Genetic Considerations for Germplasm Preservation of Clonal Materials

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The present diversity of plants used for the needs of society is immense and could expand greatly with the development of new crops and more extensive use of plants producing useful pharmacological compounds. We are relatively familiar with problems associated with germplasm preservation of major food crops, but such information is lacking for many minor crops. Aspects of genetics, physiology, and phenology must be considered to develop a useful and efficient means of germplasm preservation (29).

Elite clones have been used in agriculture for many years, and preservation is essential for continued development of new cultivars with improved characteristics. Two major considerations for preservation of clonal materials are genetic stability of the line and application of maintenance procedures to diverse arrays of genotypes. Problems with genotypic specificity can occur at any stage of the preservation process, and dealing with this is crucial to establishment of an efficient, usable system.

The manner in which clonal germplasm is preserved is examined here, especially in light of developments in plant microculture and cryobiology. Adequacy of the genetic base is also an important issue, but will not be addressed.

ACTIVE AND BASE COLLECTIONS

The concept and importance of active and base collections have been amply stated and given strong emphasis by both the National Plant Germplasm System (NPGS) and the International Board for Plant Genetic Resources (IBPGR). An active collection contains plant accessions that are available for distribution, evaluation, and documentation. Thus, accessions must be regenerated and multiplied to maintain diversity in the collection and to supply plant materials to users (14). An active collection requires methods of storage that retain the viability of samples for short to moderate periods of time (i.e., from a few weeks to several years). For active collections, clonal lines are usually maintained as field collections in either orchards or plantations or in screenhouses or greenhouses. The use of in vitro methods for maintaining plants has been advocated as an alternative route or as a safety backup for field materials. Distribution of materials from active collections is in the form of seed, budwood, cuttings, divisions, in vitro plants, and, perhaps, even as collected pollen.

A base collection, by contrast, is intended for long-term conservation of diversity (gene pool) and materials are not intended for extensive distribution. It serves, rather, as a duplicate store of diversity for a given accession should the active collection be depleted (14). Preservation of base collections requires methods that retain viability for long periods of time to minimize loss of diversity during the regeneration phase and reduce costs of regeneration and frequent viability testing.

PRESERVATION

Historically, many crops have been maintained clonally for reasons related to cultural aspects or the failure to form viable seeds. In some instances, clones are more productive than seed-derived lines, especially where standardization and uniformity are desired. Reasons for clonal maintenance of lines from the standpoint of germplasm preservation usually involve considerations of one or more of the following (16): a) lines do not form viable seeds (e.g., garlic); b) seeds produced are recalcitrant—that is, they have short storage lifetimes and do not tolerate much drying (13); c) seed sizes are so large that storage of sufficient numbers would require large amounts of space (e.g., coconut); d) lines possess long juvenile periods, hence flowering and seed formation are delayed (woody plants); e) clones are normally heterozygous (and the species heterogeneous), hence continued crossing will break up unique genetic combinations that have evolved (16).

Methods have been developed over the years so that clonal materials in active collections are in an available form for distribution and use by breeders. Different approaches are desirable for efficient and effective preservation of active vs. base collections. Strategies for maintaining clonal base collections are not as well-developed compared to those for seed. Some consensus, however, is emerging. Apical or axillary buds from in vivo or in vitro plants could be stored at cryogenic temperatures to maintain the clone (28, 35, 47, 63). However, long-term storage also could be accomplished through production of a seed population if plants are fertile and have no major incompatibility problems and if generated seeds are orthodox (33, 53). Orthodox seed are easy to handle, and viability can be maintained for many years through control of seed moisture and storage temperature. The genetics of many clonal crops are becoming known, but, compared to major agronomic crops, population structures (wild/domesticated) are still poorly defined, which complicates collection and maintenance strategies. Whether seed or clonal preservation would be most desirable has to be examined on a crop-by-crop basis. Many considerations must be assessed. For some crops, crossing to produce a seed population may lead to loss of genetic information if lethal factors are exposed. Herbaceous clonal lines are usually easier to maintain as seed populations than are woody lines. Overcoming graft incompatibilities and long juvenile periods may facilitate using seed materials from woody lines for some breeding projects. Numerous other examples could be given. The acceptance of seeds for base collection preservation of a given crop may reduce efforts into development of other methods for long-term preservation (i.e., growth-restrictive conditions for in vitro plants).

CLONAL PRESERVATION

There are many genetic issues related to collection and maintenance of species that we hold as clones, but this discussion will stress maintenance. Collection is, in principle, the same among different crops, assuming something is known of the species’ breeding system and population structure. There are, however, many practical considerations that differ in obtaining adequate sampling among individuals and populations. Actual collections of diversity for many crops, particularly those of minor importance or whose members lie within areas not easily explored, probably are not an adequate sample of existing diversity (9, 29). Adequacy of collection differs considerably among species.

Once materials have been collected, the genetic composition must be maintained by the best preservation and propagation procedures available. This statement is obvious, but the balance between practical and optimal often is based on costs and time available.

Accurate characterization of the clone is required to answer questions related to genetic stability. Characterization and evaluation would include data on morphology and phenology, agronomic or horticultural performance, physiological aspects, and electrophoretic analyses. The latter might be the most useful for germplasm purposes (40, 51, 55). For example, nonspecific protein and isozyme analyses have been used to identify and distinguish cultivars within many species (55). Methods are also being developed for electrophoretic and probe analyses of restriction enzyme fragments.
were much more extensive. There is a low level of duplication of job of maintaining clonal integrity is being done. This information also is necessary to determine frequencies of mutation (or other types of change) that might occur.

What type of change can one expect to see in a clone over time? This question, although simple in concept, is not answered easily if one is concerned with the exact nature and with the rate or frequency of change. The variation that is observed in a clone usually is due either to mutation, rearrangement of a chimera, or epigenetic change (57). In addition, changes in disease status of a clone (either becoming disease-free or disease-infected) can produce individuals with an altered appearance. Expression of mutations depends on ploidy and the nature of the mutation. Some may only be expressed in the progeny; others may express themselves in the affected individual, but only after several budbreak generations. Estimates of frequency depend on the ability to discern change. A number of quantitative traits may also be altered, but go undetected (taste, growth rates, etc.). Determination of the type of variant one has requires detailed genetic analysis and knowledge of the pattern of inheritance. Frequencies of occurrence in populations or within clones are usually not determined, and, hence, it is difficult to compare frequencies and types of change observed in field collections with those that might occur in in vitro collections.

The uncertainty of whether change has occurred also is manifest in preservation strategies. Since any single individual (or isolated propagule) may be a variant, isolates from different representatives should be made if a desired clone is to be preserved. This represents the most cautious, conservative approach to maintaining the clone. Practical issues restrict the number of individuals that can be maintained within a collection.

Clonal germplasm preservation usually is accomplished ex situ, although preservation in situ is considered for populations of related wild species.

In situ maintenance

This process involves the use of natural sites for preservation of materials (22, 30). An argument for this route is the continued evolution within a species of particular traits, including resistance/tolerance to disease or stress conditions, providing sufficient land and population sizes are used. There are many problems in establishing this type of preserve, for example, cost, political issues, and size and maintenance aspects. In particular, there is need to define specific conservation objectives. One must know the diversity of materials being conserved for application and effective management. This maintenance is best considered for wild relatives of many crop and forest species, especially in subtropical and tropical areas. Loss of germplasm in these areas is occurring rapidly, and international cooperation is needed for effective management.

Ex situ maintenance

This is the usual system for clonal maintenance and encompasses field gene banks such as orchards, plantations, and greenhouse and screenhouse collections. Field gene banks assure that a ready supply of usable materials in a mature state is available for distribution, characterization, and evaluation. However, field gene banks have the potential risk of germplasm being lost due to a disaster, and large amounts of space and labor are required to maintain only a small proportion of the diversity. It is doubtful that an adequate support would be available for the effort necessary to maintain the integrity of clones and supply distribution materials if collections were much more extensive. There is a low level of duplication of clones at a location, often only two or three individuals, and minimum duplication among locations. Disease or stress could easily eliminate all individuals of the clone. Both field and greenhouse plantings are maintained for many lines to minimize possible loss and disease spread. Little information is available on the stability of lines or frequency of mutants occurring under field gene bank conditions.

Preservation of in vitro plants and the cryopreservation of axillary buds from in vivo or in vitro plants are two areas of interest for base collections.

In vitro plant preservation

In vitro techniques are advocated for germplasm preservation because of the extensive development of plant cell, tissue, and organ cultures, and their application to many species (1, 20, 50). Some clarification and cautious use of terms are necessary to state what materials should be preserved in this fashion and the exact manner of preservation.

The term in vitro culture can refer to any proliferation maintained in sterile culture. There are ample data in the literature for many species that plant regenerants derived from adventitious buds or somatic embryos formed directly on leaf, stem, root, or other explants, or formed on callus, may exhibit considerable somaclonal variation (6, 15, 18, 19, 39, 43, 49). The frequency of variants differs among lines and species and with the extent and number of passages of callus. This potential for variation poses problems for maintaining exact clonal identity through the use of undifferentiated tissues. Thus, germplasm preservation in vitro must be accomplished by use of plants that initially were derived from meristem tips, shoot tips, or buds isolated from field or greenhouse materials and in which the tip developed directly into the plant. These in vitro plants (hopefully disease-free or at least tested) then are stored in vessels and subsequently micropropagated.

The micropropagation procedure should give clear evidence that multiplication occurs from existing axillary buds and not from adventitious buds. Methods for multiplication and preservation must be applicable to a range of genotypes and not impose any selection pressures. Unfortunately, conclusive evidence for most of the aforementioned points is lacking for most systems, and this is especially true for woody materials.

In vitro plants obtained and maintained by apical or axillary bud propagation are useful for either active and base collections, or both (31, 32). Morel (42) recognized early that in vitro plants are valuable for germplasm purposes by virtue of the control of their growth, ease of multiplication, and ease of retaining the disease-free state. The advantages of in vitro plant culture for germplasm maintenance include small amounts of space for storage, possibility of freezing plants from viral or other diseases, increased rates of multiplication, micropropagation of species often difficult to propagate, availability of propagules throughout the year, storage of plantlets for extended periods, and ease of shipment. These points have been described in review articles (16, 32, 49, 62).

Even with current understanding and level of technological development, use of in vitro plants for preservation is not without problems. These problems are usually not elaborated, but need to be considered in developing germplasm preservation procedures. They include susceptibility to disaster, human error, intensive labor requirements for in vitro collections properly maintained and monitored, difficulties in establishing and maintaining cultures, genotypic specificity at all stages of the in vitro process, use of growth-restrictive conditions, and the possibility of producing aberrant plants.

The first two points bear on the safety of maintaining in vitro materials. Storage of all materials within small growth chambers or something similar may be just as susceptible to problems as storage under field conditions. For example, mite infestation or equipment failure can cause the loss of cultures. Human error in mislabeling, media preparation, or misidentification of in vitro plantlets also can occur over time, since there are few morphological characteristics to give a simple check as to reliability. Identification of materials by growing out plants would be time-consuming, especially for species with long generation times. Thus, extensive electrophoretic characterization of the in vitro plants and periodic testing are necessary for reliable maintenance. Labor involved in these processes might be considerable for a large collection if all the procedures are carried out in a critical fashion.

Establishment of an in vitro plant is often difficult, and considerations of stock plant condition, physiological stage, and maturity must be assessed. Genotypic specificity for ease of establishment often occurs at this stage, as well as for the propagation and rooting.
stages. Woody materials often have distinctive maturity stages that can be manipulated for use in establishing cultures (24).

In vitro maintenance of certain lines may be difficult. Vitrification, the glassy appearance of some shoots maintained in culture, is usually a physiological disorder (38). These plants often do not grow, can not be acclimated, and are useless for germplasm efforts. Some genotypes seem predisposed to vitrification, and the condition is not always rectified by manipulating the culture environment.

Acclimatization of some materials is also difficult, and some losses could be expected to occur during transplanting (45). The role of an active collection is to supply materials in usable form, hence, the inability to acclimatize also limits access to the germplasm. Eventually, these culturing problems may be solved successfully, but such studies require time to deal with diversity of materials.

Growth-restrictive temperatures or media formulations are proposed to lengthen the time between transfers and give enhanced storage durations, thus reducing labor and introduction of errors (32, 62). These conditions have been used successfully to date on easy-to-propagate materials such as strawberry and potato, and their eventual application to diverse lines appears feasible. Again, genetic specificity may be a problem. A crucial question is whether growth under these conditions might be a selective process, allowing loss of some lines, rearrangement of chimeras, or possible expression of abnormal types. There are few data on this point for in vitro plant cultures stored under growth-restrictive conditions, but limited experience with strawberry and potato has shown no major problems.

Most reports for meristem culture and micropropagation show production of true-to-type plants (e.g., ref. 46). But a few reports suggest that some lines produce an increased frequency of abnormal plants (34). In some instances, this increase may be due to environmental factors, and the altered appearance will be transient (17, 59). In other instances, altered field appearance may be due to increased vigor or proliferative characteristics of in vitro plants (54). Increased use of micropropagation in the floriculture and nursery industries attests to a high frequency of faithful reproduction if careful attention is paid to critical steps in the procedure. Occasional problems are noted; for example, chimeral rearrangements might occur during meristem culture (7). Some instances of extreme variability have been observed in field plantings from in vitro plants, but such information is not published, and studies appear not to have been made to explain such variability.

Cryogenic preservation

Ex situ preservation can also be accomplished by storage of buds, shoot tips, twigs, and cuttings at low temperatures, where diffusion and chemical reactions are so slow that little loss of viability is expected to occur over time (41). Liquid N (LN) temperature (−196°C) or that in the vapor phase above LN (about −150°C) are often used because it is a suitable, relatively cheap cryogen. It should be emphasized that, in dealing with hydrated tissues, as contrasted to seeds, losses in viability usually do occur in taking samples to and from these low temperatures.

The advantage of cryogenic preservation is that propagules can be stored indefinitely without the need for subculturing. Therefore, the costly processes of plant regeneration and frequent viability testing are minimized.

There have been considerable advances in the study of the cryobiology of plant materials over the past 10 to 15 years (52). There are still questions about what freezing injury is and how it is alleviated by the use of cryoprotectants (21), but empirical methods have been developed for various solutes in suspensions and cell suspensions, callus, tissue and organ fragments, and buds and shoot tips, with varying levels of success (35, 47, 48, 63). The following comments are restricted to bud and shoot-tip systems, since these are the desired propagules for germplasm preservation strategies.

Buds and shoot-tips have been frozen and plants retrieved from a range of materials, including asparagus, brussels sprouts, cassava, chickpea, pea, peach, potato, apple, currants, gooseberry, pear, raspberry, strawberry, and tomato.

A number of issues must be addressed before this technology can actually be used for germplasm preservation. There is often an unsuitable percentage of survival for buds or meristem tips after low-temperature treatment. This percentage is often 60% or lower, although considerable variation occurs among reports. What constitutes a minimum, usable percentage of survival for a base collection? Although there are no guidelines, more than about 80% seems desirable. This level would reduce the number of buds required per sample and number of samples frozen per accession.

There is considerable experimental variation in survival. This variation suggests that all of the factors involved in a cryogenic protocol have not been optimized (35, 36, 47, 63). Further examination is necessary to produce a method that is dependable and useful for a wide range of materials.

The pattern of growth of treated buds varies considerably among species. This variation ranges from direct development of the treated shoot-tip or bud into a plant [e.g., apple and some other hardy materials (48)], to development of adventitious buds or somatic embryos directly on the treated shoot-tip or bud [pea (25), carrot (61), Solanum etuberosum Lindl. (56)], to development of a small callus prior to adventitious bud initiation [potato cultivars (57)]. Potato is probably the best-studied system, and it is quite apparent that growth-media formulations affect subsequent growth response (27, 28, 57). For nonacclimated materials, there is often a differential survival of cells within the low-temperature-treated bud, and hence callus, somatic embryo proliferation, or adventitious bud formation may occur before plant regeneration. A callus phase prior to shoot initiation is undesirable, since callus potentially increases the frequency of variants. The manner of development of low-temperature-treated buds needs to be described for other species, especially woody ones. In addition, chimeras will be impossible to preserve unless the whole bud survives and develops directly into a plant.

There are few analyses of regenerants obtained from buds or shoot-tips stored under liquid N conditions. Such information is essential to determine if cryogenic and culturing protocols are effective in maintaining clonal integrity. Once in vitro plant cultures are established from a thawed propagule, the same considerations previously discussed for in vitro plant maintenance must be evaluated. Analyzing variation from regenerants formed from buds that were originally isolated from in vivo plants, but required in vitro culturing after freezing, may be more difficult than analyzing variation from buds derived from in vitro plants.

It is a lack of information on regeneration to demonstrate applicability of a cryogenic protocol for the many genotypes that would be in a collection. It is reasonable to expect genotypic specificity in response to treated buds to cryogenic and culturing regimes. In tuber-bearing Solanums, bud survival was usually high for many cultivars and some species; however, the capability to regenerate plants on a given medium was restricted (57). There is also the possibility that certain cryoprotectant combinations may be toxic to selected genotypes.

Strategies for cryopreservation must deal with materials that have varying degrees of hardiness and capabilities to acclimate. Plants often are formed directly from buds obtained from in vivo plants in an acclimated state (e.g., apple). Acclimation of in vitro plants needs further study to see if this is a feasible route for increasing cell survival within the bud (12).

Although there is concern about possible mutagenic effects from the cryogenic protocol, few reports directly deal with this issue for higher organisms. However, in bacteria, freezing and thawing in the presence of a cryoprotectant and extended storage did not increase the frequency of revertants in the Ames system (4). In mammalian cell lines, the frequency of sister chromatid exchanges was not increased (4). There was no loss of plasmids from Escherichia coli with storage in liquid N (8). Use of low-temperature-stored semen has not led to reports of demonstrable increases in abnormal individuals.

Additional research is necessary, however, because of insufficient studies. Also, observations that freeze-drying is mutagenic to bacteria (3), that dimethyl sulfoxide (DMSO, a cryoprotectant com-
monly used for many organisms, including plants) has gene activation effects in mammalian systems (4), and that DNA in situ may be damaged if no cryoprotectants are used (10, 11, 60) provide cause for caution. Information on the effects of DMSO on plants is sparse, but it is known to interfere with metabolism (5) and cytokinesis (58).

Normal DNA repair processes are not functional in materials stored at low temperatures; hence, the question exists as to what periods of storage are possible at low temperatures before background radiation causes genetic damage. The typical approach has been to use high levels of irradiation for short periods of time and then extrapolate to years of possible storage with background levels of radiation. The few studies in this area are not with plant systems, but suggest that several centuries of storage are possible before effects are noted (2, 23).

The previous discussion is to highlight questions that require answers before cryopreservation is used on a large scale. Indeed, with recent advances in understanding freezing injury and the role of cryoprotectants, the expectation is that techniques can be adequately developed for preservation of base collections.

SUMMARY

Some of the points for active and base collection maintenance of clonal materials have been listed. There is a lack of data on genetic changes that occur during preservation and, as such, it is obvious that more information is needed if systems are to be constructed that maintain genetic diversity and clonal identity. This information will have to be developed on a crop-by-crop basis. At present, in vitro cultures are not used as the sole mode of maintenance for will have to be developed on a crop-by-crop basis. At present, in vitro cultivation effects in mammalian systems (4), and that DNA in situ may be damaged if no cryoprotectants are used (10, 11, 60) provide cause for caution. Information on the effects of DMSO on plants is sparse, but it is known to interfere with metabolism (5) and cytokinesis (58).

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Some of the points for active and base collection maintenance of clonal materials have been listed. There is a lack of data on genetic changes that occur during preservation and, as such, it is obvious that more information is needed if systems are to be constructed that maintain genetic diversity and clonal identity. This information will have to be developed on a crop-by-crop basis. At present, in vitro cultures are not used as the sole mode of maintenance for clonal systems. In vitro systems are used as backup collections, but without clear guidelines of what procedures are necessary and what levels of replication and duplication are needed as adequate safeguards. These considerations, along with guidance on what diversity of materials are to be stored, must be answered by the user community. For the present and near future, clonal collections should use both field and in vitro maintenance, and at least one duplicate of the collection should be held.

Theoretical and practical problems associated with the use of cryogenics for storage of hydrated propagules require further study before application to many clonal lines is possible. Cryopreservation of buds from materials that have the physiological capacity to cold-acclimate should be feasible in the near future if there is evidence of stability in the regenerants. Guidelines for implementing a cryopreservation system for germplasm base collections, however, are needed. These would include handling, storage systems, regrowth and culturing information, replicates needed, duplication of collection, and testing and genotype verification.

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Genetic Considerations in Germplasm Collection and Maintenance: A Summary

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There are some 240,000 species of angiosperms in the world, of which 3000 or more have been used for food at one time or another. Of those, only 30 species are produced in quantities of at least 10^7 t/year, and 12 major crops supply the world’s population with most of its plant- and animal-based caloric intake. Thus, humans are dependent for food on only a few species of the many thousands of flowering plants on the earth.

The continued growth of the world’s population makes it necessary to increase production per unit area by breeding and research into more efficient farming systems. Other than present-day cultivars and germplasm collections, sources of variability for use in breeding programs exist mainly as land races being grown as unimproved cultivars by farmers in some areas, or as wild forms growing in undeveloped areas. New sources of variability for inclusion in germplasm collections are disappearing as land races are replaced by new, more productive cultivars, and as new land areas are developed for use by humans. Thus, it is important to collect and maintain germplasm for use in breeding programs before it is lost permanently.

GERMPLASM COLLECTION AND MAINTENANCE

Collection

Centers of origin were identified by Vavilov (8) as those locations in the world where certain crops originated and usually had the greatest diversity of undeveloped germplasm. The center of diversity was a concept introduced by Harlan (4) to update Vavilov’s work, using more recent findings. Centers of diversity, as described by Harlan, are usually the best locations for collecting primitive germplasm of most crop species.

Certain guides are useful when collecting germplasm from areas that have natural stands of land races and wild species. Use a small sample size for apomictic species, a larger one for cross-pollinated species, and the largest sample for self-pollinated species. Apomictic plants generally are more heterozygous and exist in more heterogeneous populations than cross- or self-pollinated types. Thus, the same amount of diversity can be represented with relatively few