Establishment of Waterhemp Tissue Culture Lines for Herbicide Resistance Research II

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Introduction

Herbicide resistance in weeds is a genetically inherited trait (Christoffers 1999). As such, researchers have explored the ability of best management practices and new technologies to prevent or delay increases in herbicide resistance gene frequencies (Norsworthy et al. 2012). One emerging technology that might be useful in the fight against herbicide-resistant weeds is called a gene drive (Esvelt et al. 2014; Neve 2018). A gene drive is a genetic system that promotes the inheritance of specific genes in a wild population, and Esvelt et al. (2014) have proposed that such a system might be used to change herbicide-resistant weed populations back to susceptibility. As proposed, the Esvelt et al. system would be based on new CRISPR (clustered regularly interspaced short palindromic repeat) gene editing technology, where the genetic sequence responsible for resistance would be edited back to the normal, wild-type susceptible version.

In order for a gene drive based on CRISPR to work within a weed population, the necessary genes would need to be designed and introduced into the population's gene pool. As highlighted by the National Academies of Sciences (2016), such alteration of wild populations requires caution. Conducting research on gene drives also requires safety considerations to ensure that any organisms carrying genetic changes are not allowed to reproduce and escape containment before full evaluation and intentional release. Because of this, we believe that initial studies investigating the application of new genetic technologies such as gene drives for weed control should preferably be done on weed tissues that do not have the capacity to escape laboratory containment through the production of seed, pollen, or other propagules. Plants grown in tissue culture as undifferentiated cells (callus and cell suspension cultures) do not have capacity to propagate outside the laboratory, yet they still maintain most of the physiological processes that are targeted by herbicides (Pierik 1987). Our current research project sought to establish waterhemp suspension cell cultures, and to evaluate the response of these cultures to herbicidal inhibitors of acetolactate synthase (ALS), in order to facilitate future research investigating emerging genetically based solutions to the herbicide resistance problem.

Materials and Methods

Objective 1: Establish waterhemp cell suspension cultures from at least four tissue culture cell lines of herbicide-susceptible waterhemp.

Seeds of four different waterhemp accessions were sterilized in 1% sodium hypochlorite 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 32 C for 1 week. A 1 cm segment was cut from the hypocotyl of each germinated seed and placed on solid media in sterile tissue culture vessels. The media contained 3% sucrose as a carbon source, 2 μM 2,4-D as an auxin plant hormone, and 0.5 mg/L 6-benzylaminopurine (BAP) as a cytokinin plant hormone. Incubation was at room temperature (22 C). Resulting calli were subdivided and placed onto fresh media every 2-3 months.

Waterhemp hypocotyl callus cell lines A8, B2, C, and C2 were used to establish cell suspension cultures by removing small pieces of callus tissue and placing the pieces into 25 ml sterile liquid suspension media in 125-ml flasks. Initial trials used callus cell line C and three different liquid suspension mediums:

- Medium 1: MS media with 1% sucrose, 1 mg/L 2,4-D, and 0.5 mg/L BAP.
- Medium 2: MS media with 3% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L BAP.
- Medium 3: MS media with 3% sucrose, 1 mg/L 2,4-D, and 0.1 mg/L kinetin.

Cultures were incubated at 25 C on an orbital shaker at 120 rpm. Every week thereafter, 6-12 ml (depending on culture growth rate), of each suspension culture was filtered through 100 μm nylon mesh and added to 12 ml of fresh liquid media of the same type in 125-ml flasks. However, when growing cultures for Objective 2 experiments, 6-12 ml of culture was sometimes added to 25 ml fresh media in 250-ml flasks. Culture growth was assessed by removing small samples of suspension culture and determining optical density at 600 nm (OD_{600}) in a spectrophotometer.

Later, medium 2 was used to establish cell suspension cultures of callus cell lines A8, B2, and C2.

Objective 2: Determine herbicide dose response curves for imazethapyr and thifensulfuron-methyl using two waterhemp cell suspension cultures.

Stock solutions of technical-grade imazethapyr and thifensulfuron-methyl herbicides were prepared commercially or by our lab using methanol as a solvent. Methanol alone was also used as a treatment in one experiment to determine the possible effects of this alcohol on suspension culture growth.

In three separate experiments, 12 ml of suspension culture from cell line C (medium 2), was added to 12 ml of fresh medium 2 and various concentrations of imazethapyr, thifensulfuron-methyl, or methanol. Final concentrations of imazethapyr and thifensulfuron-methyl were 0, 1, 10, 100, and 1000 nM, while the final concentrations of methanol were 0 and 0.1%. In the imazethapyr experiment, three separate cultures were established for each herbicide while in the methanol experiment, only two separate cultures were used for each treatment. In the thifensulfuron-methyl experiment, only one culture was established per herbicide rate due to lack of sufficient cell-line-C suspension culture at that time.

All cultures were grown in 125-ml flasks on an orbital shaker at 120 rpm. Cultures in the methanol experiment were grown at 25 C, while cultures for the imazethapyr and thifensulfuron-methyl experiments were grown at room temperature (approximately 25 C), due to lack of incubator space. Culture growth was evaluated by reading OD_{600} as in Objective 1, after 14 days for the imazethapyr experiment, and after 7 days for the thifensulfuron-methyl and methanol experiments.

Results and Discussion

Objective 1: Establish waterhemp cell suspension cultures from at least four tissue culture cell lines of herbicide-susceptible waterhemp.

Waterhemp callus culture C was used to test three liquid mediums for establishment of cell suspension cultures. Only medium 2 successfully produced actively growing cell suspension cultures (Figure 1). Cultures could not be established using mediums 1 and 3 (data not shown). It

is not clear why only medium 2 produced actively growing cultures, but we hypothesize that the increased level of 2,4-D as the auxin hormone (2 mg/L), may have been beneficial. Medium 2 is also the same medium, albeit in liquid form, that was used to produce initial callus cultures.

Medium 2 was then used to initiate cell suspension cultures from waterhemp callus lines A8, B2, and C2. While these cultures did display some active growth, they did not grow as fast as C suspension cultures and were not adequate for herbicide dose response experiments (data not shown). Even so, we are continuing to propagate these suspension cultures to see if they eventually acclimate to culture conditions.

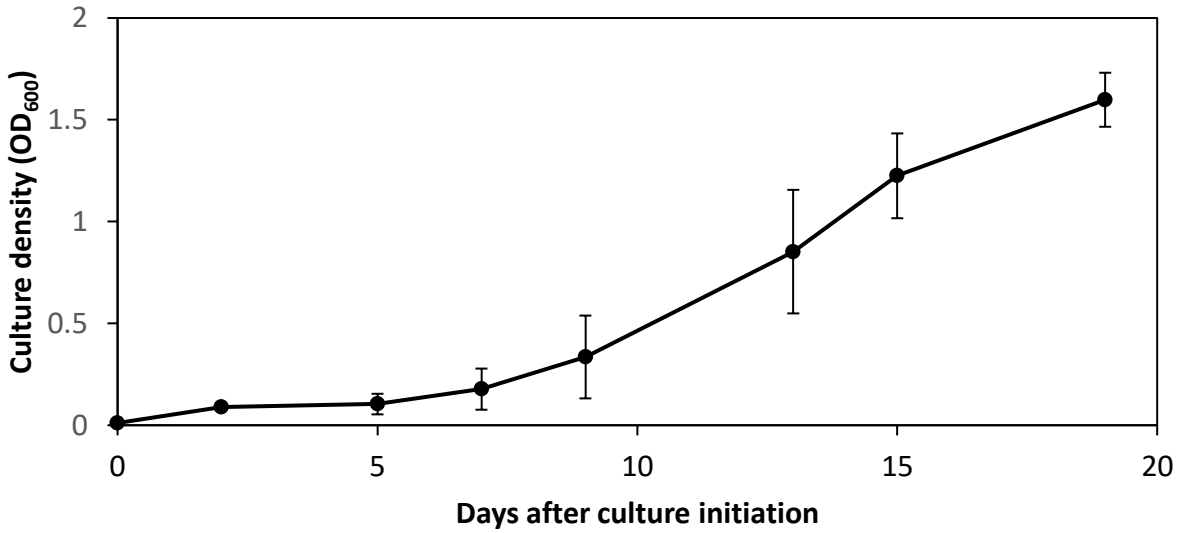

Figure 1. Growth of waterhemp cell suspension culture C in medium 2, over the course of 19 days. Optical density at 600 nm (OD_{600}) is a measure of cell density in culture and therefore increases in OD_{600} represent active growth. Bars above and below data points indicate standard error.

Objective 2: Determine herbicide dose response curves for imazethapyr and thifensulfuron-methyl using two waterhemp cell suspension cultures.

Methanol was used as the solvent for both imazethapyr and thifensulfuron-methyl herbicides, and so we tested the ability of waterhemp suspension culture C to grow in a concentration of methanol (0.1%), that would be present in herbicide experiments. After 7 days of growth, OD_{600} for control cultures (no methanol) averaged 0.774 ± 0.120, while OD_{600} for cultures in 0.1% methanol averaged 0.692 ± 0.989 (± standard deviation). While cultures grown in the presence of methanol were observed to be more variable than those grown without methanol, it was not apparent that methanol hindered growth rate. Even so, we added an equivalent amount of methanol to control (no herbicide) treatments in imazethapyr and thifensulfuron-methyl experiments.

Cell suspension culture C grew as well in the presence of 1 nM imazethapyr as the no-herbicide control (Figure 2). However, growth was noticeably decreased at 10 nM and higher concentrations of imazethapyr. These results indicate that waterhemp cells do respond to the inhibiting effects of imazethapyr herbicide while growing in suspension culture. Unfortunately, we have not confirmed the herbicide-resistance status of cell suspension culture C, and it is

possible that these results were influenced by resistance. However, sequencing of the ALS genes of culture C is in process, and this will help clarify our results. While 10 nM imazethapyr was sufficient to inhibit cell suspension culture C, we do not know how cultures produced from other waterhemp accessions may respond, regardless of resistance. But the current results support the use of suspension cell cultures to evaluate herbicide response in future research.

All tested concentrations of thifensulfuron-methyl inhibited the growth of waterhemp suspension culture C, and growth was only apparent in the control (no herbicide) cultures (Figure 3). As with imazethapyr, these results indicate that waterhemp cells growing in suspension culture do respond to thifensulfuron-methyl herbicide, and again support the use of suspension cell cultures to evaluate herbicide response. Since the lowest-tested concentration of thifensulfuron-methyl caused growth inhibition (1 nM), it is not clear what lower herbicide rate may have allowed cell suspension culture C to grow. This will need to be assessed in future research. As with imazethapyr, more analysis of suspension culture C, including sequencing of its ALS genes, will also increase the value of these results.

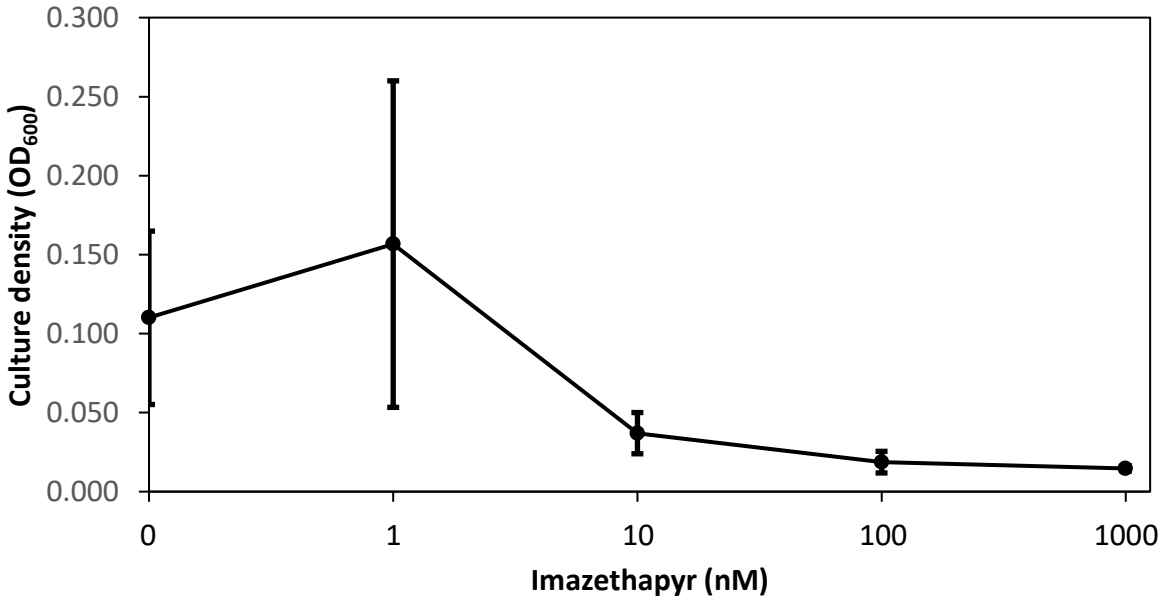

Figure 2. Growth of waterhemp cell suspension culture C after 14 days in the presence of various concentrations of imazethapyr herbicide. Optical density at 600 nm (OD_{600}) is a measure of cell density in culture and therefore higher OD_{600} readings represent more growth and less inhibition by imazethapyr, while lower OD_{600} readings represent greater inhibition by the herbicide. Bars above and below data points indicate standard error.

Conclusions

Waterhemp cell suspension cultures were successfully established from hypocotyl-derived calli using an MS medium containing 3% sucrose, 2 mg/L 2,4-D, and 0.5 mg/L BAP. However, only one cell suspension culture was found to have a growth rate sufficient for herbicide dose-response experiments. This suspension culture, derived from callus culture C and

therefore designated suspension culture C, was used to assess the response of cultured waterhemp cells to imazethapyr and thifensulfuron-methyl herbicides. Herbicidal inhibition was observed with both herbicides, but at different rates. Imazethapyr at 10 nM was necessary to inhibit the growth of suspension culture C, while no more than 1 nM thifensulfuron-methyl was needed for growth inhibition.

These results indicate that the growth-inhibitory effects of ALS-inhibiting herbicides are present when waterhemp is grown as a cell suspension culture. As such, cell suspension culture shows promise as a tool for research investigating resistance to ALS-inhibiting herbicides. This will be especially useful when evaluating emerging genetically based weed control methods, such as gene drives, because cell suspension culture provides a source of plant material that can safely be used in laboratories without risk of escape and propagation in the wild.

As a direct product of this research, a waterhemp cell suspension culture is now available for use by researchers. Further characterization of this suspension culture will increase its value in the search for viable methods of weed control and herbicide resistance mitigation for soybean growers.

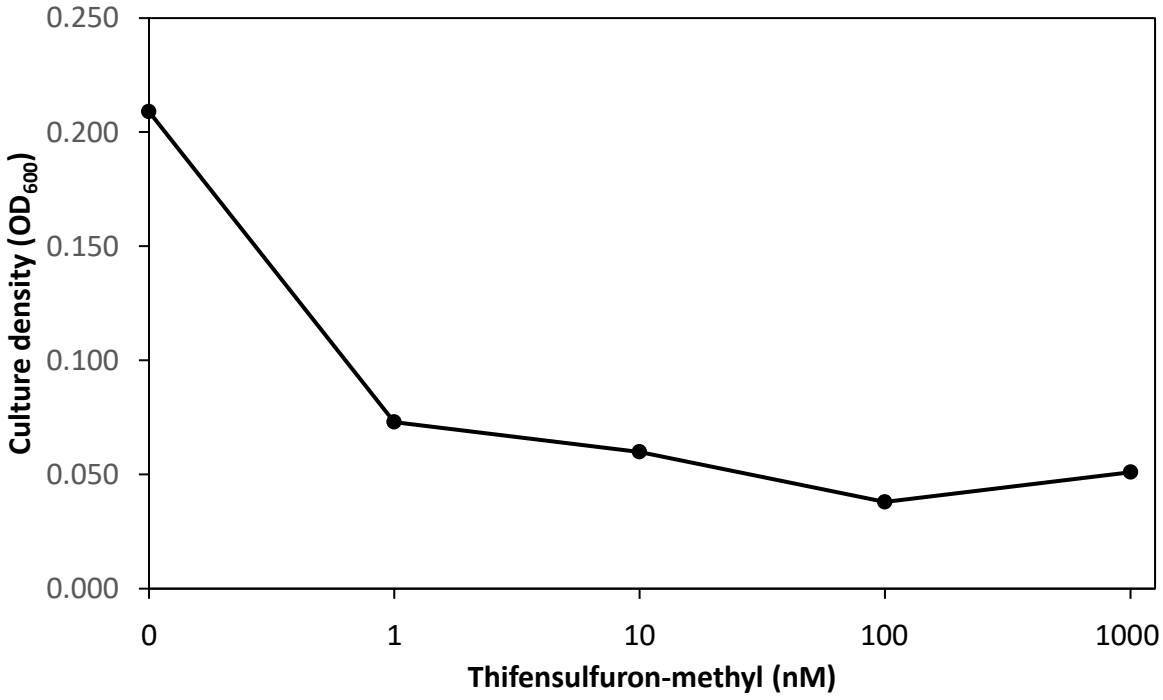

Figure 3. Growth of waterhemp cell suspension culture C after 7 days in the presence of various concentrations of thifensulfuron-methyl herbicide. Optical density at 600 nm (OD_{600}) is a measure of cell density in culture and therefore higher OD_{600} readings represent more growth and less inhibition by thifensulfuron-methyl, while lower OD_{600} readings represent greater inhibition by the herbicide.

References Cited

Christoffers MJ (1999) Genetic aspects of herbicide-resistant weed management. Weed Technol 13:647–652

Esvelt KM, Smidler AL, Catteruccia F, Church GM (2014) Concerning RNA-guided gene drives for the alteration of wild populations. eLife 2014;3:e03401

National Academies of Sciences, Engineering, and Medicine (2016) Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values. Washington, DC: The National Academies Press. 217 p

Neve P (2018) Gene drive systems: do they have a place in agricultural weed management? Pest Manag Sci 74:2671-2679

Norsworthy JK, Ward SM, Shaw DR, Llewellyn RS, Nichols RL, Webster TM, et al. (2012) Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Sci 60(special issue 1):31-62

Pierik RLM (1987) In Vitro Culture of Higher Plants. Dordrecht, The Netherlands: Martinus Nijhoff Publishers. 344 p