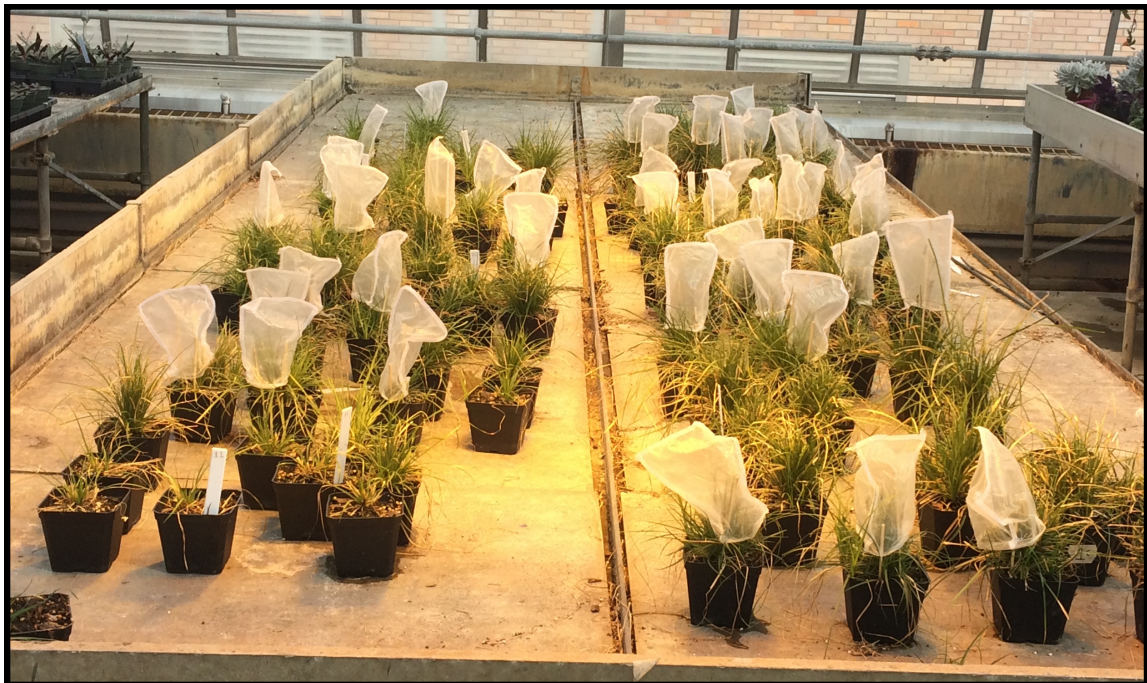
As the native plant industry continues to mature and expand production to include a greater diversity of plants for ecological restoration, researchers and practitioners must gain a deeper understanding of ecological and biological plant traits that will maximize both economic and ecological success for the native plant industry. This study has demonstrated that manipulating the breeding system of Penn sedge to facilitate outcrossing may be a promising technique to improve seed yield for this difficult to produce species. Because of the prevalence of inbreeding depression in many native plants species under natural conditions and the growing prevalence of fragmented landscapes that isolate individual populations, growers will increasingly need to explore options for cross-breeding among several populations. Seed gardens that are spatially-oriented to increase pollen transfer between genets may be a useful tool to improve the seed yield in other taxa that are underrepresented in ecological restoration projects. The benefits of continued research in the breeding systems of important restoration plant species would extend beyond viable plant production in the nursery industry. The breeding system is a strong control of population genetics in plant systems. Applied inquiries into the breeding systems of plants used in restoration could provide missing pieces of the puzzle in restoration planning, enabling managers to restore populations with the genetic capacity to persist in the long-term and the potential to evolve and keep pace with the changing conditions of our world.

**Appendix:****Figure 1:** *C. pensylvanica* seed set was significantly higher in outcrossed flowers than in selfed flowers ( $P < 0.001$ )**Figure 2:** No significant difference in seed weight ( $P = 0.719$ ) was observed between outcrossing and selfing pollination treatments in *C. pensylvanica*

**Image 1:** *C. pennsylvanica*, seen here at a sampling site in Radrick Forest, Ann Arbor MI, begins its annual growth early in the spring.



**Image 2:** Outcrossing pollination treatment in March of 2016.



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