VIRGINIA SMALL GRAINS BOARD PROGRESS REPORT, 2023

Virginia Wheat Tissue Culture Development

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This report details the work done in relation to the funding obtained from the board for research during the 2022-2023 fiscal year ($14,000). The funds were used to partially support one PhD graduate student, working with a horticulture undergraduate student, and to acquire growth media, tissue-culture consumables, and growth chamber rental. The primary objectives of the research are to develop reliable and genotype-independent plant tissue culture and regeneration methods specifically for Virginia wheat germplasm, with the aim of using them for the application of biotechnological tools for crop trait improvement. In the longer term, the goal is to implement gene editing in wheat for crop trait improvement. Below, we will describe the progress made towards these goals.

Regeneration from tissue culture.

We have successfully established a regeneration protocol that can culture shoots back from leaf-base explants. A small section of the base of seedling leaves (5 mm) is excised from the plants and cultured initially on high-auxin callus-induction medium. This leads to cell proliferation and the formation of both root and shoot/embryogenic primordia. Transfer to shoot-induction medium occasionally leads to the growth of small shoot structures. These newly formed shoots can subsequently be transferred to root-induction medium and eventually transplanted to soil where they can grow into mature plants (Figure 1). This result is a significant breakthrough as regeneration procedures from vegetative (leaf) tissue are rarely successful. Therefore, most regeneration is performed with immature embryo tissue excised from developing seeds, which is much more labor-intensive and time-consuming. Getting this procedure to work in our lab bodes well for its application in elite Virginia wheat varieties.

So far, we have only applied this method in Bob White and Chinese Spring strains. We have had issues with obtaining clean seed for the Virginia varieties. The field-grown seed we have been working with has too much contamination to be able to sterilize it sufficiently for tissue culture in Petri dishes and results in persistent growth of bacteria and fungi (Figure 2). We have worked with the breeding program to obtain seed from greenhouse-grown lines that we expect will be more amenable to our sterilization procedures.

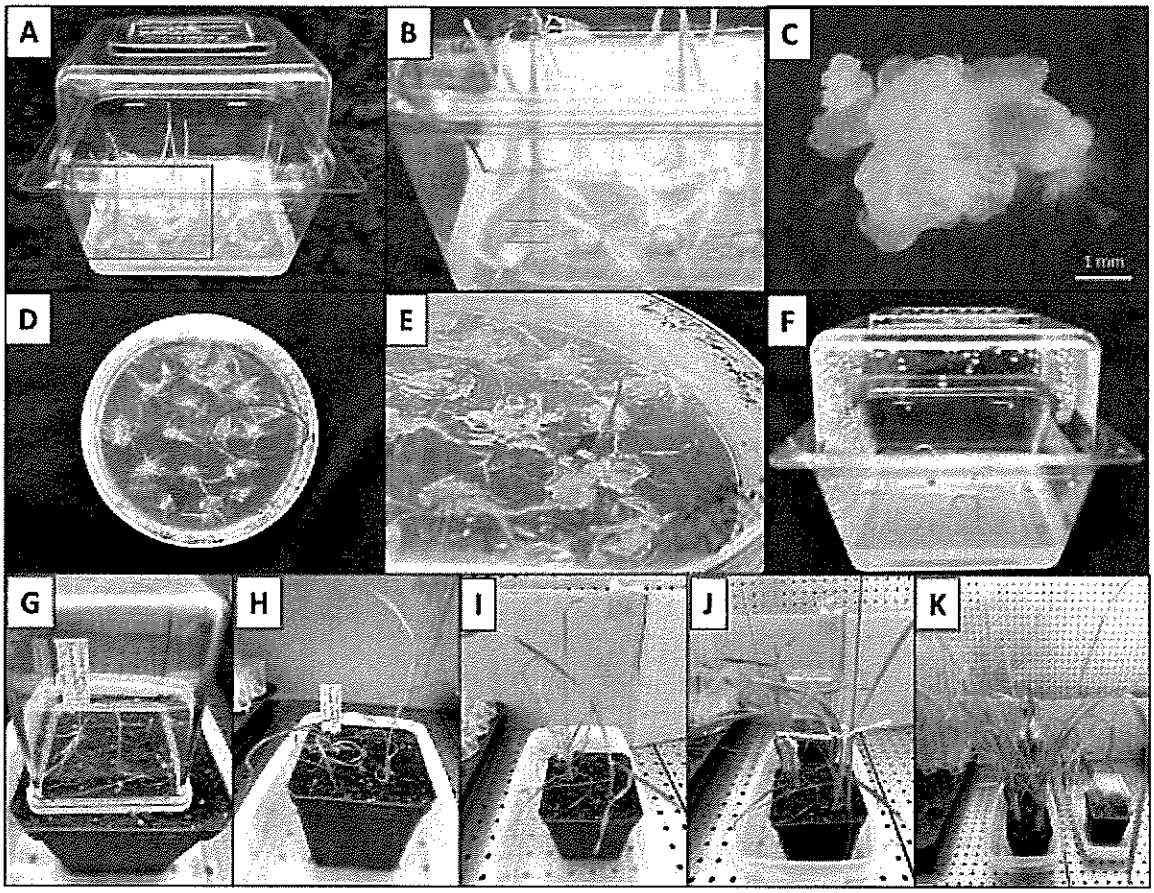

Figure 1. A) Sterilized wheat seedlings (6-7 days old) growing in a phytatray. B) Inset in A showing the leaf-base section used to obtain explants (red lines). C) Leaf-base explant after two weeks of growth on callus-induction medium; arrowhead indicates root structures, the arrow indicates embryogenic/shoot structures. D) Wheat calli two weeks after transfer to shoot-induction medium; all calli form roots (arrowhead), but some also regenerate shoots (oval). E) Inset in D showing a shoot regenerating from callus. F) A regenerated shoot one week after transfer to root-induction medium. G-K) Regenerated wheat plant after transfer to soil (two months of growth is depicted).

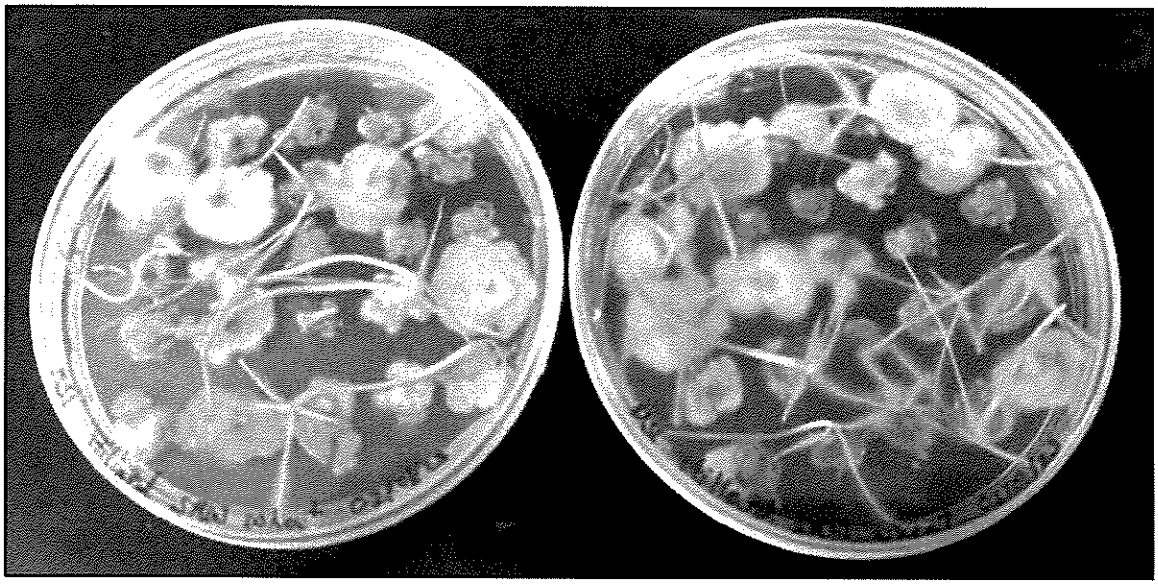

Figure 2. Attempted sterilization of field-grown seed for Hilliard. The seed showed persistent contamination with bacteria and fungus. More stringent sterilization procedures lead to a loss of germination capacity. A new trial with greenhouse-grown seeds is underway.

Stable transformation in tissue culture and transient transformation in protoplasts.
We have successfully established agrobacterium-mediated stable transformation of wheat leaf-base explants. Excised leaf segments were co-cultivated and infected with an Agrobacterium tumefaciens strain carrying a plant vector that expresses green fluorescent protein (GFP). This construct allowed us to visually confirm that there was successful transformation of plant cells through the observation of green fluorescence under a fluorescence-capably dissection microscope (Figure 3A and B). These preliminary results suggest that we can stably transform wheat leaf-base explants. This indicates that we can use this procedure to introduce the expression of morphogenic factors that can potentially enhance regeneration efficiency, especially in elite Virginia varieties.

So far, we have only performed this transformation in Chinese Spring and we need to expand this use in the Virginia varieties as tissue culture issues described above are resolved. We have shown the feasibility with a detectable fluorescent marker, the next steps would involve the combination with the established regeneration procedures and the induced expression of morphogenic factors.

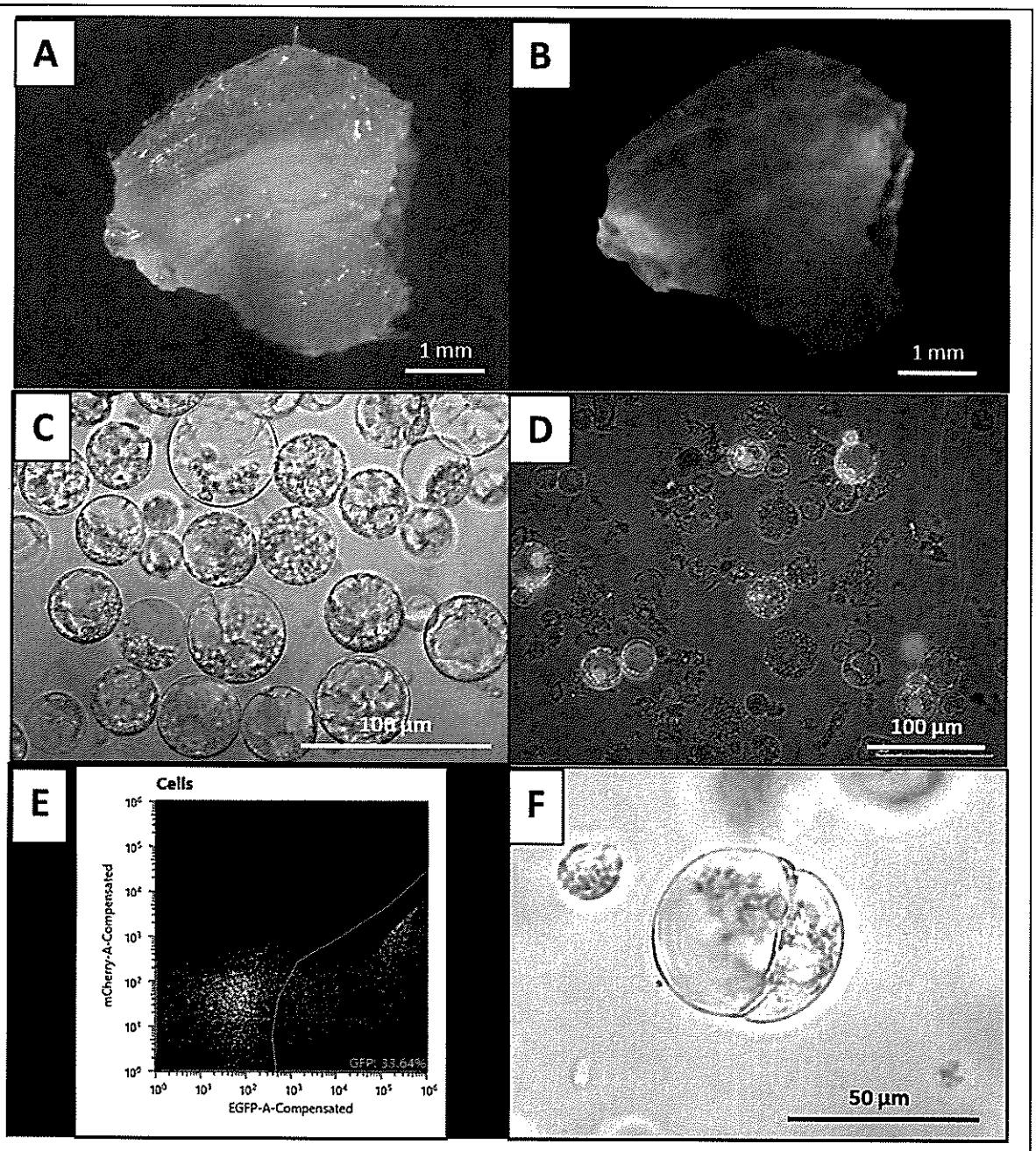

Figure 3. A-B) Stable transformation of leaf-base explants with agrobacterium-mediated green fluorescent protein (GFP) expression (A is a brightfield image and B is green fluorescence micrograph). C) Protoplast isolation from wheat seedling leaf tissue. D) Transient transformation of protoplasts with a GFP plasmid. E) Flow-cytometric quantification of protoplast transformation efficiency (33%). F) A protoplast in culture having undergone one cell division.

We have successfully established the isolation and transient transformation of wheat protoplasts obtained from seedling leaf tissue. Protoplasts were isolated from leaf tissue by incubation with cell-wall degrading enzymes and purification using filtration. Protoplasts were subsequently transformed by poly-ethylene glycol mediated plasmid transfection using a GFP expression cassette. Quantification using a flow cytometer to assess cellular fluorescence in thousands of cells demonstrated that about one third of the protoplasts were successfully transformed (Figure 3 C-E). These results indicate that we can reliably obtain and transform individual wheat cells. These methods are essential in the application of gene-editing technology in wheat as it allows us to test the gene-editing tools in a rapid and accessible manner (as opposed to having to wait for transformed plants only to find out the tolls don't work as expected and need further refinement).

Lastly, we have started preliminary experiments on the culture of wheat protoplasts. Although this is still very early on in the experimental phase, we do see promising indications that we can keep the cells alive for an extended period of time and that there are some signs of cell division (Figure 3F). Establishment of a protoplast regeneration system would allow us to implement gene-editing strategies through transient transformation procedures, which would negate the need to cross out GMO (transgenic) constructs and produce non-GMO gene-edited lines in one go.

Future directions.

Overall, we have made good progress towards our set goals. We had some setbacks with the use of Virginia cultivars due to contamination issues. However, we have a strategy in place to overcome this hurdle and are set to transfer the successes we have achieved with Bob White and Chinese Spring. Aside from the constructs we had in-hand already at the start of the project, we now have additional tools obtained from collaborators that have been shown to work independently, and specifically in enhancing monocot regeneration procedures [1]. We are eager to try these new tools in addition to the already established approaches.

1. Chen Z, Debernardi JM, Dubcovsky J, Gallavotti A (2022) The combination of morphogenic regulators BABY BOOM and GRF-GIF improves maize transformation efficiency. 2022.09.02.506370