**Establishment of Waterhemp Tissue Culture Lines for Herbicide Resistance Research**

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Introduction

Waterhemp is a problematic weed that negatively impacts soybean production, especially when its control is reduced due to herbicide resistance. Most current herbicide-resistant weed research is limited to the study of resistant biotypes that have already developed. New genetic technologies, such as those for gene editing, have potential to allow researchers to produce new weed mutations in the laboratory for study. These mutations include those that might confer herbicide resistance and those that might be useful to mitigate resistance through emerging gene drive technologies. However, unintentional escape of these new weed variants into the wild is a concern. Tissue culture provides a source of plant material that can be modified and studied without the unintentional production and spread of seeds, pollen, or other propagules. Plants grown in tissue culture as undifferentiated cells (callus cells) do not have capacity to propagate outside the laboratory, yet they still maintain most of the physiological processes that are targeted by herbicides. In other words, herbicide resistance and susceptibility are traits that can safely be studied in callus cultures. Even if gene drives never come to fruition, the ability to edit herbicide resistance genes in cultured cells would greatly facilitate other herbicide resistance studies including those correlating herbicide resistance traits with specific gene variations. Our research proposal seeks to establish waterhemp tissue culture (callus) cell lines to facilitate future research investigating possible solutions to the herbicide resistance problem.

Materials and Methods

 *Objective 1: Establish tissue culture cell lines, in the form of undifferentiated callus, from at least four accessions of waterhemp.*

 Seeds of North Dakota waterhemp accession ND297 were sterilized in 1% sodium hypochlorite (bleach) and placed on solid media in petri dishes under sterile conditions. The media contained half-strength Murashige and Skoog (MS) culture media plus 15 g/l sucrose and was solidified with 0.8% agar. Germination was performed at either room temperature (22 C) or 32 C for 1 week. A 1 cm (0.4 inch) segment was cut from the hypocotyl (stem) of each germinated seed and placed on solid media in sterile tissue culture vessels. The media contained either 0.003% or 3% sucrose as a carbon source, 2.3 μM 2,4-D as an auxin plant hormone, and either 2.3 μM kinetin or 13.3 μM BAP (6-benzylaminopurine) as a cytokinin plant hormone. Incubation was at room temperature (22 C), and the experiment was repeated. After 40 days of incubation, larger calli were sub-cultured onto fresh media with 3% sucrose, 2.3 μM 2,4-D, and 13.3 μM BAP.

 Three additional waterhemp accessions, PI 604247, PI 553086, and PI 607462, were obtained from the USDA-ARS North Central Regional Plant Introduction Station (Ames IA). Seeds of these accessions were germinated at 32 C as above, and hypocotyls were placed on media containing 3% sucrose, with 2.3 μM 2,4-D and 13.3 μM BAP as auxin and cytokinin hormones, respectively, and incubated at room temperature (22 C).

 *Objective 2: Identify any differences in callus initiation and fresh weight among waterhemp accessions and between male and female sources of tissue.*

Plants grown from accessions ND297, PI 604247, PI 553086, and PI 607462 were tested at the National Agricultural Genotyping Center (Fargo, ND) to confirm species identification. Seeds of PI 604247, PI 553086, and PI 607462 were confirmed as waterhemp, while ND297 was found to contain seeds of Powell amaranth in addition to waterhemp. Therefore, ND297 was not used for research investigating Objective 2. Initiation of cultures using seed from PI 604247, PI 553086, and PI 607462 was repeated as above, and cultured calli from both Objective 1 and the Objective 2 repeat were weighed 14 weeks after initiation.

It was observed that seedlings could not continue to grow after hypocotyls were harvested, most likely due to the very young age at which the seedlings were used. This prevented identification of cultures as male or female by observing adult plants grown from the same seedling. As an alternative method, a polymerase chain reaction (PCR)-based marker specific for male waterhemp was obtained from Dr. Patrick Tranel at the University of Illinois. Preliminary experiments using this marker were consistent with the sex of adult greenhouse-grown waterhemp, and DNA from waterhemp callus cultures was successfully isolated for analysis. However, analysis of DNA from waterhemp callus cultures using the male-specific marker has yet to be completed.

Results and Discussion

*Objective 1: Establish tissue culture cell lines, in the form of undifferentiated callus, from at least four accessions of waterhemp.*

 Seeds of ND297 germinated after incubation at 32 C, but not when incubated at room temperature. All ND297 hypocotyl sections successfully formed callus tissue when cultured on media with 3% sucrose, while none were successful with 0.003% sucrose. Of the 34 successful cultures (18 in the first replication and 16 in the second replication), half were placed on media with 2.3 μM kinetin and half with 13.3 μM BAP. Again, all cultures on 3% sucrose formed callus, so no difference in callus initiation between cytokinin hormones was observed.

 After 40 days, 6 of the larger calli were sub-cultured by dividing them into two or three pieces and placing them on fresh media with 3% sucrose and 13.3 μM BAP, plus 2,4-D. All 17 resulting sub-cultures successfully continued to grow.

Callus cultures were also successfully produced from hypocotyls of PI 604247 (four cultures), PI 553086 (two cultures), and PI 607462 (two cultures), during the first initiation experiment. Additional cultures of these accessions were obtained in Objective 2.

 *Objective 2: Identify any differences in callus initiation and fresh weight among waterhemp accessions and between male and female sources of tissue.*

In addition to the cultures obtained in Objective 1, five callus cultures of PI 604247, three callus cultures of PI 553086, and eight callus cultures of PI 607462 were obtained while repeating callus initiation for Objective 2. However, cultures of these three waterhemp accessions were affected by fungal contamination (both from Objective 1 and the repeat for Objective 2). So, a limited number of calli were available to weigh after 14 weeks of incubation (a total of four for PI 604247, three for PI 553086, and five for PI 607462). Due to the limited number of available calli, weights from Objective 1 and Objective 2 cultures were combined for analysis (Table 1). One-way analysis of variance (ANOVA) did not detect significant differences in callus weight (growth rate) among waterhemp accessions. However, high variability among calli weights within accessions may have obscured any differences.

Conclusions

 Successful germination of ND297 seeds was found to occur at 32 C, and hypocotyls successfully generated callus tissue when culture media contained 3% sucrose and either 2.3 μM kinetin or 13.3 μM BAP, along with 2.3 μM 2,4-D. However, ND297 was later determined to not be pure waterhemp, but to contain some Powell amaranth. While this may have affected the experimental results, the conditions identified using ND297 were successful in germinating seeds and initiating callus cultures from the hypocotyls of three other waterhemp accessions.

 The successful generation of callus from all tested accessions indicated that establishment of callus cultures is likely to not be limited by seed source. Significant differences in callus growth rate were also not observed among three waterhemp accessions. However, callus growth rates were highly variable within accessions. It is possible that significant differences would have been observed with larger numbers of calli.

 In conclusion, this project successfully generated waterhemp callus cultures that can be used in future research investigating herbicide resistance and emerging genetic technologies for weed control.

Table 1. Callus weights (mg) after 14 weeks of

incubation. S.D. is standard deviation.

 Waterhemp Accession

 PI 604247 PI 553086 PI 607462

 326.9 mg 69.4 1162.4

 23.5 47.2 800.0

 14.4 20.1 280.0

 8.0 251.2

 20.0

Mean 93.2 45.6 502.7

S.D. 155.9 24.7 466.2