USE OF TISSUE CULTURE IN GERMPLASM MAINTENANCE PROGRAMS

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Conventional ex-situ germplasm storage is approached in two ways, via cold storage of seed or vegetative maintenance of plants in the field. Seed storage is used for most agronomic crops and annually propagated plants. However, a large class of plants with "recalcitrant," or difficult to store, seeds must be maintained vegetatively. Recalcitrant seeds, by definition, cannot be stored for long periods of time and are usually damaged by cold storage below 0 °C. Many tropical and temperate tree fruit and nut crop species have recalcitrant seed. These species are usually maintained through vegetative propagation, usually in the field. The U.S. National Plant Germplasm System has established clonal germplasm repositories to maintain germplasm of many of these crops (19, 20).

In-vitro germplasm maintenance is an alternative to field maintenance of clones (7,21). Germplasm can be maintained in-vitro as shoot tips or meristems, as callus, or as somatic embryos. Shoot tips or meristems are the preferred tissues for in-vitro germplasm maintenance (8). Regeneration into plants is usually easier from shoot tips than from other types of tissue, questions of genetic stability and somaclonal variation are minimized, and clonal genotypes can be maintained. Callus is usually considered to be a poor choice for germplasm maintenance due to the likelihood of somaclonal variation and the difficulty of converting the tissue into plants, especially where a variety of genotypes are to be stored (11). However, callus is a compact form of storage and is the easiest type of tissue to store at -196 °C in a viable condition because of the relative ease with which cryoprotectants may be introduced into the cells. Somatic embryos are a relatively new alternative for in-vitro maintenance. They could be ideal tissues for -196 °C storage because of their small size (making infiltration of cryoprotectants easier). Somatic embryos should be as genetically stable as seeds. However, somatic embryos are usually derived from some sort of induced callus so that potential questions of genetic stability may arise. Somatic embryos have been reported to be genetically similar (identical) to the parental tissue from which they were derived (5,9). Cotyledon tissue is generally used as a source tissue in fruit crops. Therefore, direct generation of cloned somatic embryos from a vegetative parent is not possible. Somatic embryos will probably be extensively used in genetic engineering applications since the

embryos can be transformed at the single cell level and grown into whole plants, limiting the problem of transformation chimeras.

In-vitro germplasm maintenance of clonal germplasm reduces difficulties associated with systemic diseases (mycoplasmas and viruses) that can be transmitted to uninfected accessions in the field. As a consequence of a curator's ability to maintain disease-free plants in culture, distribution of germplasm as cultures can facilitate exchange where quarantine restrictions would otherwise prohibit passage of the materials. Obviously, germplasm recipients must have tissue culture facilities available to them to receive this type of material. In-vitro distribution has been successfully applied to banana, potato, and peach.

Some practical limitations to in-vitro storage as described above include the problem of genotype-media specificity. Different genotypes often have specific media requirements. Thus, for large collections with a variety of diverse genotypes, numerous media formulations may be required. This is probably the most serious limitation to in-vitro germplasm maintenance today. Reasonably broad spectrum media can be developed for species with low levels of genetic diversity (15). A question of similar importance is the genetic stability of the cultures. In-vitro maintenance procedures should be sufficient for maintenance of specific genotypes, without change, indefinitely. Any procedure involving generation of callus prior to plant regeneration has the potential for inducing or propagating genetic changes (1,6,11,17).

Although in-vitro culture has the potential for reducing environmental hazards from disease, insects, frost, etc., other risks must be considered. Power or equipment failure in storage facilities can destroy entire collections if not immediately detected. Fire, earthquake, or flood could have similarly disastrous consequences for in-vitro collections maintained in vulnerable structures. Large scale contamination through undetected sterile transfer hood failures, contaminated media, or culture mite infestations are potentially serious problems. Knowledgeable staff can minimize the latter concerns.

Discontinuities in program funding could have especially severe consequences for in-vitro maintenance programs. These programs require regular maintenance and transfer of the cultures for all nonfreezing storage temperatures. Thus, a one-year program suspension due to short term administrative decisions could result in major germplasm losses. Cultures maintained at -196 °C (liquid nitrogen temperature) are likely to be relatively secure as long as liquid nitrogen (LN_2) and minimal labor to monitor storage conditions are available.

In-vitro germplasm maintenance could be less expensive than field maintenance and, if field space is a limitation (often the case

for large tree crops), tissue culture storage may permit maintenance of a larger number of clones than field maintenance. Using eight culture tubes (20mm x 100mm) per cultivar, about 10,100 cultivars could be maintained in a 3.6m by 4.6m room (4 °C cold room for example). Assumptions: seven levels of shelving would be used with a 1.2m center aisle. With a transfer interval of one year, a technician should be able to reculture a collection of about 12,000 cultivars annually (48 cultivars recultured per day).

The cost of an in-vitro germplasm preservation program will be closely related to the frequency of transfer needed to maintain the cultures. Several possibilities for extending culture intervals have been suggested. These include storage at reduced temperature, reduction of carbon source levels (sugars), growth at reduced light levels, and addition of growth retardants to the medium.

Temperature is one of the most easily controlled variables in a tissue culture system. Tissue cultures are normally maintained at about 25 °C which is a good temperature for shoot multiplication and growth. Cultures can be maintained for four to eight weeks between transfers at this temperature; 4 °C is another common temperature for tissue culture maintenance. Fourteen examples of shoot tip or meristem derived explant maintenance for one or more years are given by Kartha (8). Several crops show improved survival at 6 °C to 9 °C (2,13,14). Cultures of mint have been successfully maintained at 2 °C in the National Clonal Germplasm Respository, Corvallis, Oregon (10). However, 0 °C to 2 °C temperatures are relatively difficult to maintain with normal refrigeration or freezer equipment (too low for refrigeration and too high for freezers).

Reduction of light levels has been suggested as a method for reduction of growth in culture. However, our experience has indicated that maintenance of peach shoot tip cultures in the dark at 25 °C results in a severe decline in vigor as well as growth. Reduced light levels had no effect when cultures were maintained at 4 °C, probably since no growth was occurring. Work by Marino *et al.* (12) has shown similar results.

Limitation of the carbon source in the media (sugars) may be expected to have results similar to reduction of light levels (which prevents CO_2). Since active growth will continue at 25 °C, tissue decline may occur. Japanese researchers have developed protocols for sugar-free media using high CO_2 and light levels to compensate for the lack of carbon in the media, demonstrating the relationship between light, CO_2, and carbon source in tissue culture systems. An alternative to using sugar-free media might be to limit CO_2 exchange. The benefits of using this approach are unknown.

The use of growth regulators in-vitro for growth restriction have not been studied to any great extent. The general emphasis in tissue culture research has been to find growth regulator combinations

that promote rather than retard various aspects of tissue growth. Abscisic acid has been reported as a growth control factor. It seems reasonable to believe that reduction of growth regulator concentrations in tissue culture media, in appropriate combinations, could effectively limit tissue growth at a variety of temperatures.

Buffered media could permit extended growth of cultures without transfer to new media by making nutrients available to the explants for a longer period before transfer to a new medium is needed. An effective nontoxic buffer (MES) is available and has been tested with several crops and tissue types (16). The effectiveness of MES buffers for extending transfer intervals has not been proven and additional work is needed to test this hypothesis.

Work in our own lab has indicated that maintenance at 4 °C could be effectively used to suspend the growth of in-vitro cultured peaches for more than 40 weeks and almonds for more than 33 weeks. Twenty-four peach cultivars were tested and although substantial variability for cultivar effects was observed, no significant declines in viability were observed. Effects of light and dark treatments were evaluated for Lovell peach after 16 weeks and a significant decline in tissue health was noted at 25 °C for the dark treatment although no differences were found at 4 °C for the two treatments. These results were generally consistent with those from Marino, *et al.* (12) with three *Prunus* rootstocks. They observed substantial loss of viability at 24 weeks in 4 °C conditions.

An ancillary experiment using three antibiotics to control bacteria inside the shoot tips demonstrated that two of these compounds, polymyxin B and rifampicin, could have significant growth limiting effects on peach shoots. Tetracycline at 6.5 mg/l did not inhibit plant growth (and was also less effective as an antibiotic). Antibiotics have previously been reported to retard shoot growth (3,4). Other compounds that could restrict tissue growth and permit extended storage are compounds such as mannitol or cryoprotectants such as sucrose or polyethylene glycol which act by changing the osmotic potential of the medium.

All of the procedures described previously for in-vitro germplasm maintenance can be classified as short to medium term methods. Periodic maintenance of the cultures is required and there is some risk of genetic change occurring during maintenance. The only technology available for truly long-term maintenance of germplasm in-vitro is cryogenic storage at or near the temperature of liquid nitrogen (-196 °C). Storage above liquid nitrogen provides a storage temperature of -130 °C to -150 °C. Metabolic activity ceases at LN_2 temperatures and the only source for mutations would be ionizing radiation or cosmic rays. Such mutational events would be

cumulative and would only be significant over a long period of time. LN_2 storage of germplasm should permit permanent storage in-vitro, without transfers or genetic changes. The technology has been tested with more than nine crop species (8). Specialized pretreatments, addition of cryoprotectants and prefreezing, are usually required for successful storage (18).

A prerequisite to the application of LN_2 preservation strategies is the ability to handle the subject tissue in-vitro, either as shoot tips, callus, or cell suspensions. Systems must also be available for generating plants from tissue-cell systems. In-vitro systems permit the introduction of cryoprotectant compounds into the tissues to prevent freezing injury.

From the preceding discussion it is apparent that the development of effective in-vitro systems will become increasingly important for germplasm maintenance programs. We must also recognize that in-vitro systems may not be appropriate for all germplasm objectives.

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