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Technologies from Agriculture to Help “Noah” Save Plants

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The agricultural context

A nexus of food, water, and energy security is rapidly approaching. More than one third of Earth’s land (1.6 billion hectares) is under cultivation and more than 70% of its fresh water is used for agriculture (FAO, 2011). Land use for cultivation ranges among countries from over 80% (i.e, Uruguay, Saudi Arabia, and Kazakhstan) to less than 4% (i.e., mostly island nations, but also Greenland, Norway, and Egypt) (Worldbank, 2019). FAO (2011) reports large disparities among countries in terms of sustainable agricultural practices, resulting in 25% of the world’s land being highly degraded and no longer productive. A warmer, wetter planet, as many are predicting, may mean longer growing season and higher agricultural productivity; but these conditions also promise changes to the virulence of crop diseases and pests in complex and unanticipated ways (Velásquez et al., 2018), further threatening food security. Agriculture is surely part of the problem, and must be part of the solution.

The encouraging news is that agriculture is rapidly changing in an effort to ease human suffering as well as impact on the environment. The ‘Green Revolution’ philosophy, begun in the 1960s, focuses on technological advancement in agriculture to increase crop productivity by integrating sciences on genetic resources, fertilizer, and pesticides. As a result, the amount of land needed to support a person is decreasing – from 0.45 hectares/yr in 1961 to 0.22 hectares/yr in 2006 (FAO, 2011). This remarkable statistic arises from increased yields – an average of 42 kg/hectare/yr for cereals worldwide (Figure 15.1), with many of the more industrialized countries enjoying double that yearly increase (Worldbank, 2019). As a result, more lands are going into production in some countries to increase food independence while other countries are taking land out of production (Figure 15.2). These shifts in land use create opportunity.

Many, if not all, the yield advancements since the 1960s (Figure 15.1) can be attributed to genetic resources of crop species. “Noah” and the speakers at the conference on species protection at the Pontifical Academy of Sciences (May 13-14) focused on ‘conservation targets’ at the species or taxonomic level (*sensu* Soulé, 1991). However, in agriculture, our conservation focus is at the population or even individual genotype level, usually to collect genetic resources that provide nature’s solutions to agricultural problems such as yield, disease resistance, drought tolerance, flavor and a host of other problems that can crop up. Hence, agriculturally-based genebanks are large in terms of number of accessions (accessions are the unique elements that comprise collections, often it is a bag of about 3,000 seed with unique identifying information), but usually small in terms of number of species included. For example, USDA’s collection is called the National Plant Germplasm System (NPGS) and currently includes about 600,000 unique accessions from about 16,000 species.[2] The strategy is to ensure that we have captured the genetic diversity of crops so that rare genes controlling important traits are available. For example, genes for resistance to Russian wheat aphid (*Diuraphis noxia*) were found within NPGS’s collection of about 55,000 unique accessions of wheat or its wild relative *Aegilops*. Plant breeders found eleven accessions with resistance to this pest, collected from the former Soviet Union and Tajikistan, where *Diuraphis noxia* originates and landraces of *Triticum aestivum* exist (Byrne et al., 2018).

The idea of collecting and preserving genetic resources ex situ for the purposes of crop enhancement is credited to the Russian botanist, Nikolai Vavilov (1887-1943), who introduced the concept of ‘Centers of Diversity’ for agronomic species, linking diversity, domestication and early human civilizations (Vavilov, 1987). Vavilov’s ideas on genetics and inheritance were considered subversive in Stalinist Russia, and so he was imprisoned and died of starvation – ironically, since his research was dedicated to feeding the world’s people.

Plants were regularly introduced to the “New World” by immigrants. In the US, formal Plant Introductions (PI) began after the Civil War when USDA was formed. The first official Plant Introduction (PI #1) is a cabbage from Siberia collected in 1898. Efforts to catalog, preserve and regenerate seeds of Plant Introductions – rather than letting them die in uncontrolled storage environments – began after World War II. This period also marks the beginning of modern-day cryobiology because of the chance discovery that spermatozoa treated with glycerol survived exposure to liquid nitrogen (Polge et al., 1949). Seeds from crops do not require additions of glycerol, or other cryoprotectants, to survive genebanking conditions. They have the remarkable capacity to survive drying, and so, unlike most biological organs and tissues, seeds can be placed in a regular freezer (-20oC)

and avoid lethal ice formation. The simplicity of the storage technology made genebanking seeds accessible to any group with reliable refrigeration. USDA's National Seed Storage Laboratory (now NLGRP) was established in 1958. Since then, genebanks storing seeds have proliferated from a handful in the 1970s to about 1750 in 2012, serving agriculture, conservation, and studies of ecology, evolution, and diversity (Hay and Probert, 2013; FAO, 2014).

We could consider plant genebanks as 'arks,' human constructs to protect plant species (or populations) from catastrophe such as a metaphorical flood. Plant genebanks that spin a 'doomsday' scenario get good publicity and public accolades. For agriculture, doomsday would be an admission of failure to produce enough food in spite of constant pressure from pathogens, pests, inclement weather, and degraded soils. So, I do not view genebanks as arks that hunker down and escape tough times. Rather, genebanks are the exact tools needed to get us through tough times, every day. They are working libraries, sharing knowledge about our biological world and providing insight about diversity, how to respect and sustainably use diversity, and consequences for humanity if we do not. In my opinion, the biggest challenge to us (and Noah) is not building an ark or loading it up. It is the 'exit plan,' that is, ensuring that the collected materials eventually get off the ark.

Loading up the Ark – What do we choose to collect and curate?

China's recent experiment to sprout seeds on the moon[3] suggests that the ark concept need not be restricted to Earth. From artists' renderings, we might envision Noah's ark to be a collection of reproductive individuals that need constant care and sustenance to ensure proliferation. These "living" collections (*sensu* Soulé, 1991) require large spaces and significant human investment in husbandry. The amount of diversity that can be concentrated is directly related to the volume occupied by individuals in combination with the amount of resources required to maintain each individual.

For plants, a living collection may be an orchard or botanical garden that grows a subset of individuals from a species or population. This is critical work to understand the growth habits and characteristics of the plant. A scientific collection is useless without these data. However, living collections are at risk from inclement weather, pests, pathogens, social unrest, and aging individuals that eventually become post-reproductive. Genetic erosion through drift, inadvertent selection, or introgression with neighboring related plants can also occur while growing or regenerating a sample. Living collections are also required to regenerate the sample, but regeneration can be expensive especially for large plants that may take years to sexually mature. The approach requires high investment in labor and land, and the return is a living specimen which is an exemplar of the species. If the goal is to keep the last remaining individuals of a species alive, this strategy buys some time.

An alternative to living collections are 'quiescent' collections that hold germplasm from organisms in a state of suspended animation. Germplasm is a small part of the organism (perhaps a single cell in the case of sperm or pollen) that carries genetic information or that can be grown into another individual. A quiescent collection exchanges the benefit of viewing a living, growing specimen for the benefit of capturing greater diversity in a compact space. Currently, NLGRP stores its collection of nearly 750,000 accessions of 3,000 seeds each in a 90x30x3 m space, essentially allowing about 3 million individuals per m3. These individuals must be stored so that viability is maintained, but they cannot be allowed to grow (discussed below). Selecting germplasm (i.e., propagules) for quiescent collections in a plant genebank requires optimization of survival to preservation stresses, processing time and cost per storage volume. Costs of processing and storage should figure significantly into the genebank's business model to determine the volume of material that can be managed effectively.

Fortunately, plants are fairly plastic in their reproductive behavior and plant genebanks have options on the propagule that embodies a pre-defined conservation target. For plants, conservation targets can be at several biological scales such as a population, an individual (or genotype), a trait, or even a particular allele (gene variant). The propagules that house the desired feature of diversity must be amenable to storage in a quiescent collection (Table 15.1). Seeds are the most commonly used propagule for plant genebanks. Usually compact, plentiful, storable, growable, and representative of maternal and pollen-donor lines, seeds might just be the ideal medium for plant genebanking. Most seeds have innate abilities to survive extreme drying and low temperature without adding cryoprotectants (Walters, 2015).

Table 15.1 Some common propagules used in plant genebanks.

propaguleadvantagesdisadvantagesexceptionsSeeds: conservation target at population and/gene level· compact

· high fecundity of some plants make it possible to collect many individuals

· highly developed, low-cost, storage technology for orthodox seeds

· efficient for propagation & regeneration & distribution

· represents progeny of extant population (can capture many genotypes and many genes)

· may present barrier to some diseases

· demonstrated ability to efficiently capture diversity

· heterogeneous traits in wild populations; multiple harvest times needed and timing can be unpredictable

· asynchronous germination can lead to poor stand establishment and drift

· long time to sexual maturity in perennials

· potentially unknown pollen source

· mating systems may preclude maintaining desired maternal traits

· non-orthodox seeds require cryogenic storage

· possible low seed production in wild due to reproductive failure (endangered species), drought, late frost, non-mast year, herbivory

Pollen: conservation target at gene level

· very compact

· available for immediate use in breeding programs

· available during flowering

· amenable to storage

· captures diverse genes

· maybe the fastest, least labor-intensive way to achieve some form of back-up

· a gamete, not an individual

· ephemeral

· difficult to harvest

· must make crosses to regenerate populations

· must be genebanked immediately after collection (short processing timeline)

shoot tips: conservation target at individual level

· compact

· captures specific genotype; OK as an exemplar of species

· amenable to in vitro culture

· preservation technologies rapidly developing

· clonal propagation reduces concern about genetic drift

· requires large amounts of quality source materials at correct phenological stage

· unexplained variation in response to growth medium among genotypes

· unexplained variation in response to growth medium among genotypes

· processing and growth is labor-intensive

· many individuals needed to capture diversity of a heterogeneous population

dormant buds or overwintering vegetative structures: conservation target at individual level

· compact

· captures specific genotype; OK as an exemplar of species

· does not require in vitro culture (less labor than shoot tips)

· preservation technologies are advancing

· clonal propagation reduces concern about genetic drift

· recovered by grafting

· plants must be winter-adapted and in acclimated state

· recovered by grafting

· many individuals needed to capture diversity of a heterogeneous population

· variable responses within and among species result from complex bud structuressomatic embryos and cell cultures: conservation target at individual level

· compact

· captures specific genotype;

· may be more amenable to preservation than non-orthodox seed

· can generate huge numbers of individuals

· successful propagation is highly genotype-specific; tends to narrow captured diversity

· high risk of soma-clonal variation

· labor intensive for establishing and processing

Source: Walters et al. (2018)*Note*: See also Havens et al. (2004) for complementary information.

Pollen is under-appreciated as a germplasm form in plants, which contrasts with animal genebanks in which semen, the counterpart to pollen, is the most commonly used germplasm form (Mazur et al., 2008). Pollen might be an effective alternative germplasm form that can capture genes of interest and deliver them to a breeding population when seeds are unavailable or have poor storage characteristics or when maintaining cuttings is cost-prohibitive. For example, pollen from oak trees is desiccation tolerant, while oak seeds tend to be recalcitrant (Franchi et al., 2011; Walters et al., 2013). Pollen is storable, but it lacks the longevity traits exhibited in seeds of the most common agronomic species (Dafni and Firmage, 2000).

Plant genebanks frequently distinguish between propagules that are sexually-derived (i.e., seeds and pollen) and those that arise from vegetative cuttings (i.e., clonally propagated). In agriculture, this distinction usually occurs because the conservation target is a specific genotype and the plant is highly heterozygous and outcrossing. For example, a genetically identical potato plant (a clone) can be regenerated from the “eye” of a potato. Clonal propagation may be necessary for plants of conservation concern if there is reproductive failure in the wild (e.g., inbreeding, no pollinators) or if population sizes are inviable (Pence, 2013). Cloning the few remaining individuals to increase demographics (but not necessarily genetic diversity) has led to successful reintroductions.

There will always be plants that appear intractable to genebanking until research finds a way. A whole class of seeds, described as ‘recalcitrant,’ were deemed impossible to store in the 1980s (Walters et al., 2013); but methods are developing to allow routine storage, albeit in liquid nitrogen. The most difficult materials for NLGRP are currently avocado (*Persea americana*) because it appears resistant to an in vitro recovery system and sugarcane (*Saccharum officinarum*) because it is riddled with endophytes and appears to lose totipotency with age of culture stock. Eliminating roadblocks for avocado is urgent as the species, like all members within Lauraceae in North and Central America, is threatened by the fungal pathogen *Raffaelea lauricola,* responsible for laurel wilt disease that is spread by the redbay ambrosia beetle (*Xyleborus glabratus*) (Kendra et al., 2013). These stories exemplify the shifting priorities of loading up an ark: save what is feasible; what is most vulnerable; what we value most?

Life on the Ark – Repository biology to aid ex situ conservation

Because of its early interests in seed biology, agriculture has made large contributions to the technological know-how for *ex situ* banking of plant genetic resources. “Orthodox” seeds, by virtue of their innate ability to survive drying, naturally achieve a state of suspended animation in which they are alive, but do not appear to be living – at least by most of our criteria of what living systems do: i.e., metabolize, grow and respond to the environment. The transition from living and growing to quiescent in seeds is associated with the change in their cells from water-based and fluid to dry and solid (Walters et al., 2010). During embryogenesis, seeds pack their cells with food reserves to provide the foundation and reinforcement for structure while concomitantly removing water.

The stabilization achieved by solidifying cytoplasm is perhaps more intuitively understood by looking at the technologies used to make plastics, stabilize dry foods and ensure that the drugs stored in our medicine cabinets deliver constant dosages up to the expiration date. These types of solids are often referred to as 'glasses,' in which the molecular organization is irregular. In the other type of solid, which we learned about in grade school, molecules are organized in a regular pattern to form a crystal, e.g., when liquid water freezes and turns to ice. The irregular molecular structure can form rather discreetly, with no discrete change in molecular structure; hence it can be quite survivable as long as the mechanical shock of shrinkage as cells lose water is avoided (Walters, 2015). Once formed, the solid can be further stabilized by lowering the temperature. The absence of water and slowed molecular motion within a solid makes lethal ice highly unlikely in orthodox seeds, and so freezer storage (also called "conventional" storage) is a standard approach to prolong viability cost effectively.

Vegetative propagules and some non-orthodox seeds (unfairly referred to as "recalcitrant") do not survive cell shrinkage during the drying process needed to solidify cytoplasm at room temperature (Table 15.1). Hence, we must engineer other methods to vitrify the cytoplasm while maintaining cell viability. Cryogenic storage for plant germplasm became accepted in the mid-1980s and routine in the mid-1990s. Successful cryopreservation involves optimization of interacting factors such as moisture, cryoprotectants and exposure rates to and from liquid nitrogen temperatures (Walters et al., 2013). There are still many species for which preservation protocols do not currently exist. This is not because we do not understand the basic principles of preservation. Rather it points out that we cannot expect diverse materials to respond to standardized treatments the same way – there is always some 'tweaking' that has to be done to achieve initial survival. With time and sufficient materials to experiment with, workable methods are available for an increasingly huge array of plant propagules to facilitate preservation of plant diversity *ex situ*. The issue is whether the current pace, set by the number of scientists working on the problem, is sufficient to meet the urgent need as water rises around the ark.

Time slows down in preserved cytoplasm, but it does not stop. The irregular structure in solidifying cytoplasm, that saved the cells initially, allows some movement to occur. As the molecules move, the cytoplasm ages. So, we must not be lulled into a false sense of security when germplasm initially survives our treatments. For most materials, survival times are long (at least 50 years), but we are observing faster than expected aging in some germplasm, such as fern spores and pollen, even at liquid nitrogen temperatures (Ballesteros et al., 2018).

The aging of quiescent germplasm during storage can seem counter-intuitive, but research in a number of apparently-unrelated disciplines is elucidating the mechanisms of change in non-crystalline solids (such as solidified cytoplasm) that eventually cause loss of function. Everyday examples include yellowing of paper, brittleness of rubber and plastics, and lost flavor in dried foods past the expiration date. For preserved germplasm, lost function usually equates to lost viability, and this occurs abruptly and without warning during storage. This is partly because only viability assays are currently available and we need to revive the germplasm (because it is quiescent) to detect aliveness. However, the inevitability that quiescent germplasm ages embodies the profound reality that chemical and physical reactions are constant degradative forces on organic matter, bringing truth to precepts that what is alive eventually returns to dust (Genesis 3:19).

It is hard to predict when the alive-dead discontinuity will occur and the constant testing for viability in quiescent germplasm consumes materials. Yet without knowing when germplasm succumbs in storage, we will not know when to use it or to regenerate it. All the effort of preserving germplasm *ex situ* will be for naught if it dies in the genebank. Therefore, we have sought to understand aging and to develop tools that indicate progress before mortality. At writing, our most successful assay monitors integrity of RNA, a class of molecules that are intermediaries between DNA (genes) and proteins (cell machinery) (Fleming et al., 2018). Based on this work, and other assays that inform about structural or biochemical changes within the solidified cytoplasm, we envision aging of preserved germplasm as a straw-that-broke-the-camel's-back process, with many small random reactions that damage any molecule within the cell, culminating in a major effect.

The increasing number of anecdotal accounts that seeds collected from the wild are harder to store are not surprising (Hay and Probert, 2013; Walters, 2015; Ballesteros and Pence, 2017). We know that plant embryo development is critical to longevity and metabolic pathways expressed during embryogenesis are key (Righetti et al., 2015; Walters, 2015). Seed quality is dependent on processes that are uncontrolled in the wild during the growing season, such as moisture availability, nutrition, competition and pathogens, and it will decline if developmental programs are not completed or extended towards germination. Phenology, fecundity, carbon partitioning, composition, seed coverings, resistance to pests and drought tolerance are all inherited traits that affect seed longevity. These traits are more uniform in domesticated plants, but vary considerably in seeds from natural populations; hence, an accession of seeds collected from the wild will be heterogeneous and this will result in differences in how individual seeds within the sample respond to genebanking conditions. One of

genbanking's major challenges is preventing domestication in wild germplasm placed under highly controlled conditions. Genebanks must preserve the wildness so that the species can eventually leave the ark.

Exiting the Ark – Benefits from plant germplasm collections

The proliferation of plant germplasm banks around the world (to more than 1700 in 2019) attests to human confidence that our ingenuity and respect for natural diversity can forestall its attrition in the face of uncertainty about the future and increasing human pressure on habitats. Moreover, this investment conveys the understanding that human fate is inextricably connected to the fate of species that also share the Earth.

Technologies to preserve diversity *ex situ* are becoming increasingly sophisticated, but they lack purpose if there is no plan beyond stockpiling germplasm. Hence, an 'exit plan' is essential to realize the benefits of investing in genebanking. Such a plan can be fraught with ethical and moral dilemmas. For example, a question during the conference in response to this paper's technology update focused on countries' ownership of genetic resources used in agriculture. Additionally, conservation groups worry about re-introducing a plant once its habitat is lost. The emerging technologies cannot address these, and many other issues, but they can 'buy time' needed for discussion. Genebanking technologies provide an available and practical strategy to temporarily forestall the rapid loss of plant biodiversity on Earth.

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END NOTES

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[3] https://www.the-scientist.com/news-opinion/china-is-growing-cotton-on-the-moon-65321