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Thinking inside the Box: Tissue Culture for Plant Propagation in a Key Ecological Species, *Andropogon gerardii*

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Abstract

Intraspecific diversity has widespread effects on ecological communities and ecosystems. To elucidate the mechanisms underlying these effects, manipulative studies require a rigorous and efficient empirical approach. Yet, replicating sufficient numbers of genetically identical individuals remains a challenge. As a result, we are limited in our understanding of the mechanisms underlying the ecological effects of intraspecific diversity. In contrast, large sample sizes are routinely produced in horticultural research using micropropagation, or tissue culture. In order to determine the potential usefulness of micropropagation technique for ecological studies, we investigated the efficiency and efficacy of micropropagation on the ecologically important non-model C4 grass species, *Andropogon gerardii*. Our preliminary results demonstrate that micropropagation is a rapid and effective technique for producing large numbers of genetically identical clones at up to 100 times the rate of traditional propagation. Key intraspecific differences among clones of *A. gerardii* were also retained through the micropropagation process. Given that traditional techniques used to test the effects of intraspecific diversity manipulations are time-limiting (greenhouse propagation) or can be biologically misrepresentative (seeds) for some species, we suggest that micropropagation might be a powerful tool for advancing ecological genetics studies in many plant systems.

Keywords

Intraspecific Diversity, Micropropagation, Perennial Grass, Ecology

1. Introduction

Intraspecific diversity of plants can have strong effects on ecosystems, from in-

fluencing community species richness and insect diversity to enhancing ecosystem function [1] [2] [3] [4]. In order to test the effects of intraspecific diversity empirically, ecologists typically assemble artificial communities of genotypes or ecotypes [4] [5] [6] [7]. Although a number of investigators have successfully performed these kinds of experiments (e.g., [8]), generating large numbers of individuals from specific genotypes remains a challenge in many species. These large numbers of individuals are necessary to draw conclusions across a range of spatial scales and different resolutions of genetic variation (within vs. among populations) with more confidence, especially when variation is high and reproducibility is a recognized need in ecology [9] [10] [11]. Understanding interdependent variables, such as the interactions between climate change, drought, and disease, will require even larger sample sizes [12] [13] [14] [15]. Thus, it is unsurprising that many integrative intraspecific diversity studies use quickly reproducing model plants instead of ecologically important non-model species. This disparity presents a substantial knowledge gap for relevant species and limits our understanding of intraspecific diversity in natural ecosystems.

Several approaches are available to test the ecological effects of intraspecific diversity in manipulative studies (**Figure 1**). These typically include genotype collection and replanting [16] [17], planting different seed accessions [4] [18] [19], and propagating clonal plants in a greenhouse environment [1] [20] [21]. Nearly all intraspecific diversity studies lack micropropagation (also known as tissue culture "in vitro" propagation), a technique widely used in crop science and horticulture to generate large numbers of genetically identical individuals for commercial planting or research [22]. For non-model species, tissue culture techniques can make commercialization possible (e.g., indouglas-fir [23]). Yet, use of micropropagated plants is rarely suggested for ecological research [24].

Investigating intraspecific diversity in ecology is especially urgent in vulnerable ecosystems faced with the looming threats brought on by climate change. For example, the tallgrass prairies of the Midwestern United States will be particularly susceptible to future droughts [25]. In this ecosystem, the C_4 grass *Andropogon gerardii* is largely responsible for ecological function, comprising up to 80% of production [26]. This species recruits primarily through tillering from underground rhizomes and rarely from seed [27] [28] [29] [30] [31]. *Andropogon gerardii* is also self-incompatible (produces genetically recombined genotypes) and has common sterile polyploid cytotypes [32] [33] [34]. These traits suggest that planting seeds of different *A. gerardii* genotypes would not be biologically representative. Genotypes are also difficult to distinguish without quantifying genetic markers, so field collection is also unreliable. Thus, any intraspecific studies performed with this and other similar species are likely to need forms of plant propagation. Greenhouse propagation can take many years [20], however micropropagation techniques could substantially reduce this timeframe.

Here, we provide evidence for the validity of the micropropagation technique within ecology by testing these methods on this ecologically important, clonally

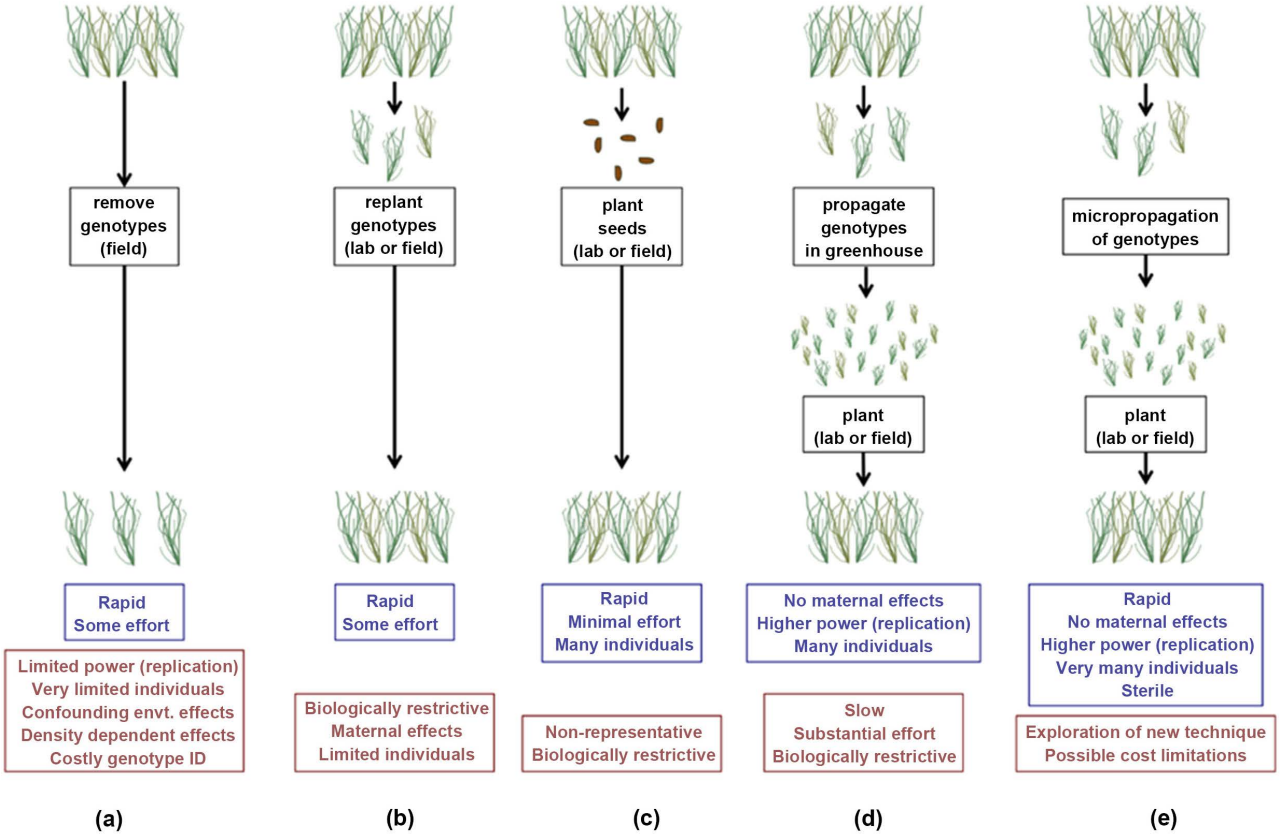

Figure 1. Experimental approaches utilized in studies manipulating plant genetic diversity typically include (a) field genotype manipulation by removal, (b) harvesting and replanting genotypes, (c) planting seeds of different genotype, (d) propagating and replanting genotypes in a greenhouse environment, or (e) propagating and replanting genotypes using tissue culture. Positive (blue) and negative (red) attributes of each approach are boxed.

reproducing species. We developed a tissue culture protocol building upon the only previously known protocol developed for *A. gerardii* [35]. Our goal was to test the efficacy and efficiency of this technique for ecological non-model species using several genotypes of *A. gerardii.* For micropropagation to be effective, a large number of individuals representing different genotypes need to be generated in a short period of time. We expected this technique to be effective for *A. gerardii* due to the success of micropropagation for many species in horticulture and *A. gerardii*'s clonal growth habit. Successful development of this protocol for an ecologically important non-model species will help encourage more efficient study of the ecological effects of intraspecific diversity on communities and ecosystems.

2. Materials and Methods

2.1. Study Species

Andropogon gerardii (big bluestem) is found throughout most of eastern North America and is the dominant species in the tallgrass prairie ecosystems of the central and eastern Great Plains [36]. Depending on fire and grazing manage-

ment, *A. gerardii* contributes a large proportion to primary producer biomass [26] and controls plant community structure [37], and is thus of great ecological interest for prairie ecosystems. Due to its ecological and commercial importance [38] [39], a number of studies have already investigated genetic diversity in this species [7] [40]-[47]. We selected three hexaploid genotypes from one population [46] to propagate in tissue culture for later use in an ecological study. The focal genotypes of *A. gerardii* have been shown to respond differentially to drier conditions and have previously been genotyped using amplified fragment length polymorphisms (AFLPs) [47]. Drought studies focused on intraspecific diversity in *A. gerardii* will be of increasing importance due to changing climate, including increased risk of climate extremes and altered timing of rainfall events in the Great Plains [25] [48] [49].

2.2. Tissue Culture

Micropropagation was started from greenhouse potted genotypes of *A. gerardii* selected by M. Avolio from a single population at the Konza Prairie Biological Station in Manhattan, KS [41]. Plants were excised below actively growing meristem using tools dipped in 70% isopropyl alcohol. Rhizome was included wherever possible. Excised plants were then sterilized using a 0.5% bleach solution with gentle shaking before transporting to a laminar flow hood with HEPA filter. Meristematic regions were identified as rhizomatous nodes and gently scrubbed with 3% hydrogen peroxide to remove any residual soil. Nodes were excised and placed on a rotary shaker in 3% hydrogen peroxide for at least 2 hours. Nodes were then separated into separate tubes and shaken for 10 minutes in sterile water with 10% bleach and 50 µL per L of Tween-20 solution. Meristematic nodes were then placed on 10 mL solid "3BX" MS-based growth media [50] for shoot initiation (**Table 1**). Due to fungal or bacterial presence, contaminated tubes were frequently swapped for fresh media during an 8 week period to establish sterile cultures.

Magenta-style tissue culture boxes containing 60 mL solid "2BX" MS-based growth media (**Table 1**) were prepared ahead of time and autoclaved for 30 minutes, after which boxes were sprayed with 70% ethanol and allowed to solidify overnight. Boxes were monitored for several days before use to ensure no contamination. Media was prepared in the Horticulture department facilities at Colorado State University. Genotypes 2 (G2), 5 (G5), and 11 (G11) [46] were the focus of this study. Names of these genotypes correspond to ranked abundance in the field (*i.e.*, G2 is the second most abundant genotype). These genotypes are abundant in the Konza Prairie Rainfall Manipulation Study with implications for ecosystem function under changing precipitation regimes [47]. Previous study has shown that G2 and G11 is drought tolerating while G5 is susceptible to water limitation [46] [47]. Five, eight, and nine plants of G2, G5, and G11, respectively, were transferred on 4 April 2014 to media boxes with flame-sterilized tools in a laminar flow hood with HEPA filter at the U.S. Department of Agriculture

Table 1. Culture media recipes used in *A. gerardii* micropropagation. MS salts: Murishage & Skoog basal salt mixture [50]. Media was prepared in 5 L Erlenmeyer flasks with a stirring bar. Once the water was hot but not boiling, salts and micronutrients were added and pH was adjusted to 5.7 using sodium hydroxide solution. The mixture was then brought to a light boil before adding sucrose and gellan gum. Media was stirred rapidly and heated until all sucrose and gellan gum was dissolved, approximately 5 - 10 minutes. All reagents were purchased from Phytotechnology Laboratories, Overland Park, KS.

Media recipe: 1 L reverse-osmosis water	Volume
3BX: Shoot initiation	
Sucrose	30 g/L
Myo-inositol	100 mg/L
MS salts	4.3 g/L
Gelritegellan gum	2 g/L
Glycine	2 mg/L
Nicotinic acid	0.5 mg/L
Pyridoxine	0.5 mg/L
Thiamine HCl	0.4 mg/L
N^6-Benzyladenine	3 mg/L
2BX: Shoot initiation	
Sucrose	30 g/L
Myo-inositol	100 mg/L
MS salts	3.4 g/L
Gelritegellan gum	2 g/L
Glycine	2 mg/L
Nicotinic acid	0.5 mg/L
Pyridoxine	0.5 mg/L
Thiamine HCl	0.4 mg/L
N^6-Benzyladenine	2 mg/L
0BX: Root initiation	
Sucrose	30 g/L
Myo-inositol	100 mg/L
MS salts	4.3 g/L
Gelritegellan gum	2 g/L
Glycine	2 mg/L
Nicotinic acid	0.5 mg/L
Pyridoxine	0.5 mg/L
Thiamine HCl	0.4 mg/L

National Center for Genomic Resources Preservation (USDA-NCGRP), Fort Collins, CO. Boxes were placed in a walk-in growth chamber at the USDA-NCGRP and provided a 16 hour photoperiod at 25˚C and 20% - 30% humidity.

Plants were allowed to propagate for 24 days, during which clumps of indi-

viduals were produced (Figure 2(a)). Individuals were then transferred with forceps to boxes containing "0BX" rooting media (Table 1; Figure 2(b)). Plants grew roots for 50 days before being transferred to misting benches at Plant Growth Facilities, Colorado State University. Transfer to the greenhouse involved rinsing residual media from plant roots with water, weighing individual fresh plants, and placing individuals into moist fritted clay media (Porous Ceramic "Greens Grade", Profile Products LLC, Buffalo Grove, IL). Height and tiller count measurements were collected on these individuals on day 6 (early) following transfer. Plants were allowed to adjust in misters for 42 days before being placed in 2.65 L cone-tainer style tree pots for further study (Stuewe & Sons, Tangent, OR).

2.3. Statistical Analyses

We tested whether genotypes differed in trait values to provide evidence for phenotypic diversity emerging from genetic diversity. We compared plant mass, height, and tiller count, genotypes using robust regression linear models (using the M estimator), a method that accommodates unequal variance. Following pairwise comparisons, we adjusted p-values using Tukey adjustments and corrections for false discovery rates. Differences in variances among genotypes were tested using two-sided F tests on each pairwise comparison with Bonferroni adjustments. All statistical analyses were performed using R (version 3.5.0, [51]) with open-source code (https://github.com/avahoffman/tissue-culture-andropogon).

3. Results

All three *A. gerardii* genotypes experienced very high rates of micropropagation. Five, eight, and nine plants of G2, G5, and G11, respectively, were transferred on 4 April 2014. These parent plants yielded 105, 110, and 121 plants of each genotype, respectively, 24 days later. For G2, we found that 38% had already developed roots after 17 days in rooting media. For G5, 76% had developed roots and for G11, we found that 45% had developed roots in rooting media. Plants with developed roots were transferred to the greenhouse on 17 June 2014, totaling 108, 118, and 135 plants of G2, G5, and G11, respectively (Table 2). The propagation rate in this study was approximately 6.1×, 4×, and 3.9× new plants for every parent plant per week. Despite some mortality during the rooting stage (28%, 4%, and 27% mortality for G2, G5, and G11, respectively), plants continued propagating in hormone-free rooting media. This resulted in a higher number of plants transferred to the greenhouse than initially started on rooting media. After tissue culture plants were transferred to the greenhouse, *A. gerardii* experienced surprisingly high rates of survival. On July 29, plants were transferred to pots for future study. A total of 108, 117, and 134 plants were transferred to pots from G2, G5, and G11, resulting in 100%, 99.2%, and 99.3% survival during the greenhouse adjustment phase in each genotype, respectively.

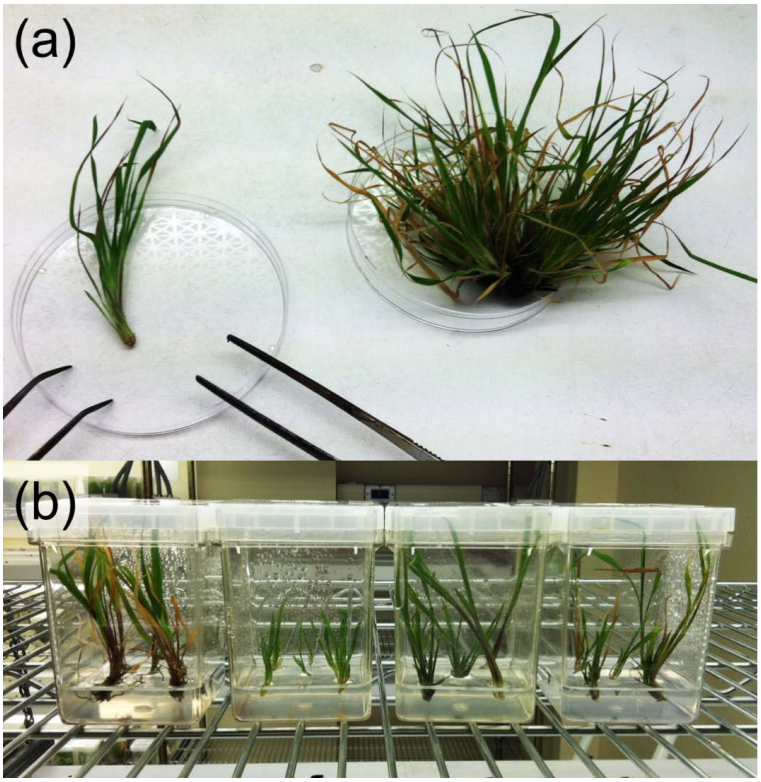

Figure 2. Micropropagation was successful in *Andropogon gerardii*. (a) Clumps of propagated plants (right) were separated into individuals (left). Individuals were then placed in rooting media (b). Boxes are arranged from left to right by genotype: G5, G1 (not used in this study), G2, and G11.

Table 2. Propagation summary for *A. gerardii* genotypes. Propagation rate refers to the number of new plants per each starting plant. "Day 17" indicates seventeen days following transfer into 0BX rooting media. "Plants transferred to greenhouse" indicates the number of plants that survived rooting and were transferred to non-sterile conditions.

Genotype	G2	G5	G11
Starting plants	5	8	9
Propagation rate	6.1/week	4/week	3.9/week
Day 17: rooting stage mortality	28%	4%	27%
Day 17: plants with roots	38%	76%	45%
Plants transferred to greenhouse	108	118	135

Differences between genotypes were examined in the greenhouse at the start of the acclimation stage, where plants were located under misters between tissue culture and placement in the ecological study. We found that genotypes varied significantly in mean starting mass, height, and tiller count (**Figure 3**). Genotype 2 had significantly greater mass than G5 ($z = -2.770$, $p < 0.001$) and G5 had significantly greater mass than G11 ($z = -3.665$, $p < 0.001$). Genotype 5 was the tallest genotype (compared to G2, $z = 4.596$, $p < 0.001$) and G11 was the shortest (compared to G2, $z = -2.282$, $p = 0.023$). Genotype 11 has significantly more

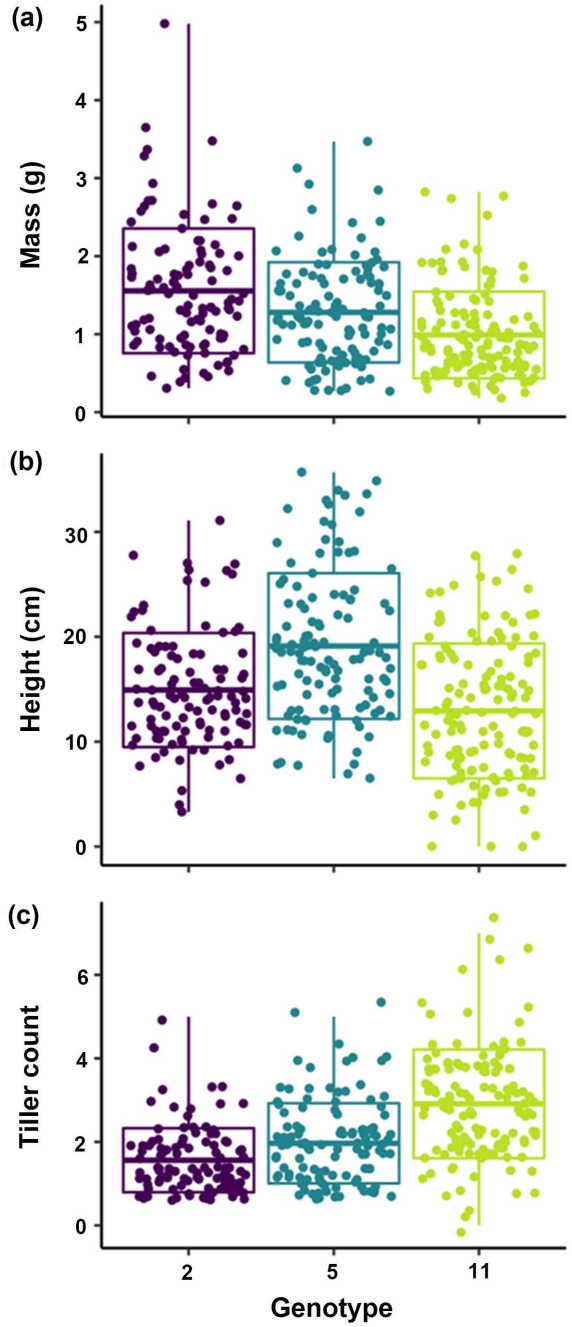

Figure 3. Traits of three genotypes of propagated plants including (a) mass, (b) height, and (c) tiller count were variable. Boxplots indicate maximum, minimum, and the mean +/− one standard deviation. Note that weight (a) is fresh weight, not dried weight.

tillers than G5 ($z = 8.162$, $p < 0.001$) whereas G2 had the fewest tillers ($z = 2.763$, $p = 0.005$). Genotypes also showed differences in trait variation. Genotype 2 showed the more variation in mass compared to G11 ($p < 0.001$) but variation between G2 and G5 ($p = 0.065$) and G5 and G11 ($p = 0.310$) were not significant. Variation in height was different between G2 and G5 ($p = 0.032$) but not differ-

ent between G2 and G11 ($p = 0.215$) or between G5 and G11 ($p = 1.000$). Lastly, G11 had the most variation in number of tillers (compared to G2, $p < 0.001$ and compared to G5, $p = 0.003$). However, G2 and G5 did not differ in tiller count variation ($p = 0.057$).

4. Discussion

In this study, we showed that micropropagation was effective and efficient for a non-model grass species with implications for other ecologically important plants. We found that we were able to propagate, root, and acclimate 360 total plants over slightly less than six months. Propagation rates for the different genotypes of *A. gerardii* were higher than traditional propagation techniques in this species (e.g., rhizome propagation in the greenhouse). Hartnett (1989) found multiplication rates for *A. gerardii* in the greenhouse were between 0.1× and 0.4× plants per week at low density. At higher density, multiplication rates were lower [52]. Commercial cultivars grown over several years yielded 0.2× plants per week [53]. We observed greenhouse multiplication rates of 0.04×, 0.1×, and 0.05× for G2, G5, and G11, respectively, when pots were kept at 25% volumetric water content over 15 months [54]. Another study examining *A. gerardii* and *A. hallii* hybrids took over three years to propagate sufficient clones [55]. In other cases, studies using rhizome propagation of *A. gerardii* did not specify rates or duration [56] or used genetically recombined seed [44]. Overall, the rhizome propagation rates are between < 1 and 11% of the rates we observed with micropropagation. This difference is substantial, but the micropropagation advantage could vary among other non-model plant species.

Because the three *A. gerardii* genotypes we examined co-occur at the plot level, we would expect them to have different ecological niches and phenotypes [42] [46] potentially leading to cascading community effects. We found that mean trait values differed among genotypes even when plants were very young. Adult G2 tended to have fewer, large tillers compared to G11, which could represent early onset of different intraspecific growth strategies and resource allocation. Differences in variance among genotypes were also common and might reflect genotype differences in plasticity, a genetic characteristic that is likely independent of propagation technique [46] [57] [58] [59]. These results demonstrate the importance of intraspecific diversity even within a single population. Phenotypic differences were evident despite growing in a very controlled tissue culture environment. Our findings also suggest the importance of controlling for genotype in statistical models even when plants appear similar.

Many genetic diversity studies focus on a few key species, such as *Oenothera biennis* [4] [60] [61] [62] [63] or *Solidago altissima* [1] [5] [64], indicating that once a system is established, subsequent studies might become more practical. However, as climate changes worsen, understanding the effects of genetic diversity on less studied species will become more critical. Some ecologically dominant species (such as *A. gerardii*, *Bouteloua gracilis*—blue grama grass, *Spartina*

alterniflora—smooth cordgrass, *Populus tremuloides*—trembling aspen, and *Taxodium distichum* baldcypress) tend to reproduce asexually, suggesting that they could be particularly amenable to micropropagation [7] [65] [66]. Micropropagation might also allow for more thorough investigation of intraspecific diversity beyond ecotype due to its rapid turn-around and potential to produce large numbers of individuals. Future study could then incorporate other levels of intraspecific diversity, such as phylogenetic relatedness among genotypes to explain variation in ecosystem function [47].

Most genetic diversity studies use seeds from different sources to represent different genotypes [6] [19] [67]-[72]. Seeds are readily attainable and minimize starting time for experiments. Yet, while model species such as *Arabidopsis thaliana* can self-pollinate and reach maturity quickly, an estimated 39% of angiosperms are self-incompatible [73] and many have long life spans, making inbred lines impossible to generate. Seeds also can be limited to commercial cultivars or vulnerable to the mismatch between the scale of genetic diversity compared to the environmental or spatial scale of the experiment [74]. In self-incompatible species, propagation techniques might be the only option for working with genotypes or ecotypes of interest. Greenhouse propagation can take months to several years [20] [75]-[81], even with added growth hormones [75] [80]. Given the issues with genetic diversity studies using traditional methods and the evidence we present above, we suggest that micropropagation is a useful alternative. Although few ecological studies exist using micropropagation, several have been successful when laboratory resources were available [7] [65].

Although micropropagation is a highly effective technique, there are a number of limitations worth acknowledging. First, plants might develop somaclonal variation. In crop development, undifferentiated callus tissue can develop novel alleles overtime [82]. Rather than use callus tissue, we took advantage of natural budding from stem and rhizome meristem in *A. gerardii*. Plants experienced rapid rates of production even with the low dose of growth hormone N^6-Benzyladenine (an artificial cytokinin). Because all genotypes began culture at the same time, we expect the amount of novel somaclonal variation to be approximately equal in each genotype, and therefore, not a confounding variable. Assuming constant mutation rates within a species, this would also be a problem for traditionally propagated plants. Furthermore, Avolio *et al.* [41] found genetic variation among clones that were physically connected by rhizome in the field. Thus, some variation could be unavoidable among clones in *A. gerardii* and potentially other species.

Micropropagation is necessarily sterile, yet many plants rely on mycorrhizae or endosymbiotic bacteria for normal function. The establishment of sterile lines in tissue culture eliminates most if not all of these important microorganisms, including within *A. gerardii*, which relies strongly on mycorrhizal fungi for phosphorusacquisition [83] [84] [85]. Although plants in micropropagation are supplemented by nutrients, transitioning back to a symbiotic relationship after

growing in sterile tissue culture might be stressful (although we observed < 1% mortality leaving tissue culture). While some stress for the plant is unavoidable, we suggest lack of microorganisms can be remedied by inoculating media with native soil following the transfer of plants from tissue culture to greenhouse or laboratory conditions. In contrast, sterility offers the opportunity to study plant performance under different microbial conditions that are not available when using field-collected individuals for propagation.

Our understanding of intraspecific diversity is still limited in non-model and ecologically important species despite the pressing effects of globalchange. Future manipulative experiments must be able to increase sample sizes and account for variation among individuals to increase statistical power and be more confident in their conclusions. We propose that micropropagation will be most useful in clonally reproducing, self-incompatible plant species where 1) greenhouse propagation is time-limiting and 2) large numbers of plants are needed. Because we were able to perform a large portion of our micropropagation collaboratively, costs were greatly reduced overall, and made tissue culture a rapid, cost-effective method for our study. Many ecological labs might be able to collaborate with crop science or horticulture labs within academic institutions. Although long-term experiments using traditional propagation are feasible, shorter experiments using micropropagation are likely more appropriate for student projects or for short-term grants. For example, this technique allowed us to complete an entire study in approximately eight months instead of years. In the future, researchers will need to evaluate the costs and benefits of different propagation methods in their species of choice while ensuring techniques are as ecologically representative as possible.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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