Dying while Dry: Kinetics and Mechanisms of Deterioration in Desiccated Organisms

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SYNOPSIS. Persistence of anhydrous organisms in nature may depend on how long they remain viable in dry environments. Longevity is determined by interactions of humidity, temperature, and unknown cellular factors that affect the propensity for damaging reactions. Here we describe our research to elucidate those cellular factors and to ultimately predict how long a population can survive under extreme conditions. Loss of viability typically follows a sigmoidal pattern, where a period of small changes precedes a cataclysmic decline. The time for viability to decrease to 50% (P50) varied among seed species and among 10 phylogenetically diverse organisms. When stored at elevated temperatures of 35°C and 32% relative humidity (RH), P50 ranged from about a week for spores of Serratia marcescens to several years for fronds of Selaginella lepidophylla. Most of the species studied survived longest at low humidity (10–20% RH), but suffered under complete dryness. Temperature dependencies of aging kinetics appeared similar among diverse organisms despite the disparate longevities. The effect of temperature on seed aging rates was consistent with the temperature dependency of molecular mobility of aqueous glasses, with both showing a reduction by several orders of magnitude when seeds were cooled from 60°C to 0°C. Longevity is an inherited trait in seeds, but its complex expression among widely divergent taxa suggests that it developed through multiple pathways.

REVIEW OF SEED LONGEVITY IN A GENEBANK

Most plants from temperate and subtropical climates produce seeds that acquire tolerance to dehydration as they mature (Vertucci and Farrant, 1995). This innate ability is the most important factor allowing humans to preserve genetic resources of wild and agricultural plants in genebanks. The USDA National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO, USA is home to over 460,000 accessions of plant and animal germplasm representing over 5,000 species. About 95% of the collection is stored as dried seeds and about one third of these have been in storage for over 30 years. The dried condition of the seed enhances its shelf-life and reduces its susceptibility to lethal ice formation, and so seeds are now routinely cryogenic stored at −18°C until 1978 and at −18°C since then. Under these storage condi-

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tions, P50s of seeds of onion, lettuce, peanut, red fescue and other species were less than 25 years, typifying species producing short-lived seeds. Species bearing seeds with medium longevities have average P50s ranging from 30 to 70 years and include poppy, sunflower, most cereal grains and eggplant (Walters et al., 2005). Values of P50 are projected to range from 80 to 500 years for species bearing seeds with exceptional longevities, such as okra, chickpea, tomato and spinach. Long-lived seeds show only minor changes in viability even after >60 years of storage at PI stations and NCGRP (Walters et al., 2005). Seeds of species from Apiaceae and Brassicaceae tended to be short-lived while those from Malvaceae and Chenopodiaceae tended to be long-lived (Walters et al., 2005). We saw no correlation between characteristic P50 values among species and the soluble carbohydrate content, sucrose content or oligosaccharide content of the seed (Walters et al., 2005), suggesting that these compounds play a minor (if any) role in the long-term stability of the seeds (as was suggested by Horbowicz and Obendorf, 1994; Leopold et al., 1994; see also Butink et al., 2000b and Butink and Leprince, 2004 who found that oligosaccharides did not affect intracellular mobility in seeds).

The difficulty of measuring sufficient losses in viability over a 30 to 40 year period in seeds stored at 5 and −18°C might suggest that detecting changes in dry seeds stored cryogenically for 20 to 30 years would be an impossible task. NCGRP’s cryogenic program, initiated in 1978, was based on the implicit assumption that the ultra-low temperatures would virtually stop the chemical and physical reactions that cause aging (Stanwood and Bass, 1978). Contrary to predictions, NCGRP monitoring data show that decreases in viability in cryogenically stored seeds can be detected within 10 to 25 years for 15 of 42 species of seeds that were placed in liquid nitrogen (−196°C) (Walters et al., 2004). Deterioration was progressive with time, demonstrating that the loss of viability resulted from aging stresses rather than initial exposure. For most plant species, the P50 of the seeds will likely increase if seeds are placed under cryogenic storage. For example, lettuce seeds, which typically have poor storage stability, have projected P50 values of 524 and >3,000 years if stored at −135 (LN vapor) and −196°C (LN), respectively; but may have a P50 value near 150 years if stored continuously at −18°C (Walters et al., 2004). For unknown reasons, aging of some species (e.g., beet, parsley, soybean and corn) sharing the same provenance progressed faster under cryogenic storage compared to conventional storage at −18°C (Walters, unpublished).

Current models of deterioration in dry biological materials do not account for the variability observed within and among seed species or the disappointing and counter-intuitive observation that cryogenic storage does not maintain viability of seeds indefinitely. The profound and unavoidable conclusion of our genebanking experiences is that deterioration continues with time, even under extremely dry and extremely cold and dry conditions. Therefore, our research objective is to understand the biology that occurs under these extreme conditions with the intention of improving genebanking efficiency with tools that predict how long an accession can survive in storage.

**Life in the Dry State**

Metabolism in dry cells is hardly detectable and is unlike cellular processes that occur in hydrated cells. In dry but viable cells, hydrated metabolism can be resuscitated, usually by simply adding water. Organisms that survive desiccation provide model systems to study how water effects chemical and physical change in cells. The most renown of these types of studies defined three levels of physiological activity in cysts of the common brine shrimp *Artemia salina* (Clegg, 1986). In similar studies using seeds, we defined five hydration levels corresponding to critical water potentials that are fairly similar among diverse plant species (Fig. 1). Fully hydrated seeds (ψ ≅ −1 MPa; Hydration Level V) can grow. Stress responses are detectable at lower hydration levels, initially as the expression of stress-related proteins and nucleic acid repair pathways (−1 ≥ ψ ≥ −3 MPa; Hydration Level IV), and then as the rampant production of free radicals and the loss of membrane integrity (−5 ≥ ψ ≥ −12 MPa; Hydration Level III). At water potentials below about −15 MPa, cells have officially entered the dry state (Hydration Level II), and most (if not all) reactions contribute to the demise of the seed over time. The rate that dry seeds age depends on their water content. Longevity first increases with dehydration (Hydration Level II) and, at some critical point, decreases with further drying (Hydration Level I). At the critical point (ψ ≅ −200 MPa, RH ≤ 22%), a change in volatile composition emitted from seeds indicates a switch in the nature of degrading reactions (Walters, 1998; Walters unpublished). The moisture level that distinguishes the two hydration levels is important because it defines the maximum longevity of the organism at that temperature.

The regulation of metabolism and degrading reactions by water has been studied in a number of organisms that tolerate and succumb to water loss (Etzler and Drost-Hansen, 1980; Leopold, 1986; Vertucci and Farrant, 1995; Crowe et al., 1997; Walters, 1998; Pammeter and Berjak, 1999; Walters et al., 2002, and ref therein). Earlier studies that pointed to “bound water” were supplanted in the 1990s by work on glass formation in aqueous systems (e.g., Burke, 1986; Koster, 1991; Leopold et al., 1994; Slade and Levine, 1991), but the unifying theme is the interactions between water and non-aqueous constituents that force changes in the properties of each (Buera et al., 2005). These interactions can cause shifts in equilibrium, making different reactions more or less likely; or they can affect molecular mobility, increasing or decreasing the rate at which equilibrium is achieved, but not affecting the conditions that define equilibrium (Walters, 2005). The
Physiological Activities at Different Levels of Seed Hydration

<table>
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<tr>
<th>Hydration Level</th>
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<th>II</th>
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<th>IV</th>
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<td></td>
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<td>catabolic activity via enzymes</td>
<td>respiration</td>
<td>osmotic excursions</td>
<td>turgor</td>
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<td>DRY AFTER-RIpening</td>
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<td>nucleic acid repair</td>
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<td>cell division</td>
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<td>‘ACCELERATED’ AGING</td>
<td>induced desiccation tolerance (if possible)</td>
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<td>Maillard Reactions</td>
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<td>GERMINATION</td>
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<td>enzyme degradation</td>
<td>membrane demixing &amp; bilayer transitions</td>
<td>“permanent wilting point” for vegetative cells</td>
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<td>recalcitrant seeds die</td>
<td>immature embryos &amp; seedlings die</td>
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<td>-1</td>
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<td>MPa</td>
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<td>10</td>
<td>50</td>
<td>90</td>
<td>99</td>
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Fig. 1. Hydration levels in seeds showing physiological activities observed as a function of the water potential of the seed. Data are summarized from Vertucci and Roos, 1990; Vertucci and Farrant, 1995; Walters, 1998, and unpublished data.

First effect of water loss, changing the driving force of reactions, is difficult to quantify in cells because the specific reactants, products, and protectants are not known; however, model studies using systems of defined composition have led to considerable insights (e.g., Karel, 1980; Labuza, 1980). The second effect of water loss, reducing the fluidity within cells, eventually leads to a solid that lacks crystalline structure, a so-called glass (e.g., Slade and Levine, 1991; Franks, 1982; Buitink and Leprince, 2004). The aqueous domain of seeds becomes glassy once water contents decline to about 0.10 g H₂O/(g dry mass – g lipid) (≈ 50% RH, assuming drying at ≤25°C) (Buitink et al., 1996; Walters, 1998; Buitink and Leprince, 2004). Hence, seeds that are stored under genebanking conditions are glassy, and yet aging reactions proceed (Walters et al., 2005) despite extreme restrictions in molecular mobility.

The properties defining glasses are also affected by temperature and the established theory is relevant to studies of the effect of water content on glass behavior. Most classically, temperature effects on kinetics are described by Boltzmann distributions of molecular energy, which is graphically represented using Arrhenius plots. The effects of temperature on molecular mobility within a glass are most generally described by the Vogel-Tamman-Fulcher (VTF) model, where viscosity increases in a double exponential relationship with decreasing temperature (Angell, 1991):

\[
\frac{\tau}{\tau_w} = \exp \left(\frac{DT_0}{T - T_0}\right) \quad \text{(Eq. 2)}
\]

and \(\tau\) and \(\tau_w\) represent viscosity at temperature \(T\) and infinite viscosity achieved at \(T_w\). The coefficient \(D\) is specific to the glass and is assumed constant with temperature. An Arrhenius plot of the VTF model shows viscosity tends toward infinity with small changes in temperature below the glass transition temperature (Tg). This tendency led to the common misconception, promulgated throughout the 1990s, that molecules essentially stop moving when a glass forms. The VTF
model is only valid for temperatures near \( T_g \), and an additional factor that accounts for structure that was fixed into the matrix when the glass formed (the fictive temperature, \( T_f \)) is included in the Adam-Gibbs (AG) model. The AG model is valid below \( T_g \) (Adam and Gibbs, 1965; Scherer, 1984; Andronis and Zografi, 1998; Shamblin et al., 1999):

\[
\frac{\tau}{\tau_0} = \exp \left[ \frac{D T_k}{T (1 - T_k/T_f)} \right] \quad \text{(Eq. 3)}
\]

where \( T_k \) is the Kauzmann temperature that defines the point at which the entropy of the supercooled liquid and crystal are equivalent (\( T_k \) is often considered equivalent to \( T_0 \)). The difference between the actual temperature of the glass (\( T_f \)) and \( T_f \) describes the residual mobility maintained by the glassy structure. If \( T_f \) equals \( T_k \), the AG model takes the form of the VTF model. If \( T_f \) is constant and \(< T_g \), the AG Model takes the form of the Arrhenius equation (i.e., temperature effects follow normal Boltzmann distributions). The terms “fragile” and “strong” were coined to describe the relative effects of temperature on molecular mobility and structure of glasses (Angell, 1991). Fragile glasses are affected by small changes in temperature (VTF behavior); strong glasses require larger temperature changes for comparable disruption of glassy structure (Arrhenius behavior).

Experimental measures of molecular mobility within biological glasses are technically difficult because of the long time spans required and the complexity of signals in biological materials. An experimentally accessible method to approximate molecular mobility makes use of the AG model and comparisons of heat capacity (\( C_p \) describes the change of entropy with temperature) (Andronis and Zografi, 1998; Shamblin et al., 1999; Walters, 2004). In seeds containing 0.07 g H\(_2\)O/g dry mass, \( T_g \approx 28^\circ \text{C} \), \( T_k \approx -40^\circ \text{C} \) and \( T_f \) changed with temperature near \( T_g \) and then remained constant at \( 17^\circ \text{C} \) with further reductions of temperature (Walters, 2004). Combining these constants with equilibrium relationships between RH, water content and temperature in seeds (Vertucci and Roos, 1993; Buitink et al., 1996; Walters, 1998), it can be suggested that glasses are fragile in seeds within Hydration Level II (Fig. 1) and strong in seeds within Hydration Level I.

The change in the temperature dependency of glass structure between Hydration Levels II and I may be related to observations that molecular mobility increases under severely dry conditions (Buitink et al., 2000a). Within Hydration Level II, molecular mobility decreased by 5 orders of magnitude in seeds when temperature was reduced from 60 to 0°C (Walters, 2004). In a similar temperature range, pea aging rates decreased by about 4 orders of magnitude. The strong effect of water content and temperature on seed longevity within Hydration Level II supports the idea that molecular mobility is an important factor controlling aging kinetics at this hydration level (i.e., Vertucci and Roos, 1990; Buitink et al., 2000a). The close correspondence between the effects of temperature on seed aging rate and molecular mobility appears to break down as temperatures decrease further and glasses become strong (Walters, 2004; Walters et al., 2004).

### Comparisons among Diverse Organisms

Broader insights of the biology that occurs under extreme conditions will be gleaned through comparisons of diverse organisms. Correlations of cellular constituents, and the environmental and genetic factors that regulate their production, will give insights on the key processes occurring in hydrated organisms that influence long term survival under extreme dry and cold. It is unknown whether the processes that permit desiccation tolerant organisms to survive the initial stress of dehydration also confer long term stability, or if a different suite of protecting mechanisms exists. A severe challenge when studying longevity at near optimum storage conditions is the timescale required to detect viability changes; however, establishing relationships between water content, RH, temperature and molecular mobility will allow us to extrapolate from simulated conditions. In this paper, comparisons are drawn from deterioration time courses measured using ten phylogenetically-diverse, desiccation-tolerant organisms. We believe that these data will be useful to future studies that address why and how organisms die under extreme conditions that preclude most chemical and biochemical reactions.

### Methods

Samples of desiccation tolerant organisms were obtained locally or purchased from seed companies or Carolina Biological Supply Company (Burlington, NC) between 1995 and 1998. All samples were received dry. Leaves of Myrothamnus flabellofolius were generously donated by Dr. Jill Farrant, Univ of Cape Town, Cape Town, So. Africa in 1997. We studied examples of plants (seeds of many species, pollen of Typha latifolia, spores of the fern, Polytrichum sp., and the moss, Woodwardia sp., and leaves of Myrothamnus flabellofolius and Selaginella lepidophylla), animals (cysts of Artemia salina), fungi (spores of Aspergillus niger and cells of Saccharomyces cerevisiae [baker’s yeast]) and bacteria (cells of Serratia marcescens). Samples were placed in RH chambers at 35°C and other temperatures ranging from \(-18\) to \(45^\circ\text{C}\) depending on availability (samples placed at \(-18^\circ\text{C}\) were held in RH chambers at \(25^\circ\text{C}\) for 2–3 weeks, then packaged in foil laminate bags). Relative humidity in RH chambers was controlled by different saturated salt solutions (Vertucci and Roos, 1993) and ranged from 0 to 90%.

Samples were tested for viability upon receipt and then during storage. Viability was evaluated as the proportion of seeds, spores or pollen grains that germinated, cysts that hatched or colonies that were produced when spores or cells were cultured under ap-
proportion of survivors ($N/N_0$ in Eq. 1) was expressed as the number of individuals that germinated, hatched, colonized or greened ($N$) divided by the value that was measured at the onset of the experiment ($N_0$). The time to decrease viability to 50% of the initial value (P50) and the time coefficient $\phi$ were calculated from Avrami coefficients.

Results and discussion

Samples from different species were viable at the beginning of the experiment and maintained a high proportion of viability after they had been dried almost completely (Fig. 2). The initial high rates of survival are a clear demonstration that tolerance to desiccation is a general feature of the organisms studied. Our data demonstrate that at least some bacteria and fungal cells survived the drying treatments; however, our measurements quantifying survival by counting the number of colonies produced do not reveal the proportion of cells from the original population that survived and hence the strength of selection for tolerance.

Viability decreased with time (Fig. 3). We detected sigmoidal decay patterns for several samples, indicating biphasic deterioration typical for freshly-harvested seeds. The rate of aging varied with RH and temperature of storage (Fig. 3A gives representative data for $S. cerevisae$) and among organisms (Fig. 3B gives data for $35^\circ C$ storage and 32% RH for representative species). Values for P50, calculated from time courses similar to those in Figure 3, show the range of longevity timescales (Fig. 4). The P50s presented in Figure 4 reflect aging kinetics at $35^\circ C$. At this elevated temperature, P50 ranged from ~6 years for Selaginella
The range of longevities measured from samples of different species that were stored at 35°C and 32% RH. Longevity is expressed as the time taken for viability of samples to decrease to 0.5 and was calculated using Avrami time courses given in Figure 3 and comparable time courses for samples not depicted in the figure.

Fig. 5. The effect of RH during storage at 35°C on the longevity of different samples. Values of P50 are calculated from time courses similar to those in Figure 3.

lepidophylla to 7 days for Serratia marcescens. Longevities of seeds from different species under these storage conditions ranged from about 6 years (cucumber) to about 2 years (peanut) (Walters, unpublished). Clearly, the long lifespans exhibited by some organisms presents an opportunity to identify innate factors and a challenge to measure their effects. The short time spans of Serratia marcescens, Aspergillus niger and Saccharomyces cerevisae must be viewed with some caution, as there is little way to know the extent of deterioration before we received the samples.

The RH at which samples were stored affected P50 values. An optimum RH between 10 and 20% was observed for most samples (representative data given in Fig 5 for storage at 35°C). A detrimental effect of overdrying has been reported previously for seeds and pollen and is believed to be associated with a change in water properties (Vertucci and Roos, 1990; Buitink et al., 1998; Walters, 1998). Leaves of Selaginella lepidophylla (Fig. 5A) exhibited a broad optimum between 8 to 43% RH and Aspergillus niger (Fig. 5D) exhibited no optimum at all, increasing in viability with progressive drying. The decrease in P50 as samples were stored below the critical RH implies a limit to the desiccation tolerance of most organisms. This limit is not exhibited during early exposure to dryness, but is manifested by prolonged exposure. In this context, one could define maximum desiccation tolerance as a cell or organism that survives forever under completely dry conditions.

The value of P50 was reduced 2 to 10 fold for all samples except A. niger by storing them under extremely dry conditions of about 1% RH or by storing samples at RH between 40 and 45%. A temperature–RH combination of 35°C and 40–45% RH is near (but below) the glass transition of seeds (Walters, 1998), which suggests that all the variation measured occurs for samples that are vitrified, assuming they have similar Tg characteristics as seeds. The different aging rates in response to RH may imply that drying within the glassy state affects molecular mobility differently in diverse organisms. Relative longevity remained constant among samples (ranking depicted in Fig. 4), despite differences in observed response to storage RH, mostly because the effects of RH were minor compared to the greater than two orders of magnitude variation in longevity among different organisms studied (compare ordinates among samples in Fig. 5). The basis of this variation is likely an intrinsic factor of the organism and identifying it will be a critical focus of future work.

The effect of temperature on aging kinetics is graphically depicted in Arrhenius plots of the Avrami time coefficient f (Eq. 1) (Fig. 6) for samples stored at 25–40% RH. We used comparable RH values rather than water contents to simplify comparisons among species, even though viscosity models (Eqs. 2, 3) compare temperature effects for samples with similar composition (Walters, 2004). Data for lettuce seed (Lactuca sativa) are given because the 30 year time courses that are available provide confidence in calculations of longevity at −18°C (Walters et al., 2004). Our treatment results in a series of near parallel lines with slopes rang-
The Arrhenius relationship reported in Figure 6 departs from linearity at about $-20^\circ$C in lettuce seeds (Walters et al., 2004). It is not known whether a similar break in behavior occurs in the other samples because the data are not currently available. Differences in Arrhenius behavior and the deviation temperature (if any) among species will allow us to infer information about the mechanisms of deterioration in these dried systems.

**CONCLUSIONS**

Desiccated organisms do not show many signs of life, but they are obviously alive if they can be resuscitated with water. The ability to survive while dry is temporary and eventually all organisms tested to date succumb with time. The time scale for these aging reactions varies with the conditions of storage (RH and temperature) and with the organism. The RH and temperature affect the intracellular molecular mobility, a factor that regulates reaction kinetics. It is likely, then, that molecular mobility may be the central thermodynamic parameter that enables aging. The reactants and protectants that contribute to the size of the driving force of aging reactions are not well known, but the broad range of longevity measured among organisms provide experimental systems to elucidate these factors.

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