Storage of Rare and Endangered Plants in *ex situ* Germplasm Banks

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Abstract

The genetic diversity of native plants can be preserved by placing large numbers of seeds or explants in *ex situ* genebanks. Seeds remain viable for decades if stored at optimum water contents in the freezer or liquid nitrogen. The quality of the seed collected influences the storage procedures used and the longevity achieved. Genebanks play no role in conservation at habitat or ecosystem levels and should be regarded as supplemental to *in situ* conservation strategies.

Keywords

seeds, pollen, recalcitrant, ultradry, cryopreservation

Introduction

Conservation programs are needed to stave off the unprecedented loss of our planet's biological diversity. Social and political pressures threaten land used for *in situ* reserves. *Ex situ* reserves provide a supplemental means to preserve the genetic diversity of species whose habitats have been disturbed or fragmented (Brown and Briggs 1991).

In an *ex situ* reserve, a "captive" population is established and maintained as either a collection of living and growing organisms or a collection of living but quiescent organisms. Sampling strategies to establish the captive population depend on whether 1) the collection consists of quiescent organisms or not, 2) the goal is to preserve rare alleles or allelic frequencies representative of the wild population, and 3) the genetic diversity is located within or among wild populations (Brown and Briggs 1991, Guerrant 1992, Dixon 1994). Living and growing collections (botanical gardens, orchards, plantations cell and tissue cultures) are expensive in terms of labor and space. Only a few individuals or specimens of a given species can be maintained and required periodic regeneration carries the risk of genetic shifts (Brown and Briggs 1991, Guerrant 1992, Dixon 1994). Living collections are also susceptible to natural disasters.

Collections of living but quiescent organisms provide a low-risk option for conserving genetic diversity of many species. In quiescent collections, propagules are stored in a state of "suspended animation." Thousands of individuals can be maintained in a tiny space with few labor requirements. When good storage practices are used, high viability can be maintained for decades -maybe centuries- obviating the need for regenerations and reducing the risks of genetic shifts within an accession (Walters et al. 1998). Quiescent collections of plants are usually seed banks such as those of the CPC or the National Seed Storage Laboratory (NSSL).

Principles of Seed Storage

The same techniques are used to establish quiescent collections of cultivated and non-cultivated species. The basic principle is to keep cells alive while stopping metabolism by drying and/or cooling. Seeds of many species become quiescent naturally when they undergo maturation drying. Exceptional seed longevities can then be achieved by exploiting this natural ability to tolerate drying. When survival for brief periods is desired, either the temperature or water content at which seeds are stored need be manipulated to maintain a desired germination percentage. The general relationship is summarized by Harrington's "100 Rule" which states that adequate seed viability can be maintained for about 5 years if the sum of the temperature in °F and the relative humidity (RH) does not exceed 100 (Justice and Bass 1978). More elaborate descriptions of the effect of water content and temperature on seed longevity are modeled in the Viability Equations where survival time is an exponential function of seed moisture content and a quadratic function of storage temperature (Ellis and Roberts 1980).

The Viability Equations served as the basis for IBPGR's recommended conditions for long-term seed storage: water contents of seeds should be adjusted to 5 ± 2% and temperatures should be kept at -18C (the temperature obtained from a single stage compressor) (IBPGR 1985). At the time, there was very little information on the fate of seeds stored at water contents less than 5%, but "the drier the better" was a presumption based on Harrington's Thumb Rules and the Viability Equations. The ±2% water content was intended to represent variation among species differing in chemical composition. However, there were no guidelines to tell which seeds should be stored at 3% and which should be stored at 7% and so the ±2% water came to signify a statistical variation, with 5% water being the target moisture level (Walters and Engels 1998).

More recent experiments show that there is no benefit of drying seeds to water contents less than 5±2%, and that overdried seeds age more rapidly (Walters and Engels 1998). The critical water content varies among species according to the lipid content of seeds (Walters and Engels 1998). For example, optimum water contents for seeds of pea (2% lipid) and Pacific yew (*Taxus brevifolia*, 71% lipid) are 6% and 1.4%, respectively (Walters 1998, Walters-Vertucci et al. 1996). The correlation between optimum water content and lipid content suggest that there is a specific amount of water required in the cytoplasm to minimize aging reactions. Optimum water contents for oily seeds are proportionately lower since most of the water present is in the cytoplasm and very little is held by oil droplets. Water activity (a_w) describes the amount of water available in the cytoplasm and is directly related to equilibrium relative humidity ($a_w \approx$ RH/100). The relationships between RH and water content for seeds are described by water sorption isotherms drawn at a series of temperatures (Fig 1 in Walters 1998). As expected, seeds with high lipid contents have lower water contents at a given RH and temperature than seeds with low lipid contents. While the optimum water content for seed storage varied among seeds, optimum RH is fairly constant between 15 and 25% RH (Walters 1998, Walters and Engels 1998). This RH range also corresponds to the moisture level at which cellular viscosity is minimum (Buitink et al. 1998).

In the past, it was assumed that the critical water content was constant with temperature. However, research has shown that the optimum water content for seed storage increases as the temperature for storage decreases (Walters 1998, Walters and Engels 1998). The results from storage experiments are consistent with predictions based on measurements of water sorption (water contents increase as temperatures decrease (Walters 1998) and cel-

lular viscosity (Buitink et al. 1998). To achieve maximum longevity, moisture content of seeds must be adjusted to an optimum which is a function of the lipid content of the seed and the storage temperature. However, the value of the optimum RH for storage is fairly similar among all species and all storage temperatures. This discovery is a boon to genebank operators who prefer standard protocols for seed storage over procedures that are specific for each seed type at each storage temperature. In general, optimum water contents for storage at 5°C, -18°C and -150°C can be achieved by equilibrating seeds at 20°C to RHs of 30%, 38% or 58%, respectively.

Lowering the temperature at which seeds are stored extends seed life-spans (Justice and Bass 1978, Roos et al. 1996, Walters et al. 1998). Seeds are not damaged by water freezing if they are equilibrated to optimum moisture levels prior to exposure to subfreezing temperatures. The exact benefits of temperature are not known since the experiments require decades to complete. The limited data available suggests that Q_{10} for aging is between 1.8 and 2.3 (Walters 1998). Thus, a reduction in storage temperature from 20°C to -20°C can reduce aging rates 10 to 30 fold. In other words, seeds that can survive for 5 to 20 years at ambient conditions may survive for 50 to 600 years in the freezer.

Storage at liquid nitrogen temperatures (-120° to -196°C) may allow seeds to survive millennia. Over a hundred species of seeds have been shown to survive initial exposure to liquid nitrogen, although some may be susceptible to damage by rapid cooling if they are very dry (Roos et al. 1996). The kinetics of aging at such extreme temperatures are not well understood and little information exists on optimum moisture conditions or packaging. The relatively high water content predicted to be optimal for liquid nitrogen storage (in equilibrium with about 58% RH at 20°C) may seem counter-intuitive, but if studies conducted at higher temperatures are extrapolated, sever damage by overdrying is predicted. Small seeds notorious for poor longevity (e.g. lettuce and onion) are routinely stored in liquid nitrogen at the NSSL; but the technique should be regarded as experimental. Curators may wish to store extremely rare seeds under conventional storage (-18°C) until the benefits and risks of liquid nitrogen storage are understood more completely.

Documented Longevities of Seeds

Records of seed longevity are often anecdotal but relay remarkable potential for long-term survival. In some surveys, seeds survived 30 to 40 years in unrefrigerated conditions (Priestley 1986, Roos and Davidson 1992, Roos et al. 1996, Christensen et al. 1998, germination results from NSSL available upon request to www.ars-grin.gov/nssl). Species from Malvaceae, Onagraceae, Polygonaceae, and Leguminosae tend to produce long-lived seeds while species from Compositae, Papaveraceae, and Liliaceae tend to produce short-lived seeds. In some of the surveys, seeds were buried in soil and it is unclear whether these longevity results can be applied to longevity of seeds stored in *ex situ* seedbanks.

Surveys of seed longevity have limited value for genebank curators who must ensure that each accession maintains high viability throughout storage. Usually there is tremendous variability in longevity among cultivars of a species and among harvest years of the same cultivar, suggesting that there are genetic and environmental components controlling longevity (Walters 1998). The variability is likely to be even greater for seeds collected from wild populations, as we also expect variability among individuals in the population. Current research suggests that immature seeds, common in harvests from wild populations, age more rapidly that their fully mature counterparts. Some of the seed quality can be restored if immature seeds are held in their pods for several days (Walters, unpublished). Aside from evaluating the maturity status of the collected material there are few tools to predict whether a particular accession will have good or poor keeping quality relative to the general performance of the species. Until those tools are available, the viability of stored seeds should be periodically monitored using germination tests.

Recalcitrant Seeds

Even when mature, some seed species have very short storage lives. Many of these seeds do not survive the extreme

drying that is required to stop metabolism and so the procedures described above to prolong storage life for "orthodox" seeds are not applicable. These seeds are called "recalcitrant." Embryogenesis is similar in orthodox and recalcitrant seeds until post-abscission when, within a few days, cell organelles dedifferentiate and protective compounds accumulate in the cytoplasm of orthodox embryos (Vertucci and Farrant 1995). These processes occur to only a limited extent in maturing recalcitrant embryos.

Recalcitrance is often found in species from tropical rainforests or aquatic areas, although seeds from several temperate tree species also exhibit the behavior (e.g. *Quercus* spp.)

Species producing recalcitrant seeds are found in widely divergent taxa (VonTeichman and vanWyk 1994), but also among congeners with orthodox behavior (Hong and Ellis 1995) (e.g. *Acer* spp.). Several endangered species indigenous to the US produce recalcitrant seeds (e.g. *Zizania texan*a and *Howellia aquatilis*), but, in general, very little is known about the physiologies of seeds from rare species. In a survey of seeds from over 200 rare or threatened Hawaiian species, more than 85% were considered non-recalcitrant (Walters, Crane, Hill, unpublished).

Recalcitrant seeds are identified by whether they survive drying. Drying should be rapid (over a few days), as slow drying allows metabolic imbalances that damage cells (Walters and Farrant 1995). The level of recalcitrance can be determined by evaluating survival after seeds have been equilibrated to various RH. Seeds that do not survive drying to < 75% RH are fully recalcitrant and to < 5% RH are likely to be intermediate between recalcitrant and orthodox. Seeds with intermediate behaviors (e.g. coffee, citrus, papaya) can usually dry to very low water contents, but they deteriorate rapidly if equilibrated at RH ≥ 30%. Measuring survival as a function of water content rather than relative humidity led to incorrect classification of lemon (*Citrus limon*) seeds (they are intermediate (Walters and Crane, unpublished)) and Pacific yew (*Taxus brevifolia*) seeds (they are orthodox (Walters-Vertucci et al. 1996)).

Recalcitrant seeds can be preserved using vitrification procedures where the water content of embryonic axes are optimized to limit ice crystallization and then axes are cooled rapidly to limit ice crystal growth (Roos et al. 1996)

Other Propagules

With proper sampling technique, a collection of seeds from a wild population of plants can adequately represent the genetic composition of that population. Sometimes it is not possible to harvest the proper amount of seed (seed production or viability is low) or cryopreservation of seeds is exceptionally difficult (some recalcitrant seeds). In these cases, genebank curators may wish to clone individuals from a population. Procedures for *in vitro* culture of apical shoot tips and cryopreservation by vitrification have been developed for a number of accessions (Dixon 1994, Roos et al. 1996), but survival varies markedly among clones. For some woody species, dormant vegetative buds can be cryopreserved and then grafted to root stock (Dixon 1994, Roos et al. 1996). Preservation of vegetatively propagated materials offers distinct advantages for conserving the genetic diversity of species with few individuals (Dixon 1994).

In conjunction with other conservation strategies, preservation of pollen may also help to conserve the genetic diversity in wild populations, especially of trees or shrubs. Like seeds, different species of pollen may have orthodox or recalcitrant storage behaviors. Storage protocols for pollens vary depending on their physiology, but are essentially the same as those outlined for seeds (e.g. Roos et al. 1996).

Conclusions

The biodiversity of threatened populations can be conserved using a combination of *in situ* and *ex situ* reserves. With appropriate sampling, genetic variability of plant populations can be preserved in living but quiescent collections for decades and maybe centuries. A variety of propagules can be used and the techniques for proper handling are mostly known. Maintaining quiescent collections requires little labor and few resources compared to other conservation strategies.

Literature Cited

Brown, A.H.D. and J.D. Briggs. 1991. Sampling strategies for genetic

variation in *ex situ* collections of endangered plant species.. In Genetics and Conservation of Rare Plants. Pp99-119. D.A. Falk and K. E. Holsinger, eds. Oxford University Press, New York.

Buitink, J., M.M.A.E. Claessens, M.A. Hemminga, and F.A. Hoekstra. 1998. Influence of water content and temperature on molecular mobility and intracellular glasses in seeds an pollen. Plant Physiology 118: 531-541.

Christensen, T.L., E.E. Roos, D.A. Davidson, and R.J. Reinsvold. 1998. Germination of seed after fifty years in vacuum storage. American Journal of Botany 85: 87-88.

Ellis, R.H. and E.H. Roberts. 1980. Improved equations for the prediction of seed longevity. Annals of Botany 45: 13-30.

Dixon, K. W. 1994. Towards integrated conservation of Australian endangered plants - the Western Australian model. Biodiversity and Conservation 3: 148-159.

Guerrant, E.O. 1992. Genetic and demographic considerations in the sampling and reintroduction of rare plants. In Conservation Biology: The Theory and Practice of Nature Conservation, Preservation and Management. Pp321-344. P.L. Fiedler and S.K. Jain, eds. Chapman and Hall,

Hong T.D. and R.H. Ellis. 1995. Interspecific variation in seed storage behaviour within two genera Coffea and Citrus. Seed Science & Technology 23: 165-181.

IBPGR. 1985. International Board for Plant Genetic Resources Advisory Committee on Seed Storage: Report on the Third Meeting, Rome.

Justice, O.L. and L.N. Bass. 1978. Principles and Practices of Seed Storage. Agriculture Handbook No. 506. US Government Printing Office, Washington, D.C.

Priestley, D.A. 1986. Seed Aging. Comstock Publishing Associates, Ithaca, NY.

Roos, E.E. and D.A. Davidson. 1992. Record longevities of vegetable seeds in storage. HortScience 27: 393-396.

Roos, E.E., L.E. Towill, C.T. Walters, S.A. Blackman and P.C. Stanwood. 1996. Preservation techniques for extending the longevity of plant tissues. In Sampling the Green World: Innovative Concepts of Collection, Preservation, and Storage of Plant Diversity. Pp. 157-204. T.F. Stuessy and S.H. Sohmer. Columbia Univ Press, New York.

Vertucci, C.W. and J.M. Farrant. 1995. Acquisition and loss of desiccation tolerance. In Seed Development and Germination. Pp237-271. J. Kigel, J. and G. Galili, ed. Marcel Dekker, NY.

Von Teichman, I. and van Wyk, A.E. 1994. Structural aspects and trends in the evolution of recalcitrant seed in dicotyledons. Seed Science Research 4: 225-239.

Walters, C. 1998. Understanding the mechanisms and kinetics of seed aging. Seed Science Research 8, 223-244.

Walters, C. and J. Engels. 1998. The effects of storing seeds under extremely dry conditions. Seed Science Research 8 (suppl): 3-8.

Walters, C., E.E. Roos, D.H. Touchell, P.C. Stanwood, L.Towill, L. Wiesner, and S.A. Eberhart. 1998. Refrigeration can save seeds economically. Nature 395: 758.

Walters-Vertucci, C. J. Crane and N.C. Vance. 1996. Physiological aspects of *Taxus brevifolia* seeds in relation to seed storage characteristics. Physiologia Plantarum 98: 1-12.