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Evaluation of Hall's Panicgrass (*Panicum hallii* Vasey) as a Model System for Genetic Modification of Recalcitrance in Switchgrass (*Panicum virgatum* (L.))

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I am submitting herewith a thesis written by Joshua Nathaniel Grant entitled "Evaluation of Hall's Panicgrass (*Panicum hallii* Vasey) as a Model System for Genetic Modification of Recalcitrance in Switchgrass (*Panicum virgatum* (L.))." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Charles N. Stewart, Major Professor

We have read this thesis and recommend its acceptance:

Scott C. Lenaghan, Max Chen

Accepted for the Council:
Dixie L. Thompson

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(Original signatures are on file with official student records.)

Evaluation of Hall's Panicgrass (*Panicum hallii* Vasey) as a Model System for Genetic Modification of Recalcitrance in Switchgrass (*Panicum virgatum* (L.))

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Joshua Nathaniel Grant
May 2017

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DEDICATION

The enclosed work is dedicated to my wife, Sarah, my son, Silas, and my parents, Jerome and Veronica.

ACKNOWLEDGMENTS

I would like to acknowledge the work of Thomas Juenger at the University of Texas at Austin, who brought *Panicum hallii* out of obscurity and into the mainstream. Additionally, I would like to thank Jonathan D. Willis, Charleson Poovaiah, and Taylor Frazier for their wisdom and guidance.

ABSTRACT

While switchgrass (*Panicum virgatum* (L.)) has long been recognized as a viable bioenergy feedstock, it and other plants have cell walls with recalcitrance to processing. Recalcitrance is recognized as a major barrier to broad adoption of switchgrass and other feedstocks for cellulosic bioenergy. In an effort to reduce recalcitrance, transgenic plants have been generated with altered cell wall phenotypes such as reduced lignin. Unfortunately, stable transformation of switchgrass and other C_4 grasses is time intensive, costly, and genetic analysis is further complicated by polyploid genomic structures. Unlike switchgrass, which can be tetraploid to octoploid, a closely related species, Hall's panicgrass (*Panicum hallii* Vasey), is diploid, and has a much smaller genome. In addition, Hall's panicgrass is a smaller plant with a faster generation time and is capable of self-fertilization. In the present study, germplasm from two inbred populations of Hall's panicgrass, FIL2 and HAL2, were selected to assess the feasibility of using Hall's panicgrass as a model for switchgrass. Included in this work was the development of methods using seeds immediately harvested from plants grown in the greenhouse for germination, sterilization, callus induction, transformation, and regeneration. Seed germination was optimized on NB medium at 70 ±[plus or minus] 11% for FIL2 and 82 ±[plus or minus]3.0% for HAL2. Callus induction was optimized on MS-OG medium at 51 ±[plus or minus]29% and 81 ±[plus or minus]19% for HAL2. Shoot regeneration was optimized on REG medium at 11.5± [plus or minus] 0.8 shoots/gram for FIL2 and 11.3 ±[plus or minus]0.6 shoots/gram for HAL2. Root regeneration occurred at 100% frequency for all callus expressing roots on Diet-MSO. In addition to a complete tissue culture system, a suspension culture system was also developed to more rapidly produce tissue for cell-based experiments. Cell suspensions of Hall's panicgrass, both FIL2 and HAL2, generated more callus after 16 weeks of culture (141 ±[plus or minus] 22% for FIL2; 302 ±[plus or minus] 54% for HAL2) than the solid-medium culture system.

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CHAPTER I – INTRODUCTION

The need for a C_4 model

Switchgrass (*Panicum virgatum* L.) is a warm-season perennial C4 grass from the subfamily *Panicoideae*, and is native to North America where it has evolved to thrive in many different environments (Lewandowski et al. 2003). Switchgrass has widely been considered as a lignocellulosic biofuel feedstock with strong potential for net carbon sequestration (Sanderson et al. 2006). Switchgrass has diverged into two ecotypes: upland and lowland (Zhang et al. 2011b; Porter Jr 1966). Several key characteristics of switchgrass have piqued research interest as an emerging bioenergy crop: a high biomass yield (McLaughlin and Adams Kszos 2005), the need for low agronomic input (Moser and Vogel 1995), and C_4 metabolism (Vogel 2004). The interest has led to the sequencing of the switchgrass genome, which is currently in a draft stage (Nordberg et al. 2014; *Panicum virgatum* v1.1, DOE-JGI, http:://www.phytozome.net/panicumvirgatum). Additionally, tissue culture systems for switchgrass have been around since the mid-1990s (Denchev and Conger 1994), and genetic transformation systems utilizing *Agrobacterium tumefaciens* were reported in the early-2000s (Somleva et al. 2002). The development of elite tissue culture lines that produce high rates of somatic embryogenesis, high frequency of shoot and root regeneration, and high susceptibility to transformation have greatly improved efficiency and reduced time cost (Xu et al. 2011; Li and Qu 2011). In addition to *Agrobacterium*-mediated transformation, biolistic transformation using accelerated gold particles was first produced in the early 2000s (Richards et al. 2001), and has since been demonstrated to be quite effective (King et al. 2014). However, switchgrass requires a long and arduous process for the establishment of transgenic plants, usually taking about six months from callus induction to soil-grown plants (Xi et al. 2009). Breeding experiments are frequently conducted in the field, requiring a year to generate F_1 progeny (Casler 2012; Bouton 2007). Additionally, the polyploid nature of switchgrass makes genetic experiments difficult as most specimens are tetraploid or octoploid (Costich et al. 2010). Even among populations, ploidy levels can vary between tetraploidy and octoploidy (Wullschleger et al. 1996). Further, switchgrass is self-incompatible, making it difficult to fix traits to homozygosity (Martinez-Reyna and Vogel 2002). Therefore, studies utilizing an evolutionarily similar model C_4 plant can greatly aide our understanding of switchgrass cell wall biosynthesis pathways.

Model systems are excellent tools for plant biologists, with *Arabidopsis thaliana* being the archetype of a plant genomic model (Meinke et al. 1998). Other plant models have been used such as rice (*Oryza sativa)* for photosynthesis (Ye 2007) and stem elongation (Kende et al. 1998), and *Brachypodium distachyon*, a model for functional genomics in C_3 grass species (Draper et al. 2001). However, there is a lack of model C_4 plants. A C_4 plant species would have the same desirable characteristics of other model plants to enable its use as a research model for switchgrass and other grass or cereal crops: possess desirable physical characteristics and low maintenance cost, exhibit self-compatibility with a fast life cycle, be diploid with a small genome, and be amenable to reverse genetics, i.e., transformable.

A small plant footprint is desirable for model plants, as more plants can be grown in closer quarters. Heights for model plants are varied, but they are generally compact.

Arabidopsis ranges from 20 to 25 cm (Meinke et al. 1998), *Brachypodium* ranges from 15 to 20 cm, and an average height of rice is 100 cm (Brkljacic et al. 2011). Low maintenance requirements are also necessary as researchers must not focus on keeping a model plant alive, which is why most model systems have minimal care requirements. Both *Arabidopsis* and *Brachypodium* can be grown almost despite researcher involvement, rice has more demanding requirements (Brkljacic et al. 2011). A fast life cycle would allow for more generations to be established more quickly (Brkljacic et al. 2011), while self-compatibility would facilitate homozygosity, allowing for the easy identification and selection of traits (Bergelson et al. 1996). *Arabidopsis* is one of the fastest growing model plants, with a four-week life cycle (Pruitt and Meyerowitz 1986). Historically, model plants are self-compatible, as is the case with *Arabidopsis* (Meinke et al. 1998). Plants which are self-compatible are hermaphroditic and can produce viable offspring from self-pollination (Jarne and Charlesworth 1993; Barrett 2003). However, model plants also need to be able to undergo crossing experiments to create double mutants for genetic studies (Tzafrir et al. 2004). Therefore, a model plant would need to exhibit both self-fertilization and be capable of outcrossing. All three of these model examples are efficiently crossed and prefer self-fertilization (Brkljacic et al. 2011). The small footprint and low maintenance coupled with a fast life cycle and self-compatibility allow for easy initiation, effortless care, the ability to cultivate a wide selection of phenotypes.

In consideration of a plant as a model system, genetic attributes such as a small, diploid genome that has been sequenced and evolutionary relationship to the intended plant are greatly desired. Model plants are generally diploid (Izawa and Shimamoto 1996; Meinke et al. 1998; Doust et al. 2009; Brutnell et al. 2010; Brkljacic et al. 2011) with small genomes, thereby reducing the number of homologous genes and making knockouts and knockdowns easier (Husband and Sabara 2004), as well as aiding in genetic analysis (Vogel et al. 2010). Also, the genomes of these organisms have been sequenced are relatively small: the *Arabidopsis* genome contains 119 Mb (Kaul et al. 2000), the *Brachypodium* genome comprises 272 Mb (Vogel et al. 2010), and the rice genome has 382 Mb (Project 2005). Closely related species between a model and an organism of interest allows for the knockout or knockdown of genes in the model with analogues or orthologues in crops of interest (Oshlack et al. 2007).

Finally, the model system needs to be amenable to genetic manipulation. Transformation methods exist for all three model plants: *Arabidopsis* (Zhang et al. 2006; Bent 2006; Clough and Bent 1998), *Brachypodium* (Vogel et al. 2006), and rice (Ozawa 2009). The preferred method of transformation is with the use of *Agrobacterium*, however other genetic modification methods, such as particle bombardment, have been utilized (Li et al. 1993) during early stages of model system development. One of the oldest model plant systems, *Arabidopsis*, also has one of the simplest transformation protocols, making the skill required for experimentation negligible: the floral-dip method (Zhang et al. 2006; Clough and Bent 1998). This method allows for the immediate generation of transgenic seeds, thereby bypassing a tissue culture stage and resulting in swift production of T_1 progeny. Susceptibility to *Agrobacterium*-mediated transformation has been genetically analyzed to reveal several key genes relating to

susceptibility such as the transcription factors VIP1 (Tzfira et al. 2001) and *MTF1* (Sardesai et al. 2014). Likewise, genes relating to resistance have also been discovered in *Arabidopsis,* such as the flagellin receptor *FLS2* (Zipfel et al. 2004) and mutations in *RAT1* (Gaspar et al. 2004).

However, these aforementioned model plants fall short in their relevancy to serve as a model for switchgrass and many grain crops in one or both of two key areas: cell wall type and photosynthesis. *Arabidopsis* has type 1 cell walls, whereas *Brachypodium* and rice have type 2 cell walls (Brkljacic et al. 2011). Type 1 cell walls are found both in dicots and non-commelinid monocots and are comprised of equal parts cross-linking xyloglucans and cellulose, while type 2 cell walls are primarily found in commelinid monocots and consist of cellulose and glucuronoarabinoxylan (Carpita and Gibeaut 1993). The molecular mechanisms behind cell wall extension differ between the two types (Carpita and Gibeaut 1993), and as such, the genes controlling cell wall construction differ as well (Darley et al. 2001), preventing studies involving genes from a cell wall type 1 plant to be relatable to a cell wall type 2 plant. Switchgrass is a commelinid grass, and therefore contains type 2 cell walls (Sarath et al. 2008). Both *Brachypodium* and rice are C_3 plants, therefore their ability to accurately model carbon sequestration in C_4 plants is suspect. Therefore, to model recalcitrance in switchgrass, a plant possessing cell wall type 2 and C_4 photosynthesis would be preferable.

While cell wall type is important, especially when trying to design strategies to increase cellulose content or decrease lignin content (Donaldson 2007), the type of carbon sequestration utilized in photosynthesis is equally important as C_4 photosynthesis requires a different leaf anatomy (Kranz anatomy) than C_3 photosynthesis. Even between closely related C_3 and C_4 plants, there are significant changes to expression levels of thousands of genes, mostly related to leaf development (Bräutigam et al. 2011; Gowik et al. 2011). It has been posited that the secondary cell walls of C_4 grasses are not well modeled by C_3 grasses (Nelson 2011). For instance, the brown midrib mutant (*bmr*) is a classic example of the difference between C_4 and C_3 plants in that this mutation does not occur in C_3 plants (Sattler et al. 2010). These *bmr* mutants exhibit browning around in the leaf midrib and stem of mutant plants (Kuc and Nelson 1964; Porter et al. 1978; Marita et al. 2003; Sattler et al. 2010). All three of these models exhibit C_3 photosynthesis, therefore studies utilizing these models to relate to a C_4 plant cannot consider leaf development or photosynthesis, as even among C_4 plants there is a variety of biochemical pathways generating C_4 photosynthesis.
Since the three above-mentioned model plants differ from switchgrass in both the cell wall biosynthesis and polysaccharide makeup, biochemical methods in which they form cell walls, they are suboptimal models for switchgrass and many C_4 grain crops.

Panicum hallii as a C_4 model

Panicum hallii Vasey (Hall's panicgrass) is a perennial C_4 grass plant in the family Poaceae. *P. hallii* has some notable features that make it a good candidate for a model C_4 system: diploidy ($2n = 2x = 18$) (Waller 1976), small genome size (Anderson et al. 2011), and physical characteristics such as small stature and favorable reproductive traits. A transcriptome analysis and gene expression atlas has been

reported for *P. hallii* var. *filipes* (Scribn.) (Meyer et al. 2012), and an early draft of the genome is available (*Panicum hallii* v1.1). However, no study has evaluated *P. hallii* as a model system nor reported successful genetic transformation. However, a tissue culture and regeneration medium has been developed for multiple grass species, and Hall's panicgrass was among the grass species evaluated for response to tissue culture (Seo et al. 2010; Seo et al. 2008). *P. hallii* has been characterized into two distinct ecotypes (Waller 1976): the upland variety, *P. hallii* var. *hallii*, and the lowland variety, *P. hallii* var *filipes*. Additionally, *P. hallii* has been identified as having an evolutionary relationship to other *Panicum* species, namely switchgrass (Zhang et al. 2011a). Furthermore, the large number of seeds produced per plant and a seed-to-seed time of eight weeks (Lowry et al. 2012) can accelerate breeding programs and generation of transgenic progeny. *P. hallii*'s model characteristics and its similarity to switchgrass make it an excellent candidate for a C_4 model system for gene-to-phenotype studies.

Currently, two inbred populations (FIL2 & HAL2) are undergoing sequencing by the Joint Genome Institute (*Panicum hallii* v1.1 ; Nordberg et al. 2014). Unlike the large genomes of other lignocellulosic biofuel grass crops such as switchgrass (1230 Mb (*Panicum virgatum* v1.1, DOE-JGI, http:://www.phytozome.net/panicumvirgatum)), *Miscanthus* x *giganteus* (6846 Mb) (Rayburn et al. 2009), and *Sorghum bicolor* (730 Mb), the genome of *P. hallii* is relatively small: about 550 Mb for HAL2 (Lowry et al. 2012) and 453 Mb for FIL2 (*Panicum hallii* v1.1). In comparison to other model and bioenergy plants (Figure 1), both HAL2 and FIL2 have smaller genomes than most bioenergy crops. The smaller genome of *P. hallii* allows for easier genome mapping and sequencing due to *P. hallii*'s relatively small genome in comparison with polyploid panicoids like switchgrass.

In contrast to the large stature of switchgrass, with can reach to 2.7 meters in height (Silzer 2000), *P. hallii* is much smaller, growing to an average height of 65.7 cm (FIL2) or 35.6 cm (HAL2) at maturity (Lowry et al. 2014). Some reproductive characteristics of *P. hallii* also make it an excellent choice for a model system, such as its preference for self-fertilization and the production of hundreds (HAL2) to thousands (FIL2) of seeds per plant (Lowry et al. 2012). The tendency for *P. hallii* to prefer self-fertilization (Lowry et al. 2012) to obligate outcrossing provides several benefits: simplified Mendelian segregation, homozygous line establishment, and zygosity analysis. While *P. hallii* exhibits selfing, outcrossing experiments can be performed, for instance in microsatellite studies in which FIL2 was crossed with HAL2 (Zhang et al. 2011a). Switchgrass has a slow seed to seed time when compared with *P. hallii* and grown under similar conditions (Table 1). The quick seed to seed time and smaller stature of *P. hallii* when compared to switchgrass means that more plants can be cycled more quickly in the same amount of space that fewer switchgrass plants could be grown and cycled. The plant cell wall type of switchgrass is a cell wall type 2 (Sarath et al. 2008), and since other closely related forage grasses have cell wall type 2 (Akin 2008) it can be assumed that *P. hallii* exhibits cell wall type 2, but chemical testing will be needed to confirm. Additionally, both plants exhibit C_4 photosynthesis (Waller and Lewis 1979).

Current practices on transformation in switchgrass are time-consuming and can take around four months for the establishment of callus (Denchev and Conger 1995), three months for regeneration of soil-based transgenic plants (Li and Qu 2011), and one-and-a-half years for production of T1 plants (Casler et al. 2011). Therefore, identification of a C_4 plant with a small genome, relative ease in transformation, and a fast and efficient life cycle can greatly increase productivity when experimenting with functional genomics in a C_4 grass. Therefore, the development of a transformation system for *P. hallii* would allow for functional genomics studies in a plant closely related to switchgrass, with fewer gene repeats and a faster life cycle.

From an evolutionary perspective, *P. hallii* has many of the characteristics a model plant. Tissue culture protocols have previously been reported for *P. hallii*, with callus induction frequencies for mature seeds ranging from 49.9-96.7% depending upon accession (Seo et al. 2008). Shoot regeneration frequency has been reported as 8.3 ±4.2% resulting in an average of 1.50 ±0.29 shoots per callus (Seo et al. 2010). These studies leave room for improvement as well as optimization for immature seeds or endosperm derived tissue. Moreover, a transformation system capable of producing regenerate transgenic plants with relative ease is necessary for the consideration of *P. hallii* as a potential C_4 model. Additionally, a database of mutant model plants would provide an easily accessible resource for scientists studying switchgrass, in much the same way as the *Arabidopsis* mutant database (Parinov et al. 1999). The development of a swift and optimized tissue culture system and high frequency transformation procedure would cement *P. hallii* as a model C_4 system.

P. hallii possesses the necessary evolutionary proximity to switchgrass, shares cell wall type 2 architecture and C_4 photosynthesis, and boats traits similar with current model plants. However, the potentiality of *P. hallii* as a model system for switchgrass studies has not been tested. To be properly considered as a model, tissue culture of *P. hallii* will need to be optimized to be competitive with switchgrass in both callus initiation and plant regeneration. After optimization of tissue culture, susceptibility to genetic transformation can be evaluated. The optimization of *P. hallii* tissue culture methods are the first step in evaluating *P. hallii* as a model system for switchgrass.

CHAPTER II – TISSUE CULTURE

Joshua N. Grant, Jason N. Burris, C. Neal Stewart, Jr., and Scott C. Lenaghan were the authors for this paper. All authors contributed to the writing of the manuscript that has been submitted for publication to BMC Biotechnology. Joshua N. Grant, developed and carried out the experiments. Jason N. Burris assisted in designing experiments and evaluating success and failure. C. Neal Stewart, Jr. provided funding, and guidance. Scott C. Lenaghan provided input on statistical tests, experimental design, and guidance. The manuscript is presented below in its submitted form.

Improved tissue culture conditions for the emerging C_4 model *Panicum hallii*

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Abstract

Background

Panicum hallii Vasey (Hall's panicgrass) is a compact, perennial C_4 grass in the family Poaceae, which has potential to enable bioenergy research for switchgrass (*Panicum virgatum* L.). Unlike *P. hallii*, switchgrass has a large genome, allopolyploidy, self-incompatibility, a long life cycle, and large stature—all suboptimal traits for rapid genetics research. Herein we improved tissue culture methodologies for two inbred *P. hallii* populations: FIL2 and HAL2, to enable further development of *P. hallii* as a model C_4 plant.

Results

The optimal seed-derived callus induction medium was determined to be Murashige and Skoog (MS) medium supplemented with 40 mg L^{-1} L-cysteine, 300 mg L^{-1} L-proline, 3% sucrose, 1 g L^{-1} casein hydrolysate, 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 45 µg L^{-1} 6-benzylaminopurine (BAP), which resulted in callus induction of 51 ±29% for FIL2 and 81 ±19% for HAL2. The optimal inflorescence-derived callus induction was observed on MP medium (MS medium supplemented with 2 g L^{-1} L-proline, 3% maltose, 5 mg L^{-1} 2,4-D, and 500 µg L^{-1} BAP), resulting in callus induction of 100 ±0.0% for FIL2 and 84 ±2.4% for HAL2. Shoot regeneration rates of 11.5 ±0.8 shoots/gram for FIL2 and 11.3 ±0.6 shoots/gram for HAL2 were achieved using seed-induced callus, whereas shoot regeneration rates of 26.2 ±2.6 shoots/gram for FIL2 and 29.3 ±3.6 shoots/gram for HAL2 were achieved from inflorescence-induced callus. Further, cell suspension cultures of *P. hallii* were established from seed-derived

callus, providing faster generation of callus tissue compared with culture using solidified media (1.41-fold increase for FIL2 and 3.00-fold increase for HAL2).

Conclusions

Aside from abbreviated tissue culture times from callus induction to plant regeneration for HAL2, we noted no apparent differences between FIL2 and HAL2 populations in tissue culture performance. For both populations, the cell suspension cultures outperformed tissue cultures on solidified media. Using the methods developed in this work, *P. hallii* callus was induced from seeds immediately after harvest in a shorter time and with higher frequencies than switchgrass. For clonal propagation, *P. hallii* callus was established from R1 inflorescences, similar to switchgrass, which further strengthens the potential of this plant as a C_4 model for genetic studies. The rapid cycling (seed-to-seed time) and ease of culture, further demonstrate the potential utility of *P. hallii* as a C_4 model plant.

Keywords

C_4 model; tissue culture; *Panicum hallii*; *Panicum virgatum*; regeneration; recalcitrance; suspension culture

Background

Switchgrass, *Panicum virgatum* L., is a perennial C_4 grass native to North America, which has shown promise as a cellulosic bioenergy feedstock (Sanderson 2006). As a feedstock, switchgrass is attractive in that it produces high biomass (McLaughlin and Adams-Kszos 2005) with relatively low farmer input in a wide range of temperate climates (Moser and Vogel 1995). The bioenergy potential of switchgrass has led to the development of numerous tissue culture and transformation protocols (King et al. 2014; Ramamoorthy and Kumar 2012; Li and Qu 2011; Xi et al. 2009; Solmleva et al. 2002; Denchev and Conger 1995), along with a draft genome available from the United States Department of Energy (DOE) Joint Genome Institute (JGI, http://www.jgi.doe.gov/genome-projects). Transgenic switchgrass plants have been developed for improved cell wall biosynthesis traits for biofuel production, for example, the overexpression of transcription factors (Shen et al. 2012) and the use of RNAi-mediated knockdowns (Fu et al. 2011). However, like many crops, switchgrass transformation, while reliable, takes around six months from callus induction to regeneration of plants (Xi et al. 2009). Further, switchgrass is self-incompatible, which, along with its large genome (Casler 2012) and allopolyploidy result in complicated genetic analysis scenarios (Lu et al. 2013). Therefore, a reverse genetics pipeline could be enhanced by the identification of an appropriate fast cycling C_4 model plant to speed the development of the next-generation switchgrass.

As a potential C_4 model plant, *P. hallii* displays many desirable qualities: it is small in stature (average mature heights of accessions are 35.6-65.7 cm), has a small genome (453- 550 Mb), and a rapid life cycle (seed-to-seed time of 40-90 d) (Lowry et al. 2015; Lowry et al. 2013). Further, *P. hallii* can produce somatic embryogenic callus from seed within 35-50 d, compared to 120 d for switchgrass (Somleva 2007). Previous

studies on *P. hallii* have focused on the development of microsatellite markers (Lowry et al. 2012), analysis of gene expression and transcriptomics (Meyer et al. 2012), exploration of biodiversity within the species (Lowry et al. 2013), and the genetic divergence of ecotypes (Lowry et al. 2015). Additionally, a tissue culture (Seo et al. 2008) and regeneration system (Seo et al. 2010) for mature seeds (> 1 year old) of *P. hallii* has been developed and compared with other *Panicum* species. The goal of the current study was to develop facile and robust tissue culture methodologies for *P. hallii* using inflorescences, fresh seeds (< 6 months old), and cell suspension cultures.

Methods

Plant material and reagents

Seeds from inbred populations of *P. hallii* var. *filipes* (Scribn.) Waller (PAHAF) and *P. hallii* Vasey var. *hallii* (PAHAH), designated FIL2 and HAL2 were kindly donated by Dr. Tom Juenger and colleagues at the University of Texas at Austin (Lowry et al. 2015). Plants generated from these seeds were grown in greenhouses, selfed, and their progeny yielded seeds for subsequent experiments. All plants were grown under a 16 h photoperiod, and mature panicles were lightly shaken to assist self-fertilization and seed set. Seeds were collected and plated on various media in a randomized block design. For inflorescence-derived callus, inflorescences were collected from plants at the onset of bolting before panicle emergence. Callus generated from inflorescences of a tissue culture elite switchgrass control, Performer 605 (PVP-605), was used for comparison in all experiments.

Basal media components complete with vitamins of Murashige and Skoog (MS), Kao & Michayluk (KM8), and Chu's N6 (NB) were obtained from PhytoTechnology Laboratories (Shawnee Mission, KS, USA). Media components for LP9 (Burris et al. 2009) and AA (Muller and Grafe 1978) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All media components were mixed and contained 30 g L^{-1} of sucrose (Thermo Fisher Scientific, Waltham, MA, USA) or maltose (Sigma-Aldrich, St. Louis, MO, USA). The plant hormones used in the following experiments were 2,4-dichlorophenoxyacetic acid (2,4-D) (PhytoTechnology Laboratories), 6-benzylaminopurine (BAP) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), and gibberellic acid (GA3) (Sigma-Aldrich, St. Louis, MO, USA). For solidified media, Phytagel (3 g L^{-1}, Sigma-Aldrich, St. Louis, MO, USA) was added before autoclaving, and 15 mL were poured into Petri dishes and solidified under aseptic conditions in a laminar flow hood.

Seed germination and sterilization

Seeds immediately harvested from greenhouse grown plants and seeds stored for > 1 year were tested for germination efficiency with and without seed coat removal (Seo et al. 2008). To remove the seed coat, chaff was manually separated from seeds, and 300 grit sandpaper was used to abrade the seed coat (Juenger, personal communication). Three replicates consisting of 33 seeds per plate were used to determine the germination efficiency. Prior to plating on MS medium with no sugar or

hormones (Diet-MS), seeds were suspended in 0.5 mL of either sterile water or a filter-sterilized solution of 1.44 μM GA3. Seeds were then pipetted onto plates and incubated at 24˚C in either the dark or the light. Coleoptile emergence was monitored weekly for three weeks; germination frequency was calculated as the number of seeds with an emerging coleoptile divided by the total number of seeds on the plate. After determining the best method for germination, surface sterilization methods were tested using two treatments: a combination of 5% dilution of commercial sodium hypochlorite bleach and 70% ethanol (Treatment 1, Juenger, personal communication) or a modified chlorine gas protocol (Treatment 2, (Muller and Grafe 1978)). For Treatment 1, seeds were immersed in 5% bleach and agitated for one minute, then transferred to 70% ethanol and agitated for one minute before being washed five times with sterile water. For Treatment 2, seeds were placed into 1.5 mL microfuge tubes up to the 0.1 mL mark. Tubes, with their caps open, were then placed in an air-tight chamber with 33 mL of bleach in a fume hood. Next, 1 mL of 12 N HCl was added to the bleach before sealing the air-tight chamber. Seeds were left in the chamber for 16 h before being transferred to a laminar flow hood for de-fumigation for 15 min. Seeds were then immediately placed onto Diet-MSO. Seed sterilization efficiency was determined by calculating both germination frequency and scoring the seeds for the presence or absence of contamination around an individual seed after six weeks. To determine significance between the two treatments, Student's T-tests were conducted, as described in the statistical analysis.

Media optimization

We assessed the performance of *P. hallii* callus induction and proliferation using media defined from the monocot tissue culture literature: AA (Toriyama and Hinata 1985) and KM8 (Kumlehn and Nitzsche 1996), LP9 (Burris et al. 2009), MS (Murashige and Skoog 1962), MS-OG (Lee et al. 2006), MS-BH (Shatters and Wheeler 1994), MS-PM (Oldach et al. 2001), MS-SC (Larkin 1981), MS-SEO (Seo et al. 2008), MP (Li and Qu 2011), and MP-PAH, a novel medium developed in this work based on preliminary experimentation with *P. hallii* (Table 2). Germination efficiency, percent callus induction, callus type (I-IV), callus proliferation, and regeneration frequency were determined for each medium. Callus induction frequency was calculated in triplicate using 33 seeds per plate per medium. Plates were examined weekly for callus formation from each individual seed, and the number of seeds producing callus was recorded. The type of callus was scored on the following scale: type I was hard, compact, and white; type II was friable, hard, and light yellow; type III was fast-growing, mucilaginous, and yellow to white; type IV was spongey and slow-growing. Callus proliferation at a range of temperatures (20, 24, 28, 32, and 36˚C) was measured on four replicates, each containing 3 g of callus. The fresh weight of callus was taken 4 weeks after induction. Callus induced on each medium was subdivided into three replicates of 3 g each to conduct growth rate analysis. Callus growth was measured after four weeks by mass gained. The plant regeneration experiment tallied the number of shoots from three replicate plates, each containing 1 g callus, by medium (Table 3). All regeneration media were based on MS at pH 5.8, with a few modifications: REG contained 30 g L^{-1}

maltose, 40 mg L^{-1} BAP, 485 µg L^{-1} GA3; REG-SEO contained 30 g L^{-1} maltose, 4.8 mg L^{-1} naphthalene acetic acid (NAA), and 990 µg L^{-1} GA3 REG-R contained 4.8 mg L^{-1} NAA and 485 µg L^{-1} GA3**;** REG-SEO-R contained 4.8 mg L^{-1} NAA; diet-MS contained no sugars or hormones. Regeneration frequency was calculated as number of shoots per callus piece and number of shoots per gram. The optimal medium was determined by evaluating the performance of each medium for callus induction rate, callus type, and plant regeneration.

Suspension culture

All media used in the tissue culture experiments were evaluated for establishment of suspension cultures. Suspension cultures were initiated by placing 2.5 g of macerated, heterogeneous callus into 100 mL flasks, containing 30 mL of each medium type, with weekly subcultures for 4 weeks until suspension cultures were established. Initial subcultures were conducted by allowing cells to settle at room temperature for about 10 min, removing supernatant, and resuspending them in 30 mL of fresh medium. Flasks containing 30 mL of medium with no tissue were used as a control for media evaporation, with media being exchanged weekly. All experiments were performed in triplicate. Cell suspension characteristics were analyzed using the following methods: dissimilation curves to measure growth characteristics (Schripsema et al. 1990), packed cell volume to quantify total growth after 30 d (Ho and Vasil 1983), cell viability through fluorescein diacetate-propidium iodide (FDA-PI) simultaneous double-staining (Jones and Senft 1985), and cell size distribution using image analysis of micrographs (Ibaraki and Kenji 2001).

Dissimilation curves were measured for 30 d by comparing the daily evaporation relative to the sentinel flasks to the daily mass change in the inoculated flasks. The average evaporation of each control flask was taken daily and added back to the difference between the previous day and current day mass for each corresponding media. $D = (S_P - S_C) + (C_P - C_C)$, where D is the dissimilation of carbon from the sugar source, S_C is the sample's current day mass, S_P is the sample's previous day mass, C_P is the control's previous day mass, C_C is the control's current day mass. Subcultures were made every two weeks by transferring to a 50 mL Falcon tube, centrifuging for 10 min at 150 x *g* at room temperature, removing spent medium, and resuspending with 30 mL of medium. Packed cell volume (PCV) was measured by taking three 1 mL aliquots for each medium after 30 d and centrifugation for 10 min at 150 x *g* at room temperature and measuring the volume of the cell pellet. Cell viability was confirmed after 30 d by taking 1 mL aliquots from each flask and staining with 10 µL of a 0.1% FDA solution and 5 µL of a 0.2% PI solution. Eppendorf tubes were covered with aluminum foil and vortexed on low speed for 30 seconds. After incubation in the dark for 5 min two 10 µL replicates were examined on a hemocytometer. This method generated three biological replicates with two technical replicates for each treatment. Cell viability was calculated as a percentage of live cells out of the total number of cells. Cell size distribution was calculated by placing 10 mL of cell suspensions from each flask into a canted 25 cm^2 flat-bottomed flask and observing 100 cells using an inverted microscope. Then, 100 cells were measured using the image analysis software package FIJI (Schindelin et al.

2012). Cells were analyzed for total area and length to width ratio. Length to width ratio was calculated by taking the larger measurement as length and the smaller measurement as width.

Regeneration of plants from suspension cells was conducted by two treatment methods. Treatment 1 involved removing all media from suspension cells, washing the cells three times in a medium containing no hormones followed by resuspension of cells in one of two regeneration media: REG or REG-SEO. A secondary regeneration experiment was performed by transferring intact callus pieces back onto solid MS-OG for two weeks before attempting regeneration on solidified medium. Populations were analyzed separately and treatments were compared using a two-way ANOVA controlling for initiation medium and regeneration medium.

Direct comparison with published methods

The tissue culture methods developed in this work were directly compared to previously published methods across three parameters: callus induction, callus proliferation, and regeneration. Seeds stored for greater than one year and seeds immediately harvested from the greenhouse were sterilized using chlorine gas and plated onto either MS-OG or MS-SEO and analyzed weekly for eight weeks to score callus induction. Callus was then weighed and checked for proliferation by weighing callus at each subculture for four weeks. Eleven pieces of type II callus were selected for regeneration from each medium. Regeneration was scored weekly for four weeks. All statistics were analyzed by population type and controlling for medium using a one-way ANOVA. If significant differences were observed in the ANOVA at $p=0.05$, then mean separation was calculated using Tukey's Honest Significant Difference.

Callus induction from inflorescences

Callus was induced from inflorescences using previously described methods (Alexandrova et al. 1996), in which immature inflorescences were surface sterilized using 5% dilution of commercial bleach for 30 min and 70% ethanol for 10 min, before being cultured for two weeks on MSB (MS supplemented with 4 mg L^{-1} BAP, 3% maltose, and 2 g L^{-1} Phytagel). After two weeks, pre-cultured inflorescences were chopped into < 5 mm pieces and transferred onto MP medium (Li and Qu 2011) or MS-OG (Lee et al. 2006) medium, and callus was transferred bi-weekly after an initial four weeks of culture. After a total of eight weeks, eleven callus pieces were weighed and placed onto REG medium to determine the regeneration efficiency.

Statistical analysis

All statistical analysis was performed using R 3.3.0 (R Core Team, Vienna, Austria). Tukey's Honest Significant Difference was calculated using the package AGRICOLAE.

Results and discussion

Seed germination and sterilization

Grass species typically have mechanisms of seed dormancy (Simpson 2007), which often require seed coat scarification to break dormancy (Adkins et al. 2002). The most effective method for breaking seed dormancy of HAL2 seeds was found to be the removal of the seed coat with 300-grit sandpaper and germination in the dark (45.8 ±2.4%; $p < 0.05$). Seed coat removal did not affect germination of FIL2 seeds. The chlorine gas sterilization procedure appeared to be effective each instance, whereas minor microbial contamination was observed in cultures after the bleach treatment. Therefore, chlorine gas was used subsequently for seed sterilization. Our standard germination procedure was established to remove seed coats from HAL2, but not from FIL2 before sterilizing with chlorine gas, followed by germination in the dark.

Media optimization

Experiments with various media indicated that NB medium promoted germination in HAL2 better than any other media type (Figure 2A, Table 4), with a rate 81.8 ±1.7%, $p < 0.05$. However, seeds plated on NB failed to produce any callus (Figure 2B). Seeds germinated on LP9 at 19.7 ±3.9% (FIL2) and 10.1 ±2.3% (HAL2), whereas callus was induced at 54.6 ±12.0% (FIL2) and 64.1 ±3.6% (HAL2); seeds germinated on MS-OG at 67.7 ±2.7% (FIL2) and 17.2 ±7.9% (HAL2) and callus was induced at 67.7 ±2.7% (FIL2) and 81.8 ±8.0% (HAL2). Seeds placed on MS-SEO had a high germination rate (50.5 ±10.7% for FIL2; 45.5 ±3.5% for HAL2), and a high induction rate (52.5 ±5.3% for FIL2; 53.5 ±6.1% for HAL2).

Next, callus type was scored and calculated as a percent of total callus induced for each media type (Figure 2C). The apparent best medium for type II callus induction for FIL2 was MS-Sucrose (66.7 ±1.7%) with a $p < 0.05$. The top performers of type II callus induction for HAL2 was MS-OG (70.7 ±23.5%), MS-Sucrose (67.7 ±3.6%), MP (53.0 ±7.6%), and LP9 (42.3 ±2.6%), with no significant differences among those treatments. No type IV callus was induced during this experiment, so the analysis only focused on callus types I, II, and III. For the next experiments, only LP9, MP, MP-PAH, MS-BH, MS-Maltose, MS-OG, MS-PM, MS-SEO, and MS-Sucrose were selected, since they resulted in the production of type II callus in both populations. In addition, the optimal temperature of callus production was 24-28 ˚C using MS-OG medium with a significant increase ($p < 0.05$) in mass of 2.14 ±0.17 g (FIL2) and 2.36 ± 0.22 g (HAL2) compared to other temperatures tested.

More shoots per callus were produced in MS-OG medium: 3.8 ±0.3 shoots per callus for FIL2 and 4.6 ±1.0 shoots per callus for HAL2 (Figure 2D), which was significantly different from all other treatments ($p < 0.05$). MS-OG and LP9 media were optimal for FIL2 callus growth (7.9 ±0.3 g and 6.2 ±0.44 g, respectively). HAL2 callus responded to multiple media with no significant difference among the top four media: MS-OG, LP9, MP, and MS-Sucrose (Figure 2E). Even though MS-OG medium was equivalent to those media just listed, it was superior in type II callus induction, and

resulted ultimately in more regenerated shoots than the other media tested. Therefore, we chose MS-OG medium for subsequent experiments.

MS-OG medium was used then to test effects of various 2,4 D concentrations on callus growth (Figure 3). After 35 d, 0.75 mg L^{-1} 2,4-D, with callus subcultured weekly, performed better than all other auxin treatments for FIL2, which produced a callus area of 9.6 ±1.2 cm^2 when comparing populations separately under a one-way ANOVA controlling for treatment and analyzed at $p < 0.05$. However, HAL2 produced the same callus areas under treatments of 0.75 mg L^{-1} 2,4-D auxin with weekly callus subculture (8.7 ±1.3 cm^2), 3 mg L^{-1} 2,4-D auxin with bi-weekly callus subcultures (8.8 ±1.4 cm^2), and 3 mg L^{-1} 2,4-D with no callus subcultures (8.8 ±1.3 cm^2). HAL2 callus generated from the 0.37 mg L^{-1} 2,4-D auxin treatment was derived mainly from the coleoptile, therefore these results might have skewed the analysis. The treatment of 0.75 mg L^{-1} 2,4-D subcultured weekly led to increased callus production in FIL2 (9.6 ±1.2 g) (Figure 3A), but there was no significant difference for this treatment and the 3 mg L^{-1} 2,4-D treatment for HAL2 (6.8 ±1.4 g, FIL2; 8.7 ±0.7 g, HAL2). Analysis of the callus type induced for each treatment (Figure 3A) indicated that after 35 d, the highest percentage of type II callus was obtained using the 3 mg L^{-1} 2,4-D auxin concentration (23.7 ±2.3%, FIL2; 24.7 ±2.4%, HAL2) regardless of subculture frequency. Most callus induced by the 0.75 mg L^{-1} 2,4-D treatment, subcultured weekly, was type III callus (88.1 ±7.5%, FIL2; 73.3 ±6.6%, HAL2). Therefore, the optimal protocol for tissue culture of *P. hallii* was to induce callus for two weeks on MS-OG containing 3 mg L^{-1} 2,4-D auxin, and then subculture bi-weekly indefinitely on the same medium.

The type of callus (I-IV) is perhaps the most important factor in tissue culture methods. In grasses, type II callus has optimal embryogenic capacity (Burris et al. 2009; Denchev and Conger 1994). In our experiments, we determined that two callus types readily produced shoots: type I and type II. Type III callus rarely led to plant regeneration and type IV callus never regenerated (Figure 3B). The auxin 2,4-D is used in the tissue culture of grass species in varying concentrations: 20 mg L^{-1} for *Paspalum scrobiculatum* (Vikrant 2003), 5 mg L^{-1} for switchgrass (Burris et al. 2009), and 10 mg L^{-1} for *Panicum maximum* (Lu and Vasil 1982), thus our results are on the low end of the requirement for panicoid grasses.

Prolonged subculturing of HAL2 callus introduced a mucilaginous covering of callus cultures after twenty weeks that appeared to be associated with decreased callus proliferation (Figure 4). While HAL2 callus proliferated more quickly than FIL2, it also declined in proliferation between the 18th and 20th weeks (Figure 4), suggesting that the tissue should not be used after this time. FIL2 callus biomass doubling per week after week 24, while HAL2 began doubling in biomass after week 14.

Suspension culture

Dissimilation curve data (Figure 5) generated from suspension cultures established on each medium from the earlier screen demonstrated that MS-OG provided the best tissue growth. PCV data indicated that MS-OG was the optimal medium for suspension cultures (Figure 6). MS-OG appeared to be ineffective just after culture establishment, however, MS-OG enabled cultures to metabolize the most

amount of carbon when compared with cultures on other media after 30 d of culture (3.84 ±0.2 g, FIL2; 4.58 ±0.3 g, HAL2). Further analysis of the packed cell volume (Figure 6A**)** indicated that MS-OG (0.72 ±.023 mL), LP9 (0.66 ±0.041 mL), MP-PAH (0.65 ±.046 mL), & MS-SEO (0.56 ±0.023 mL) were not significantly different for the FIL2 population while suspensions maintained in MS-OG had the greatest packed cell volume for the HAL2 population at 0.82 ±0.029 mL ($p < 0.05$). Dual staining with PI-FDA (Figure 7) indicated that MS-OG had the highest viability at the tested time-point for FIL2 (57.2 ±3.4%, $p < 0.05$) and that there was no significant difference in LP9 (42.1 ±3.6%), MP (44.7 ±2.6%), MP-PAH (44.3 ±3.1%), and MS-OG (48.0 ±2.1%) for the HAL2 population. Unfortunately, plant regeneration from suspension culture in liquid medium was not observed in any treatments. However, shoot regeneration was observed when callus was re-established post cell culture by placing suspension cultures onto their corresponding medium followed by transfer to regeneration medium.

Suspension cultures allow for the generation of clonal variation within a single genotype (Larkin and Scowcroft 1981) more quickly than tissue culture. Plant suspension cultures provide both faster growth than tissue culture and the ability for simple production, isolation, and purification of foreign proteins (Hellwig et al. 2004). Suspension cultures can be synchronized (Kumagai-Sano et al. 2007) to obtain a homologous population of cells, thereby allowing experimentation on cell physiology, biochemistry, and metabolic events at the cellular level. A cell suspension culture can also aid in mutagenesis studies using CRISPR/Cas9 (Mercx et al. 2016) or chemicals such as ethyl methanesulfonate (Acanda et al. 2014). The only downside to the system proposed here is that plant regeneration cannot occur directly from suspension cultures, which requires an extra solidified tissue culture step prior to plant regeneration.

Direct comparison with published methods

Seeds stored over a year were not significantly different in either germination frequency or callus induction rates between MS-OG and MS-SEO for FIL2 when compared via a Student's *t*-test at $p < 0.05$, however HAL2 seeds aged > 1 yr germinated and induced callus more frequently on MS-SEO. When populations were analyzed separately under a two-way ANOVA controlling for seed age and medium, germination and induction rates for seeds aged > 1 yr were statistically similar for FIL2 yet statistically different for HAL2 when compared at $p < 0.05$ (Figure 8A&B). Seeds immediately harvested from the greenhouse were statistically different regardless of population for either medium, with MS-OG consistently outperforming MS-SEO in both germination and callus induction: FIL2 germination rates increased from 8.0 ±2.0% for MS-SEO to 23 ±1.5% for MS-OG and HAL2 germination rates increased from 11 ±2.3% for MS-SEO to 49 ±2.3% for MS-OG, while FIL2 induction rates increased from 7.0±2.1% for MS-SEO to 32 ±2.5% for MS-OG and HAL2 induction rates increased from 10 ±1.5% for MS-SEO 49 ±2.3% for MS-OG (Figure 3A&B). L-proline has been shown to promote somatic embryogenesis in maize (Armstrong and Green 1984; Vasil and Vasil 1986) and rice (Chowdhry et al. 1993), and MS-OG contains 300 mg L^{-1} L-proline while MS-SEO contains none. There was no statistical difference among the callus types generated in MS-OG at either seed age (Figure 8C). While the freshly

harvested seed callus induction rates (51 ±29% for FIL2 and 81 ±19% for HAL2) were not as high as those previously published on mature seeds (49.9% for accession CPI.68864 and 96.7% for accession 85 B-1) (Seo et al. 2008), the method developed in this work allowed for seeds to be used within a week of harvest. The seeds used in previous studies were obtained from the National Institute of Livestock and Grassland Science, Tochigi, Japan and had been preserved at 4˚C for an undisclosed amount of time (Seo et al. 2008). Seed age has been documented as affecting germination (Shaidaee et al. 1969). Since *P. hallii* was evaluated for use as a model system, a yearlong delay to gain an incrementally higher germination rates is not feasible.

A shoot regeneration screen (Figure 9) indicated that REG and Diet-MS were the best media for shoot and root regeneration, respectively, regardless of either callus type I or II. Student's *t*-test indicated that there was no significant difference between FIL2 and HAL2 when evaluated at $p < 0.05$. However, callus type did differ significantly within populations when evaluated with Student's *t*-test at $p < 0.05$. Callus type I could induce shoots on REG at 27 ±1.7 shoots/callus for FIL2 and 26 ±1.73 shoots/callus for HAL2, and callus type II was able to induce shoots on REG at 3.3 ±0.5 shoots/callus for FIL2 and 4.2 ±0.5 shoots/callus for HAL2. Shoot regeneration on REG outperformed the other medium in this experiment, as REG-SEO was only able to produce 0.92 ±0.2 shoots/callus FIL2 and 0.92 ±0.1 shoots/callus for HAL2 for callus type II, which was significantly less ($p < 0.05$) than shoot regeneration on REG when populations and callus types were analyzed separately under a one-way ANOVA controlling for regeneration medium (Figure 9A). For root regeneration (Figure 9B), Diet-MSO optimally induced roots compared with other media tested, with a rooting frequency of 100 ±0% for all callus with shoots for both populations. The data indicated that REG medium was statistically better at shoot induction than REG-SEO, and that Diet-MSO was statistically better at rooting than compared to either other medium when compared at $p < 0.05$.

For MS-SEO, some germinating seeds did not produce callus; conversely for MS-OG, callus was induced from seeds with no germination. Seed-derived callus for most grass species tend to produce callus from a germinated seed, such as with *Poa pratensis* (van der Valk and Zaal 1989). The ability for the callus to be induced without seed germination may occur from endosperm tissue as seen in rice (Nakano et al. 1975) and ryegrass (Kumlehn and Nitzsche 1996). However, callus from this source can be maintained and plants regenerated similarly to meristem-derived callus. The lack of any endosperm-derived callus in MS-SEO may indicate that one of the medium components of MS-OG is necessary to initiate endosperm-derived callus, but this could also be the result of the high ratio of auxin to cytokinin found in MS-OG.

Callus induction from inflorescences

Callus induced from inflorescences performed significantly better ($p < 0.05$) when placed onto MP media under a one-way ANOVA controlling for medium (Figure 10A), with FIL2 proliferating 3.7 ±0.3 g additional weight for MP and only 1.7 ±0.2 g for MS-OG and HAL2 producing 4.1 ±0.1 g for MP and only 1.8 ±0.1 g for MS-OG. MP was further confirmed as a better medium in allowing more shoots per callus piece to be

induced (Figure 10B): FIL2 yielded 11 ±1.7 shoots per callus piece for MP and 3.7 ±2.3 shoots per callus piece for MS-OG while HAL2 trended similarly with 9.8 ±2.4 shoots per callus piece for MP and 1.3 ±0.4 shoots per callus piece for MS-OG ($p < 0.05$). High levels of L-proline are commonly used in media maintaining inflorescence callus (Armstrong and Green 1985; Holme et al. 1997). MP contains 2 g L^{-1} of L-proline, while MS-OG contains 300 mg L^{-1}. In addition, further experimentation should utilize callus induced from inflorescences, as this callus would be genotypically identical to the mother plant as opposed to seeds, which will have genetic variability.

Conclusions

Both inbred populations of *P. hallii* can be cultured using semi-solid medium or liquid suspension cultures. For both populations, the best medium for tissue or suspension culture was MS-OG. These cultures can undergo shoot regeneration on semi-solidified REG medium as quickly as one week for HAL2 and two weeks for FIL2. Root induction occurs with ease when Diet-MSO is used as rooting medium, with 100% of plantlets producing roots. Therefore, the speed with which our system can produce callus from both freshly harvested seed and inflorescences further demonstrates the potential of *P. hallii* as a model C_4 plant. Additionally, this tissue culture procedure can be used to develop a transformation system in which seeds immediately harvested from the greenhouse or inflorescences cut from the plants can be used as explants, thus greatly increasing the speed of experiments. The specific impact of this work is the increased speed with which callus can be generated from either seed or plants.

Declarations

List of abbreviations

2,4-D – 2,4-dichlorophenoxyacetic acid
BAP – 6-benzylaminopurine
FDA – fluorescein diacetate
FIL2 – inbred population of *Panicum hallii* var. *filipes*
HAL2 – inbred population of *Panicum hallii* var. *hallii*
MS – Murashige and Skoog Medium
MSB – MS supplemented with 13.3 μM BAP, 3% maltose, and 2 gL^{-1} Phytagel
PAHAF – Panicum hallii var. filipes
PAHAH – Panicum hallii var. hallii
PI – propidium iodide

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data used in this paper will be available in a single Excel spreadsheet.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

JNG, JNB, CNS, and SCL contributed to the writing of the paper. JNG developed and carried out the experiments. JNB and SCL assisted in experimental design and data analysis.

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CHAPTER III - CONCLUSIONS

Model plants: the past, present, and future

Arabidopsis thaliana was the first widely adopted plant model. Experimentation with *Arabidopsis* began in 1907 with the publication of Friedrich Laibach's Ph.D. dissertation on *Arabidopsis*, however, interest in experimentation and functional genomics did not arise in this species until the mid-1990s with simplified and inexpensive gene cloning methods (Meyerowitz 2001). Nevertheless, *Arabidopsis* had already been in use as a model in fundamental plant biology because of its four week life cycle, and the relatively small genome size of five chromosomes (Rédei 1975; Pruitt and Meyerowitz 1986). As the cost of molecular biology tools declined and their use became more mainstream, funding for molecular research focused on *Arabidopsis* became more available, culminating in the release of its genome sequence in late 2000 (Kaul et al. 2000). The success of the *Arabidopsis* genome sequencing project trumpeted a new era of plant biology: since the release of the *Arabidopsis* genome, many other plant genomes have been sequenced. There was an exponential rise in data describing assembled plant genomes from 2010 to 2014 (Figure 11).

Currently, model plants are used in a variety of ways. Novel genome editing methods such as CRISPR/Cas9 have been incorporated into *Nicotiana benthamiana* (Nekrasov et al. 2013) and *Nicotiana tabacum* BY-2 suspension cultures (Mercx et al. 2016). However, *Arabidopsis* appears to still the preferred model in fundamental plant biology research. The future of model plants is solidified. As technology advances, more complicated experiments can be conducted with models. While there is an opinion that the future of model plants may actually reside in non-model plant species, as current research into single-nucleotide polymorphisms (SNPs) is being conducted on non-model plant species to enhance selection of desired traits in the target organism (Christmas et al. 2016). This technology would assume the target organism's gene regulation follows tendencies established in that of previously reported model plants, thereby negating the further development of new model systems. However, these experiments are dependent upon the organism under review, and that organism may be slow growing, needy, or genetically complex. Further research into plant proteomics and signaling pathways will become more common with technological advancement.

Panicum hallii: potential model for switchgrass and other C_4 grass crops

Panicum hallii can be used for functional genomic studies in C_4 perennial grasses. Our findings strengthen the case for a *P. hallii* C_4 model in several key areas: (1) an improved method for generating somatic embryogenic callus from seeds directly harvested from the greenhouse, (2) improved shoot and root regeneration media, (3) and the ability to be maintained in both solid and liquid cultures. Somatic embryogenic callus can readily be produced from both inbred populations. Our improvement of the existing tissue culture method for *P. hallii* (Seo et al. 2008), includes ability to use seeds fresh from the greenhouse, allowing us to induce callus in about half of the seeds. The ability to use fresh, immature seeds decreases the amount of time required to establish transgenic or mutagenic generations by about a year. Our shoot regeneration method improves on the previous study (Seo et al. 2010), with our method increasing number of shoots per callus by 280% for FIL2 and 220% for HAL2. *P. hallii*'s capability to be

maintained in either solid or liquid cultures demonstrates the flexibility when it comes to culture care and maintenance. Additionally, the liquid culture was able to outperform solid cultures by 420% in mass gained. These accomplishments provide a major stepping stone toward establishing *P. hallii* as a model C_4 plant.

Our results indicate that seed-derived callus can be obtained within one week of seed harvest, and callus can be regenerated as quickly as six weeks after initiation. The ability for genotypes to remain constant in a tissue culture system is a must. Callus can be initiated from inflorescences, thereby providing genotypically identical callus as the parent plant. This callus can then be maintained in either solid or liquid cultures, thus providing an abundance of tissue. The isolation of single genotype cultures for chemical mutagenesis would involve selection of single pieces of somatic embryogenic callus. A single piece of callus would be proliferated into multiple grams of a single genotype. Part of these callus pieces could be subjected to chemical mutagenesis, and then all callus could be regenerated into whole plants. An unmutated genotype would exist with plenty of mutants with which to compare. The ability for *P. hallii* to be maintained in both liquid and solid cultures allows for a variety of experimentation: isolation of single genotype cultures for chemical mutagenesis, single cell transformation, and high-throughput analysis of transgenic or mutant lines.

Further work

Further work could be done to optimize auxin and cytokinin levels after induction of somatic embryogenic callus. The regeneration medium could also be optimized, as the number of shoots per callus piece in switchgrass regeneration are much higher, with hundreds of shoots per callus piece (Liu et al. 2015; King et al. 2014). It is likely that both further optimization of medium components and advanced improvements in germplasm could increase regeneration rates. Additionally, successful *Agrobacterium*-mediated transformation in either a tissue culture, suspension culture, or floral-dip setting could greatly increase the candidacy of *P. hallii* as a model organism. Moreover, biolistic transformation of *P. hallii* could be implemented until *Agrobacterium*-mediated transformation is successful. Currently, switchgrass is primarily transformed in callus tissue cultures with *Agrobacterium* (Li and Qu 2011). However, biolistic transformation of switchgrass has been reported with a high frequency of success (King et al. 2014). Development of a floral-dip method much like that used with *Arabidopsis* would enable a quick generation of transgenic progeny with very little input. Overall, the development of a transformation system is absolutely necessary in order to advance *P. hallii* as a model system.

In addition to a transformation system, genotypes need to be screened for susceptibility to tissue culture. These genotypes would be designated as elite tissue culture lines. Development of elite tissue culture lines could further increase somatic embryogenic callus induction and transformation efficiency. These elite tissue culture lines could be selected based on biolistic or *Agrobacterium*-mediated transformations in addition to further development of both the tissue and suspension culture protocols.

Conclusions

The work herein provides the basic groundwork for development of transformation systems and elite tissue culture lines. Continual cycling of seed to callus to plant to seed will allow for the genetic variation needed to screen for high somatic embryogenesis. Once lines are selected, callus of the desired genotype can be readily produced from inflorescences. One plant could provide a multitude of tissue, with which the transformation systems can be applied. The outlook for *P. hallii* is a positive one, and future experimentation would be quite rewarding.

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APPENDIX

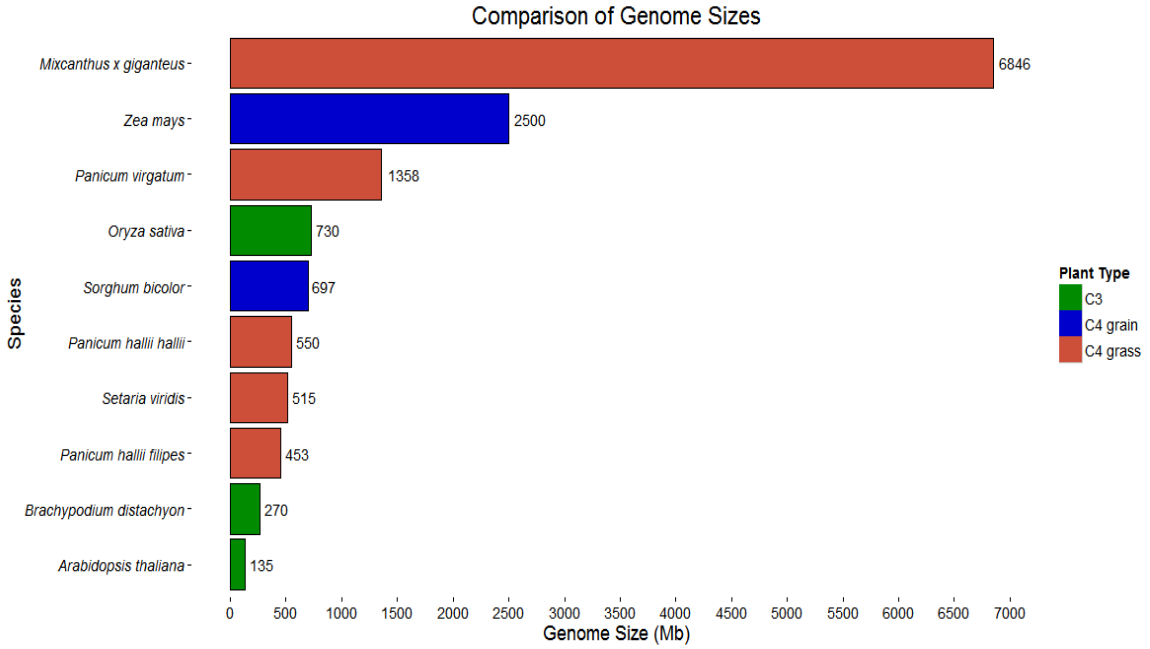

Figure 1. A comparison of genome sizes for bioenergy crops and model plants.

Table 1. Days to seed for two inbred populations of *P. hallii* and the 'Alamo' cultivar of switchgrass for plants grown in the greenhouse. Plants were analyzed separately using a one-way ANOVA (p<0.01). Mean separation was analyzed using Tukey's Honest Significant Difference. Data represents three replicates.

Plant	Days to Seed		Height (cm)	
Panicum hallii var filipes 'FIL2'	87 ± 22	b	75.8 ± 13.85	b
Panicum hallii var hallii 'HAL2'	40 ± 12	c	58.9 ± 14.8	b
Panicum virgatum 'Alamo'	160 ± 32	a	186.2 ± 12.3	a

Table 2. Definition of all media used during the experiment.

		AA	KM8	LP9	MS-OG	MS-BH	MS-PM	MP-PAH	MP	MS-SEO	MS-SC	MS-Sucrose	MS-Maltose	NB
Iron (mg Macro (mg L^{-1})	(NH4)2SO4	130		460										460
	CaCl2*2H2O	150	600	370	590	590	590	590	590	590	590	590	590	380
	KCl		300											
	KH2PO4		160	390	1700	1700	1700	1700	1700	1700	1700	1700	1700	390
	KNO3	2500	1900	2800	1900	1900	1900	1900	1900	1900	1900	1900	1900	2800
	MgSO4*7H2O	1500	300	1300	370	370	370	370	370	370	370	370	370	1300
	NaH2PO4*H2O	150												
	NH4NO3		600		1600	1600	1600	1600	1600	1600	1600	1600	1600	
	FeSO4*7H2O	28	28	29	29	29	29	29	29	29	29	29	29	18
	Na2 EDTA	37	47	41	41	41	41	41	41	41	41	41	41	26
Micro (mg L^{-1})	CuSO4*5H2O	0.025	0.025	0.024	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	H3BO3	3	3	3	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	3
	KI	0.75	0.76	0.76	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.75
	MnSO4*H2O	8.9	10	6.8	15	15	15	15	15	15	15	15	15	6.8
	Na2MoO4*2H2O	0.242	0.243.2	0.242	0.248.6	0.248.6	248.6	0.248.6	0.248.6	0.248.6	0.248.6	0.248.6	0.248.6	0.242
	ZnSO4*7H2O													

Table 2. Continued.

		AA	KM8	LP9	MS-OG	MS-BH	MS-PM	MP-PAH	MP	MS-SEO	MS-SC	MS-Sucrose	MS-Maltose	NB
	BD Bacto Casamino Acids (g/L)			0.5										0.3
	Aspartic Acid	0.27												
	Calcium Pantothenate		1.0											
	Cholecalciferol		0.01											
	Choline Chloride		1.0											
	Citric Acid		40											
	Cyanocobalamin		0.01											
	D-Biotin		0.01											
	DL-Malic Acid		40											
	Folic Acid		0.4											
	Fumaric Acid		40											
	Glycine	7.5												
Organics (mg L^{-1})	Inositol	100	100	100	100	100	100	100	100	100	100	100	100	100
	L-Ascorbic Acid		2											
	L-Cysteine				40						34			
	L-Arginine	170												
	L-Glutamine	880		500							500			500

Table 2. Continued.

		AA	KM8	LP9	MS-OG	MS-BH	MS-PM	MP-PAH	MP	MS-SEO	MS-SC	MS-Sucrose	MS-Maltose	NB
Organics (mg L^{-1})	L-Proline	2000		500	300			2000	2000			2000	2000	
	L-Tryptophan						42				82			
	Nicotinic Acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	p-Aminobenzoic Acid		0.02											
	Pyruvic Acid, Potassium Salt		20											
	Pyridoxine HCl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Niacinamide		1.0											
	Riboflavin		0.2											
	Sodium pyruvate		20											
	Thiamine HCl	10	10	10	10	10	10	10	10	10	10	10	10	10
Sugars (g L^{-1})	Maltose							30	30		30	30		
	Sucrose	20	30	30	30	30	30			30			30	30

Table 2. Continued.

		AA	KM8	LP9	MS-OG	MS-BH	MS-PM	MP-PAH	MP	MS-SEO	MS-SC	MS-Sucrose	MS-Maltose	NB
Hormones (µM)	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.99	4	5	3	2	2.5	2.2	5	3.6	6.7	5	5	2
	6-Benzylaminopurine (BAP)				0.045			2.3	0.5		1.4	0.5	0.5	
	GA3	0.1												
	IAA						1.0							
	Kinetin	0.2					0.5							
pH			5.7	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.7	5.8	5.8	5.8

Table 3. A comparison of regeneration media used.

Component	REG	REG-SEO	REG-R	REG-SEO-R	Diet MSO
Basal Salts	MS	MS	MS	MS	MS
Vitamins	B5	MS	B5	MS	MS
Maltose (g/L)	30	30			
Naphthalene Acetic Acid (NAA) (μM)		26	26	26	
N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea (TDZ)		4.5			
Gibberellic Acid (GA3) (μM)	1.4		1.4		
6-Benzylaminopurine (BAP) (μM)	177.6				
pH	5.8	5.8	5.8	5.8	5.8

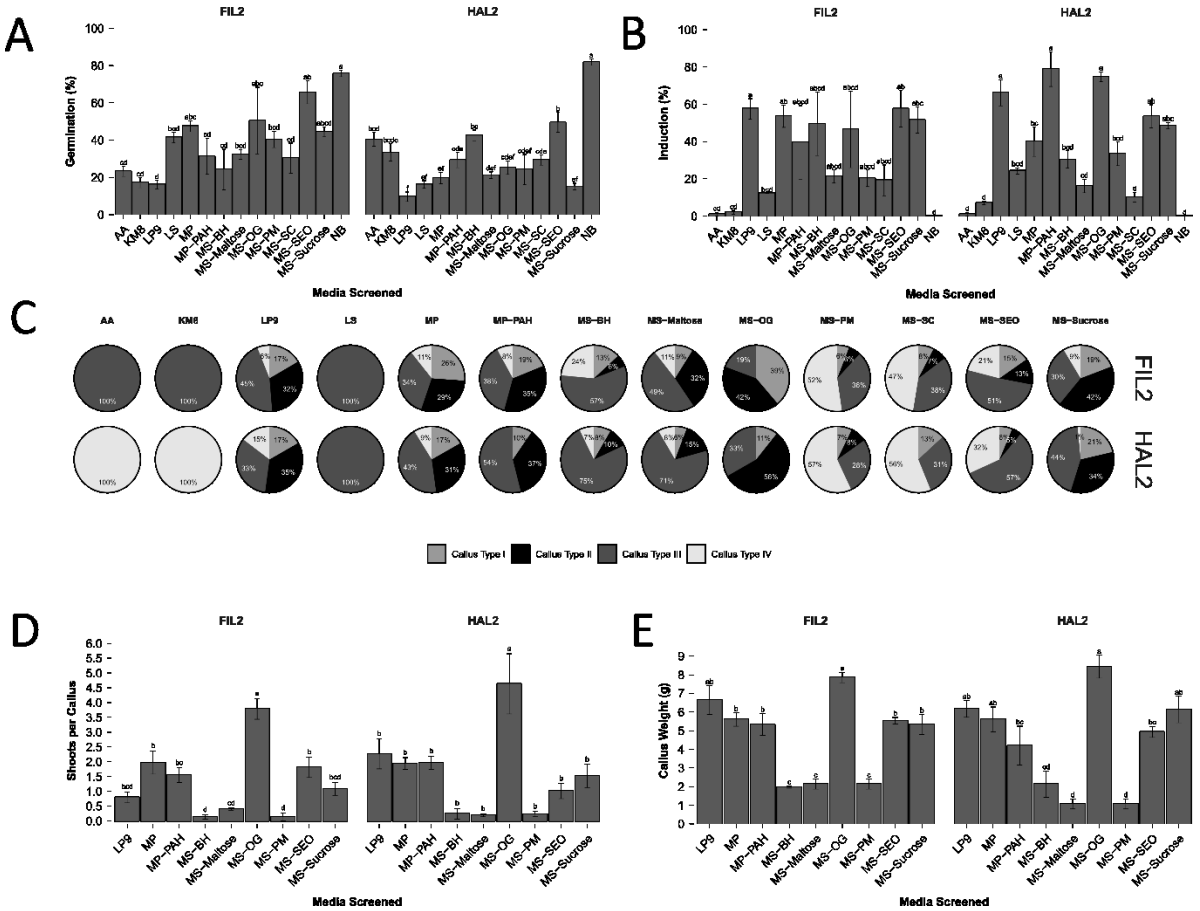

Figure 2. Media screen results. (A) Effect of media on germination. Data represent three replicates of 33 seeds. Populations were analyzed separately under a one-way ANOVA controlling for medium. (B) Effect of media on callus induction. (C) Pie chart showing callus types generated from each medium. (D) Regeneration effect scored as shoots per callus piece for callus induced on each medium. Data represent three replicates of one gram of callus (7-11 pieces). (E) Callus proliferation measured in grams for each medium. Data represent three replicates of two grams of callus. (A, B, D, E) Populations were analyzed separately under a one-way ANOVA controlling for medium. ANOVA tests showed differences among treatments for both HAL2 and FIL2 ($p < 0.01$). Mean separation was analyzed with Tukey's HSD, standard error is shown.

Table 4. Media screen ranking system based on mean separation scores. Induction score was calculated as 'a'=5, 'ab'=4, 'abc'=3, 'abcd'=2, 'bc'=1, and all other rankings = 0. Proliferation score calculated as 'a'=5, 'ab'=4, 'b'=3, 'bc'=2, 'c'=1, and all other rankings = 0. Regeneration score calculated as 'a'=5, 'ab'=4, 'b'=3, 'bc'=2, 'c'=1, and all other rankings = 0.

Population	Medium	Induction Score	Proliferation Score	Regeneration Score	Total Score
FIL2	LP9	5	4	0	9
	MP	4	3	3	10
	MP-PAH	2	3	2	7
	MS-OG	5	5	5	15
	MS-SEO	3	3	3	9
	MS-Sucrose	3	3	0	6
HAL2	LP9	5	4	3	12
	MP	0	4	3	7
	MP-PAH	5	2	3	10
	MS-OG	5	5	5	15
	MS-SEO	4	2	3	9
	MS-Sucrose	3	4	3	10

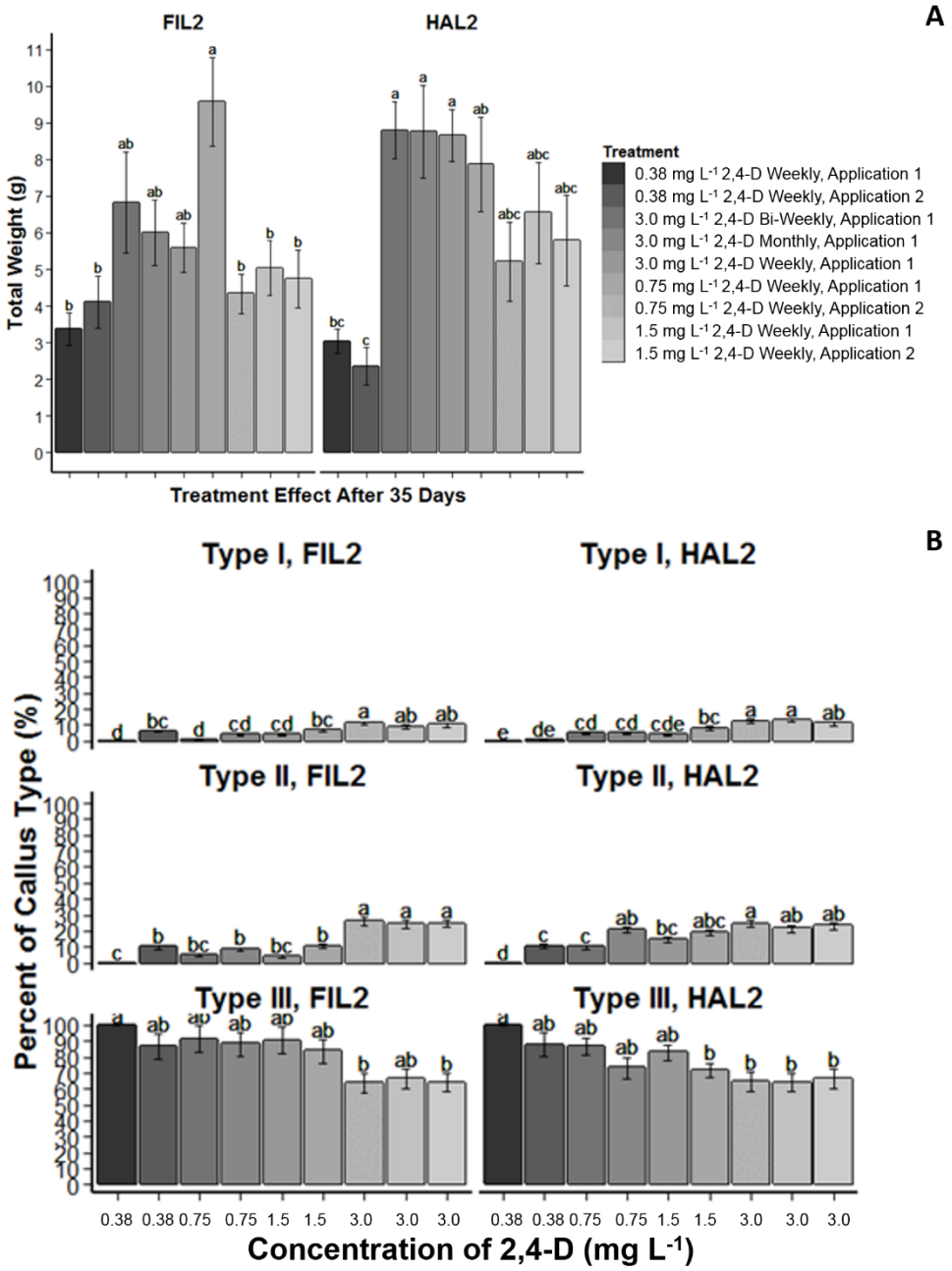

Figure 3. Effect of 2,4-D on callus. (A) Total weight in grams of callus after 35 days of 2,4-D treatment. (B) Type of callus induced on each treatment. Data represent three replicates of 33 callus pieces per replicate. ANOVA test showed differences ($p < 0.05$). Mean separation was analyzed using Tukey's HSD. Standard error is shown.

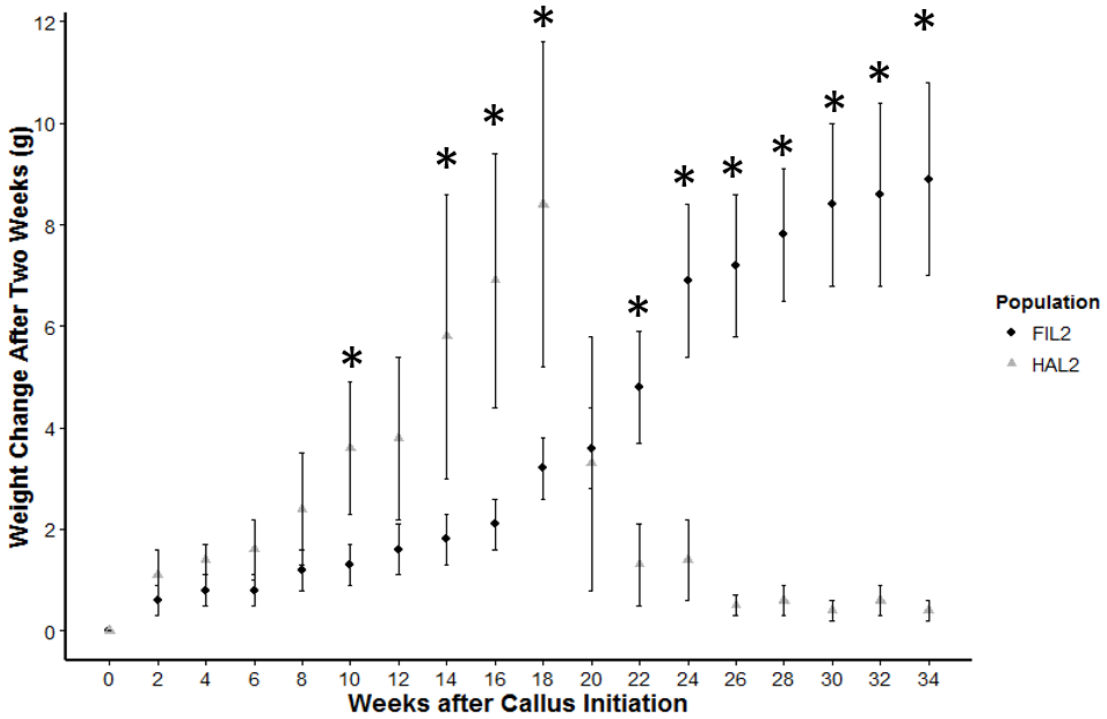

Figure 4. A comparison of weight change every two weeks between FIL2 and HAL2. Each week was analyzed separately under a one-way ANOVA controlling for population. ANOVA test showing differences among populations are marked with an asterisk ($p<0.01$). These data represent ten replicates of three grams of callus at each subculture.

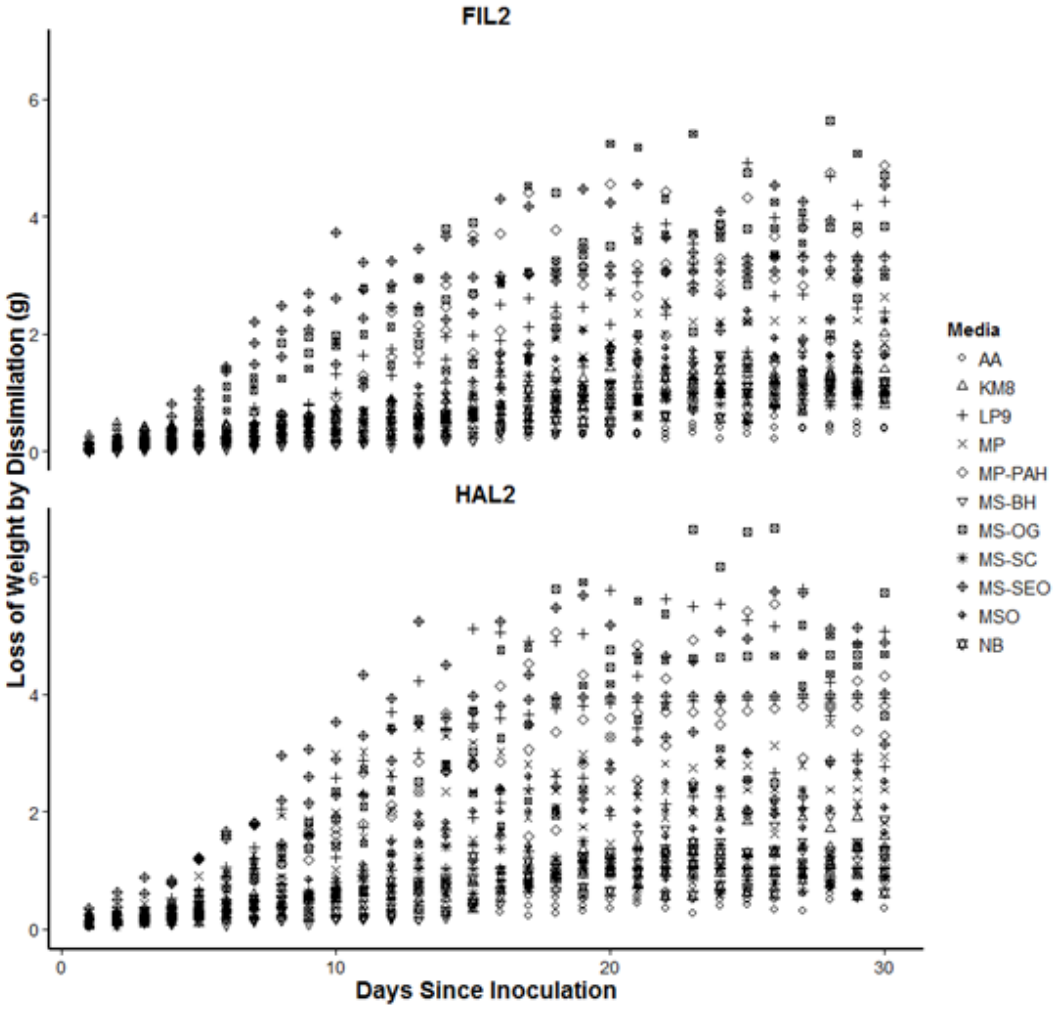

Figure 5. Dissimilation curve. Each point represents one replicate of each measurement.

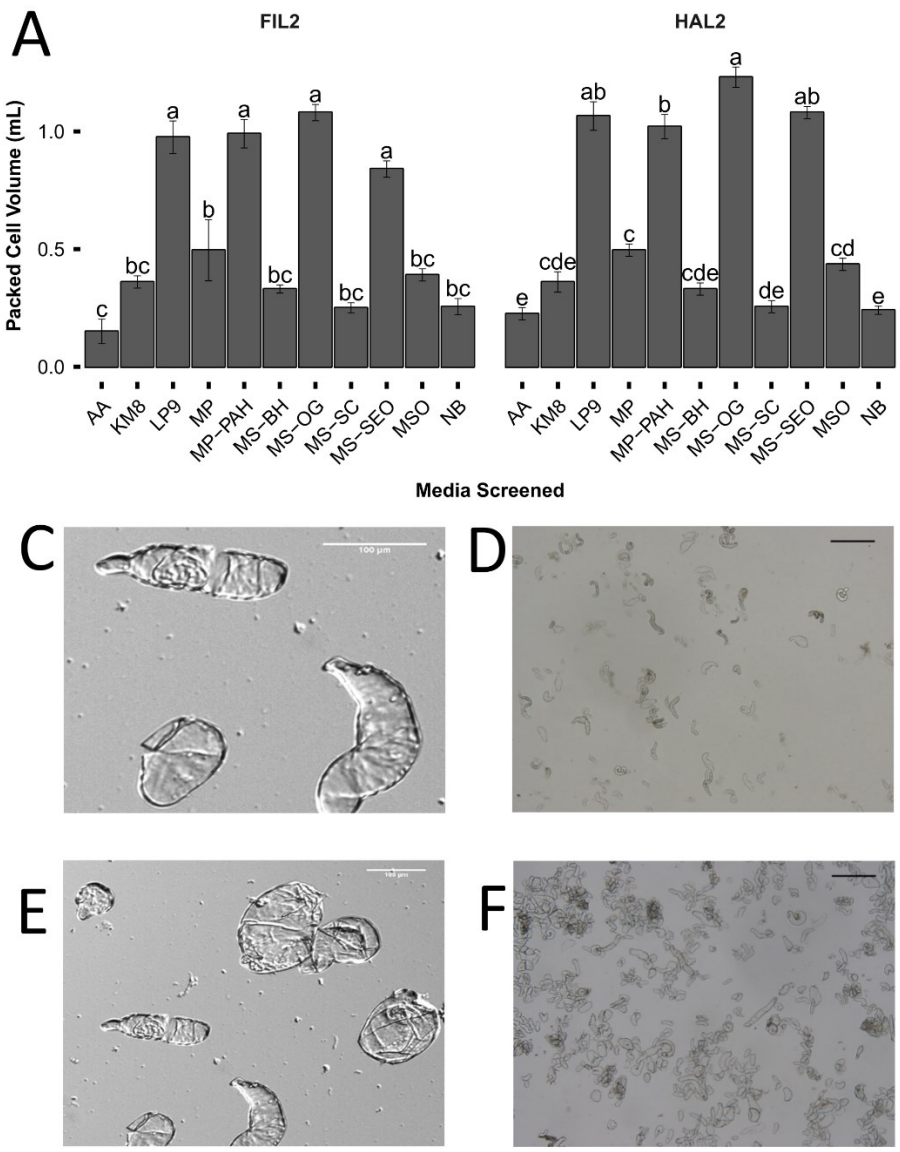

Figure 6. Suspension results. (A) Packed cell volume in mL of three replicates of 1.5 mL suspensions. Populations were analyzed separately under a one-way ANOVA controlling for medium. ANOVA test showed differences among treatments ($p < 0.01$). Mean separation was analyzed using Tukey's HSD. Standard error is shown. (B & C) FIL2 suspension cells. (D & E) HAL2 suspension cells. (D-E) Scale bars represent 100 µm.

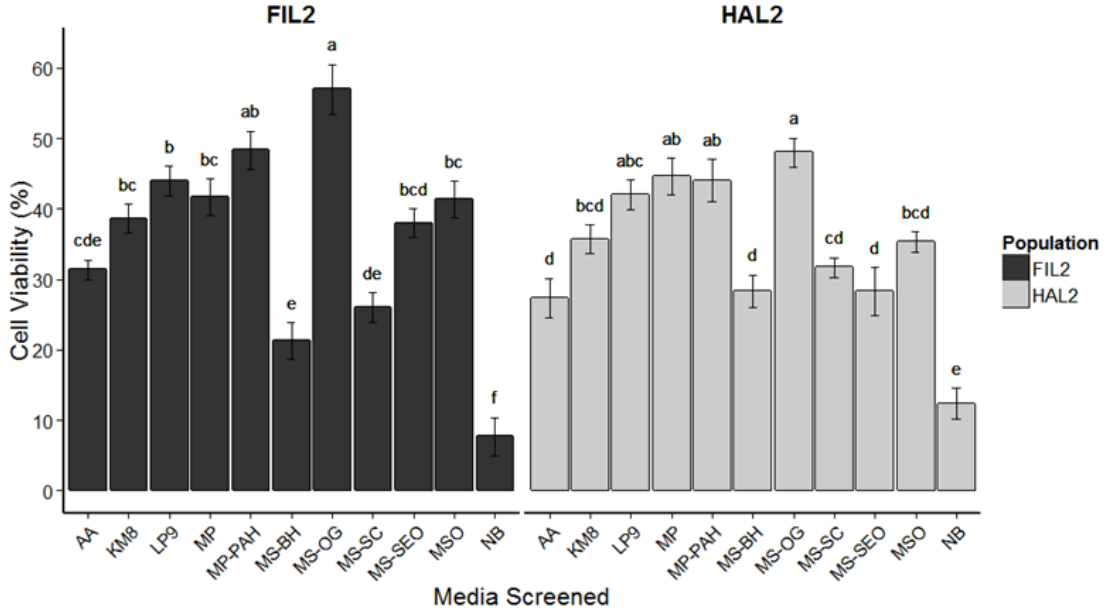

Figure 7. Cell viability as measured by dual staining with FDA and PI. Populations were analyzed separately under a one-way ANOVA controlling for medium ($p < 0.05$). Mean separation was analyzed using Tukey's HSD. Data represent two technical replicates of three flasks. Standard error is shown.

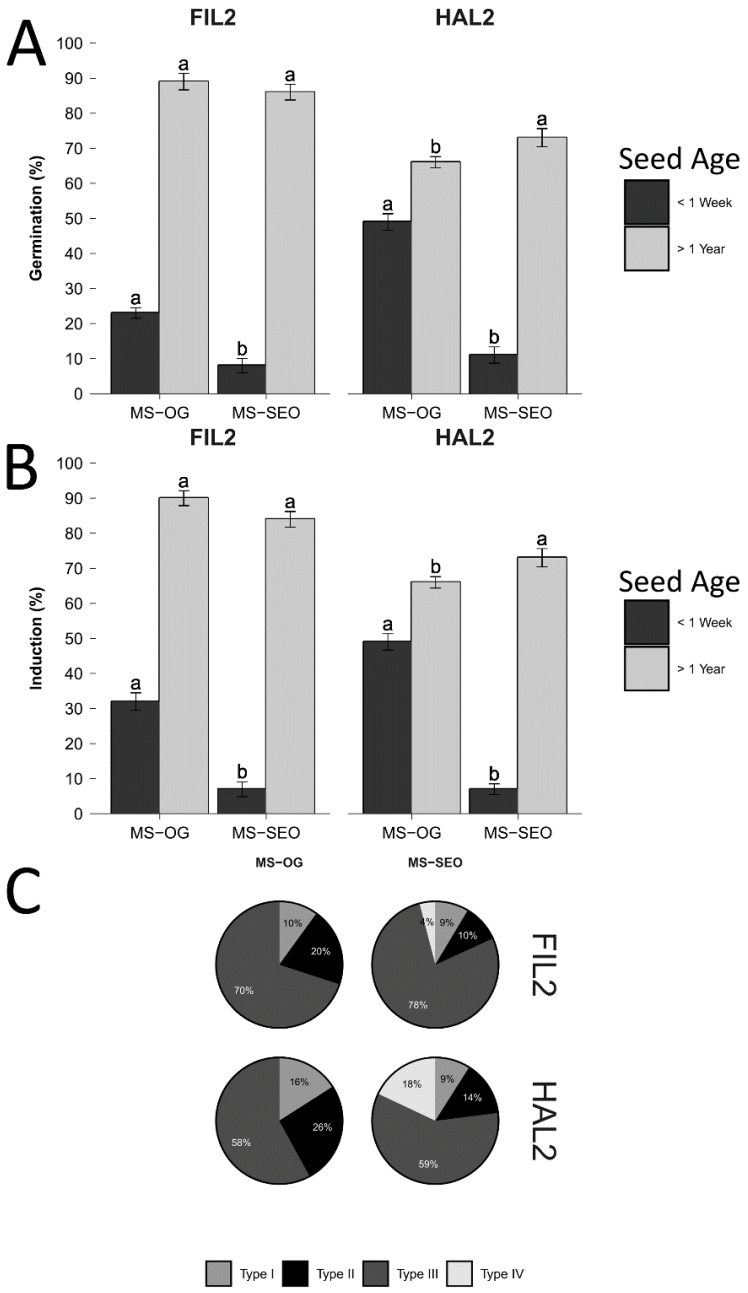

Figure 8. Direct comparison results. (A) Germination results. (B) Callus induction results. (C) Pie graph showing type of callus induced. Populations were analyzed separately under a one-way ANOVA controlling for medium ($p < 0.05$). Mean separation was analyzed using Tukey's HSD. Data represent ten replicates of ten seeds per replicate. Standard error is shown.

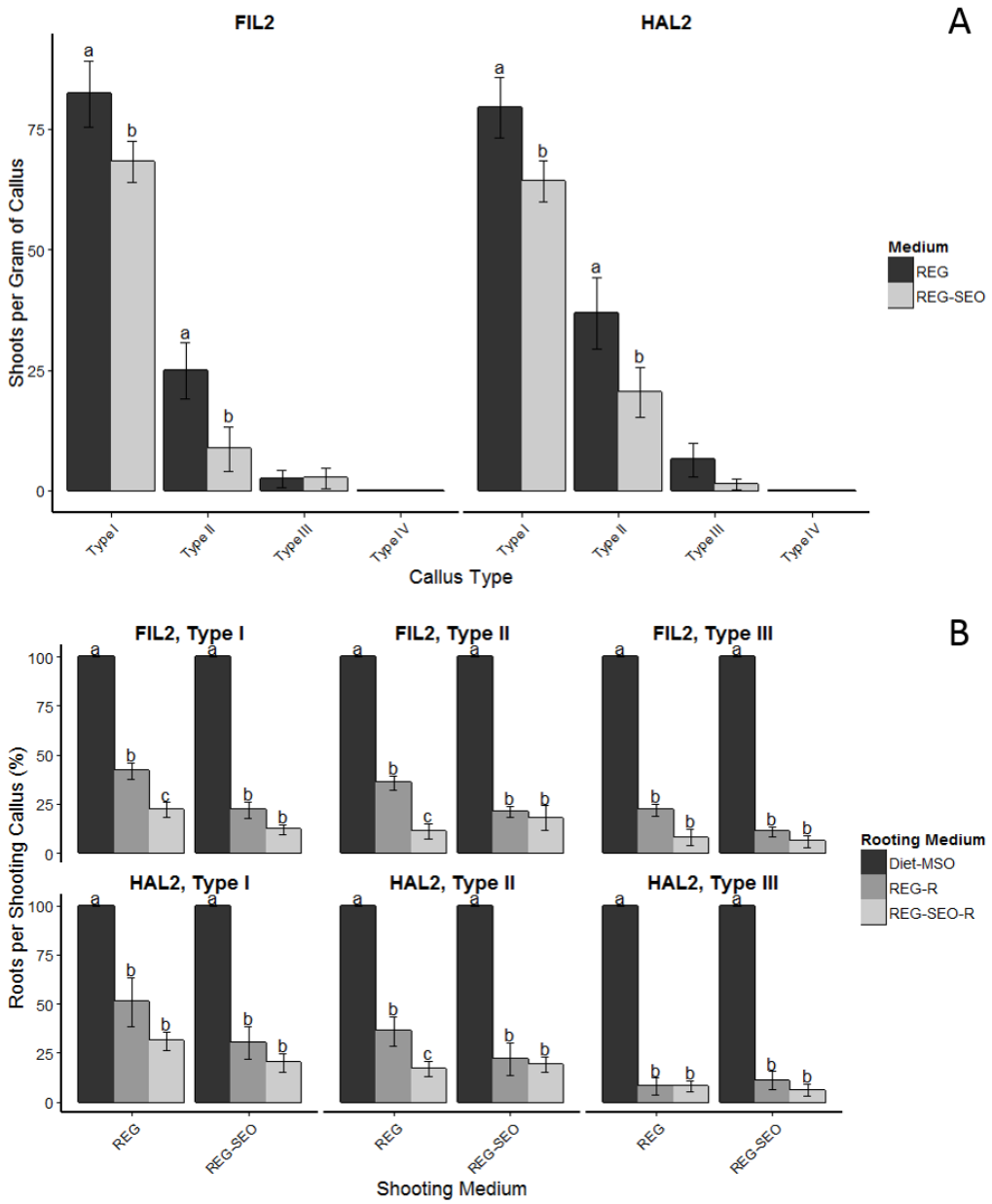

Figure 9. Regeneration results. (A) Regenerating shoots per gram on differing callus types. (B) Root regeneration percentages for shoot regenerated callus on two shoot regeneration media. Populations and callus types were analyzed separately under a one-way ANOVA controlling for shoot regeneration medium. Differences are reported ($p < 0.05$) where mean separation is reported. Mean separation was analyzed using Tukey's HSD. Standard error is shown.

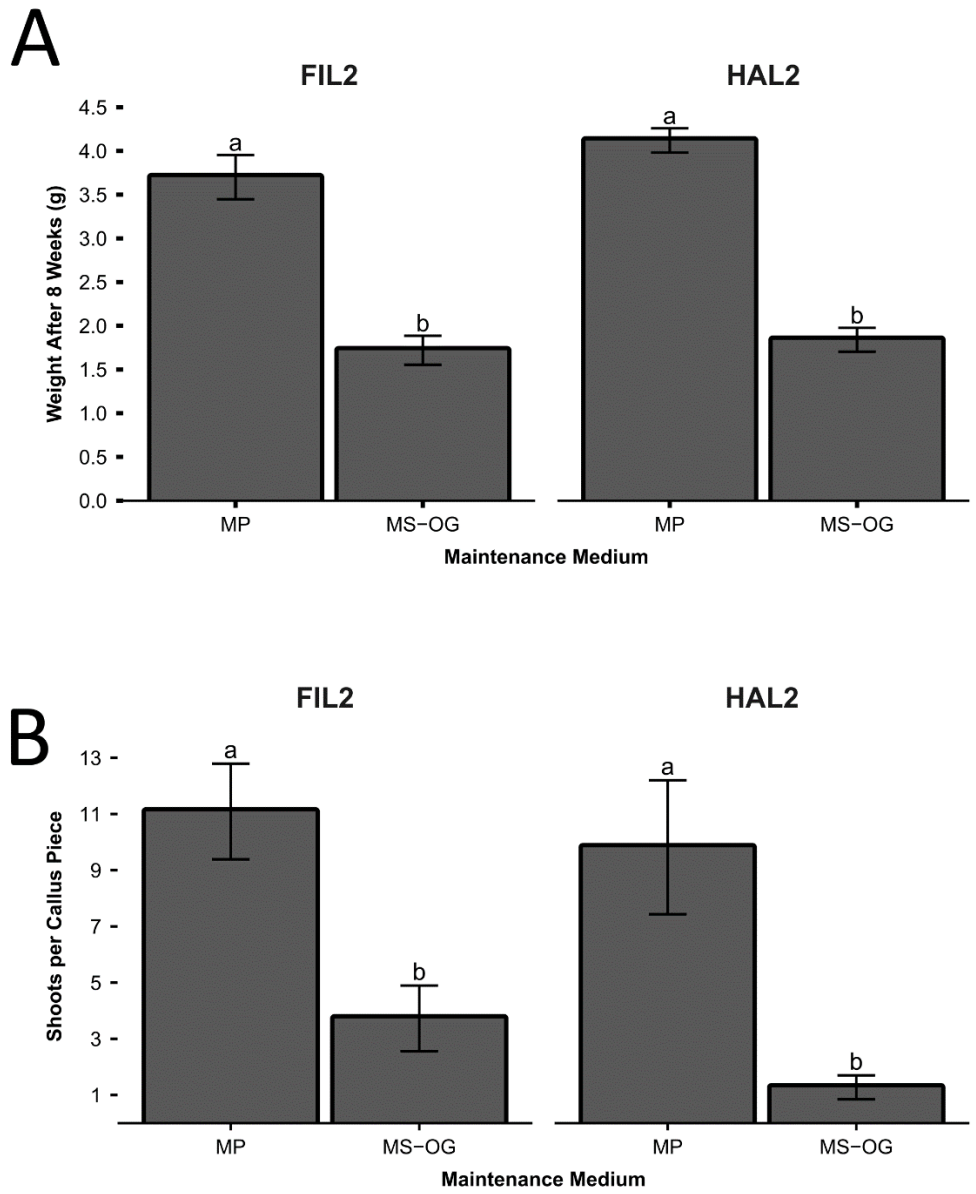

Figure 10. Callus induction from inflorescences. (A) Callus weight at 8 weeks on maintenance medium. Data represent ten replicates of ten callus pieces per replicate. (B) Shoots per callus piece on REG medium. Data represent ten replicates of ten callus pieces per replicate. Populations were analyzed separately under a one-way ANOVA controlling for medium ($p < 0.05$). Mean separation was analyzed using Tukey's HSD. Standard error is shown.

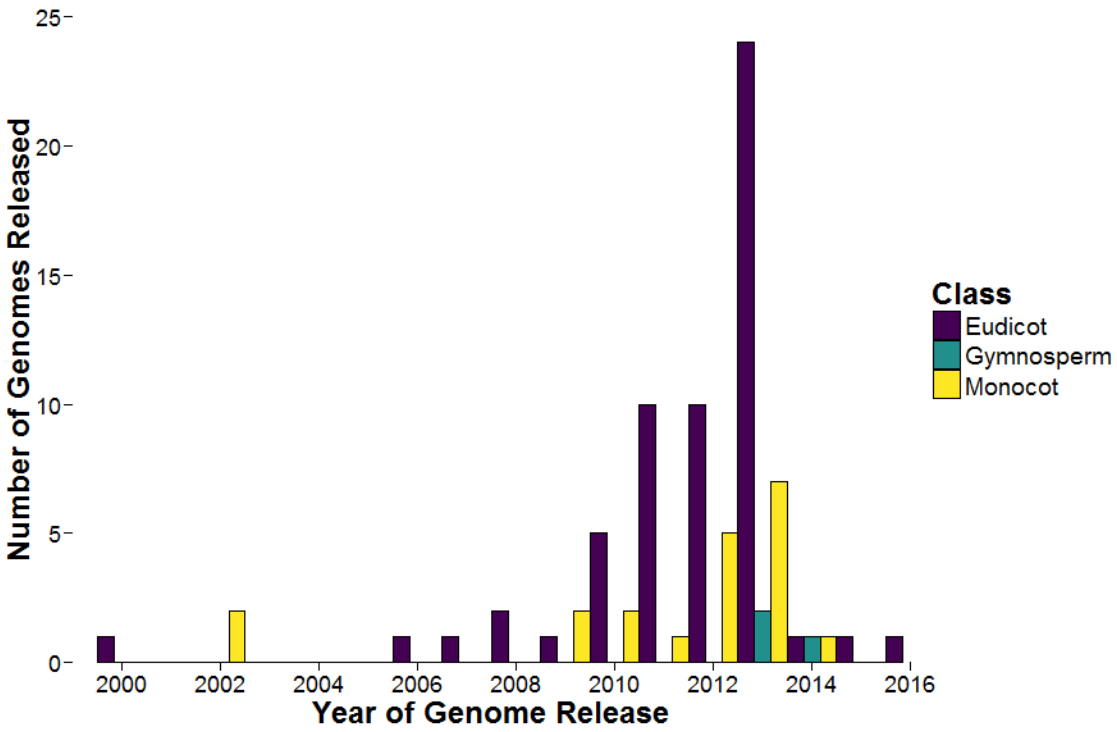

Figure 11. The release of sequenced plant genomes by year. Data was pulled from the DOE-JGI and PubMed articles.

VITA

Joshua N. Grant was born in 1985 in Seneca, South Carolina. He graduated from the University of Tennessee in 2014 with a Bachelor's of Science Degree in Plant Sciences with a concentration in Plant Biotechnology. He currently works as a Bioinformatician with Microbial Insights, Inc., where he has optimized Next Generation Sequencing data to be presented to clients in a clear, informative manner.