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In Vitro Production Methods for *Harrisia aboriginium,* an Endangered Florida Endemic Cactus

Reed Gilmore
Florida Gulf Coast University, Department of Department of Ecology & Environmental Studies, Fort Myers, Florida, USA

John L Griffis, Jr
Florida Gulf Coast University, Department of Department of Ecology & Environmental Studies, Fort Myers, Florida, USA

ABSTRACT
***Harrisia aboriginium* is an endangered cactus endemic to Southwest Florida. This study developed a protocol for sterile micropropagation of this species. The disinfestation protocol this research determined as a best practice for seeds of this species included a 15-minute wash in soapy water followed by a quick dip in 95% ethanol. This was followed by a 15-minute wash in 1.875% sodium hypochlorite solution supplemented with Tween® 20 and finished with three rinses in sterile deionized water. There was vigorous shaking of the vessel containing the seeds and various solutions at all times. Several hundred seeds were introduced into sterile culture successfully using this protocol with a 0% contamination rate and a 98.6% germination rate. The *H. aboriginium* seedlings germinated and grew well on a medium containing half-strength modified version of the nutrients and vitamins described by Murashige and Skoog, supplemented with 15g/L sugar. The medium was solidified with a combination of 4.1g/L agar and 1.1g/L CP Kelco Gelzan®. To induce shoot multiplication, *H. aboriginium* seedlings were transferred to the same medium supplemented with various types and concentrations of plant hormones; it was determined that the media supplemented with 6-BAP gave the best multiplication results. Increasing concentrations of 6-BAP in the medium caused increased shoot formation, with the highest rate of 6-BAP addition evaluated (3 mg/L), resulting in an average of 12.2 shoots being produced in four months. Whole shoot clusters or individual separated shoots readily formed roots without the need of supplemental rooting hormone. Plants with roots were transplanted into a substrate composed of half commercial potting soil mix and half perlite v/v. Plants were moved into greenhouse conditions, watered when dry, about once a week, and fed with a diluted Miracle-Gro® Water Soluble All Purpose Plant Food 24-8-16 fertilizer at a concentration of one teaspoon per liter with every watering. Acclimating plants from culture was straight-forward with a 100% survival rate.**

Keywords: tissue culture, germination, micropropagation, conservation.

INTRODUCTION
Harrisia aboriginium is an endangered cactus that is endemic to niche coastal scrub habitats in Southwest Florida. This plant forms tall, cylindrical, sprawling stems with large white flowers. The United States Fish and Wildlife Service (USFWS) lists the species as endangered on their Environmental Conservation Online System. As of 2021, this plant is only found in 13 locations,

across four counties, and at least one known inhabited location has been completely extirpated, according to McCourt, Chambers, and Holst [1].

The goal of this research project was to develop a reliable micropropagation method to produce large numbers of strong, nursery-ready, *Harrisia aboriginium* plants. For this project, germinating seeds in sterile conditions and optimizing shoot multiplication of the seedlings through direct organogenesis seemed to be the best path forward. Using seeds collected from plants *in situ* allows us to preserve any genetic diversity that exists in the remaining plants. In vitro micropropagation methods are commonly used in horticulture to produce exponentially increasing numbers of uniform, disease-free, clonally propagated plants. These methods take place in completely controlled environments and are repeatable and scalable for a wide variety of situations. These production methods can assist restoration projects of endangered species by supplying these rare plants in the quantities required. Development of endangered species micropropagation protocols may also increase the supply of plant species in the ornamental market; this could lessen the value of and demand for poached plants.

The Marie Selby Botanical Gardens (MSBG) and the United States Fish and Wildlife Service (USFWS) are currently engaged in a multi-year restoration project for this species and have contracted our lab to develop in vitro propagation methods and to supply their project with plants. We have been able to produce a large number of plants from each seed in a relatively short period of time compared to seedling production using conventional propagation methods. The micropropagation methods utilized rely on clonal propagation starting with seeds to scale up to larger numbers of clones. Starting with seeds and tracking propagation for each seed allows for preservation of existing genetic diversity.

METHODS

Selection of Plant Material

This protocol was established using nursery produced seeds donated by James Freeman of Cactus Island Nursery. We are grateful for this donation as this research would not be possible without it. Multiple fruits were harvested from his nursery and processed by removing the pulp with water and drying the seeds on paper towels. These dry seeds were used for the next steps. Once the methods are developed, we will be provided with additional seeds collected by MSBG staff from the various locations still inhabited by the endangered cactus.

Seed Disinfestation Trial

Seeds were shaken in a jar with tap water supplemented with a drop of Tween 20 for ~15 minutes to wet the surface of the seeds and remove any debris. The seeds were poured into a strainer and rinsed again with tap water, then rinsed quickly with 70% isopropyl alcohol. A standard commercial bleach product containing 7.5% sodium hypochlorite was diluted at a 1:3 ratio (25%), yielding a final concentration of 1.875% sodium hypochlorite. The diluted bleach solution, seeds, and a drop of Tween® 20 were added to a vessel that was sealed and vigorously shaken for either 15 minutes or 20 minutes; this provided two different sterilization times. From this point onward, the sealed vessels were not opened unless it was within a sterile laminar flow hood. All remining steps took place under sterile conditions to prevent contamination. The seeds were next rinsed with sterile deionized water for five minutes; this

rinse procedure was repeated three times to remove any sodium hypochlorite and surfactant. Once the disinfestation was complete, the seeds were left to dry on a sterile paper plate in the laminar flow hood.

Seed Germination Media Trial

Sterile seeds were placed on two different germination media. The two germination media evaluated were a modified Murashige and Skoog medium (MSM) [2] and a Lloyd & McCown Woody Plant Basal medium (WPM) [3]. Both of these pre-mixed nutrient media products were supplemented with vitamins as listed on the product labels. The MSM (Phytotech® product M541) medium was modified from regular MSM medium by replacing potassium phosphate monobasic with 300mg/L sodium phosphate monobasic, replacing ferrous sulfate and disodium EDTA with ferric sodium EDTA and including 150mg/L adenine hemisulfate and 1000mg/L casein. The WPM medium (Phytotech® product L145), a medium lower in nutrients than the MSM medium, was selected as a comparison medium because *H. aboriginium* naturally grows in areas with limited soil nutrients.

The MSM medium was prepared at half-strength by adding 2.845 g of that powder mix to each liter of deionized water. The WPM medium was prepared at half-strength by adding 2.3 g of that powder mix to each liter of deionized water. After dissolving each powdered medium in deionized water, both MSM and WPM media solutions were supplemented with 15 g/L sucrose, also half the conventional rate added to many media. The pH of each solution was adjusted to 5.7 using potassium hydroxide. Next, 4.1g of micropropagation grade agar (Phytotech® product A296) and 1.1g of CP Kelco Gelzan® gellan gum were added to each liter of nutrient solution and the solutions were heated to boiling in a microwave oven to dissolve the solidifying agents. Using a peristaltic pump, ~11ml of each medium was distributed into 25mm x 150mm round bottom glass test tubes and the tubes were closed with polypropylene caps. Test tubes containing media were autoclaved for 16 minutes at 121° C and 15 PSI; then the test tubes were transferred to the laminar flow hood where they cooled and the media solidified.

For each of the four treatments (two different bleaching time periods, two different germination media), 35 seeds were sown, each seed in an individual test tube. All cultures were maintained in a growth chamber (Percival® CU-36L4) with a 12-hour photoperiod at ~170 μmoles/m^2/s using LED lights. The temperature in the chamber was maintained at 25°C during the 12-hour light period and 23°C during the 12-hour dark period. Weekly for three months, researchers examined the number of seeds contaminated, the number of seeds germinated, and the number of seeds ungerminated for each treatment. Then, 20 individuals were removed from each medium, and the length in millimeters from cotyledon to apical tip was recorded to compare overall growth on the two different media.

Direct Shoot Organogenesis Trial

After three months in the germination stage (Fig. 1), all germinated seedlings were ready for the multiplication stage of the project. All seedlings were single, columnar individuals with no visual evidence of axillary bud initiation. In the laminar flow hood, seedlings had their roots removed by pulling them off with sterile scalpels. These rootless seedlings were moved onto one of six different multiplication media. These six media were based on the same half-strength

MSM medium used in the germination stage, but each multiplication medium included the addition of a single cytokinin to induce the formation of basal axillary shoots. Each of the six cytokinin media treatments were replicated for 15 seedlings. A stock solution was prepared for each cytokinin by weighing out 10mg of cytokinin and dissolving each one in a small amount of ethanol in a 10ml volumetric flask and then bringing each solution to volume with DI water. These cytokinin stock solutions allowed for volumetric dosing to create equimolar treatments. The treatments compared in this stage were 4.44 μM, 8.9 μM, or 13.3 μM of 6-Benzylaminopurine (BA), 4.44 μM Kinetin (KIN), 4.44 μM 6-(y,y-dimethylallylamino) purine (2iP), and 4.44 μM Thidiazuron (TDZ). For example, in the lowest BA treatment, 1ml of 1mg/ml BA stock solution was added to 1 liter of half-strength MSM medium, resulting in a BA concentration of 1mg/liter. BA has a molar weight of 225.2492 g/mol, so the final solution has a BA concentration of 4.4 μM. KIN has a molar weight of 215.216 g/mol so to create an equimolar treatment, the same 1mg/ml concentration stock solution was added at a rate of 0.94ml/L resulting in a KIN concentration of 4.4 μM. All of the multiplication media were prepared exactly as the MSM germination medium previously described, except the various cytokinin stock solutions were added to separate solutions before the pH adjustments. The multiplication media were also distributed into test tubes, autoclaved and cooled as before. This array of hormone treatments allowed us to compare the effects of including several different concentrations of BA in the multiplication medium while also allowing us to compare incorporating low doses of other common cytokinins into half-strength MSM medium at rates that are equimolar to the lowest BA treatment. After four months on multiplication medium, all seedlings had new roots removed again in the laminar flow hood and the plants were transferred into Phytotech Culturejar™ G9 containing ~35ml of the same half-strength MSM medium (without hormones) that was used for seed germination. The transfer of seedlings to these larger vessels without the addition of cytokinin allowed any buds formed/stimulated by each cytokinin treatment to grow out into shoots. After four more months in this half-strength MSM medium, the number of shoots produced by each seedling plant was recorded.

Root Induction Trial

After the shoot numbers were recorded in the previous stage, any roots were removed from individual plants (single or clusters of shoots) in the laminar flow hood and the plants were placed into Magenta G7 vessels with 50 ml of the same half-strength MSM medium as used in the germination medium with the addition of one of two different auxin treatments to induce rooting. The two treatments used were low doses of the common rooting hormone, Indole-3-butyric acid (IBA) at concentrations of 0.1mg/L (0.49 μM) or 1mg/L (4.9 μM).

Acclimation Trial

Plants with roots were acclimated out of culture as clumps or single columns as short as 2.5cm in height. Roots were gently rinsed in warm tap water to remove any remaining gel and plants were placed in a well-drained soil mix. The potting substrate used was 1-part soil (Miracle-Gro® Cactus, Palm & Citrus soil), 1-part medium grain size perlite. The two substrate components were mixed, autoclaved, and distributed into nursery plug containers. For small single columns, standard nursery cell trays were used with humidity domes. For larger clumps of shoots, or for up-potting cell plugs, 7.62cm x 7.62cm x 20.32cm forestry pots were used to accommodate deeper root development (Fig. 2). Plants were watered when dry, once every 7-

10 days, until cell or pot was thoroughly saturated. After each watering, plants were fed ~15 ml of a dilute all-purpose fertilizer solution. This solution was made by mixing one teaspoon of soluble fertilizer (Miracle-Gro® 24-8-16 water soluble all-purpose fertilizer) per one Liter of water. Plants were grown 8 inches below two parallel 42W LED grow lights on a wire rack.

RESULTS

Seed Surface Disinfestation Results

Complete surface disinfestation was achieved with our methods, with no contamination for any of our 140 trial seeds (35 seeds each for the four different treatment groups). The 15-minute surface sterilization time resulted in a 97.15% germination rate, and the 20-minute surface sterilization time resulted in a germination rate of 100%.

Seedling Growth Media Comparison Results

Equally healthy-appearing plants were formed in both the half-strength MSM medium and the half-strength WPM medium. The measurements of 20 seedlings from each treatment showed no statistical difference in growth rates. The plants in the MSM treatment had an average height of 30.05 mm and the plants in the WPM treatment had an average height of 30.1 mm.

Shoot Multiplication Treatment Results

The results from the multiplication stage are shown in Table 1. and demonstrate that of the cytokinin treatments compared, either MSM or WPM half-strength medium with the addition of 3ml/L of BA stock solution (a final medium concentration of 13.3 µM BA) was the most productive for the induction of shoots. This concentration of BA yielded an average of 12.2 axillary buds per initial singular column. The lower 2ip and Kinetin concentrations tested were ineffective multiplication treatments for this species. Although the TDZ treatment did promote a number of axillary buds, the species had an unfortunate reaction to this growth regulator. The buds formed remained small and did not grow out into healthy shoots like the buds in the BA treatments. Plants grown on the TDZ treatment medium had slower, stunted growth and they did not regrow their roots easily, unlike seedlings growing in the other multiplication treatment media.

Table 1: Harrisia aboriginium bud formation after 4 months in various in vitro cytokinin treatments.

Comparison of Cytokinin Treatments		
Cytokinin	Concentration (mg/L)	Average number of buds formed
BA	1 mg/L	2.9
BA	2 mg/L	9.6
BA	3 mg/L	12.2
Kin	0.94 mg/L	1
2iP	0.89 mg/L	1
TDZ	0.97 mg/L	7.75

Root Induction and Acclimatization Results

All *H. aboriginium*, except plants in the TDZ treatment, regrew root structures in under four months after they were removed and placed in either final concentration (0.49 or 4.9 µM) of

IBA. Columns and clumps of this species reliably regrew their roots in the half-strength MSM media at every stage, including media without auxin or cytokinin. 100% of *H. aboriginium* plants were successfully acclimated from culture into standard cactus nursery conditions using the methods described.

CONCLUSIONS/DISCUSSION

In vitro production methods of *Harrisia aboriginium* are uncomplicated, especially when starting with seed (Fig. 3). For surface sterilization of seeds, using a 1.875% sodium hypochlorite solution with Tween 20, either for the 15- or 20-minute protocol described, is reliable and effective. The half strength MSM medium described was an effective growing media in all stages. Although there were no problems with the WPM in the germination stage, this study did not test the effects of it any further. The results of the multiplication stage show that 13.3 μM final concentration of 6-Benzylaminopurine was the optimal treatment of those trialed, producing an average of 12.2 shoots formed per single, columnar plant. It is possible higher concentrations of BA may result in a greater number of apical buds; however, that might also inhibit rooting. Although this study compared two auxin concentration treatments at the rooting stage, addition of auxin to growth medium was unnecessary for this species. Clumps of shoots formed in the multiplication stage were rooted easily in the MSM media without the addition of auxins. The shoots can also be removed from the clumps at around 1 inch in length and easily rooted in MSM media without the addition of auxins. All plants with roots were easily acclimated into nursery conditions using the methods described and were not overly sensitive to drying out.

Acknowledgments

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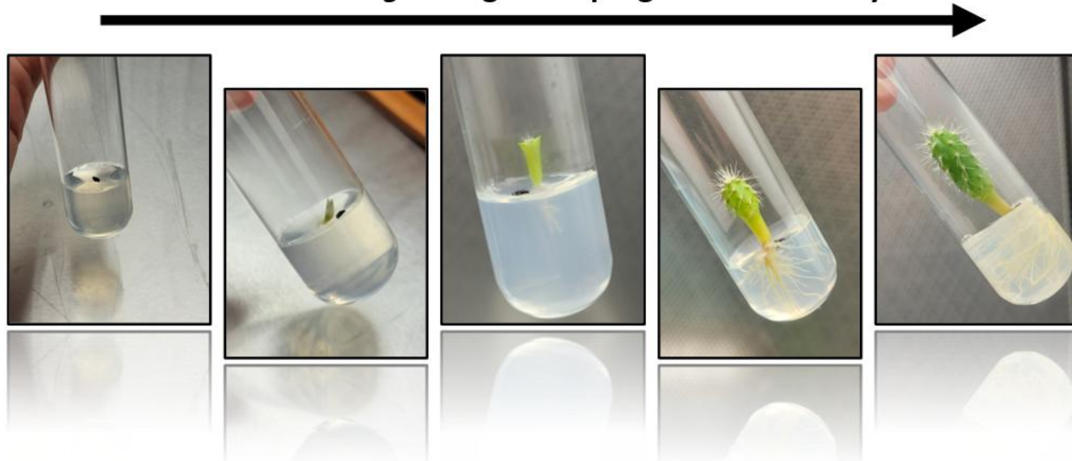

Figure 1: 90 days of growth after seed germination

Figure 2: Clumps of shoots transplanted into nursery conditions after multiplication

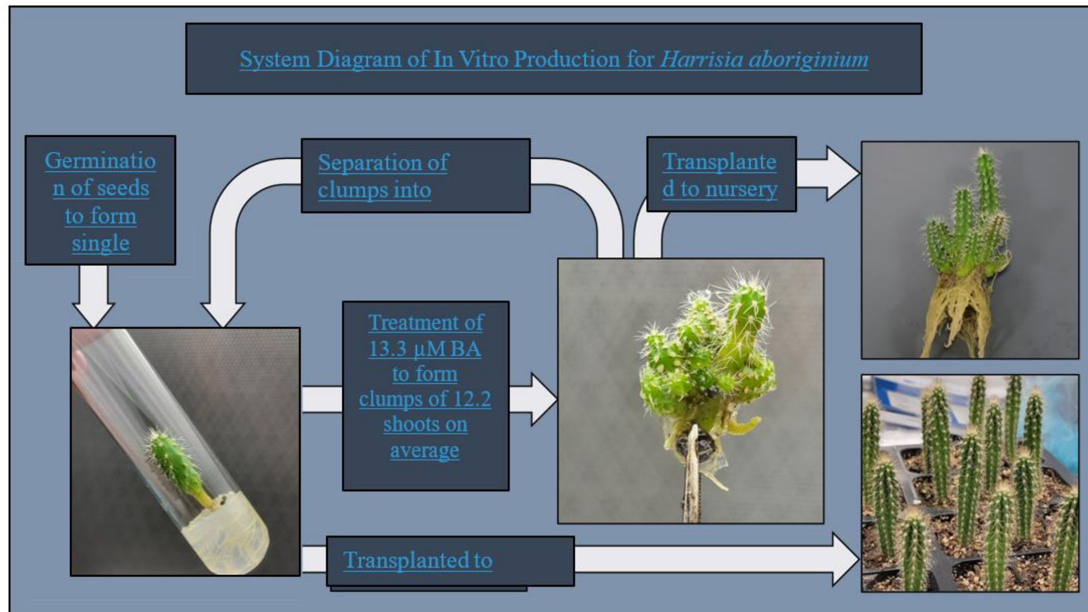

Figure 3: Production methods diagram