Optimization of Tissue Culture in *Erigeron canadensis*

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robert Andrew Petursson

December 2023

Abstract

Glyphosate resistance among weed species has been an increasing problem in crop production over the past two decades. Glyphosate resistant *Erigeron canadensis* (horseweed) has been found on multiple continents and 20 U.S. states. It appears that the resistance is of non-target type, with mechanisms of action that have yet to be elucidated at the gene level. For the first portion of the study, I focused on reproducing the original indirect organogenesis protocol but discovered biotype variability can influence regeneration success. An improved indirect organogenesis method was then developed using the previously published methods that could be used across biotypes. A DBI (DeVerna et al. 1984) based solid medium (I Macro/I Micro Murashige and Skoog (MS) salts/vitamins, 3% sucrose, 0.2% gelzan, 2 mg/L kinetin, 0.438 mg/L indole acetic acid (IAA), pH 5.8) called DBIX was statistically significant ($p<0.001$) over the several media designed and had a 77% regeneration rate in wild-type tissue. The second portion of the study was to establish a new liquid callus culture protocol that was adaptable to different biotypes. I evaluated three different published liquid media types for their effects on callus and cell growth in liquid culture using three different horseweed biotypes. With a statistical difference ($p<0.001$) in all three media, Medium 2 (MS basal salts, 3% sucrose, 1 mg/L 6-benzylaminopurine (BAP) and 0.02 mg/L indole-3-butyric acid (IBA), pH 5.8) produced healthy viable cells/callus pieces and a faster regeneration protocol over the other media types. Two new methods were developed for tissue culture using horseweed and provide a foundation for genetic engineering and gene editing.

Table of Contents

List of Figures

Chapter 1: Literature Review

Chemical herbicide usage began as early as 1896 in France with the creation of Sinox (dinitro-cresol). This herbicide that was introduced into the United States of America in 1940, which was banned by the EPA (Environmental Protection Agency) in the 1990s, due to high toxicity to humans (Norris-Trull et al. 2020). Shortly after the introduction of Sinox , 2,4-dichlorophenoxyacetic acid (2,4-D), isopropyl-N-phenylcarbamate (IPC) and 2,4,5-trichlorophenoxyacetic (2,4,5-T) were released in 1945 to control broad-leaf weeds and grass species. These herbicides were groundbreaking because of their efficacy at low dose rates compared to the older herbicides. In the 1970s, Monsanto released a broad-spectrum herbicide in which the active ingredient was N-(phosphonomethyl) glycine (glyphosate). This herbicide is applied to the foliage or roots on a variety of plant species including trees and shrubs, grasses, and broadleaf plants. Its effectiveness led to it becoming one of the world's most used herbicides in the last 5 decades.

Glyphosate-resistant genetically engineered crop plants were introduced in 1996, which allowed for weed control to be possible by glyphosate applications in crop fields during active crop growth. These glyphosate-resistant crops such as soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and corn (*Zea mays*) created a large demand for the herbicide product, resulting in a 15-fold increase of glyphosate usage shortly after glyphosate-resistant crops became widely cultivated (Benbrook 2016). The usage of glyphosate steadily increased over time as more farmers grew genetically modified (GM) crops (Figure 1). In the United States GM soybean, cotton, and corn comprise 95%, 95% and 93% of their respective acreage (USDA 2022). The agricultural trend in recent years is toward maximizing crop yields while minimizing

the amount of land usage with a focus on environmental preservation. Agriculture has produced some negative effects on the environment such as erosion, land usage and chemical applications to the fields and surrounding areas. The use of GM crops promotes environmental preservation through soil conservation practices like conservation tillage and no-till systems. These practices reduce equipment and labor use, resulting in lower gas emissions and reduced erosion in the farming community. An estimated range of 15% and 45% of acreage of cotton, soybean, corn, and wheat are grown using no-till systems (USDA 2018). No-till agriculture is compatible with glyphosate-resistant crops paired with glyphosate spraying of the fields to minimize competition. Initially, herbicide-resistant row crops offered advantages to corn, cotton, and soybean farmers. Nowadays, this technology has spread across the US, encompassing 12 different plant species (Figure 2). Glyphosate, an effective herbicide for controlling most weeds in row crop systems, faces challenges from evolved glyphosate-resistant weed species, posing threats to system sustainability (GM crops, glyphosate, and no-till practices). These resistant weeds reduce crop yields and limit resources allocated for row crops.

The mode of action of glyphosate is to specifically inhibit EPSP (5-enolpyruvylshikimate-3-phosphate) synthase, which is an enzyme that is critical within the shikimate pathway (Siehl 1997, Serra et al. 2021). The shikimate pathway is responsible for the biosynthesis of aromatic amino acids (tyrosine, phenylalanine, and tryptophan) and folates (Klaus et al. 1999). These important metabolites are imperative precursors for products such as lignin (Harborne 1964) and alkaloids (Bochkov et al. 2010). Glyphosate enters the growing regions of the plant (root,

shoot and leaf tissue) and protein production in those areas are reduced, which ultimately causes plant death.

Overuse of this effective herbicide has led to the evolution of resistance within some weedy plant species. As one example, a resistant *Lolium rigidum* biotype emerged in 1996 within an apple orchard in Australia (Heap et al. 2018). The context of biotype is in reference towards a group of plants that have similar or identical genetic makeup of another plant group within the same species (i.e., different horseweed species that differ by a resistance trait). Almost 30 years later, there have been 267 different biotypes of plants that have been identified as herbicide-resistant (Heap 2023). Out of the 267 different biotypes, there are 57 biotypes that have been found to be specifically glyphosate resistant, which includes weed species such as *Amaranthus palmeri* and *Erigeron canadensis*. Understanding the mechanisms behind the evolution of resistance is important to elucidate to address management strategies.

Erigeron canadensis was first identified to have glyphosate-resistance in Delaware, USA (2000) (VanGessel 2001). This population was found after only three years of glyphosate repeated usage within the agricultural industry within the state. The wide spread identification of glyphosate-resistant horseweed began and currently has identified resistant populations in states such as California (Hanson et al. 2009), Indiana (Davis et al. 2008), Ohio (Beres et al. 2018) and more. It is unclear on how these populations have spread throughout the United States (Figure 3), but it is possible that some of the populations have evolved independently from one another while others were brought into states that bordered resistant populations. Finding different populations of glyphosate-resistant horseweed across the United States, it was clear that identifying the mechanisms of this resistance was a source of inquiry. Finding the

mechanism of this resistance can provide the basis for gene editing experiments or resolving the glyphosate resistance issue within horseweed.

Glyphosate resistance is grouped into two categories: target-site and non-target-site resistance. Target-site resistance includes EPSP synthase mutations, overexpression of the enzyme or gene copy number variation (CPV) of the *EPSP synthase* gene. Non-target-site resistance occurs in gene evolution other than that of EPSP synthase. Target-site resistance has been identified in multiple species such as *Eleusine indica* (Zhang et al. 2021) that was revealed to show an increased copy numbers of the *EPSP synthase* gene in addition to the *EPSP synthase* gene having single nucleotide polymorphisms (SNPs). A mutated EPSP synthase might prevent the glyphosate molecule from properly interfering with the enzyme and this would allow for normal cell functions to occur. An overexpressed *EPSP synthase* or increased gene CPV can enable cells to tolerate the presence of glyphosate. Increased EPSP synthase prevents glyphosate from disrupting the shikimate pathway; these plants would then survive glyphosate treatment. This type of resistance has been documented and established as the first step in attempting to identify glyphosate resistance within weed species. The investigation of target-site resistance has identified >20 biotypes of weed species in the last two decades (Heap 2023). However, the more potentially problematic resistance is derived from the non-target-site resistance. Two different types of non-target-site resistance mechanisms have been identified: 1) vacuole compartmentalization of glyphosate and 2) cell efflux of glyphosate. Both types of outcomes are likely mediated by membrane-bound active transporters. Vacuole sequestration permanently removes glyphosate from the cytoplasm within source tissues, which prevents glyphosate movement into sink tissue (Feng et al. 2004, Koger et al. 2005, Ge et al. 2010). This

type of mechanism (glyphosate sequestering in source cell vacuoles) has been identified in glyphosate resistant weed species such as *Lolium* spp. (ryegrass) (Ge et al. 2012). Treated cells may be injured or killed but the treated weeds survive. This mechanism is mediated by active transport via transmembrane substrate pumps that are driven by ATP (adenosine triphosphate), e.g., ABC transporters (Ge et al. 2014). The other identified non-target resistance mechanism is the removal of glyphosate from cellular matrix by active transmembrane transporter proteins, like the proteins for vacuole compartmentalization. ABC (ATP-binding cassette) transporters, such as an ABCC transporter discovered in a glyphosate-resistant *Echinochloa colona* in Australia, transport glyphosate out of cells (Pan et al. 2021). The ABCC transporter (*EcABCC8*) was confirmed to confer glyphosate-resistance by overexpressing the gene in susceptible plants. The ABCC transporter protein group has been characterized as a multidrug-resistant group within land plants (Remy et al. 2014). These proteins have evolved to provide internal defense against substrates like heavy metals, secondary metabolites (Kovalchuk et al. 2015) and most recently, glyphosate. The ABCC group has also been described to have detoxification properties (Lane et al. 2016, Banasiak et al. 2021), which has been confirmed through overexpression of *AtABCB25* that endowed cadmium and lead tolerance in *Arabidopsis* root tips (Kim et al. 2006). The vacuole sequestration and efflux pumps mechanisms may be mediated by ABCC-type proteins, but little data exist to support that notion. One weed species that has been a focus of glyphosate-resistance is *Erigeron canadensis* (horseweed, marestail, fleabane), originally called *Conyza canadensis*, which has at least 256 ABC transporter members, which is more than twice that of other plant species, such as *Arabidopsis* and rice (Peng et al. 2014).

Within *Erigeron canadensis*, several studies have attempted to identify the genes responsible for the glyphosate resistance through genome-wide screening and transcriptome sequencing (Peng et al. 2010, Yang et al. 2021). These studies identified candidate genes for subsequent functional studies to potentially elucidate the mechanism in glyphosate-resistant horseweed (GRH) when glyphosate is applied. GRH has been compared with glyphosate-susceptible horseweed (GSH) to potentially identify a new gene expression within the resistant populations. Denoted in Peng et al. 2010, gene candidates were labeled *M6*, *M7*, *M10*, and *M11* as they were discovered during the draft genome stage and unannotated but identified later as ABC-like transporter genes. *M11* and *M10* had the greatest level of fold-change upregulation upon glyphosate treatment when compared to the controls (Peng et al. 2010). These two genes were found to be upregulated in resistant biotypes when treated with glyphosate across studies (Nol et al. 2012, Yang et al. 2021, Bo et al. 2021). With the recent addition of a more complete genome assembly (Laforest et al. 2020), characterization could occur for these genes (*M11* and *M10*). Using the Basic Local Alignment Search Tool (BLAST) and the NCBI (National Center for Biotechnology Information) database, *M10* was found to be an ABCC-like gene that produces a vacuole bound protein and *M11* is an ABCC-like gene that produces a cellular membrane bound protein. The proteins match with the non-target resistance mechanisms (vacuole compartmentalization and cellular matrix efflux) and have been shown to have upregulation during glyphosate treatments within horseweed. With evidence showing that these genes are upregulated during glyphosate treatments and the protein sequence like other glyphosate-resistance proteins, I hypothesize that one or both genes are responsible for glyphosate resistance in *Erigeron canadensis*. However, despite the

genomic studies and gene expression in glyphosate treatments, tissue culture protocols need to be optimized before genome engineering or gene editing can occur.

There have been only two published studies (Scheiber et al. 2006, Halfhill et al. 2007) that describe protocols for tissue culture with horseweed and only one of them was successful in regenerating transgenic horseweed (Halfhill et al. 2007). The lack of tissue culture studies within horseweed has generated a bottleneck in furthering the investigation of glyphosate resistance. Thus, the first goal of this thesis research was to optimize tissue culture conditions among three horseweed genotypes using indirect organogenesis for regeneration. The second goal was to generate a novel liquid callus protocol that worked with the different biotypes of horseweed. Lastly, we tested these tissue culture protocols with transformation and gene editing experiments.

Chapter 2: Establishing and Optimization of Tissue Culture work within *Erigeron Canadensis*

Introduction:

Non-target glyphosate resistance mechanisms in weeds have largely remained unknown. Discovering the evolution mechanisms would be helpful for effective management of resistant biotypes. Glyphosate resistant horseweed began to arise in the early 2000s (locations include Delaware, Tennessee, Ohio, Kentucky, Maryland, Michigan) and continued to increase over time, resulting in over 40 different occurrences worldwide in two decades (Heap 2023). Horseweed's seed production (over 200,000 seeds per plant (Bekech et al. 1993)) and self-pollination traits have allowed this species to thrive in no-till systems, which has only promoted glyphosate-resistant populations. Several studies have investigated gene expression changes (upregulation or downregulation) among different glyphosate-resistant biotypes, which indicate that genes *M11* and *M10* may be involved (Peng et al. 2010, Yang et al. 2021, Bo et al. 2021). *M11* and *M10* have been identified to have the greatest fold-change in gene expression within studies that have glyphosate treatments on resistant biotypes. These genes of interest in glyphosate studies have the potential to be responsible for glyphosate resistance.

Horseweed transformation procedures were developed by Halfhill et al. (2007). *Agrobacterium tumefaciens*-mediated transformation was used to engineer leaf-derived callus to stably engineer a green fluorescent protein gene construct into a single germplasm (western Tennessee biotype, Halfhill et al. 2007). Unfortunately, there appears to be genotype-dependence within tissue culture conditions, given that the published method has not been successful on multiple horseweed genotypes (Tennessee susceptible (middle Tennessee

biotype, Yang et al. 2021), Tennessee resistant (middle Tennessee biotype, Yang et al. 2021), multiple Delaware biotypes (Courtesy of Dr. Mark VanGessel) and others). This type of event has been reported in different plant species in which biotype variation can play a role in tissue culture success (Fiuk et al. 2010, Mikuła et al. 2011, Machczyńska et al. 2015, Orłowska et al. 2020, Bednarek et al. 2021). This variation can be responsible from gene expression, epigenetics, biochemistry, or morphological responses. Thus, finding protocols that can overcome this obstacle can be extremely beneficial for tissue culture practices.

Transient expression for transgene expression has been performed among genotypes. While transient expression is helpful, it does not substitute for stable transformation (Fehér 2019). Stable transformation practices allow for testing the effects of altering the expression of these genes in various horseweed genotypes. If it was possible to downregulate both genes of interest followed with a glyphosate treatment, we would potentially find out if either gene are connected to glyphosate resistance. Improved tissue culture is required for routine genetic engineering and plant regeneration in horseweed.

The aim of my study is to develop more consistent tissue culture protocols that can be used with different biotypes of horseweed. The objectives were: 1) Produce a regeneration protocol for indirect organogenesis; 2) Establish a novel liquid culture protocol to increase tissue amount and regeneration; 3) Implement transformation protocols for stable regeneration in leaf tissue and callus that is required to subsequently test candidate glyphosate resistance genes. Multiple horseweed genotypes- both resistant and susceptible to glyphosate treatment- were tested.

Material and Methods:

Seed Sterilization:

Horseweed seeds were rinsed with 10% household bleach and 1% tween solution for 2 to 3 minutes. After the bleach solution was removed, a 70% ethanol solution was used for a maximum time of 35 s with aggressive shaking. Following the ethanol rinse, sterilized DI water was used for three separate rinses between 3 to 5 min for each to ensure that the seeds were clean. Seeds were spread on MSO medium (Murashige and Skoog medium, Murashige and Skoog 1962) for germination and plantlets remained on those plates for 1 to 2 weeks before being transferred to fresh MSO medium.

Indirect Organogenesis Media Testing:

Based on the regeneration protocol in Halfhill et al. (2007), a DBI medium (DeVerna and Collins 1984) was used for the initial phase of testing. However, DBI was not effective in regenerating Tennessee susceptible (TNS), Tennessee resistant (TNR) biotypes as well as the Delaware accessions used in the current project. Therefore, I tested 6 different media: DBI, DBIX, DB, CBN1, CBN10, and SIM with 500 explants (biotypes used: Tennessee Susceptible (middle Tennessee), Tennessee Resistant (middle Tennessee)(Peng et al. 2014) and Delaware) per media type (composition in Table 1). The sample size of 500 explants per media type consisted of 100 explants for Delaware biotype, 200 explants for Tennessee Susceptible and 200 explants for Tennessee Resistant. For the context of explants for this experiment, explants are pieces of leaf tissue that were excised and cut into 2 to 3 cm leaf tissue strips. Explants used 8-12 leaf stage and 13-18 leaf stage for all the media and biotypes used. For each plate of

media, there were 20 explants (i.e., Delaware had a total of 5 plates for DBIX testing, TNR had a total of 10 plates for DBI testing).

All the media that were generated differed from the original DBI medium by changing the hormone concentrations or the hormone types. DBIX used a higher ratio of kinetin to IAA (indole-3-acetic acid) (2 mg/L: 0.438 mg/L), DB used a concentration of 2 mg/L kinetin with no IAA, and SIM used 0.4 mg/L of BAP(6-benzylaminopurine) with no auxin added. For CBN1, I used 1.125 mg/L BAP and 0.931 mg/L NAA (1-naphthaleneacetic acid) with an addition of 62.5 mg of charcoal for an increased carbon availability for the callus development. CBN10 contained 62.5 mg of charcoal with a higher ratio of BAP to NAA (2.25 mg/L BAP: 0.186 mg/L NAA).

For statistical analysis, I used a one-way ANOVA to compare the regeneration frequency among the 6 different media. Using the sample size of 25 plates (10 plates for TNS, 10 plates for TNR and 5 plates for DEL) and α=0.05, for post-analysis I used Tukey's honest significant difference (HSD) test to determine if one medium's results are statistically significant when compared to the rest of the media (SAS 9.4, Cary, N.C., U.S.A.).

Expansion of Target Genes:

The long-term goal of this research is to understand what gene or genes are responsible for endowing glyphosate resistance in horseweed populations. I used the *M10* and *M11* genes that were found to be upregulated in the presence of glyphosate in TNS/TNR populations (western, middle, and eastern Tennessee biotypes, Korea, and Greece) (Nol et al. 2012, Peng et al. 2010, Yang et al. 2021, Bo et al. 2021). I used previously obtained genomic sequences from

Dr. Yongil Yang to expand on the *M11* contig for primer design. After expanding on M11, I compared this contig with the Canadian fleabane genome (Laforest et al. 2020) to find similar gene sequences. Based on the previous sequences, primers were designed (Supplementary Table 1) and ordered from Integrated DNA Technologies for amplification of *M11*, which was sent to Eurofins Scientific for sequencing.

Construct Assembly:

Constructs were cloned into pAGM4723 (Weber et al. 2011) backbone that was modified by Dr. Alex Pfotenhauer to include a CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) with a Cauliflower Mosaic 35S promoter (CaMV 35s) and a GFP (green fluorescent protein) insert with a separate CaMV 35S promoter. The guide RNA and guide RNA scaffold are driven by an *Arabidopsis* U6-26t promoter. The gRNAs were designed based on the sequencing previously mentioned, focused on the first 100-200 base pairs of exon 1 for *M11* to generate a knockout of this gene. Golden Gate cloning was used to produce constructs containing guide RNAs flanked with Bsa1 sites. After Golden Gate cloning, vectors were transformed into Top10 *Escherichia coli* cells and plated on to LB with kanamycin plates for selection. After 2 days in a 37°C incubator, a single colony was selected, and PCR verification occurred on the colony as well as growing the colony out for DNA extraction. Following the PCR verification of the correct plasmid, *Agrobacterium tumefaciens* st. EHA105 transformation was performed. *A. tumefaciens* strain EHA105 was used due to availability and effectiveness. This *Agrobacterium* strain has for generating positive transgenic plants (Chetty et al. 2012, Debernardi et al. 2020, Li et al. 2022). I used *A. tumefaciens* strain EHA105 to remain consistent across all transformation testing in leaf and callus testing.

Indirect Organogenesis for Transgenic Regeneration:

Leaf tissue was used for the process of indirect organogenesis for transgenic regeneration. The leaf transformation protocol was derived from Halfhill et al. (2007), in which leaf tissue from the 8-12 leaf stage. The usage of a different growth stage (13-18 leaf stage) was included to test if development of leaf tissue was a factor. The leaf tissue was excised and cut into 1 cm by 2-3 cm strips that would be soaked with an *Agrobacterium* culture in a shaking incubator set at 28°C for two days prior. The culture was moved into a 50 ml test tube and the culture was pelleted at 4,000 x g for 10 minutes in a table-top centrifuge. The culture was resuspended using a liquid DBIX medium for an OD600 of 0.7, which was poured onto the leaf explants to be soaked. The explants were then hand shaken every 5 minutes for a total of 40 minutes in the culture solution. The explants were then moved to solid DBIX medium and placed in dark conditions for the next 3 days, allowing for the *Agrobacterium* to incubate. After the third day, the leaf explants were washed 3 times in 50 ml tubes containing liquid DBIX and TIMENTIN® (400 mg) to decrease *Agrobacterium* overgrowth. Explants were then placed on selection medium within a long photoperiod light room (16 hr light and 8 hr dark with temperatures at 24°C) that contains kanamycin (50mg/L) and TIMENTIN® (400 mg/L). The use of 50 mg/L kanamycin over 200 mg/L (Halfhill et al. 2007) was chosen due to how the wild types reacted towards the lower dosage and the attempt to reduce exogenous stress factors on the developing callus. Depending on the constructs used as well as the biotype used, callus development ranged from 1-3 months to determine if callus was transgenic. To generate a statistical power of significance for the transformation and regeneration of transgenic protocol, I used a power analysis to determine the minimum of explants needed for this protocol to be

considered significant. With the original method generating two transgenic plants (Halfhill et al. 2007), I expected a 10% transgenic regeneration rate with the new protocol based on the 77% regeneration rate derived from Figure 4. With these terms defined, the power analysis with a $p<0.05$ and a predicted transgenic regeneration rate of 10%, a minimum sample size of 200 explants was required.

Liquid Callus Culture Protocol:

The focus for this experiment was focused on producing sterile callus cultures that can be sub-cultured as well as be used for regeneration purposes. The objective was to increase the number of callus pieces being used for transformation experiments along with providing a readily available source of tissue for testing. Medium 1 was comprised of MS basal salts, 3% sucrose, 1 mg/L BAP and 1.98 mg/L 2,4-D for the switchgrass culture medium (Ondzighi-Assoume et al. 2019). Medium 2 was comprised of MS basal salts, 3% sucrose, 1 mg/L BAP and 0.02 mg/L IBA for the petunia culture medium (Kang et al. 2020). Medium 3 is a commercial liquid culture (Kao and Michayluk Basal Salt Mixture 10 L, PhytoTech Labs, Inc., Lenexa Kansas) that consisted of macro and micro-nutrients along with multiple hormones: 0.2 mg/L Zeatin, 1 mg/L NAA, and 0.1 mg/L 2,4-D (Kang et al. 2020) (Table 2).

The callus culture material was derived from callus generated by placing leaf tissue on solid DBIX for 2-3 weeks to produce friable callus (Sidorov et al. 2006) or type II callus, which is white in appearance with a crispy texture. For testing the different media and comparing the growth of callus, all cultures were started with 0.5 grams of horseweed friable callus (3 biotypes were used with all the media) and were placed into 30 ml of media in a 125 ml Erlenmeyer flask

for one month. Tissue was cultured in total darkness with a temperature range of 22°C to 26.5°C on a rotary shaker at 90-100 rpm. During the month period, “feeding” (this refers to removing 5 mL of supernatant and replacing it with new medium) of media occurred every five to seven days. Viability checks occurred every two days for two weeks to make sure the callus cultures were developing; viability was determined by FDA (fluorescein diacetate) staining (10 µl in 1 ml culture supernatant) and viewed under FITC (fluorescein-5-isothiocyanate) spectrum (excitation peak 491 nm/emission peak 516 nm).These cultures were allowed to develop and grow for one month before all the callus pieces were removed and final weights (FW) were taken and recorded.

This experiment was designed to test the different media (Medium 1, 2, 3) along with comparing biotypes (TNR, TNS, DEL). Each media had a flask for each biotype with a replication factor of three, so a sample size of nine cultures per media type. One-way ANOVA ($p<0.05$) was performed to determine if one of the media generated statistically different results (final weight of callus pieces) from the other media. Tukey’s HSD test was used on the data to mark significant differences between the media types with a $\alpha=0.05$ (SAS 9.4, Cary, N.C., U.S.A.). Afterwards, a two-way ANOVA was used to test differences between the biotype and media factors.

Liquid Callus Culture Transformation and Regeneration Trial:

To confirm that the callus pieces were healthy and prepared for transformation, 1 ml of supernatant along with a small piece of callus that was crushed up are viability checked with FDA. Two days prior to transformation, the 50 mL *Agrobacterium* cultures are placed in a

shaking incubator set at 28°C. After confirmation that both the supernatant contains healthy cells and healthy callus pieces, the *Agrobacterium* cultures were centrifuged at 4,000 g for 10 minutes. The culture was resuspended in the same callus culture medium that the callus was grown in for an OD of 0.5. The callus to be transformed was then moved onto sterile petri dishes with 20-25 ml of the *Agrobacterium* suspension and placed in a vacuum chamber for 20 minutes. The callus pieces were moved from the suspension culture onto HSIM (MS basal salts, 3% sucrose, 0.2% gelzan, pH 5.8 with 0.05 mg/L IAA and 7 mg/L kinetin) medium and wrapped with surgical tape to be incubated in the dark for 3 days. The callus pieces were moved onto selection plates for shoot regeneration in a light room with a long photoperiod (16 hr light and 8 hr dark (Halfhill et al. 2007) with temperatures at 24°C).

Results:

Indirect Organogenesis Media Testing:

Out of the six different media types, DBIX and DBI were capable of successfully regenerating shoots and were statistically different from the rest of the media types (Figure 4) (α=0.05). There was a statistical difference between DBIX's shoot average mean (15.40 shoots per plate) and DBI's shoot average mean (2.76 shoots per plate) (Figure 4) (p<0.001). Another comparison was made to determine if biotypes were significantly different within those two media. Ultimately, biotypes were considered not different from one another in this experiment (p=0.0971, Figure 5A). The other factor in this experiment was leaf tissue stage, as half the total samples used were from plants at the 8-12 leaf stage tissue and the other half used were from plants at the 13-18 leaf stage. After comparing the shoot averages by leaf stage (DBIX and DBI data only), it was determined that stage of tissue was not statistically different (p=0.7344)

(Figure 5B). Overall, it was determined that the shoot averages were statistically significant based on which media (DBI and DBIX) was used, and not from tissue stage or biotype.

Rooting data was not taken during these experiments as any shoots retrieved from the media testing was placed onto MSO medium for growth and root development. Those same shoots were grown into 8-12 leaf stages or 13-18 leaf stages for transformation experiments for indirect organogenesis testing or to continue the leaf tissue pipeline. An observation for rooting: no issues producing roots with the shoots that were generated from either DBI or DBIX.

Expansion of Target Genes:

After receiving the sequences back from Eurofins (Figure 6), *M11* was found to be a part of a gene annotated as *CcABBC3*. The gene contains 10 exons, 9,384 base pairs in length and translates a protein size of 1312 amino acids that forms a transmembrane protein. Using the Basic Local Alignment Search Tool (BLAST), the protein was matched with ABCC-like transporter proteins across multiple species. BLAST is a program that can identify similar sequences (DNA, RNA, or proteins) in closely related species or different organisms by searching publicly available sequence databases. The *CcABCC3* protein is comprised of two transmembrane domains (TMD) and two nucleotide binding domains (NBD) that contain the site for binding of substrates (secondary metabolites, amino acids, liposomes, and drugs) to transport inside or outside the cell (Sun et al. 2021).

Indirect Organogenesis for Transgenic Regeneration:

Attempting to regenerate transgenic horseweed through indirect organogenesis, nine different constructs were used during this experiment: pBIN-mGFP5-ER (Supplementary Figure

1), pSKEA401 and seven CRISPR-Cas9 constructs that contained different gRNA sequences. The pSKEA401 and pBIN-mGFP5-ER constructs contained a kanamycin resistance gene which provided a selection for transgenic plants. TNS, TNR and Delaware biotypes were used along with the different tissue stages (8-12 leaf stage and 13-18 leaf stage) using the previously stated *Agrobacterium* transformation protocol on >200 explants per construct. Despite developing a regeneration medium that worked with non-transgenic plants, there were no positive transgenic shoots that developed in any of the media (DBIX and DBI were used with selection). All putative transgenic shoots (derived from pBIN-mGFP5-ER or pSKEA401) that developed on selection media (kanamycin 50 mg/L) (Scheiber et al. 2006) were inspected under a microscope with GFP filters (excitation 469 nm /emission 525 nm) for GFP expression. No GFP expression was found in any of the putative shoots even though the shoots were growing and developing on selection media. The positively transformed (GFP expression) callus either continued to develop or would begin to die and fail to produce healthy shoots. The low dosage of kanamycin may have allowed non-transformed callus to out-compete the transformed callus before it had an opportunity to develop.

The CRISPR-Cas9 constructs contained only a GFP insert that would assist in indication of positively transformed callus. Screening using a GFP filter would occur after 8 weeks of initial transformation and callus that expressed GFP would be transferred to new media while non-transgenic callus would be thrown away. Shoots that developed out of GFP-expressing callus would be screened with the GFP filter yet there was no evidence that those shoots were expressing GFP either. Overall, there were no transgenic plants that regenerated from indirect organogenesis using leaf tissue.

Liquid Callus Culture Establishment:

Medium 1 (comprised of MS basal salts, 3% sucrose, 1 mg/L BAP and 1.98 mg/L 2,4-D) could promote TNS and DEL callus growth (Figure 7G/H) but did not result in additional callus pieces. TNR callus did not grow and were determined to be dead after 2-weeks in the culture (Figure 7 A/B). Medium 3 (Kao and Michayluk Basal Salt Mixture 10 L, Phytotech Labs) provided a healthy growth condition for TNS, as this biotype showed increased callus weight with newly formed free-floating cells in the supernatant (Figure 7K/L). While medium 3 had positive results with TNS, TNR (Figure 7E/F) and DEL did not react in a similar manner as these cultures grew very little in comparison and did not produce more callus pieces. Medium 2 (comprised of MS basal salts, 3% sucrose, 1 mg/L BAP and 0.02 mg/L IBA) facilitated increased callus weight in all three biotypes along with increasing the number of callus pieces. We experienced biotype variability within this experiment as TNS developed in all three media while TNR had three different reactions towards the different media used (medium 1: TNR cells died and did not increase FW, medium 2: healthy cells and increased FW, medium 3: did not increase FW), as this was evident in the viability checks that occurred (Figure 7).

A two-way ANOVA was performed (media type and biotype) (Figure 8), and it was determined that the interaction was significant ($p=0.0028$). To understand which factor (biotype/media type) is responsible for the significance, I used one-way ANOVAs on each one to find their individual significance. The one-way ANOVA for media revealed that there was a statistical difference in mean final weights of callus between at least two of the media ($F(8,26) = 180.85$, $p=<0.001$). After using Tukey's HSD test, a significant difference was found between the effect of media on callus final weight averages with a 95% confidence (Figure 9A). The one-

way ANOVA for biotype determined that there was no significant difference between the biotypes (p=0.3092) (Figure 9B). With these factors separated and analyzed, it was concluded that media type was responsible for the final weight differences for the callus pieces. This experiment showed how each biotype reacted to the different media compositions and showed that medium 2 produced the most callus and was effective across different biotypes.

After the establishment of a callus culture protocol, I used medium 2 to generate more callus cultures to test how long these cultures could be sustained in liquid culture with regular subculturing. Viability checks were done at 2-3 weeks after starting these cultures, as cells will begin to shed from the starting callus at this point (Figure 10 C). Using FDA stain on samples from these cultures provided the indication of healthy development (Figure 10), cells in the supernatant and a crushed callus piece. With "feeding" occurring on a weekly basis, these cultures can be kept alive and developed for a minimum of 3 months (oldest culture as of date being 3 months old) (Figure 11). The oldest culture filled the entire culture (30-50 mL) with callus pieces and floating cells in the supernatant before needing subculturing, when using medium 2. Figure 11A shows a culture that has been developing for 2 months and has filled the flask with viable cells/callus as shown in Figure 11B/C.

Liquid Callus Culture Transformation and Regeneration Trial:

Transformation trials were attempted with the viable callus pieces that were derived from TNS,TNR and DEL callus cultures. Transforming with *A. tumefaciens* st. EHA105 (construct: pBIN-mGFP5-ER) to test for transformation capability and regeneration of transgenic plants, as these callus pieces can produce multiple shoots from a single callus piece (Figure 11G). With

multiple shoots being regenerated at a higher rate, this potentially could regenerate transgenic horseweed. Preliminary transformations have occurred (Callus Culture Transformation and Regeneration Trial Method), but there have been some obstacles with this such as *Agrobacterium* overgrowth during the incubation time and the light room (16 hr light/ 8 hr dark) despite being on selection media (TIMENTIN® 400 mg/L). Further optimization is needed for callus transformation. Non-model plant (switchgrass and petunia) protocols led to the protocol used in my research. While my research represents early-stage optimization, my protocol might be adapted to generate cell suspension cultures that could possibly lead towards transformation and regeneration of transgenic horseweed.

Discussion:

Glyphosate-resistant horseweed is problematic for sustainable crop production using no-till practices and glyphosate-resistant crops (Varah et al. 2020). With the growing need to understand non-model plants such as *Erigeron canadensis* and *Amaranthus palmeri* for non-target glyphosate-resistant, tissue culture protocols need to be expanded. Even under limited use of tissue culture protocols, several studies have provided insight on possible mechanisms that could be responsible for endowing glyphosate-resistance in horseweed (Peng et al. 2010, Yuan et al. 2010, Nol et al. 2012, Bo et al. 2021, Yang et al. 2021).

Despite the studies to identify glyphosate-resistance in horseweed, there are very few tissue culture studies focusing on horseweed regeneration. Very little success has occurred in tissue culture studies with horseweed, only two studies (Halfhill et al. 2007, Scheiber et al. 2006) have published the methods on shoot regeneration. One of the greatest challenges with

working with a weed species such as horseweed, is biotype or genotype specificity in tissue culture responses. Despite my use of geographically similar biotypes to Halfhill et al. (2007), they are not the exact same plant lines (TNR and TNS) from that study due to horseweed's seed viability of 2-3 years, in which the original lines were lost. This biotype difference can be an obstacle when attempting tissue culture experiments within the horseweed populations, as it has been identified in other tissue culture experiments (Machczyńska et al. 2015, Orłowska et al. 2020). However, finding protocols that can be applied to a species instead of a specific biotype within the species offers the chance for further development of research.

The regeneration media testing resulted in providing further insight on how horseweed biotypes react to different hormones in tissue culture. The solid medium DBIX was found to be statistically significant over the rest of the media by producing shoots at a higher and more consistent rate (77% shoot regeneration, $p<0.001$). Despite having an improved indirect organogenesis process for horseweed, regeneration of transgenic horseweed still was not accomplished with this protocol. One possible explanation for the lack of success in transgenic plant regeneration may stem from the utilization of insufficient selection pressure applied post-transformation, allowing non-transgenic callus to outcompete the transgenic cells. The selection media contained a low dosage of kanamycin (50 mg/L) to reduce stress on shoot development, resulting in nontransgenic escapes and the lack of transgenic horseweed shoots. For future experiments, a higher level of selection may result in successful transgenic horseweed while using the optimized tissue culture medium (DBIX). Overall, with the testing of indirect organogenesis, it was clear that biotype variation can be managed with the proper hormone concentration.

Replacing the indirect organogenesis method (strictly solid medium) with liquid medium based tissue culture environment is not unique as there have been a multitude of studies done to produce a liquid culture system for non-model organisms such as *Petunia hybrida* (Kang et al. 2020), *Phyllanthus acidus* (Duangporn et al. 2009), *Scrophularia striata* (Khanpour-Ardestani et al. 2015), and *Brassica oleracea* (Shafiq et al. 1999). After comparing different media compositions, it became clear that only one of the compositions had positive results (healthy callus development and cell proliferation) across biotypes. The results for final weight in callus cultures for medium 2 results were statistically significant (Figure 8, Figure 9A) when compared to the other media types. Given the success of tissue regeneration in the *Asteraceae* family using BAP and IBA at similar concentrations (1 mg/L BAP and 0.07 mg/L IBA) as reported by Blando et al. in 2021, there was potential for horseweed to respond positively. Using a callus culture suspension method offers an opportunity for transgenic regeneration for horseweed especially since the timeline for regeneration is shortened and biotype variability is not a factor (Figure 9B). After testing indirect organogenesis and callus culture regeneration, a comparison was made to assess timeline and advantages/disadvantages with one another (Figure 12). The comparison offers two different methods for regeneration instead of a single approach. Having a system that can reduce the regeneration time in half provides the base for large amounts of transgenic screening potentially. While indirect organogenesis has regenerated transgenic shoots from horseweed leaf tissue (Halfhill et al. 2007), it might be beneficial to use liquid media for gene editing experiments in horseweed.

Conclusion:

The current study provides groundwork in producing two different tissue culture systems that are not affected by biotype variability within horseweed. Previously, there were only two published methods for regeneration (Scheiber et al. 2006, Halfhill et al. 2007) though indirect organogenesis, which was optimized in this study through solid DBIX. We were also able to establish a new protocol for liquid callus cultures using medium 2, which has never been reported before in *Erigeron canadensis*. Both experiments generated statistical evidence that solid DBIX and liquid medium 2 worked the best in their respective experiments for regenerating wild-type horseweed. To conclude, this study provided a novel liquid callus protocol along with an optimized indirect organogenesis medium. These experiments provided the basis for further tissue culture studies to be conducted such as genome engineering or gene editing experimentations.

Chapter 3: Conclusion and Recommendation

Achieving reproducible indirect organogenesis regeneration was a step towards achieving stable transformation with horseweed. With horseweed, which can take months to develop wild-type plants within tissue culture, tissue culture methods needed to be optimized if stable transformation is the goal. The first challenge of optimization was finding the correct concentration of hormones for indirect organogenesis along with overcoming biotype variation in tissue culture. We achieved this by modifying the initially published approach to be more encompassing when dealing with diverse horseweed biotypes. A biotype-independent protocol is the goal. Despite success in indirect organogenesis, stable transformation was elusive for all biotypes tested.

Establishing a novel liquid callus culture in horseweed was shown to have a variety of advantages that were not true for indirect organogenesis. The callus culture protocol was found to work across biotypes, like the indirect organogenesis protocol, but also can produce more tissue and cut down the timeline for regeneration in half. This protocol offers the chance to generate a pipeline of tissue for gene editing experiments with a constant pipeline of readily available tissue. Another aspect of callus suspension culture is the convenience and low maintenance required for this method. This protocol shown in the thesis has the potential for high throughput testing of constructs for horseweed gene editing experiments or establishing a cell suspension culture.

Taking all these factors into account, it was necessary to reevaluate and enhance the tissue culture foundation in horseweed research before stable transformations can be achieved. With these protocols, the opportunity for mass gene testing is possible and provides

the chance for further exploration within glyphosate resistance. Both protocols have shown improvements for regeneration and testing with stable transformations can be built, using these protocols as step one for that process.

List of References

Banasiak J, Jasiński M. (2021) ATP-binding cassette transporters in nonmodel plants. *New Phytologist* 233(4): 1597-1612

Bednarek P, Pachota K, Dynkowska W, Machczyńska J, Orłowska R. (2021) Understanding in-vitro tissue culture-induced variation phenomenon in microspore system. *International Journal of Molecular Sciences* 22(14)

Bekech M, Bhowmik P. (1993) Horseweed (*Conyza canadensis*) seed production, emergence, and distribution in no-tillage and conventional-tillage corn (*Zea mays*). *Agronomy* (*Trends Agriculture Science)* 1: 67-71

Benbrook C. (2016) Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe* 28(1): 3

Beres Z, Ernst E, Ackley B, Loux M, Owen M, Snow A. (2018) High levels of glyphosate resistance in *Conyza canadensis* from agricultural and non-agricultural sites in Ohio and Iowa. *Scientific Reports* 8

Blando F, Rizzello F, Durante M, De Paolis A, Caretto S, Mita G. (2021) In vitro adventitious regeneration of *Artemisia annua* L. influencing artemisinin metabolism. *Horticulturae* 7(11): 438

Bo A, Jia W, Le T, Won O, Khaitov B, Cho K, Park I, Park K. (2021) Mechanisms of glyphosate-resistant horseweed (*Conyza canadensis*) collected from tangerine orchards in Korea. *Horticulture, Environment, and Biotechnology* 62: 995-1002

Bochkov D, Sysolyatin S, Kalashnikov A, Surmacheva I. (2012) Shikimic acid: review of its analytical, isolation, and purification techniques from plant and microbial sources. *Journal of Chemical Biology* 5(1): 5-17

Chetty V, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas M. (2012) Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. *Plant Cell Reports* 32: 239-247

Davis V, Gibson K, Johnson W. (2008) A field survey to determine distribution and frequency of glyphosate-resistant horseweed (*Conyza canadensis*) in Indiana. *Weed Technology* 22: 331–338

Debernardi J, Tricoli D, Ercoli M, Hayta S, Ronald P, Palatnik J, Dubcovsky J. (2020) A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nature Biotechnology* 38(11): 1274-1279

DeVerna J, Collins G. (1984) Maternal haploids of *Petunia axillaris* (Lam.) B.S.P. via culture of placenta attached ovules. *Theoretical and Applied Genetics* 69: 187-192

Duangporn P, Siripong P. (2009) Effect of auxin and cytokinin on Phyllanthusol A production by callus cultures of *Phyllanthus acidus* skeels. *American-Eurasian Journal of Agricultural and Environmental Sciences* 5:258–263

Fehér A. (2019) Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? *Frontiers in Plant Science* 10

Feng P, Tran M, Chiu T, Sammons R, Heck G, CaJacob C. (2004) Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): Retention, uptake, translocation, and metabolism. *Weed Science* 52(4): 498-505

Fiuk A, Bednarek P, Rybczyński J. (2010) Flow cytometry, HPLC-RP, and metAFLP analyses to assess genetic variability in somatic embryo-derived plantlets of *Gentiana pannonica* Scop. *Plant Molecular Biology Report* 28:413–420

Ge X, d'Avignon D, Ackerman J, Sammons R. (2010) Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. *Pest Management Science* 66: 345-348

Ge X, d'Avignon D, Ackerman J, Collavo A, Sattin M, Ostrander E, Hall E, Sammons R, Preston C. (2012) Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (Lolium spp.) from Australia, South America, and Europe: A 31 P NMR investigation. *Journal of Agricultural and Food Chemistry* 60(5): 1243–1250

Ge X, d'Avignon D, Ackerman J, Sammons R. (2014) In vivo ^{31}P-nuclear magnetic resonance studies of glyphosate uptake, vacuolar sequestration, and tonoplast pump activity in glyphosate-resistant horseweed. *Plant Physiology* 166(3): 1255–1268

Halfhill M, Good L, Basu C, Burris J, Main C, Mueller T, Stewart C. (2007) Transformation and segregation of GFP fluorescence and glyphosate resistance in horseweed (*Conyza canadensis*) hybrids. *Plant Cell Reports* 26: 303-311

Hanson B, Shrestha A, Shaner D. (2009) Distribution of glyphosate-resistant horseweed (*Conyza canadensis*) and relationship to cropping systems in the Central Valley of California. *Weed Science* 57: 48-53

Harborne J. (1964) *Biochemistry of Phenolic Compounds*. Academic Press

Heap I. (2023) The International Herbicide-Resistant Weed Database. Retrieved April 27, 2023, from https://www.weedscience.org/

Heap I, Duke S. (2018) Overview of glyphosate-resistant weeds worldwide. *Pest Management Science* 74(5): 1040-1049

ISAAA. (2023) International Service for the Acquisition of Agri-biotech Applications. Retrieved July 31, 2023, from https://www.isaaa.org/

Kang H, Naing A, Kim C. (2020) Protoplast isolation and shoot regeneration from protoplast-derived callus of *Petunia hybrida* Cv. Mirage Rose. *Biology (Basel)* 9(8):228

Kao K, Michayluk M. (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta* 126:105-110

Khanpour-Ardestani N, Sharifi M, Behmanesh M. (2015) Establishment of callus and cell suspension culture of *Scrophularia striata* Boiss.: an in vitro approach for acteoside production. *Cytotechnology* 67(3): 475–485.

Kim D, Bovet L, Kushnir S, Noh E, Martinoia E, Lee Y. (2006) *AtATM3* is involved in heavy metal resistance in *Arabidopsis*. *Plant Physiology* 140(3): 922-932

Klaus H, Weaver L. (1999) The shikimate pathway. *Annual Review Plant Physiology Plant Molecular Biology* 50: 473-503

Koger C, Reddy K. (2005) Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Science* 54:84-89

Kovalchuk A, Kohler A, Martin F, Asiegbu, F. (2015) Diversity and evolution of ABC proteins in mycorrhiza-forming fungi. *BMC Evolutionary Biology* 15: 249

Laforest M, Martin S, Bisaillon K, Soufiane B, Meloche S, Page E. (2020) A chromosome-scale draft sequence of the Canada fleabane genome. *Pest Management Science* 76(6): 2158-2169

Lane T, Rempe C, Davitt J, Staton M, Peng Y, Soltis D, Melkonian M, Deyholos M, Leebens-Mack J, Chase M, Rothfels C, Stevenson D, Graham S, Yu J, Liu T, Pires J, Edger P, Zhang Y, Xie Y, Zhu Y, Carpenter E, Wong G, Stewart C. (2016) Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. *BMC Biotechnology* 16(1): 47

Li M, Wang D, Long X, Hao Z, Lu Y, Zhou Y, Peng Y, Cheng T, Shi J, Chen J. (2022) *Agrobacterium*-Mediated Genetic Transformation of Embryogenic Callus in a *Liriodendron* Hybrid (*L. Chinense* × *L. Tulipifera*). *Frontiers in Plant Science* 13

Machczyńska J, Zimny J, Bednarek P. (2015) Tissue culture-induced genetic and epigenetic variation in triticale (× *Triticosecale* spp. Wittmack ex A. Camus 1927) regenerants. *Plant Molecular Biology* 89(3): 279–292

Mikuła A, Tomiczak K, Rybczynski J. (2011) Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi)genetic uniformity of regenerants. *Plant Cell Reports* 30:565–574

Murashige T, Skoog F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497

Nol N, Tsikou D, Eid M, Livieratos I, Giannopolitis C. (2012) Shikimate leaf disc assay for early detection of glyphosate resistance in *Conyza canadensis* and relative transcript levels of EPSPS and ABC transporter genes. *Weed Research* 52: 233-241

Norris-Trull D. (2020) The History of Herbicides and Other Pesticides Prior to & During WWII. *Management of Invasive Plants in the Western USA*. Retrieved April 27, 2023, from https://www.invasiveplantswesternusa.org/

Ondzighi-Assoume C, Willis J, Ouma W, Allen S, King Z, Parrott W, Liu W, Burris J, Lenaghan S, Stewart C. (2019) Embryogenic cell suspensions for high-capacity genetic transformation and regeneration of switchgrass (*Panicum virgatum L*.). *Biotechnology for Biofuels*. 12: 290

Orłowska R, Bednarek, P. (2020) Precise evaluation of tissue culture-induced variation during optimisation of in vitro regeneration regime in barley. *Plant Molecular Biology* 103(1-2): 33–50

Pan L, Yu Q, Wang J, Han H, Mao L, Nyporko A, Maguza A, Fan L, Bai L, Powles S. (2021) An ABCC-type transporter endowing glyphosate resistance in plants. *Proceedings of the National Academy of Sciences of the United States of America* 118(16)

Peng Y, Abercrombie L, Yuan J, Riggins C, Sammons R, Tranel P, Stewart C. (2010) Characterization of the horseweed (*Conyza canadensis*) transcriptome using GS-FLX 454 pyrosequencing and its application for expression analysis of candidate non-target herbicide resistance genes. *Pest Management Science* 66(10): 1053-1062

Peng Y, Lai Z, Lane T, Nageswara-Rao M, Okada M, Jasieniuk M, O'Geen H, Kim R, Sammons R, Rieseberg L, Stewart C. (2014) De novo genome assembly of the economically important weed horseweed using integrated data from multiple sequencing platforms. *Plant Physiology* 166(3): 1241–1254

Remy E, Duque P. (2014) Beyond cellular detoxification: A plethora of physiological roles for MDR transporter homologs in plants. *Frontiers in Physiology* 5

Scheiber P, Tran M, Duncan D. (2006) Tissue culture and transient transformation of Marestail (*Conyza canadensis* (*L.*) *Cronquist*). *Plant Cell Reports* 25(6): 507-512

Serra L, Estienne A, Vasseur C, Froment P, Dupont J. (2021) Review: mechanisms of glyphosate and glyphosate-based herbicides action in female and male fertility in humans and animal models. *Cells* 10(11): 3079

Shafiq A, William S. (1999) Effects of NAA and BAP on callus culture and plant regeneration in curly kale (*Brassica oleraces* L.). *Pakistan Journal of Biological Sciences* 2: 109-112.

Sidorov V, Gilbertson L, Addae P, Duncan D. (2006) *Agrobacterium*-mediated transformation of seedling-derived maize callus. *Plant Cell Reports* 25: 320–328

Siehl D. (1997) Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis. *Herbicide activity: toxicology, biochemistry and molecular biology* 37-67

Sultana M, Frazier T, Millwood R, Lenaghan S, Stewart C. (2019) Development and validation of a novel and robust cell culture system in soybean (*Glycine max* (L.) Merr.) for promoter screening. *Plant Cell Reports* 38(10): 1329–1345

Sun N, Xie Y, Wu Y, Guo N, Li D, Gao J. (2021) Genome-wide identification of ABCC gene family and their expression analysis in pigment deposition of fiber in brown cotton (*Gossypium hirsutum*). *PloS one* 16(5)

United States Department of Agriculture (Economic Research Service) (2022) *Recent Trends in GE Adoption*. Retrieved May 5, 2023, from https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-u-s/recent-trends-in-ge-adoption/

Varah A, Ahodo K, Coutts S, Hicks H, Comont D, Crook L, Hull R, Neve P, Childs D, Freckleton R, Norris K. (2020) The costs of human-induced evolution in an agricultural system. *Nature Sustainability* 3(1): 63-71

VanGessel M. (2001) Glyphosate-resistant horseweed from Delaware. *Weed Science* 49: 703–705

Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S. (2011) A modular cloning system for standardized assembly of multigene constructs. *PLoS One* 6(2)

Yang Y, Gardner C, Gupta P, Peng Y, Piasecki C, Milwood R, Ahn T, Stewart C. (2021) Novel candidate genes differentially expressed in glyphosate-treated horseweed (*Conyza canadensis*). *Genes* 12(10): 1616

Yuan J, Abercrombie L, Cao Y, Halfhill M, Zhou X, Peng Y, Hu J, Rao M, Heck G, Larosa T, Sammons R, Wang X, Ranjan P, Johnson D, Wadl P, Scheffler B, Rinehart T, Trigiano R, Stewart C. (2010) Functional genomics analysis of horseweed (*Conyza canadensis*) with special reference to the evolution of non–target-site glyphosate resistance. *Weed Science* 58(2): 109-117

Zhang C, Yu C, Yu Q, Guo W, Zhang T, Tian X. (2021) Evolution of multiple target-site resistance mechanisms in individual plants of glyphosate-resistant *Eleusine indica* from China. *Pest Management Science* 77: 4810-4817

Appendix

Table 1: Media compositions and hormone concentrations for solid media testing. All concentrations are based on 1-liter formulations.

Media Names:	Hormone Concentrations		Extra Additives
DBI	2 mg L^{-1} kinetin	1 mg L^{-1} IAA	N/A
DBIX	2 mg L^{-1} kinetin	0.438 mg L^{-1} IAA	N/A
DB	2 mg L^{-1} kinetin	N/A	N/A
CBN1	1.125 mg L^{-1} BAP	0.931 mg L^{-1} NAA	62.5 mg L^{-1} charcoal
CBN10	2.25 mg L^{-1} BAP	0.186 mg L^{-1} NAA	62.5 mg L^{-1} charcoal
SIM	0.4 mg L^{-1} BAP	N/A	N/A
All Media contained I Macro/I Micro salts, 3% sucrose, 0.2% gelzen, pH 5.8			

Table 2: Callus culture media compositions and hormone concentrations for liquid media testing. All concentrations are based on 1-liter formulations.

Media Names:	Hormone Concentrations		
Medium 1	1 mg L^{-1} BAP	1.98 mg L^{-1} 2,4-D	N/A
Medium 2	1 mg L^{-1} BAP	0.02 mg L^{-1} IBA	N/A
Medium 3	0.2 mg L^{-1} Zeatin	1 mg L^{-1} NAA	0.1 mg L^{-1} 2,4-D
Medium 1: Murashige and Skoog (MS) basal salts, 3% sucrose, pH 5.8 (Ondzighi-Assoume et al. 2019) Medium 2: Murashige and Skoog (MS) basal salts,3% sucrose, pH 5.8 (Kang et al. 2020) Medium 3: KM Macro and Micro salts and others (Kao et al. 1975) (Kang et al. 2020)			

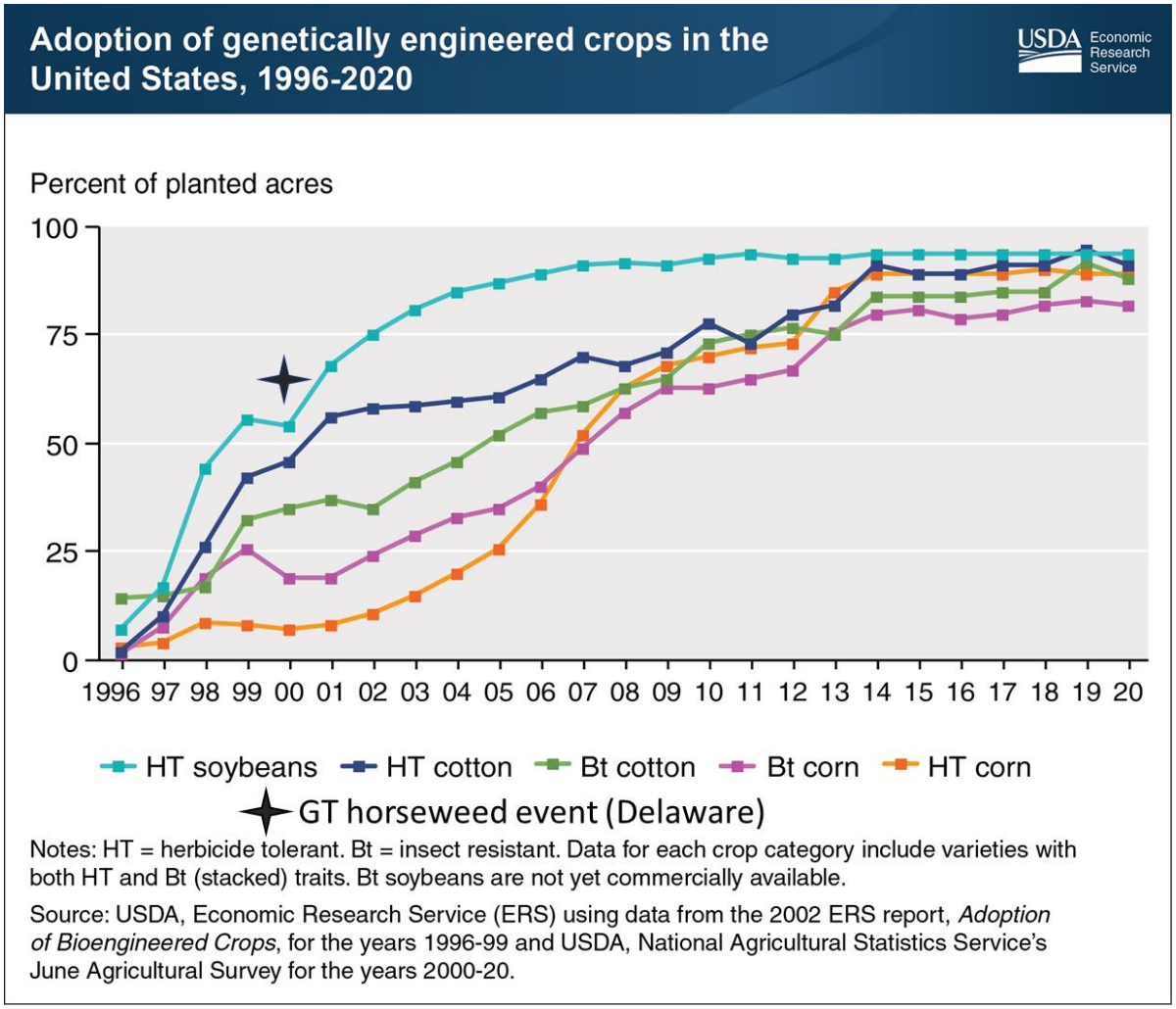


Figure 1: Trendlines on genetically modified (GM) crops within the United States from 1996 to 2020, based on percentage of plant acres. The first glyphosate tolerant (GT) horseweed event is marked with a star in 2000. The informatic was adapted from the USDA, ERS website.

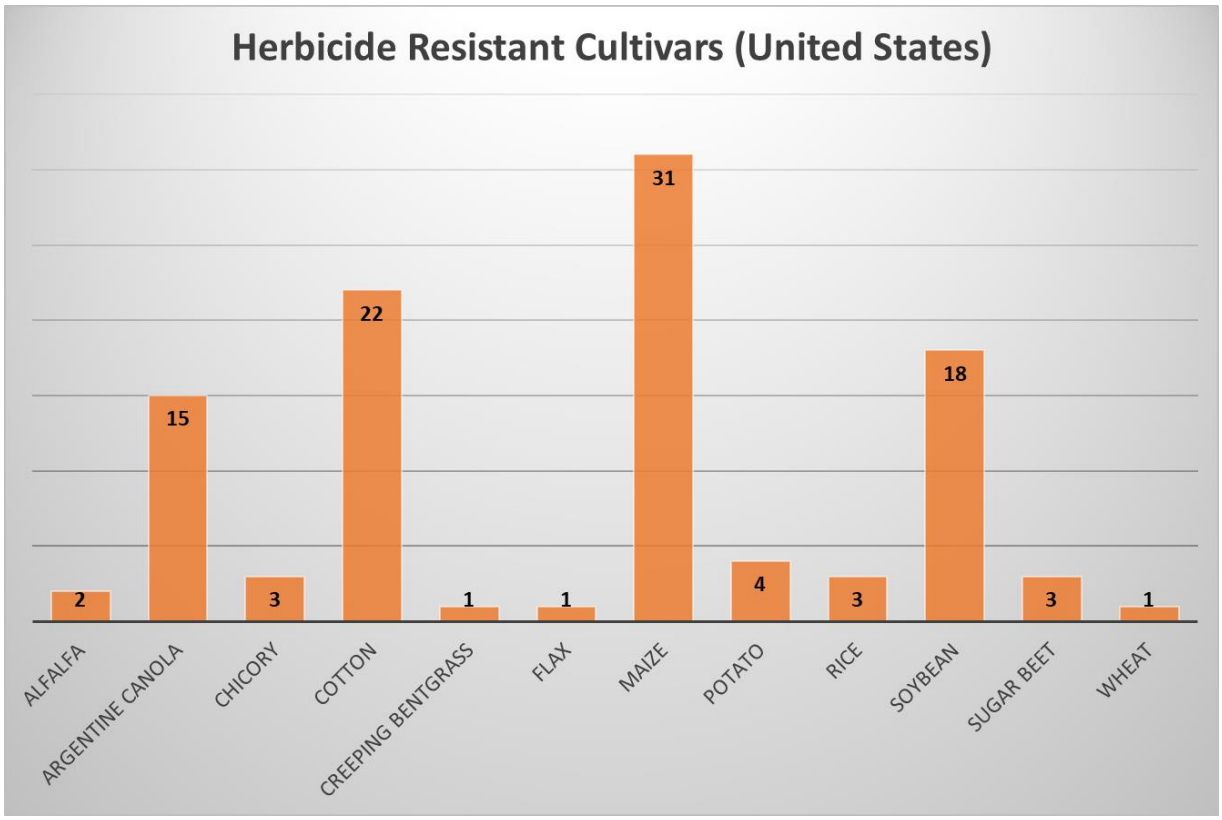


Figure 2: Current approved herbicide resistant cultivars within the United States of America. This is an infographic that shows what plants contain approved herbicide tolerant genes that are allowed for food and/or feed consumption within the United States of America, with a total of 104 events across 12 plant species. This material is published by International Service for the Acquisition of Agri-biotech Applications (ISAAA) (www.isaaa.org).

Figure 3: Map of the United States of America with stars representing locations that glyphosate-resistant horseweed has been identified within the state borders. Delaware (which is represented by a golden star) was the first state to have glyphosate resistant horseweed identified in the United States and over the course of 20 years, we have identified more than 20 other events. The data on location of glyphosate resistant horseweed was derived through the International Herbicide-Resistant Weed Database (Heap 2023).

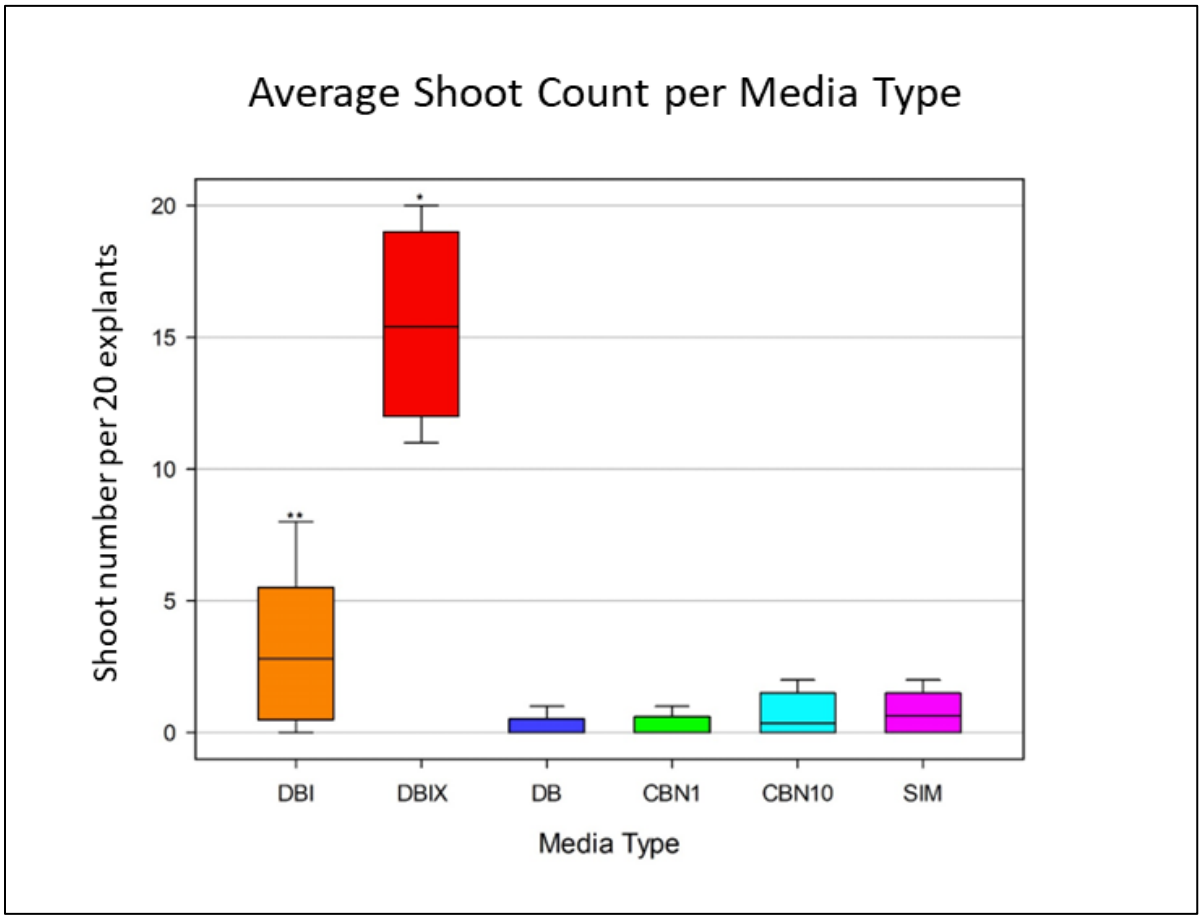


Figure 4: The effects of different solid media types on average shoot regeneration. Each box plot represents the average shoot count recorded for each medium. Shoots were tallied out of 20 as there were 20 explants per plate. Statistical analysis of regeneration media testing using a one-way ANOVA to compare the six different media (SAS 9.4, Cary, N.C., U.S.A.) (Media types denoted with same symbol indicates there is no significant difference according to Tukey's HSD test, α=0.05). DBIX and DBI demonstrated significant differences (p<0.001) compared to the other tested media, individually.

Figure 5: Comparison of shoot regeneration counts between the solid media, DBIX and DBI. Shoots were tallied out of 20 as there were 20 explants per plate. (A) Comparing the shoot averages when dividing the biotype tissue material between the 13-18 leaf stage and 8-12 leaf stage when using DBIX and DBI (p=0.7344). (B) Comparing the shoot averages of the different biotypes when using DBIX and DBI (p=0.0971). Statistical analysis of regeneration media testing using a one-way ANOVA to compare the solid media for both figures (SAS 9.4, Cary, N.C., U.S.A.) (Media types denoted with same symbol indicates there is no significant difference according to Tukey's HSD test, α=0.05).

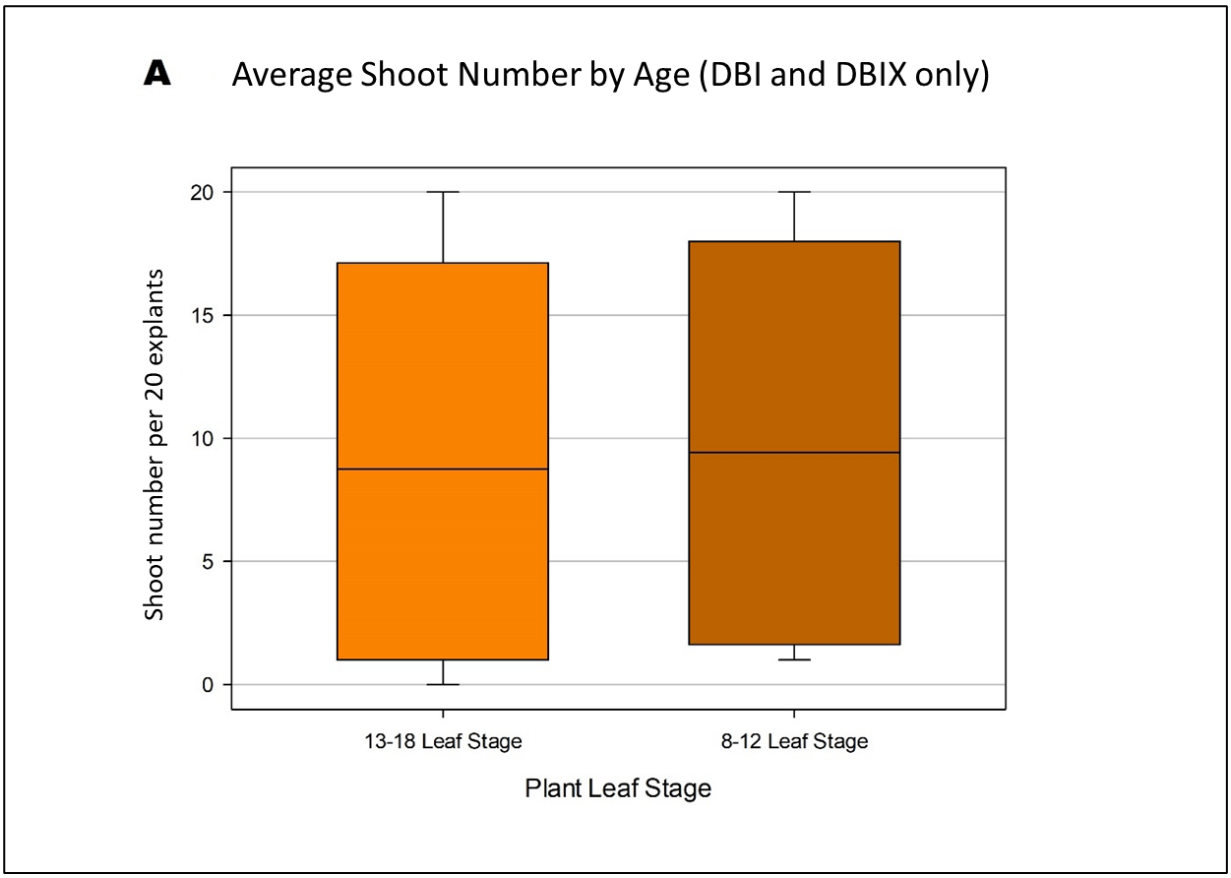


Figure 5 continued

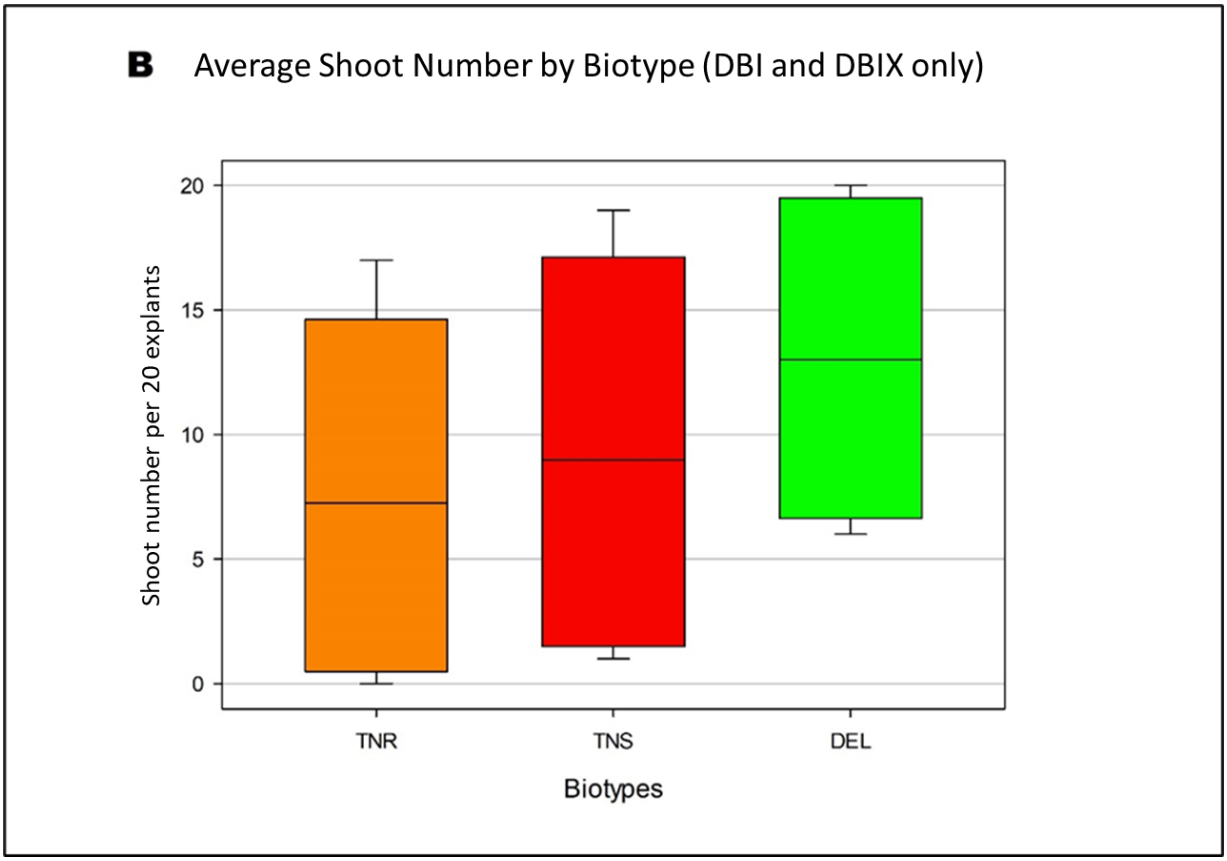


Figure 5 continued

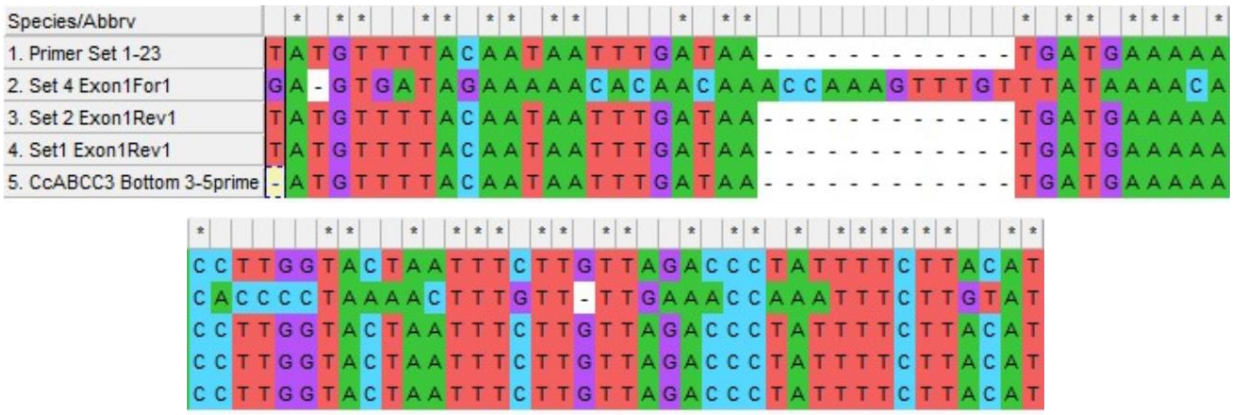


Figure 6: Sequencing data received by Eurofins for *M11*/*CcABCC3*. The shown sequence are the primer sets with the draft sequence. This alignment was performed in MEGAX MUSCLE alignment, with all reads matching a base pair denoted with *. The sequence above is the beginning of exon 1 for *M11*/*CcABCC3*, which this gene in total is 9,384 basepairs in length with a total of 10 coding regions or exons.

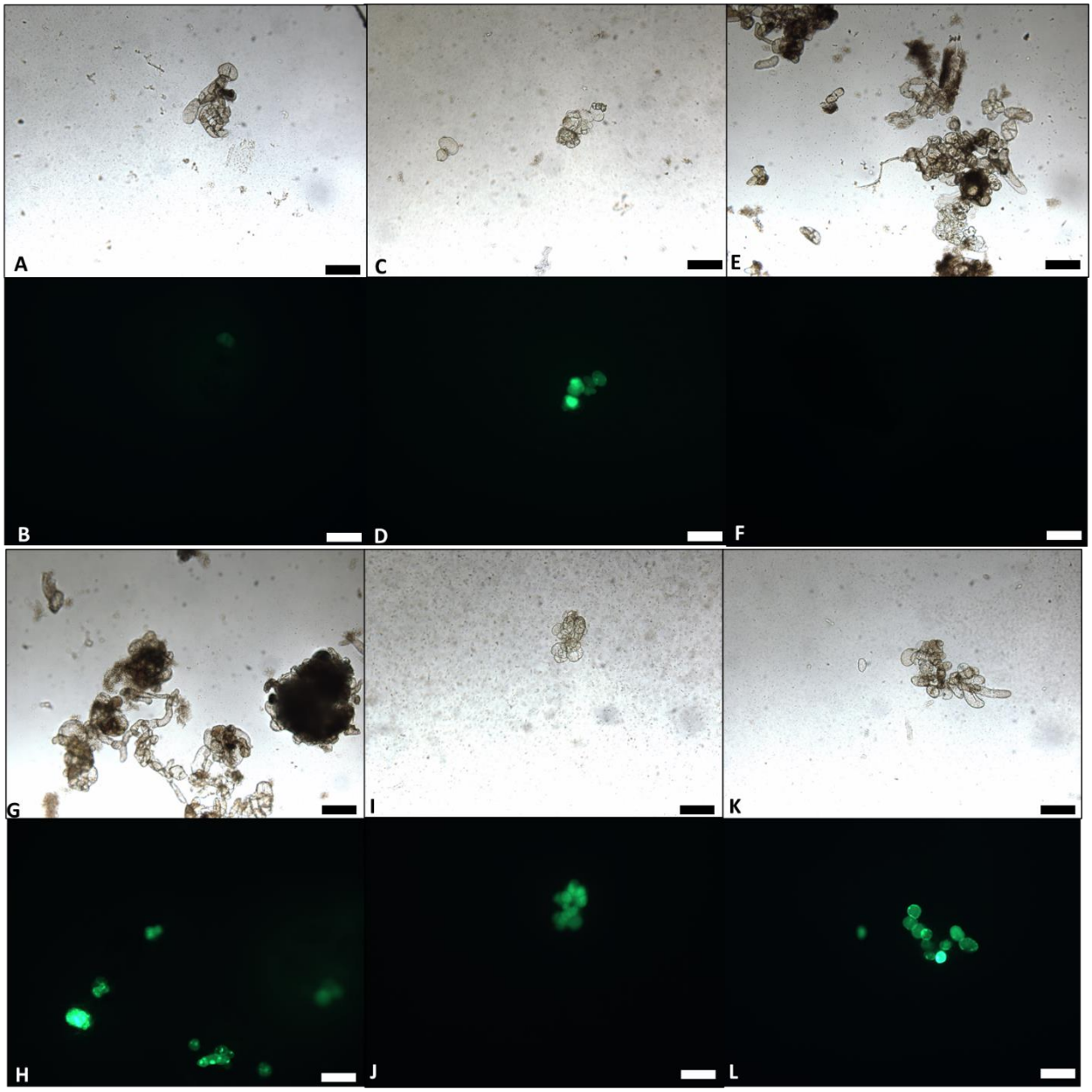


Figure 7: Cell viability assessments using supernatant samples from liquid callus culture media. TNR cells in Medium 1 (MS basal salts, 3% sucrose, 1 mg/L BAP, and 1.98 mg/L 2,4-D) were stained with FDA, as shown in slides **A** and **B** (brightfield 12 ms and FITC filter 300 ms, respectively). Similarly, TNR cells in Medium 2 (MS basal salts, 3% sucrose, 1 mg/L BAP, and 0.02 mg/L IBA) were stained with FDA and displayed in slides **C** and **D**. Slide **E** and **F** illustrate TNR cells in Medium 3 (Kao and Michayluk Basal Salt Mixture with multiple hormones: zeatin, NAA, and 2,4-D from Phytotech Labs) stained with FDA. For TNS cells in Medium 1, FDA staining results are shown in slides **G** and **H** (brightfield 12 ms and FITC filter 300 ms, respectively). For TNS cells in Medium 2, the FDA-stained image is presented in slides **I** and **J** (brightfield and FITC filter). Slide **K** (brightfield) and **L** (FITC filter) depicts TNS cells in Medium 3 stained with FDA. The size bars for slides A-L are 50 µm.

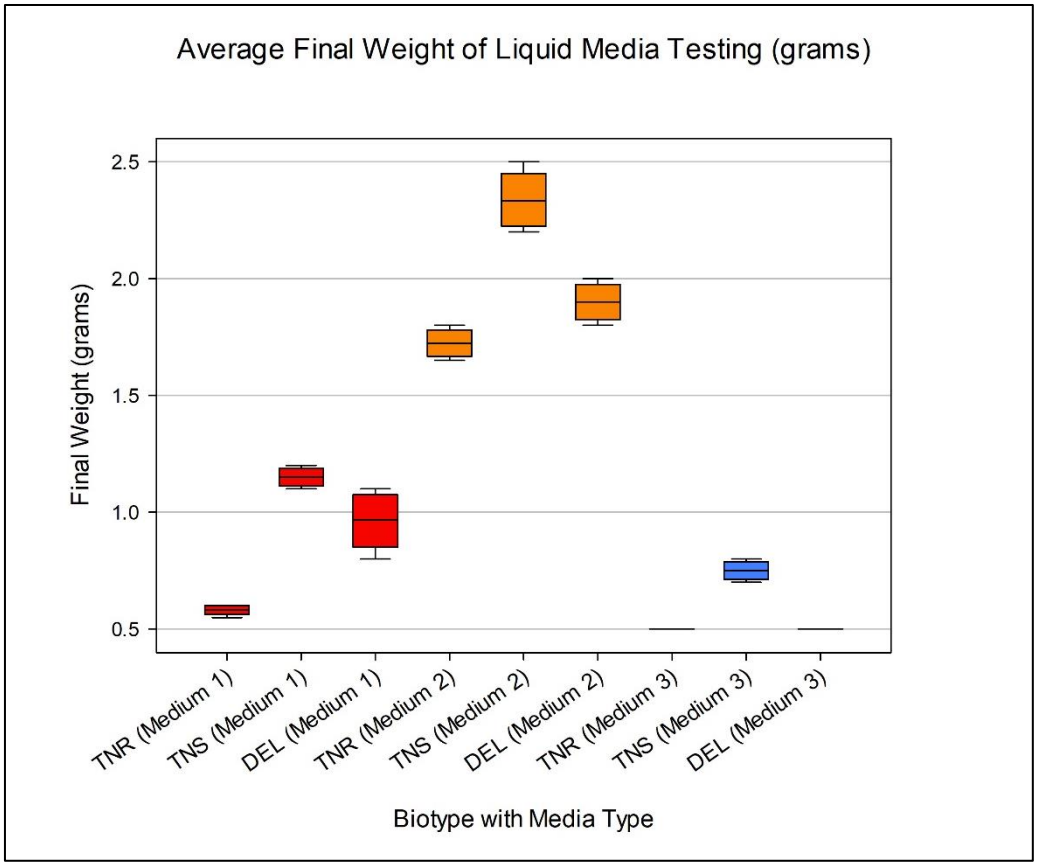


Figure 8: Comparing the final weights (FW) of callus between the different liquid media types along with biotypes. Each biotype and media combination were replicated 3 times, n=27 for the entire study. These weights are recorded after one month of development for the callus within the different media, the starting weight of callus was 0.5 grams. The biotypes are Tennessee Resistant (TNR), Tennessee Susceptible (TNS), and Delaware (DEL) with Medium 1 (MS basal salts, 3% sucrose, 1 mg/L BAP and 1.98 mg/L 2,4-D), Medium 2 (MS basal salts, 3% sucrose, 1 mg/L BAP and 0.02 mg/L IBA) and Medium 3 (Kao and Michayluk Basal Salt Mixture with multiple hormones: zeatin, NAA, and 2,4-D (from Phytotech Labs)). For the statistical analysis of liquid media testing, a two-way ANOVA was employed using SAS 9.4 (Cary, N.C., U.S.A.). Utilizing Tukey's HSD test with a confidence level of α=0.05, the results revealed a statistically significant difference within the interaction of biotype and media (p=0.0028).

Figure 9: The effect of media on different biotypes: Tennessee Resistant (TNR), Tennessee Susceptible (TNS) and Delaware (DEL). The sample size for each group was 9 cultures for a total sample size of 27. (A) final weights of the callus after one month in different media. All cultures started with 0.5 grams of callus and were placed into total darkness on a rotary shaker (90-100 rpm) for one month at a temperature range of 22°C to 26.5°C. A one-way ANOVA test was used with an α=0.05 to identify any significant differences (p<0.001) (Media types denoted with the same symbol indicates there is no significant difference). (B) is compiling the final weights of the different media and only separating them by biotypes. The one-way ANOVA on biotypes was used to identify any statistical difference (p=0.3092) (SAS 9.4, Cary, N.C., U.S.A.) (Biotypes denoted with different symbols indicates there is a significant difference).

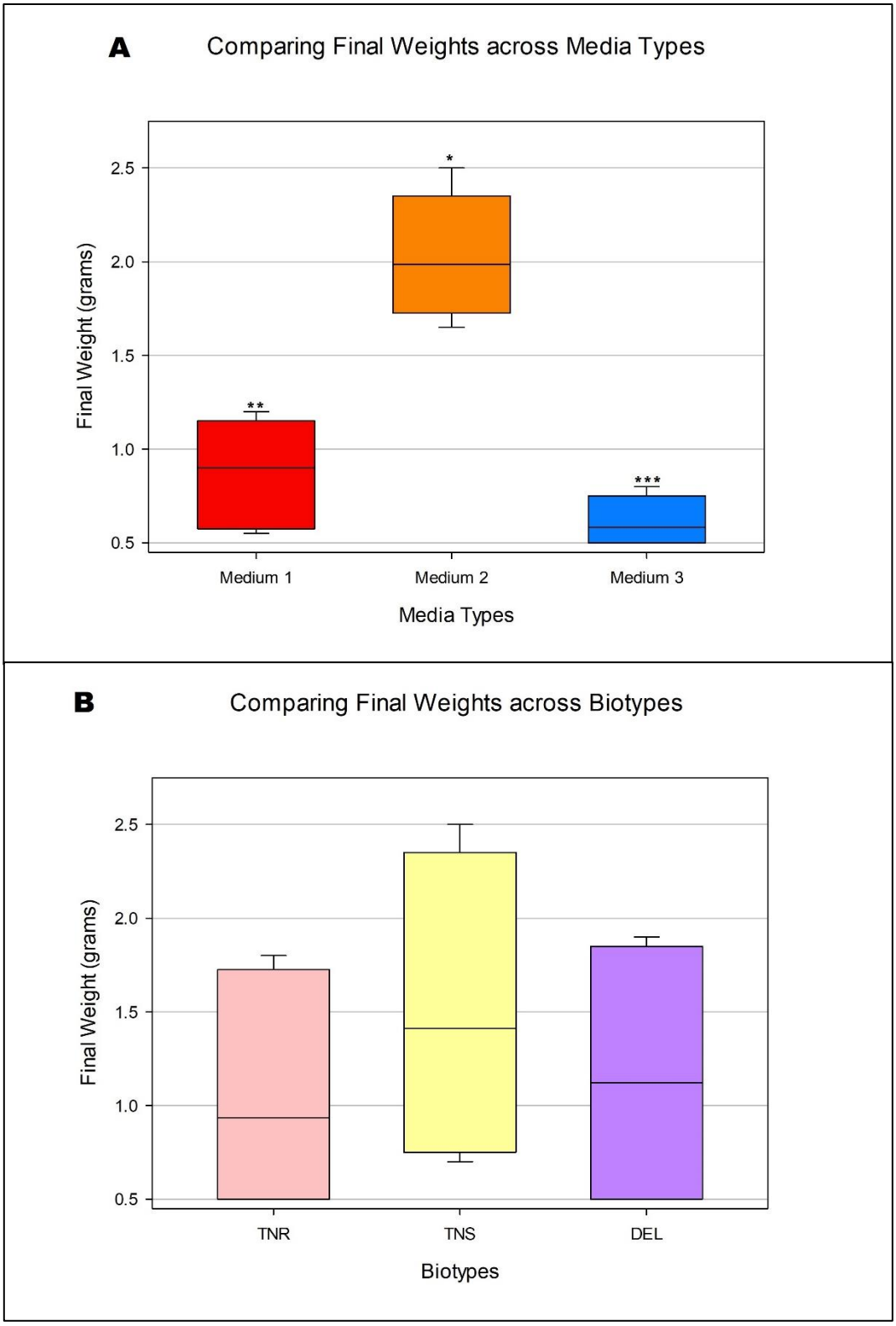


Figure 9 continued

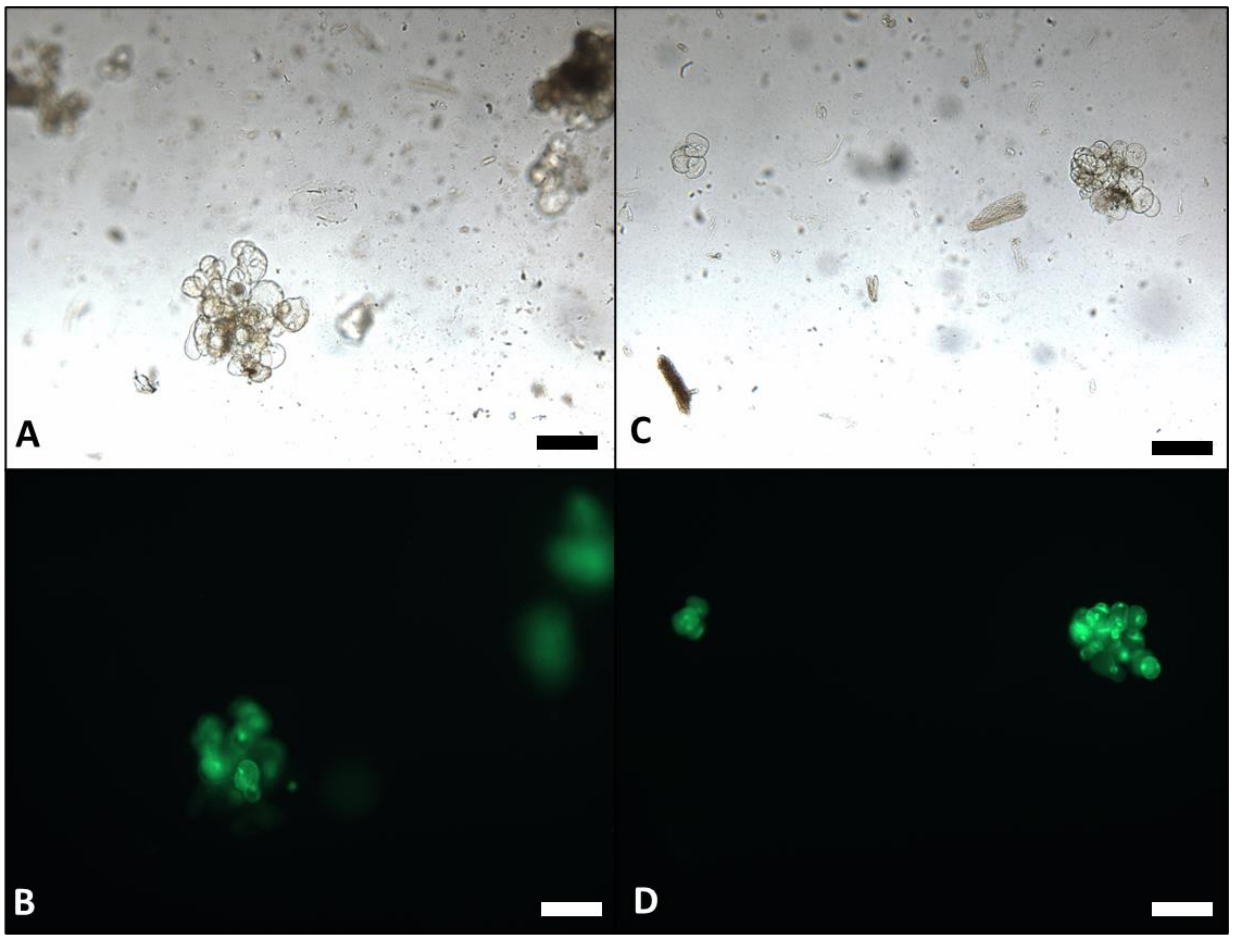


Figure 10: Samples from liquid callus cultures for cell viability assessments from Delaware cultures in Medium 2. These were prior to attempting transformation with *A. tumefacien*s st. EHA105 containing pBIN-mGFP5-ER to test a transformation protocol. **A** (brightfield 12 ms), **B** (FITC filter 300 ms) are crushed callus 3-week-old Delaware callus stained with FDA. **C** (brightfield 12 ms), **D** (FITC filter 300 ms) show supernatant 3-week-old Delaware callus stained with FDA. The size bars are 50 µm for slides A-D.

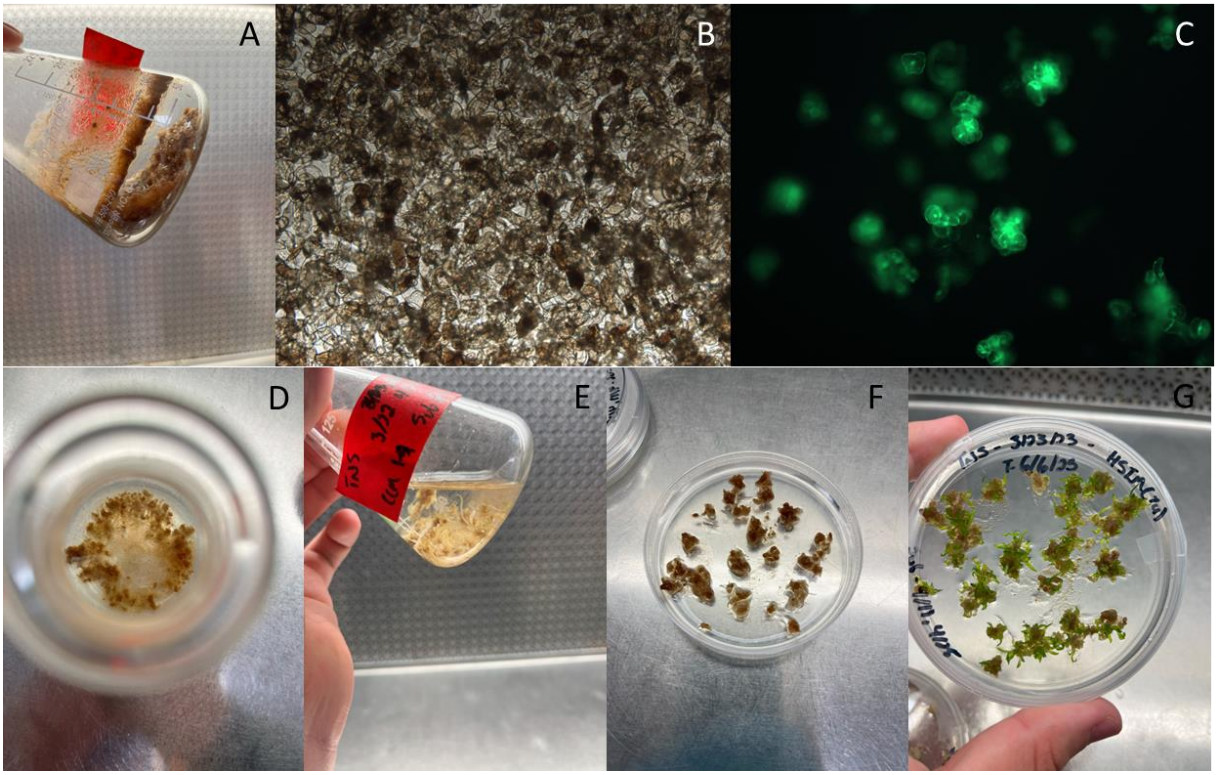


Figure 11: Progression timeline of callus culture paired with regeneration using medium 2. **A** mature callus culture of Tennessee susceptible cells that have been growing for 3 months and have reached a peak density level. **B/C** aliquot of the supernatant of the mature culture to assess cell viability using FDA before getting subculture (**B** brightfield 12 ms, **C** FITC 100 ms) (FITC filter: excitation peak 491 nm/emission peak 516 nm). **D** starting subculture from the mature culture with roughly 1 gram of tissue in a 30 ml culture. **E** subculture after 5 days, with large growth and development in the 30 ml culture. **F** callus pieces on HSIM (MS basal salts, 3% sucrose, 0.2% gelzan pH 5.8, 7 mg/L kinetin and 0.05 mg/L IAA) to produce shoots. **G** one week after the initial plating of callus explants onto solid media with multiple shoots being produced with each callus piece.

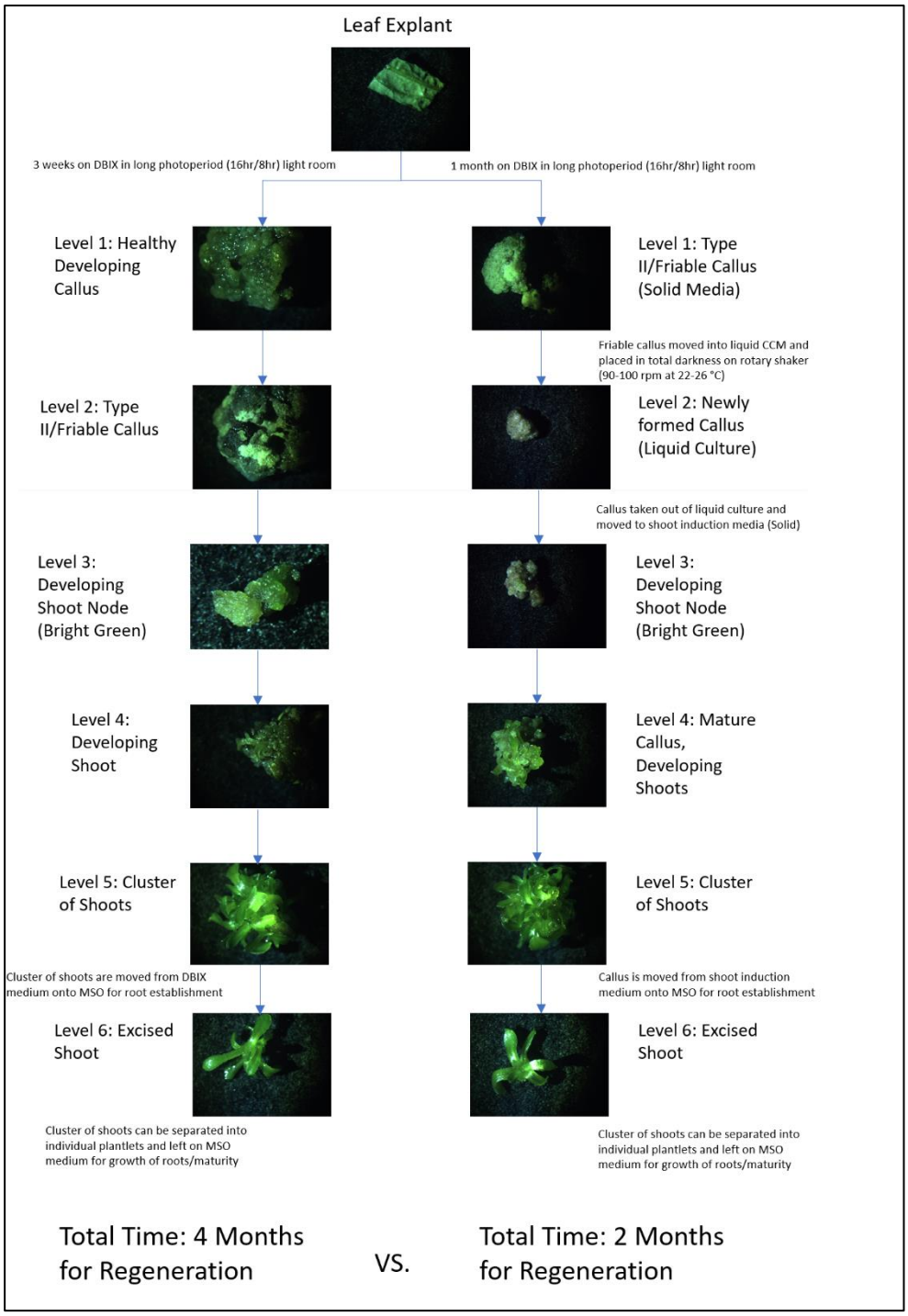
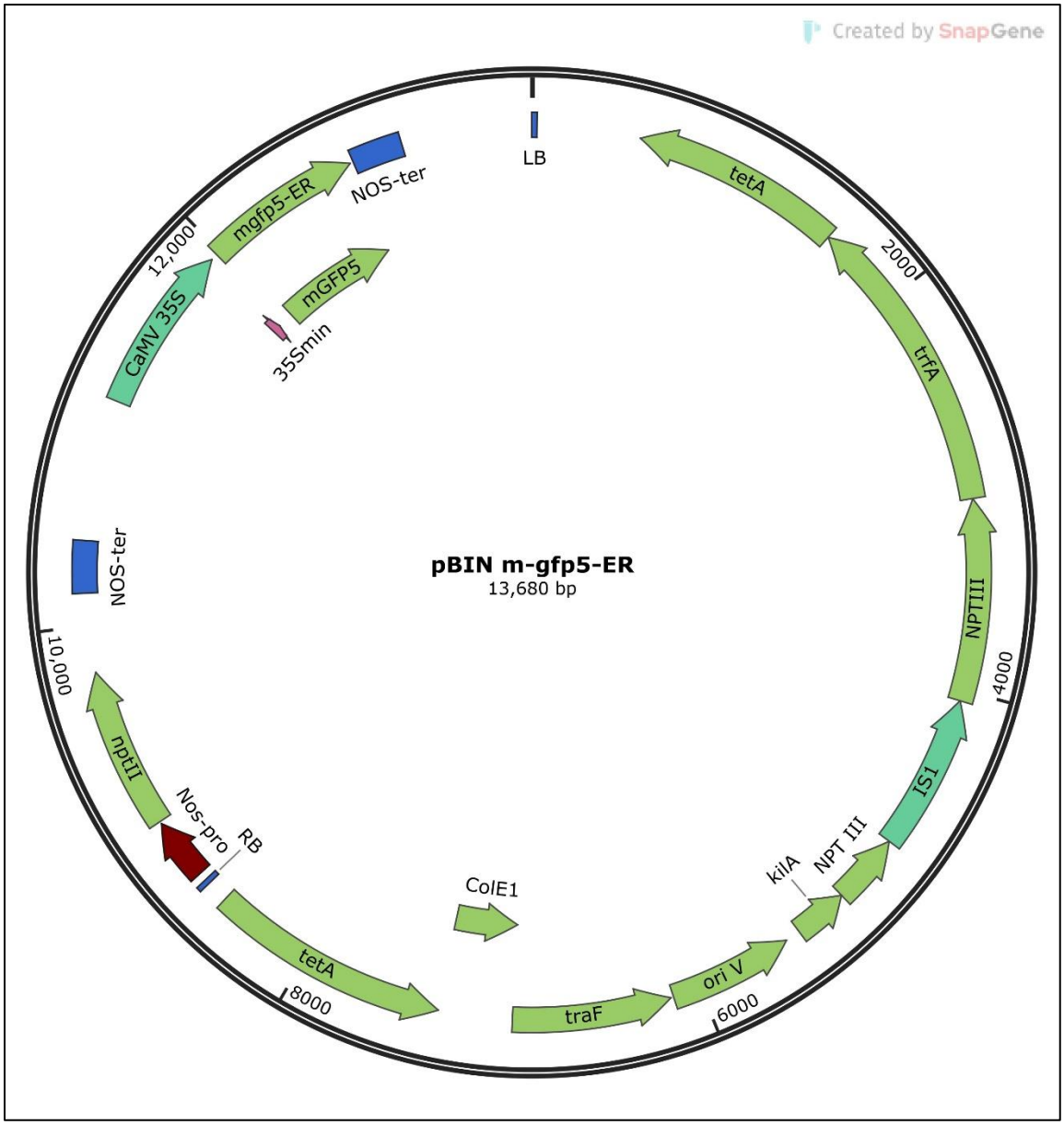


Figure 12: Regeneration timetable comparing indirect organogenesis (solid media) with callus culture regeneration (liquid media). The left side of the flow chart depicts the time and steps for regeneration using leaf tissue. Leaf tissue regeneration takes ~4 months for total regeneration. The right side of the flow chart are the different phases for regenerating plants through the callus culture method. The liquid callus culture method takes ~2 months for full regeneration of plants. All images are taken under a bright field condition, despite the vibrant light green appearance of shoots in some panels.

Supplementary Table/Figures:

Supplementary Table 1: List of primers designed for sequencing *M11/CcABCC3* in horseweed.

Primers for Sequencing *M11/CcABCC3*	
1.23_For_RAP	CAAACGTTGTTGTCTCCGTCT
1.1_Rev_RAP	TGTCACTGAGCCGAAGATATG
1.4_Rev_RAP	CACTGAACGGGTCATCAAAA
2.3_For_RAP	GGCTCGGGGAAGTCTAGTTT
2.1_Rev_RAP	CAACTAGCCGTGCCATTTCT
2.4_Rev_RAP	GTTCGCTGGGGATAGATGAA
3.3_For_RAP	TGGTTAGGCATACGTTTGGA
3.4_Rev_RAP	AAAGGGTCAACAAAGTTGGCTA
4.3_For_RAP	CCTGCATATTTTGCCACATC
4.1_Rev_RAP	TGAACATTGGCATCACCATT
5.2_For_RAP	CTCCATATGGGCGTCGTAGT
5.1_Rev_RAP	AGAACTTCGCAACCCCTTTC
5.3_Rev_RAP	GTCGCGATTTGGTAGACCTC



Supplementary Figure 1: The original construct used in the regeneration of transgenic horseweed (Halfhill et al. 2007). This was transformed into *A. tumefaciens* st. EHA105 to stay consistent with the rest of the constructs used in this study.

Vita

Robert Andrew Petursson was born in 1998 in Virginia. He graduated from North Carolina State University in 2020 with a Bachelor of Science Degree in Horticulture Science and a Bachelor of Science Degree in Animal Science with a minor degree in Biotechnology. He is currently pursuing his master's in Plant Science with a concentration in Biotechnology from the University of Tennessee, Knoxville. He is expanding the tissue culture protocols used with horseweed, which may be useful in identifying glyphosate resistance within this weed species.