



Variation of desiccation tolerance and longevity in fern spores



Daniel Ballesteros*, Lisa M. Hill, Christina Walters

USDA-ARS National Laboratory for Genetic Resources Preservation, 1111 South Mason Street, Fort Collins, CO 80521, USA

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ABSTRACT

This work contributes to the understanding of plant cell responses to extreme water stress when it is applied at different intensity and duration. Fern spores are used to explore survival at relative humidity (RH) < 85% because their unicellular nature eliminates complexities that may arise in multicellular organisms from slower drying and variable responses of different cell types. Fern spore cytoplasm solidifies between 30 and 60% RH and spores survive this transition, but subsequently lose viability. We characterized the kinetics of viability loss in terms of the fluid to solid transition using concepts of water activity (i.e., sorption) and glass transition (T_g), two concepts that dominate studies of food and pharmaceutical stability. For all fern species studied, longest survival times were observed in spores placed at about 10–25% RH and mortality rates increased sharply above and below this moisture level. A RH of 10–25% corresponds well to sorption behavior parameters and is below the glass transition, measured using differential scanning calorimetry. Though response to RH was similar among species, the kinetics of deterioration varied considerably among species and this implies differences in the structure or mobility of molecules within the solidified cytoplasm. Our work suggests that desiccation damage occurs in desiccation tolerant cells, and that it is expressed as a time-dependent response, otherwise known as aging.

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1. Introduction

The ability to survive extreme desiccation is an ancestral trait originally expressed in unicellular organisms and, later, in the first land plants (Dekkers et al., 2015; Gaff and Oliver, 2013). In vascular plants, this trait is usually only expressed in reproductive cells – spores of ferns and pollen and seeds of gymnosperms and angiosperms (Franchi et al., 2011; Gaff and Oliver, 2013; Hoekstra, 2005), though there are occasional examples of species producing desiccation-tolerant somatic or vegetative cells (Farrant and Moore, 2011; Gaff and Oliver, 2013). Once dried, organisms may survive for a few days or several millennia, depending on a host of known and unknown factors (e.g. Walters et al., 2005; see seed survival under increasingly dry conditions predicted by Ellis and Roberts (1980) viability models at <http://data.kew.org/sid/viability/percent1.jsp>). The increasing attention to desiccation tolerance and longevity in the plant literature attests to its importance in a range of contexts from persistence of weed seeds in the soil to

synthesis of bioproducts. Our lab's specific interest is an ex situ conservation mandate, for which we seek measures to sustain cell viability indefinitely through drying or cooling, and usually both. A major challenge for us is the ability to predict how much desiccation stress (sensu Levitt, 1980) can be tolerated by an organism and how long the organism can survive in the desiccated state. This challenge embodies a tacit paradox: we attempt to preserve viability using lethal stresses.

Considerable molecular work has been dedicated to discovering the underlying genetic and metabolic mechanisms that enable plant cells to survive desiccation and to persist in the dry state (e.g. Dekkers et al., 2015; Farrant et al., 2015; Sano et al., 2016; Verdier et al., 2013). Currently, desiccation tolerance and longevity are viewed as separate but related traits that develop consecutively during late embryo development (Verdier et al., 2013). Desiccation tolerance is most often treated as a binary trait, measured as short-time survival, or not, to a single, perhaps arbitrary water stress challenge of 50% RH (e.g. Alpert, 2005; Leprince and Buitink, 2015) or 0.08 g H₂O g⁻¹ dry weight (Hong and Ellis, 1996; Pritchard et al., 2004). By definition, longevity is a time-dependent response; however, it is most frequently characterized under stressful, but not necessarily desiccated, conditions (e.g. Rajjou et al., 2008). Through physiological studies using diverse organisms and a broad range of treatments, we know there is large variation of response to

* Corresponding author. Present address: Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Wellcome Trust Millennium Building, Ardingly, West Sussex RH17 6TN, United Kingdom.

E-mail addresses: d.ballesteros@kew.org (D. Ballesteros), lisa.hill@ars.usda.gov (L.M. Hill), christina.walters@ars.usda.gov (C. Walters).

water stresses applied for short and long durations (Hoekstra, 2005; Probert et al., 2009; Royal Botanic Gardens Kew, 2016; Walters et al., 2005). These studies provide useful phenotyping resources to support molecular studies. In turn, molecular studies promise to provide markers to guide preservation technologies.

The process of desiccation fundamentally transitions cytoplasm from fluid to solid. One can imagine that the molecular crowding required in this transition creates considerable mechanical stress and reorganization of molecular complexes (Meryman, 1974; Walters, 2015). When solidification occurs, responses to desiccation are expressed both temporally and spatially. This is because the compressive forces persist and molecules continue to relax, albeit slowly because of steric effects, into more efficient packing structures (Ballesteros and Walters, 2011; Shamblyn et al., 1999; Walters, 2004). The proximity between molecules and the relaxation rate are believed to drive solid-state reactions (Walters et al., 2010 and references therein). Interactions between composition, processing methods and time drive functional properties of plastics, preserved foods and pharmaceuticals (e.g. Guo et al., 2013; Menard, 2008; Roos and Drusch, 2015). These interactions are rarely explored in studies of desiccation tolerance and longevity, but they provide an opportunity to relate metabolic switches to changed physiological capacity.

The nexus of desiccation tolerance and longevity is poignantly illustrated by the so-called 'intermediate' category of seed storage behavior (Ellis et al., 1990; Walters, 2015). Intermediate seeds do not respond to moisture or temperature as expected by seeds in either orthodox or recalcitrant categories (Roberts, 1973). Intermediate seeds tend to age faster if dried or cooled within the solid matrix or may simply age faster than expected despite the stabilizing benefits of a solid matrix (Walters, 2015). Observations of so-called intermediate behavior may explain lower perceived desiccation tolerance or faster aging in other organisms, such as more-or-less desiccation tolerant pollens (Franchi et al., 2011; Hoekstra, 2005), mosses (Hoekstra, 2005; Koster et al., 2010; Oliver and Wood, 1997), yeasts (Morgan et al., 2006; Walters et al., 2005) and nematodes (Grewal, 2000; Perry et al., 2012).

A cross-cutting issue is the increasing recognition that cell responses to moisture and temperature are highly complex and might vary among tissues of a multi-celled organism (e.g. Müller et al., 2010; Scheler et al., 2015). Averaging response across all cells in a complex sample could dilute cell-specific responses. Unraveling the major factors involved in survival and stability of desiccated cells may be facilitated by using simple systems where cell differentiation is not a variable.

In this paper, we consider questions of desiccation tolerance and longevity within fern spores. Fern spores are unicellular haploid organisms, which eliminates complications of interpretation arising from studies of multicellular organisms. Fern spores naturally disperse from fertile leaves after maturation and drying to between 0.04 and 0.17 g water/g fresh mass, depending on species and ambient conditions (Gabriel y Galan and Prada, 2010). Species are sometimes categorized by the color of spores at maturity: green (or chlorophyllous) and non-green (or non-chlorophyllous). Green spores typically have thin walls, germinate and lose viability faster than non-green spores (Lloyd and Klekowski, 1970). The short life spans of green spores have led to the widely-held belief that they have limited tolerance to desiccation (Ibars and Estrelles, 2012; Perez-Garcia et al., 1994), though conflicting evidence suggests green spores can tolerate substantial water loss (Hoekstra, 2005; Lebkuecher, 1997; Li and Shi, 2014; Mikula et al., 2015; Pence, 2000). Moisture responses of non-green spores appear similar to orthodox seeds (Ballesteros, 2010; Ibars and Estrelles, 2012; Walters et al., 2005); however actual studies of survival under controlled moisture conditions are rare. Faster than expected ageing of some species stored at temperatures between -10°C and -30°C

may implicate intermediate storage behavior in non-green spores (Ballesteros, 2010 and references therein).

A premise of this paper is that better predictions of short (i.e., desiccation tolerance) and long (i.e. longevity) term responses to desiccation require understanding the biology of solidified cytoplasm. To that end, the objectives of this work were to explore variation in how fern spores respond to water loss. To what moisture levels can green and non-green spores survive? Does expression of damage increase with progressive drying (i.e., a characteristic of desiccation tolerance), with progressive time (i.e., a characteristic of aging), or both? We wished to relate physiological response to desiccation to biophysical parameters that measure change of the cytoplasm from fluid to solid and hypothesized that green and non-green fern spores survive the transition to solid-state, but succumb at different rates even though cytoplasm is a solid matrix. Support of this hypothesis implies differences in the structure or mobility of molecules within the solid matrix. The unicellular feature of fern spores, then, becomes a powerful tool to explore the relationship of these properties to cell survival following extreme water loss.

2. Material and methods

2.1. Plant materials

Basic information about fern spore characteristics and longevity were gleaned from the literature (Lloyd and Klekowski, 1970) and guided selection of species producing either green or non-green spore types. Mature fronds from five unrelated fern species were collected from wild populations or botanic gardens in USA, Spain and Portugal (Table 1). To release spores, fronds were pressed onto glossy paper and allowed to dry. After sporangial dehiscence, spores were collected from the paper, sieved and subsequently stored at 4°C until used, which was usually within 1 day and 1 week after spore collection for green spores and non-green spores respectively. Spores from Spain and Portugal were packed in an insulated box and mailed via expedited post to Fort Collins, CO, USA, arriving within 3 days after harvest.

2.2. Moisture control during storage and water sorption isotherm construction

Interactions of moisture, temperature and time on fern spore viability was measured by storing spores in relative humidity-controlled chambers at 25 and 5°C in the dark. For each treatment, about 500 mg of spores was spread in a thin layer on an open Petri dish (35 mm diameter), which was placed in a jar containing a saturated salt solution that maintained relative humidity (RH). Eleven different salt solutions were used to give RH ranging from 0.5 to 85% (Ballesteros and Walters, 2007a). RH was monitored and confirmed using Hobo dataloggers, model # U12-011 (Onset Corporation, Cape Cod, Massachusetts, USA). In addition, *E. hyemale* spores that had been adjusted to different RHs at 25°C were hermetically sealed into a series of 20 mg aluminum sample pans (Perkin Elmer, Norwalk, CT, USA) and stored at -18°C .

Fresh mass of subsamples were measured daily to monitor water content changes. These subsamples (about 2–10 mg) were placed into preweighed aluminum foil packets or Perkin Elmer sample pans that were heated at 95°C for 36 h to obtain dry weight (DW) measurements. Mass was measured to the 0.001 mg place using a Cahn C-31 microbalance (Thermo Electron Corp, Waltham, MA, USA). Water content was calculated from the difference in fresh and dry weight and is expressed on a dry weight basis as $\text{g H}_2\text{O g}^{-1}$ DW. Most changes in water content occurred within 1 or 2 days in the RH chamber and small changes in samples placed at 0.5, 75 and

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