MICROPROPAGATION: AN IMPORTANT TOOL IN THE CONSERVATION OF ENDANGERED HAWAIIAN PLANTS

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Abstract

About half the taxa of native Hawaiian vascular plants are endangered in the biological sense. Some 25% have already been federally listed as endangered or threatened (272 endangered, 10 threatened); 49.5% of federally listed endangered plant species native to the U.S. are Hawaiian species. About 300 more have been significantly depleted and are currently treated as Species of Concern. Many endangered Hawaiian plants are exceedingly rare. Eleven taxa are currently known from only a single specimen of each remaining in the wild; more than 100 species currently have 20 or fewer plants remaining in the wild. Many rare Hawaiian plants have recalcitrant seeds, and standard seed storage techniques will not provide satisfactory long-term storage for maintenance of genetic diversity.

In 1991 Lyon Arboretum initiated a project to apply any appropriate micropropagation techniques to the conservation of rare Hawaiian plants. These techniques include tissue culture and cloning, as well as embryo culture, immature and mature seed culture. When material is available, embryo and seed cultures are the techniques of choice, in order to maximize genetic variability. The objectives of the project include (a) prevention of extinction of Hawaiian plant taxa, (b) propagating plants for use in approved restoration and reintroduction projects, and for garden use, and (c) maintaining an *in vitro* genetic safety net for the most critically endangered taxa. Lyon Arboretum works cooperatively with four other Hawaiian botanical gardens in the Center for Plant Conservation's network, various state and federal agencies officially concerned with plant conservation and endangered species, private conservation agencies such as The Nature Conservancy, environmental organizations, and major private landowners, in joint conservation efforts.

To date more than 80 federally listed endangered taxa have been successfully grown at Lyon Arboretum using micropropagation techniques. For several taxa specimens produced by micropropagation have been used for restoration / reintroduction proj-

ects. Advantages of micropropagation include relatively low cost long-term storage of large quantities of material, and maintenance of whatever total genetic variability exists in very small populations. For taxa with 20 or fewer plants remaining, our goal is to propagate every individual. Some taxa, when reduced to small population sizes, exhibit inbreeding depression manifested in various ways. In two species studied which each had one remaining wild individual, the plants flowered regularly but viable seeds were never produced. Both species produced embryos, which aborted spontaneously before maturity. In both cases embryo culture resulted in production of healthy seedlings.

Introduction

Hawaii, an isolated archipelago in the middle of the Pacific, is a favorite vacation spot for many around the world. Hawaii is known for its wondrous natural displays such as erupting volcanoes, pristine white sand beaches, clear blue seas and also its unique flora and fauna. What is surprising to many, is that the plants that have become symbolic of Hawaii, such as the Bird of Paradise, orchids, plumeria and even the pineapple are not natives but recent human introductions. In fact, the about half of all the plants growing without cultivation in Hawaii today are of foreign origin, and have been introduced by human activity, mostly within the past 200 years.

Currently, there are approximately 1000 native Hawaiian species of Angiosperms, of which 90% are endemic, and 150 Pteridophytes of which 70% are endemic. The Angiosperm numbers represent the highest percentage of endemism found within a single large island group anywhere in the world. Hawaii's high rate of plant endemism can be attributed to its geographical isolation from continents and other islands, isolation between and among the individual islands, and its highly diverse microclimates. In the islands elevations range from sea level to 4,205 meters (13,796 feet), and average annual rainfall ranges from 178 to over 11,430 millimeters (from 7 to over 450 inches). Following the advent of human discovery and colonization, significant decline and in many cases extinction of native Hawaiian plant taxa has occurred.

Today, over one half of all the Hawaiian vascular plants are biologically endangered with approximately 25% federally listed as endangered or threatened (272 endangered, 10 threatened) and another 25% significantly depleted to be treated as species of concern (approximately 300). Eleven taxa are so rare that they are currently known only from a single specimen remaining in the wild. In addition, there are a more than a hundred species that are known from 20 or fewer remaining wild individuals. Many of the remnant populations have been reduced so far that normal regenerating capabilities have been impaired or rendered nonfunctional.

Plant micropropagation is a technology that has been developing and redefined continuously over the past 30 years. It has become an indispensable tool in the areas of biotechnology, genetic engineering, and in plant propagation, for the production of mass quantities of clonal material for the commercial agricultural industry. Another area where micropropagation has been receiving an increasing amount of interest is that of plant genetic conservation.

Micropropagation at Lyon Arboretum

In 1991, Lyon Arboretum initiated the rare Hawaiian plant project utilizing micropropagation as a tool for plant genetic conservation. The objectives of this project were to (a) prevent further extinction of Hawaiian plant taxa, (b) propagate plants for use in approved restoration and reintroduction projects, and also for garden use, and (c) initiate and maintain an *in vitro* germplasm collection of the critically endangered plants included within the genetic safety net listing. Lyon Arboretum works cooperatively in joint conservation efforts with four other Hawaiian botanical gardens in the Center for Plant Conservation's network, and with various state and federal agencies officially concerned with plant conservation and endangered species, private conservation agencies such as The Nature Conservancy, environmental organizations, and major private landowners.

Theoretically, there exists a potential to produce entire plants from small pieces of plant tissue, or explants. When explants are placed *in vitro*, literally meaning under glass, in an environment free of microorganisms and onto an appropriate nutrient rich medium, they can regenerate into plants. Plant

explants for micropropagation are either vegetatively or sexually derived. The most commonly used vegetative explants include apical, axillary and root meristems, stem nodes, stem internodes and leaves. They produce plantlets called clones, which share an identical genotype with each other as well as the original parent. These clones can eventually be multiplied to produce more plants in a process known as cloning or clonal propagation. Sexually derived explants such as seed, embryos, ovules, and pollen produce plantlets with unique genotypes.

One of the goals for all Hawaiian taxa that have 20 or fewer plants in the wild is to vegetatively propagate the remaining individuals for the purpose of germplasm collection and storage. Seeds are also collected from these individuals when available but in many cases, inbreeding depression is exhibited within these small populations and result in plants, which produce no seed, or seed that abort spontaneously before they reach maturity. In the case where no viable mature seed are produced, the immature seed, before they abort, can be collected and embryo or ovule culture attempted. Another problem encountered with Hawaiian plants is that many have recalcitrant seed and standard seed storage techniques have proved to be inadequate. In these cases, *in vitro* germination and storage of the seedlings is currently the only way to preserve the genetic diversity contained within the seeds. Once the clones or seedlings are established in *in vitro* culture, the plantlets continue to grow miniaturized leaves and stems and remain in a juvenile or juvenile-like state. Therefore, it is possible to maintain these plants for periods of several months to a few years in small vessels within controlled environments.

In the micropropagation of Hawaiian natives, it is of utmost importance to preserve the integrity of the original plant genotype; therefore any variables which may jeopardize the genetic stability during the culturing process must be minimized. The ability to establish *in vitro* cultures of plants that can regenerate normally and maintain a high degree of genetic stability is dependent upon several factors. The selection of suitable plant material and explants, proper surface disinfestation, plant medium and culture conditions should all be considered. At Lyon Arboretum, the majority of the plant material submitted is wild collected and the establishment of an *in vitro* culture is often times hampered by this fact.

The selection of suitable plant material not only pertains to the type of explant used for culturing, but also to the time of harvest, juvenility of the material and the general health of the plant. Harvesting of vegetative cuttings should be timed to coincide with the plant's active vegetative growth cycle. Many times this is not possible due to two main factors, which are inadequate information on the plant's horticultural requirements, and difficulty in obtaining plant material due to limited access. Micropropagation of juvenile plants is often much easier than with their mature counterparts. While many of the plants in the wild are not juvenile but mature adults, suitable material can still be collected from certain areas where the tissues mature less rapidly than in the rest of the plant. Plant tissues that tend to be more juvenile are generally found on the lower branches of the plant. Suckers arising from the stem, root, or stump also display delayed maturation.

Apical and axillary meristems, when induced to produce direct shoot regeneration have been known to possess greater genetic stability. Plants derived from unorganized callus cultures originating from non-meristematic tissue such as stem internodes and leaves are generally thought to be more genetically unstable and their use discouraged. Sometimes non-meristematic explants are the only kind available for micropropagation and callus culture the only way to regenerate plants. In this case, organogenic or shooty type callus is preferred due to its greater genetic stability. Viable mature seeds are generally propagated using conventional greenhouse practices but are placed into *in vitro* culture if germplasm storage is requested. The optimal time to collect seed is just before fruits dehisce or fall from the plant. Seeds collected in this manner are not only assured of their parentage, but also easier to disinfest due to less microfloral contamination.

Initiation of *in vitro* cultures are usually more successful if explants are taken from plants which are in reasonably good health. Any decline caused either by disease or senescence tends to be irreversible and continue to progress in the explants during micropropagation.

Many of the critically endangered Hawaiian plant populations are very small, fractured, older and in decline due to a multitude of environmental disturbances resulting in premature death of existing plants and the lack of regeneration within the natural population. The poor condition of these species makes collection of viable material suitable for *in vitro* culture and successful micropropagation extremely difficult.

Techniques Employed

When material is collected for micropropagation, it is important that it is received in the lab as quickly as possible. Once in the laboratory, the material is trimmed, washed and prepared for disinfestation and any extra material is sent to the greenhouse for propagation. Almost all of the vegetative cuttings and seed that are sent to Lyon are wild or field collected. Primarily due to the high level of microflora found on and within wild collected plant tissue, they must be subjected to a harsh chemical regimen before clean explant material can be obtained for sterile culture. Sometimes the explants are lost during the disinfestment treatment before the material can be sufficiently cleaned. If wild collected vegetative cuttings can be established in the greenhouse, the new growth is used as starting material for micropropagation since they are much cleaner and easier to disinfest.

Treatments and soaking times will vary according to how dirty the plant material is and also on the sensitivity of the plant tissue to the cleaning agent. Careful monitoring of the plant material throughout the disinfestation process must be done to prevent over sterilization. Very dirty material may require one or more pretreatments before the actual sterilizing of the final explant. The pretreatments we use are long water rinses, 3% hydrogen peroxide dips, 70% ethanol dips and Physan 20 soaks. The most common disinfectant is sodium hypochlorite, which is found as a 5% solution in household bleach. For disinfestation, the soaking solutions are prepared fresh by diluting the bleach to a 5 (1 part bleach to 4 parts water) and 10 (1 part bleach to 9 parts water) percent dilution with 1 drop of Tween20 added for every 100 ml of solution made. Vegetative propagules are usually soaked initially in the10% bleach solution for 15-30 minutes. In a petri dish containing 5% bleach solution, the explants are trimmed further into final explant pieces with the aid of a dissecting microscope. The final explants are placed into a 5% solution for 1-15 minutes, rinsed well with sterile water then placed onto the appropriate plant medium.

Intact immature fruits are usually soaked in a 10% bleach solution for 30 minutes to 3 hours. The containers with the fruit are brought into the transfer hood and the seeds excised under aseptic conditions. The seeds are either left whole, or ovule or embryo culture is performed. Mature seeds are extracted prior to sterilization if they are still in the fruit. The seeds are washed well in water then soaked in a 10% bleach solution for 15 minutes to overnight. To enhance germination, the seeds may be scarified or dipped in 95% ethanol and quickly flamed prior to placing onto the germination medium.

The media formulations used at Lyon Arboretum for micropropagation are based on the Murashige and Skoog medium, commonly known as MS. This medium was originally developed for the rapid growth of tobacco tissue cultures, but has become one of the most utilized medium formulations in the tissue culture of many kinds of plants and plant tissue worldwide. Different variations of this medium are made for the purpose of inducing specific plant responses at specific stages of *in vitro* plant growth. In Hawaii, the plant genetic conservation effort encompasses a considerably large and diverse number of plant species where very little or no previous propagation information is known. In almost all cases, there is no time or sufficient plant material to optimize the culture conditions, which makes appropriate media selection difficult. Media selection is usually determined by the plant itself and the type of culture desired. There are basically three different types of tissue culture performed at the Lyon Arboretum micropropagation lab; seed, embryo and organ culture.

Whole seeds may be sown *in vitro* to produce intact plants. Seeds are germinated and maintained on a medium containing only one half of the MS macro and micronutrients (1/2 MS) with no hormones. Seeds that are immature, very small or rare benefit from *in vitro* germination. Embryo culture entails the excision of the embryo from the seed coat. Embryos are

placed onto a 1/2 MS medium with no hormones but may contain a higher sugar concentration, coconut water or charcoal.

The regeneration of plants from vegetative plant material that possess meristematic or non-meristematic tissue is accomplished through organ culture. Organogenesis is encouraged, which is the development of plant organs directly on the explant surface or upon an intervening callus phase. A 1/2 MS medium supplemented with hormones such as auxins and cytokinins is used to induce shoot and root growth. The ratio of auxin to cytokinin is often manipulated to promote one type of growth as opposed to the other. In some cases, auxin is omitted during the initiation of the cultures to promote shoot growth and an auxin added later. Once whole plants are regenerated, they are maintained on a hormone free medium.

To date more than 80 federally listed endangered taxa have been successfully grown at Lyon Arboretum using micropropagation techniques. For several of these taxa, specimens produced by micropropagation have been used for restoration and reintroduction projects. In addition about 80 more species of concern have been grown successfully. Ware also concerned with conservation of genetic resources of old Hawaiian crop plants, and are successfully maintaining *in vitro* about 70 unique Hawaiian cultivars of kalo (taro), *Colocasia esculenta*.

Examples of Restoration of Endangered Hawaiian Species Using Micropropagated Plants

We present four examples of successes attained:

1. *Delissea undulata*. This species is a woody lobeliad with a single palm-like unbranched stem that may reach 10 meters in height and 5 centimeters in diameter, with a crown of leaves each about 20 centimeters long. Populations were known originally from four islands; three of these disappeared during the nineteenth century. The Hawai`i Island population was known to have persisted in small numbers until 1971 but occurred only in an area devoted to cattle ranching, and was thought to have disappeared in the early 70's. It is listed as "Extinct" in the 1990 *Manual of the Flowering Plants of Hawai`i*. In 1992 at Pu`uwa`wa`a Jon Giffen, a wildlife biologist with the Hawai`i State Division of Forestry and Wildlife found a single plant, 2 meters tall, that had been knocked over by cattle but survived growing horizontally over a deep hole that was the entrance to a lava tube. He propped up the plant, fenced it to keep cattle away, and shortly afterward it began flowering. This single individual proved highly fertile. Immature fruits were collected before birds or insects could damage them, and over 300 plants were produced in the micropropagation lab by in-vitro ovule culture. Several plants have been returned to the site where they are planted in a fenced area, and many others are being grown successfully in a nursery devoted to endangered plant culture at Volcano on the island of Hawai`i. With continued protection from cattle chances of restoration appear to be good.

2. *Cyanea pinnatifida*. This lobeliad is a sparsely branched shrub to 3 meters tall, with pinnately lobed leaves as much as 60 centimeters long. It occurred in the southern Wai`anae Mountains of O`ahu. Although it was last collected in 1965, it was known to survive as a single plant in the Palikea area. This plant had been observed for several years growing on a nearly vertical slope that offered some protection from feral goats. It had never flowered and no natural reproduction had been observed. Dr. Gregory Koob, then in charge of the Lyon micropropagation lab, collected two lateral buds from this plant and eventually produced clonally more than 500 new plants. The site with the remaining wild plant is now a preserve managed by The Nature Conservancy of Hawai'i, and when a management plan is adopted several plants produced by micropropagation will be returned to the wild. In the meantime two plants flowered in the Lyon green-

house in 1995. These were hand pollinated and several seeds were produced, although they aborted before reaching maturity. However a few new seedlings were produced from embryos taken from very young seeds. Also, several clonally produced plants were sent to a private reserve on the island of Kaua`i, where they have thrived, flowered, and produced viable seeds. Thus, to the extent that the original plant was heterozygous it now seems possible to restore at least a small, amount of genetic variability to this species.

3. *Cyanea superba*. Plants of this species are palm-like trees to 6 meters tall, with leaves to 1 meter in length. Cream-colored flowers to 8 centimeters long are produced on 35-centimeter long pendent racemes. The species is confined to the northern Wai`anae mountains of O`ahu. Five plants are known to remain in the wild, in the Pahole State Natural Area Reserve. Many plants were produced in the micropropagation lab using immature seed culture; about 100 have now been returned to the wild, and the first flowers have just been produced on these plants.

4. *Cyanea asarifolia*. This lobeliad is a shrub less than one meter tall, with small leaves and white flowers about 3.5 centimeters long. It was described as a new species from a collection made in 1970 at Anahola Valley on Kaua`i. The only known colony contained about 10 plants. After Hurricane `Iniki in 1992 only two plants remained, both badly damaged. However, prior to the hurricane the Lyon micropropagation lab obtained young fruit and produced about 500 seedlings from immature seeds. Several have been returned to the State Division of Forestry and Wildlife and are being planted on the original site.

Conclusions

These examples demonstrate that species can be rescued from extinction, multiplied using various micropropagation techniques, and restored in the wild, when appropriate management of their habitats can be undertaken. But, given the magnitude of the problem, work has just begun.

Literature Cited

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