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

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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).

 We previously developed tissue cultured cell lines from waterhemp seedling hypocotyls, and used these cell lines to establish cell suspension cultures. The resulting cell suspension cultures were found to respond to acetolactate synthase (ALS)-inhibiting herbicides in a dose-dependent manner, and represented a renewable source of plant material for genetic research investigating waterhemp. However, individual plant cells in suspension cultures still have cell walls that are barriers to the introduction of biochemicals necessary for CRISPR-based gene editing. In contrast, protoplasts are plant cells in which the cell wall has been removed, usually through the use of enzymes that specifically digest cell wall components. Such protoplasts are more amenable to the introduction of biochemicals. The current research sought to develop a method of generating protoplasts from established waterhemp cell suspension cultures. After successfully generating protoplasts, we also worked to establish a method of isolating individual protoplasts in a culture medium that would allow regeneration of cell walls and subsequent cell division. Such a system would allow future production of genetically pure cultures after CRISPR-based gene editing.

Materials and Methods

 *Objective 1: Successfully generate and maintain healthy protoplasts (cells after removal of cell walls), from a waterhemp cell suspension culture (plant tissue grown in liquid media in a laboratory).*

 The waterhemp tissue culture cell line used in the current work was originally derived from the hypotocyl of a germinating waterhemp seedling. Callus cell tissue was obtained from the hypocotyl after growth on agar-solidified half-strength Murashige and Skoog (MS) culture media plus 15 g/L sucrose, 2 μM 2,4-D, and 0.5 mg/L 6-benzylaminopurine (BAP). This callus culture was used to establish cell suspension cultures grown in liquid media containing 3% sucrose and the same 2,4-D and BAP concentrations as the callus culture above.

Using these cell suspension cultures, the conditions necessary to enzymatically digest/remove cell walls from individual cells, in order to generate protoplasts, was investigated. In the first trial (Trial 1a), 5 ml of a 1-month-old waterhemp cell suspension culture was centrifuged to pellet the cells, and the supernatant was replaced with 5 ml of the following filter-sterilized enzyme solution: 0.55 M sorbitol, 0.6% cellulase R-10, 0.2% hemicellulase, and 0.2% pectinase, pH 5.8. Cells were incubated at room temperature for 5 hours with gentle agitation, filtered, and washed 6-7 times with resuspension in 0.55 M sorbitol. The trial was then repeated (Trial 1b) using a younger 3-day-old waterhemp cell suspension culture with incubation at room temperature for 7 hours using the same enzyme solution.

 In Trial 2, a different enzyme solution was prepared by dissolving 0.2 g driselase in 10 ml of 0.4 M mannitol, pH 5.2. Because driselase is poorly soluble in aqueous liquids, the solution was centrifuged to remove undissolved enzyme and the supernatant was collected. Waterhemp cells from a 5-day-old cell suspension culture were then incubated in this driselase solution at 30 C for 90 minutes with gentle agitation.

 Trial 3 used an enzyme solution of sorbitol, cellulase R-10, hemicellulose, and pectinase similar to Trial 1, but with increased cellulase R-10 (0.8%). A 10-day-old cell suspension culture was incubated in this enzyme solution for 4 hours at 30 C.

 For Trial 4a, previous enzymes were replaced with 0.1% pectolyase Y23 and 1% cellulase Onozuka RS in 0.4 M mannitol, pH 5.5. Incubation was for 45 min at 30 C with agitation, followed by a wash and resuspension in 0.4 M mannitol. This trial was repeated (Trial 4b), but with the incubation period increased to 90 min.

 Trial 5 was a repeat of Trial 1a, but with the addition of driselase prepared in 0.55 M sorbitol (see Trial 2 above for details on driselase preparation).

 Trial 6 then used a significantly different enzyme solution of 6.5% mannitol, 0.1% CaCl2 • H2O, 4% cellulase R-10, and 0.2% macerozyme R-10. The waterhemp cell suspension culture was 4 days old, and the incubation period was 90 min at room temperature in a petri dish on an orbital shaker (60 rpm), followed by another 90 min incubation at 32 C. After incubation, the cell suspension was centrifuged at 1000 rpm for 3 min, and cells were resuspended in 6.5% mannitol and 0.1% CaCl2 • H2O. Trial 6 was repeated a total of three times.

 For all Objective 1 trials, the presence of protoplasts was initially determined by microscopic observation without staining. Samples were then stained with calcofluor white, which stains plant cell walls, to confirm that cell walls were no longer present on the putative protoplasts. After protoplast confirmation, percent protoplast viability was determined via staining with Evans blue and/or fluorescein diacetate (FDA). Evans blue stains dead cells but not cells that are alive, and FDA stains only live cells.

 *Objective 2: Establish micro-colonies (small clumps of cells), from single waterhemp protoplasts.*

 Waterhemp protoplasts prepared from cell suspension cultures using the Trial 6 protocol above (6.5% mannitol, 0.1% CaCl2 • H2O, 4% cellulase R-10, and 0.2% macerozyme R-10) were used to investigate the separation and growth of single protoplasts in solid media. The initial concentration of protoplasts in liquid culture was determined by counting with a hemocytometer. The protoplast suspension was then centrifuged at 1000 rpm for 3 min, and protoplasts were resuspended in an MS suspension solution containing 1.2% agar, 10 mM CaCl2, 11% glucose, 0.1 mg/L 2,4-D, and 0.1 mg/L BAP, pH 6.0. Resuspension took place prior to agar solidification after autoclaving, so that protoplasts would be distributed throughout the final solidified medium. Still prior to agar solidification, the protoplast suspension was distributed into a cell-plating dish with individual wells, one drop per well.

 After agar solidification, six wells were supplemented with 0.5 ml additional liquid cell suspension culture containing live waterhemp cells, and six other wells were supplemented with 0.5 ml conditioned medium (liquid media from waterhemp cell suspension cultures, but with the cells removed). Twelve additional cells containing protoplasts in solidified media were not supplemented with additional liquid culture/media. Dishes were then sealed, incubated at room temperature in the dark, and examined after one week.

Results and Discussion

 *Objective 1: Successfully generate and maintain healthy protoplasts (cells after removal of cell walls), from a waterhemp cell suspension culture (plant tissue grown in liquid media in a laboratory).*

 Six distinct sugar/enzyme combinations were evaluated for their ability to generate waterhemp protoplasts from cell suspension cultures (Table 1). The primary purpose of the sorbitol or mannitol sugars in each digestion solution was as an osmoticum to keep protoplasts from bursting after removal of cell walls. The enzymes in each solution were for the digestion/removal of the cell walls. The only solution found to remove waterhemp cell walls and generate protoplasts used 4% cellulase Onozuka RS and 0.2% macerozyme R-10 in 6.5% mannitol (see Trial 6 of Table 1). Because Trial 4 also used cellulase Onozuka RS and mannitol, but did not generate protoplasts, it is likely that macerozyme R-10 is a key enzyme that is necessary for protoplast generation from cell suspension cultures in waterhemp. Macerozyme R-10 is an enzyme that was used by Toriyama and Hinata (1987) to generate protoplasts from rice cell suspension cultures.

 Trial 6 was repeated three times, and protoplasts were successfully generated each time. Lack of cell walls was confirmed by staining with calcofluor white. The only cells where cell walls remained intact after digestion were those that were clumped together with other cells, most likely due to decreased exposure to the enzyme solution. Protoplast viability was measured after each trial via staining with Evans Blue and/or FDA, with viability ranging from 72-78%. This indicates that the majority of protoplasts are alive after removal of cell walls, which is necessary for subsequent CRISPR-based gene editing in future experiments.

Table 1. The type and amount of sugar (osmoticum) and the cell wall digestion enzymes used in each trial, and whether or not protoplasts (cells without cell walls) were successfully produced.

 **Trial**

 1a/1b 2 3 4a/4b 5 6

**Sugars (osmoticum)**

 Mannitol - 0.4 M - 0.4 M - 6.5%

 Sorbitol 0.55 M - 0.55 M - 0.55 M -

**Enzymes**

 Cellulase R-10 0.6% - 0.8% - 0.6% -

 Cellulase Onozuka RS - - - 1% - 4%

 Driselase\* - 20 mg/ml - - 20 mg/ml -

 Hemicellulase 0.2% - 0.2% - 0.2% -

 Macerozyme R-10 - - - - - 0.2%

 Pectinase 0.2% - 0.2% - 0.2% -

 Pectolyase Y23 - - - 0.1% - -

**Protoplasts produced?** No No No No No Yes

\*Driselase is poorly soluble in aqueous solution and precipitates are removed during preparation of the driselase digestion solution. Therefore, the final concentrations of driselase were likely less than those given.

 *Objective 2: Establish micro-colonies (small clumps of cells), from single waterhemp protoplasts.*

Waterhemp protoplasts generated using the enzyme solution of Trial 6 above were observed for cell wall regeneration and cell division after being suspended in a solidified growth medium. After one week, the integrity of individual protoplasts in solidified growth medium was maintained, based on microscopic observations. However, cell wall regeneration and cell division was not observed. Media in which cell suspension cultures are growing or have grown can sometimes be used to stimulate growth of individual protoplasts. However, neither the addition of a small amount of actively growing waterhemp cell suspension culture nor the addition of culture media from which cells had been removed was observed to stimulate cell wall regeneration and/or cell division.

Regeneration of suspension cell cultures after CRISPR-based gene editing of protoplasts is necessary for planned experiments using waterhemp cell cultures. Therefore, lack of protoplast cell wall regeneration and division will need to be overcome in future research. It is possible that a longer period of time is necessary to begin these processes, or that the growth medium used will need to be changed. The growth medium used in Objective 2 was based on that of Folling and Olesen (1999), for use in the regeneration of *Lolium* spp. from protoplasts. However, it may be beneficial to use a medium more similar to that which we are using to grow our waterhemp cell suspension cultures.

Conclusions

 Waterhemp protoplasts were successfully generated from cell suspension cultures, providing a protocol for use in future CRISPR-based gene editing experiments. This advance will allow researchers to perform a wide variety of genetic experiments in cell culture, which is advantageous because plant cell cultures cannot reproduce outside of laboratory conditions. Thus, genetic experiments can be done in waterhemp without risk of laboratory escape.

 The majority of waterhemp protoplasts generated in this research were confirmed to be alive and viable. However, regeneration of cell walls and cell division were not observed under the tested conditions. More research is necessary to develop a protocol for waterhemp protoplast regeneration.

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