Rehydration from Desiccation: Evaluating the Potential for Leaf Water Absorption in X. elegans

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Abstract: Desiccation tolerance is the ability to survive through periods of extreme cellular water loss. Most seeds commonly exhibit a degree of desiccation tolerance while vegetative bodies of plants rarely show this characteristic. Desiccation tolerant vascular plants, in particular, are a rarity. Although this phenomenon may have potential benefits in crop populations worldwide, there are still many gaps in our scientific understanding. While the science behind the process of desiccating has been widely researched, the process of recovering from this state of stress, especially in restoring xylem activity after cavitation is still relatively unknown. Although plants normally gain water through their roots, this influx of water may not provide enough pressure to refill the entire plant’s vasculature, raising question to the role of leaf water absorption in the refilling process. This study will first provide a brief overview of the desiccation tolerant angiosperm *Xerophyta elegans*. Data comparing desiccated and hydrated states will then be presented to add to the body of literature as a foundation for investigating for the role of leaf water absorption on rehydration. Specifically, changes in length and width of the leaf, scanning electron microscopy of the leaf surface, and hydrophobicity measurements will be used to examine surface structures and morphology to characterize water relations on the leaf surface. Comparisons between histochemical staining at the middle and petiole of the leaves in desiccated and hydrated cross sections provides information on internal biochemical changes that may enhance water absorption along the leaf surface.

Introduction

Documented global temperature increase has been linked to increased aridity on land across the globe (Dai 2012). As the 21st century continues, there is an increased risk of prolonged drought characterized by decreased precipitation and increased evaporation. Already, the increasing frequency and intensity of drought in recent years has been linked to crop insecurity, among other problems. A study by Lesk, for example, identified that the 10% decrease in cereal crop production over the past decade displays enough of an impact to raise questions about our global food security in coming years (Lesk 2015). As these climate changes continue, water availability will continuously need to be addressed.

Drought events dramatically impact plant species. Plants are sessile and must survive and adapt to environmental stressors without movement. Consequently, plants have evolved many adaptations for drought tolerance including altering photosynthetic pathways and developing unique anatomical features to maximize water conservation. These plants are found in arid environments worldwide. A more rare phenomenon exists in which plants can survive desiccation. Termed desiccation tolerance, water contents within cells equilibrate to surrounding air and the organism enters a state of dormancy. A large majority of cellular water (80-90%) is
often lost. Also known as resurrection plants, some of these organisms have even been known to equilibrate to 0% relative humidity (Gaff 1977). Once water is reintroduced, often months to years later, normal functioning and photosynthetic activity continues.

It is thought that desiccation tolerance was a basal-trait for terrestrial plant life (Mishler and Churchill 1985). Surviving states of low water availability may have been crucial in the initial colonization of land from aquatic environments, which may explain the high number of bryophytes that display desiccation tolerance. As plants developed more complex structures and physiology this survival mechanism may have been lost or silenced, as tolerance places limitations on metabolism and growth (Oliver et al. 2000). Current cases of desiccation tolerance especially in tracheophytes, vascular plants with cellular conduits for fluid flow, may therefore be cases of re-evolution in specific environments, especially xeric and rocky niches (Gaff 1971).

Many plants utilize desiccation tolerance in seeds, pollen, or spores that remain dormant until a variety of environmental conditions initiate germination. Vegetative bodies of plant species display this tolerance much less often. The majority of species that do display vegetative desiccation tolerance are normally non-vascular such as bryophytes, algae, and lichens that can easily regain water throughout their tissues through diffusion (Oliver et al. 2000). Vascular plants, specifically angiosperms, are a small minority of desiccation tolerant species, as these organisms use cellular tubing to conduct water and minerals throughout their tissues. Xylem and phloem cells carry water and photosynthetic metabolites, respectively, from roots to the leaves through a continuous stream of fluid. Transpiration, or evaporation and loss of water through the leaves, provides a negative pressure to pull water against gravity (Dixon 1914). This process known as the cohesion-tension theory can be disrupted if bubbles form within the xylem tissue, termed cavitation. Cavitation occurs when pressure exceeds the tensile strength of the xylem tubing, breaking the continuous stream, or when low water availability in the soil cannot provide an adequate water source to maintain filled xylem conduits.
As xylem cells are interconnected by pits, or perforations in the cell walls, fluid flow can normally circumvent cavitated xylem sections. Additionally, the effects of cavitation can often be overcome through production of new xylem from meristematic tissues (Ameglio et al. 2002) or through xylem refilling in which water droplets form along xylem vessels and diffuse into the conduits (Brodersen and McElrone 2013). However, when cavitation is overwhelming, as is the case in extreme drought, water conductance through the tissues halts completely and water availability is too low for the majority of plants to regain enough water to effectively refill xylem conduits in the ways described (Roo 2016).

Desiccation tolerant species, on the other hand, can overcome drastically low levels of water in which the majority of xylem conduits cavitate. When this occurs, molecular and genetic expression changes, as the plant enters a state of dormancy. Generally, in order to survive desiccation, plants must limit the damage of drying to a repairable level, maintain adequate physiology in the dormant state, and utilize repair mechanisms upon water introduction to achieve normal photosynthetic functioning (Bewley 1979). A variety of unique biochemical pathways ensue to accomplish these necessities. Literature has described these mechanisms in detail. Generally, desiccation tolerant plants exhibit dramatically increased sugar production in the leaves and begin producing a variety of antioxidant molecules to combat oxidative stress and light damage. Hormonal changes, especially ABA and cytokinins, have been documented. Cells shrink and cell wall compositions are often altered (Alpert 2005).

Resurrection plants fall into two major categories based on general mechanisms of surviving the desiccated period. Some focus on cellular protection during the dormant state while others focusing on repair mechanisms after water is reintroduced (Oliver et al. 2000). More specifically, some resurrection plants will retain chlorophyll throughout desiccation, termed homoiochlorophyllous, while others will lose chlorophyll and re-synthesize it upon hydration, termed poikilochlorophyllous. Homoiochlorophyllous species can generally recover normal
photosynthetic activity much faster, but have higher risks of photodescriptor and oxidative
damage by maintaining chlorophyll pigments (Luttge et al. 2011).

Researchers have given substantial attention to these biochemical processes and
differences that ensure survival. However, less research has been conducted on the rehydration
process, specifically the physical patterns of water reentry into plant tissues. When water is
reintroduced, resurrection plants must refill their embolized xylem tissues in order to regain
normal functioning, a feat that desiccation sensitive plants cannot accomplish. This requires an
influx of water substantial enough to achieve a continuous stream from the roots to the leaves.
The need to refill xylem against forces of gravity may be a large factor in the documented size
limitations in desiccation tolerant species (Alpert 2005).

Water enters a plant mainly through the roots. This absorption creates a positive pressure
that will partially push water and solutes up through xylem cells. With normal transpiration, root
pressure helps fluid flow throughout the plant and can lead to embolized xylem repair (Wegner
2013). However, this pressure, termed root pressure, may not be enough to completely refill
xylem conduits at efficient rates especially after desiccation when cavitation is overwhelming.
(Gaff 1977). Water can also enter a plant through the leaves either by diffusion across epidermal
cells or through open stomata, microscopic openings primarily used for gas exchange along the
leaf surface. Leaf water absorption, or water entry at the leaf surface, may, therefore, serve as a
mechanism for rehydration. The leaves’ structures and physical characteristics influences water
entry into a plant by establishing differences in water potentials that ultimately determine how
water will interact on the leaf surface (Liang 2009). Some desiccation tolerant plants heavily
rely on their leaves for rehydration, some fully rehydrating only when leaves come into direct
contact with moisture (Gaff 1977). Other plants, although rehydration is possible through the
roots will exhibit slower cellular water uptake and slower physiological recovery when the
leaves are kept dry (Bernacchia et al. 1996). Specific physical characteristics of the leaves that
may impact their influence on rehydration, however, have not been clearly identified in these organisms.

Understanding the role of leaves in the rehydration of desiccated plants will be vital in understanding efficient techniques to achieve rehydration and xylem refilling. Exploring leaf surface structures and general anatomy of desiccation tolerant plants will provide important information relating to water relations at the leaf’s surface and possible mechanisms of water absorption. By analyzing leaves we can explore the patterns of water condensation along the surface of the leave, potential channels for water movement, and areas of thin surface where water may be directly absorbed. Hydrophobicity measurements, the association between the water droplets and a surface that determine water droplet retention, can provide substantial knowledge about how water interacts with the plant’s tissues (Holder 2007). However, the concept of leaf hydrophobicity has never been applied to resurrection plants in prior literature. Knowledge of desiccation tolerant species’ leaves may aid in understanding necessary steps in engineering crop plants to exhibit similar water absorption.

To first add to the general body of literature on desiccation tolerance, this report will examine in depth a desiccation tolerant angiosperm from South Africa, *Xerophyta elegans*. Since this plant has not received individual attention for decades, the report will provide a literature review documenting *X. elegans*’s taxonomy, biochemistry, and ecology. In addition to this, data on physical leaf surface structures and morphology, hydrophobicity, and internal biochemistry will be presented from desiccated and hydrated states to support previous literary findings as well as fill in gaps relating to the characterization of the plant’s leaves. This information may be used in the future to construct data on the leaves of different resurrection plants, to grow a body of literature focusing on the potential importance of these structures on rehydration.
Xerophyta Elegans

*Xerophyta elegans* is a monocot, homiochlorophyllous resurrection plant endemic to xeric, rocky habitats of South Africa mountains (Gaff 1978). Specifically, it is widespread in eastern South Africa from KwaZulu-Natal Drakensberg foothills to eastern Mpumalanga (Behnze ?).

Originally referred to as *Talbotia elegans*, the species has a unique and controversial position with the family *Velloziaceae*. *Velloziaceae* contains approximately 250 species found in China, Yemen, Saudi Arabia, Africa, and South America and can be divided into four genera: *Vellozia*, *Barbaceniopsis*, *Barbacenia*, and *Xerophyta* as a result of parsimonious divisions along morphological and genetic differences (Mello-Silva et al. 2011). *Vellozia*, *Barbacenia*, and *Barbaceniopsis* species are endemic to South and Central America while *Xerophyta* spp. are endemic to mainland Africa and Madagascar (Mello-Silva et al. 2011).

Early literature placed *X. elegans* in the monotypic genus, *Talbotia*, due to morphological differences in leaves and flowers (Ayensu 1974). However, opposing ideas were soon to follow, as other researchers disagreed with the distinction, inserting *X. elegans* into *Xerophyta* (Menezes 1980). Current phylogenies acknowledge the differences between *X. elegans* and the rest of the genus, but note that for taxonomic simplicity, the distinction can be ignored (Behnke et al. 2012; Mello-Silva et al. 2011).

Two recent articles examine the phylogeny and anatomical characteristics of *Velloziaceae* and therefore *X. elegans*. The older study by Mello-Silva et al. identifies 67 anatomical characteristics of Velloziaceae (Figure 1) and constructs phylogenetic trees based on morphology and total DNA. A later study by Behnke et al. identifies 22 anatomical characteristics in Velloziaceae species (Figure 2) and constructs phylogenies based on *rbcL* genes. Mello-Silva et al. identify the morphological distinctions between *Xerophyta* and the other three genuses in Velloziaceae as well as the distinctions between *X. elegans* and the rest of genus. Both *Xerophyta* and the *Talbotia* distinction are unique to other members of *Velloziaceae* in that they have
trigonous transverse sections of their ovaries (0) and basal loculicidal capsule fruit (57).

*Talbotia* is morphologically distinct from other *Xerophyta* species based on evolved traits in *Talbotia* and *Xerophyta*. *Xerophyta* has an abscission line between the sheath and lamina (10), abaxial strands in the leaves (14), apical appendages in the anthers (46), and more or less equal lengths between the style and anther (55). *Talbotia* shows evolved hypoamphistomatic stomatal distribution (10), an absence of conduction tissues in marginal bundles (16), triangular transverse sections of pedicels, anther dehiscing by a separated split (50), and stigmas above stamens (54).

Both studies’ phylogenetic trees are shown (Figure 3; Figure 4). The tree by Mello-Silva contains six *Xerophyta* species, placing *X. elegans* in its own clade. Behnke listed substantially more *Xerophyta* species, placing *X. elegans* in clade with *X. tanzaniana*. 
<table>
<thead>
<tr>
<th>Character</th>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyllotaxis</td>
<td>tristichous (0), spirotristichous (1), spirally (2)</td>
<td></td>
</tr>
<tr>
<td>Deciduousness of leaves</td>
<td>deciduous (0), persistent, at least the sheath (1)</td>
<td></td>
</tr>
<tr>
<td>Abscission line between sheath and lamina</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Distal portion of leaf blade</td>
<td>attenuate (0), truncate (1)</td>
<td></td>
</tr>
<tr>
<td>Transverse posture of leaf blade</td>
<td>arcuate (0), plane (1)</td>
<td></td>
</tr>
<tr>
<td>Longitudinal posture of leaf blade</td>
<td>involute (0), flat (1), revolute (2)</td>
<td></td>
</tr>
<tr>
<td>Furrows in leaf blade</td>
<td>absent (0), on abaxial surface only (1), on both surfaces (2)</td>
<td></td>
</tr>
<tr>
<td>Papilae in furrows</td>
<td>absent or inconspicuous (0), coronal (1), finger-like (2)</td>
<td></td>
</tr>
<tr>
<td>Leaf trichomes or emergences</td>
<td>absent (0), multicellular base, on lamina (1), multicellular base, on margins and midrib (2), un- or multicellular with unicellular base (3)</td>
<td></td>
</tr>
<tr>
<td>Stomatal distribution in leaves</td>
<td>hypostomatic (0), hypoamphistomatic (1), amphistomatic (2)</td>
<td></td>
</tr>
<tr>
<td>Subsidiary cells</td>
<td>smooth (0), ridged (1)</td>
<td></td>
</tr>
<tr>
<td>Specialized cells</td>
<td>absent (0), present on adaxial surface only (1), present on both surfaces (2)</td>
<td></td>
</tr>
<tr>
<td>Adaxial strands</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Abaxial strands</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Marginal bundle</td>
<td>rounded (0), triangular (1), absent (2)</td>
<td></td>
</tr>
<tr>
<td>Conduction tissues in marginal bundle</td>
<td>present (0), absent (1)</td>
<td></td>
</tr>
<tr>
<td>Aqueous hypodermis</td>
<td>extending to bundle sheaths only (0), extending to bundle sheaths and furrows (1), absent (2)</td>
<td></td>
</tr>
<tr>
<td>Aquiferous parenchyma between bundles</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Sclerenchyma pattern</td>
<td>Xerophyta type (0), Vellozia type (1), Barbacenia type (2), other types (3)</td>
<td></td>
</tr>
<tr>
<td>Phloem strands</td>
<td>two, separated (0), two, united at bottom (1), one (2)</td>
<td></td>
</tr>
<tr>
<td>Minor fibre-vascular bundles</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Sheath of leaf vascular bundles</td>
<td>simple (0), double (1)</td>
<td></td>
</tr>
<tr>
<td>Transfusion tracheids</td>
<td>M &amp; al.; absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Inflorcescence</td>
<td>M &amp; al.; with major axis (0), suppression of major axis (1)</td>
<td></td>
</tr>
<tr>
<td>Flower number</td>
<td>solitary or grouped (0), always solitary (1)</td>
<td></td>
</tr>
<tr>
<td>Pedicel position</td>
<td>evident (0), hidden by leaves (1)</td>
<td></td>
</tr>
<tr>
<td>Transverse section of pedicel</td>
<td>triangular (0), circular (1)</td>
<td></td>
</tr>
<tr>
<td>Vascular bundles in pedicel</td>
<td>six (0), nine (1), 12 (2), 15 (3), 18 (4), 24 (5), 36 (6)</td>
<td></td>
</tr>
<tr>
<td>Belt of sclerified cells in pedicel</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Emergence type</td>
<td>capsule (0), capsule-truncated (1), NA</td>
<td></td>
</tr>
<tr>
<td>Pedicellar emergences</td>
<td>absent (0), laxly disposed (1), densely disposed (2)</td>
<td></td>
</tr>
<tr>
<td>Ovary outline</td>
<td>longer than broad (0), as long as broad (1), broader than long (2)</td>
<td></td>
</tr>
<tr>
<td>Transverse section of ovary</td>
<td>trigonous (0), circular-trilobate (1)</td>
<td></td>
</tr>
<tr>
<td>Placentation</td>
<td>S &amp; L; axile (0), parietal (1)</td>
<td></td>
</tr>
<tr>
<td>Corona</td>
<td>absent (0), present (1)</td>
<td></td>
</tr>
<tr>
<td>Corona: present (0), undifferentiated (1), absent (2)</td>
<td></td>
<td></td>
</tr>
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<td>Papilae in furrows</td>
<td>absent or inconspicuous (0), coronulate (1), finger-like (2)</td>
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</tbody>
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Figure 1: Character analysis and coding—Mello-Silva et al. 2011. Yellow highlighted characteristics indicate the anatomical feature in X. elegans. An asterisk indicates the traits that have evolved specifically for X. elegans that have previously justified the Talbotia identification. Red highlights indicate the characteristics shared by all other Xerophyta species.
Figure 2. Character analysis and coding—Behnke et al. 2012. Yellow highlighted characteristics indicate the anatomical feature in X. elegans.

1. Leaf lamina: deciduous (0); persistent (1)
2. Leaf sheath: not distinct (0); scale-like (1); early split into fibres (2)
3. Attachment of leaf sheath: appressed (0); tip exerted/revolute (1)
4. Stomatal distribution in leaf lamina: hypostomatic (0); amphistomatic (1)
5. Leaf trichomes or emergences: absent or only at midrib and margins (0); present on lower and/or upper surface (1)
6. Arrangement of mesophyll: dorsiventral (0); isolateral (1)
7. Hypodermis: present (0); absent (1)
8. Aquifereous parenchyma between bundles: absent (0); present (1)
9. Adaxially extended bundle sheath and/or hypodermis: absent (0); present (1)
10. Furrows in leaf blade: absent (0); on abaxial surface only (1); on both surfaces (2)
11. Leaf epidermis: not elaborate (0); papillate (1); (partly) sclerified (2)
12. Trichomes in furrows: absent or inconspicuous (0); coronulate, finger-like or other (1) N/A
13. Leaf glands: absent (0); deeply sunken (0); patellar or other (1)
14. Crystals in leaf blade: absent (0); present (1)
15. Transcurrent lateral bundles: absent (0); basic Barbaonia type (1); Capillaris group type (2)
16. Nontranscurrent lateral bundles: absent (0); Vellozia type (1); Xerophyta type (2)
17. Minor fibrovascular bundles in mesophyll: absent (0); present (1)
18. Spaces between lateral bundles: narrow (0); double (1); triple or more (2)
19. Sclerenchyma bands underneath lateral bundles: absent (0); present (1)
20. Sclerenchyma strands between lateral bundles: absent (0); present (1)
21. Midvein abaxial girder: crescentiform (0); bulbiform or other (1)
22. Sclerenchyma at marginal bundle: rounded (0); triangular (1)
Figure 3: Phylogenetic tree constructed by Mello-Silva et al. utilizing a combination of morphology and total DNA differences. Species listed in orange designates the *Xerophyta* genus.
Figure 4: Phylogenetic tree constructed by Benkhe et al. 2012 constructed using rbcL gene differences.
The ecology, anatomy, and patterns of desiccation are explored much less in the literature compared to its phylogeny. Gaff in 1978 was the first to address cellular differences between hydrated and desiccated states in *X. elegans* specifically.

*X. elegans* is a monocot shrub, reaching approximately half a foot. Its leaves averaging 10-20 cm long (Behnke et al. 2012) are bright green in hydrated states and brown or purple during periods of desiccation. The purple color is the direct result of anthocyanin build up during the state of dormancy (Gaff 1978). Desiccation is paired with dramatic leaf folding along the midrib and physical shrinking, decreasing to approximately half its hydrated width (Hallum and Luff 1980). It has consistent lamina and presents teeth along the leaf edge. *X. elegans* is the only member of *Xerophyta* without longitudinal leaf furrows and instead has epidermal cells with anticlinal walls near stomata (Coetzee 1974). The cuticle is thinner than in other *Xerophyta* species, indicating a potential for water entry (Coetzee 1974). The leaves are dorsiventral and stomatal distribution is amphistomatic (Coetzee, 1974; Behnke et al. 2012; Mello-Silva et al. 2011).

As a homoiochlorophyllous species, chloroplast structures are conserved through drying. Thylakoid stacks are disorganized but membrane integrity is retained (Hallam and Capicchiano, 1974). Plastid visualization is lost, as membranes loosen (Tuba et al. 1998). *X. elegans* presents less chloroplast organization during drying than other homoiochlorophyllous species that often display staircase stacking (Hallam and Luff 1980). Generally, homoiochlorophyllous resurrection plants recover from desiccation quickly, as photosynthetic pathways can resume rapidly after water is reintroduced (Tuba et al. 1998).

Once water is reintroduced, *X. elegans* can fully rehydrate in 8 hours and begins respiration within 24 hours. The first stage of rehydration consists of physical wetting of the
plant resulting in cell walls returning to their original position. Cell walls normally collapse and fold during desiccation (Moore et al. 2008). The cytoplasm remains attached to one side of the cell initially, normally a side with high amounts of pit fields and plasmodesmata. By 2 hours of wetting, the cytoplasm consolidates especially around chloroplast lamellae. At 4 hours, chloroplast reorganization allows for visualization. After 8 hours organization returns to the cytoplasm with plasmalemma along the cell wall and distinct chloroplast double membranes. By 12 hours mitochondrial membranes are visualized and chloroplasts display better organization. After 24 hours, chloroplasts are fully structured and tightly compacted. Starch is present within the chloroplasts and fully structured mitochondria can be seen (Gaff 1978).

Although the literature, like Mello-Silva and Behnke, lists anatomical features of \textit{X. elegans} rarely do they discuss these features in the context of rehydrating from desiccation. Other than occasional discussion about leaf width and cellular changes, there isn’t much dialogue focused on the leaf surface’s impact on water entry. Similarly, there is a gap in the research describing the plant’s ecological niche, documenting normal amounts of water exposure. Hydrophobicity measurements that may potentially relate to leaf water absorption (Holder 2011; Liang 2009).

**Materials and Methods**

In order to understand and characterize the leaves of \textit{X. elegans}, multiple parameters were examined. All plants were purchased through Marina Del Rey Garden Center in Los Angeles, Ca and stored at Loyola Marymount University’s greenhouse. During analysis of the desiccation process, plants were stored at 28 °C in a VWR plant growth chamber with controlled light cycles. Some were watered twice weekly while others did not receive any hydration. Leaf
length and width were measured to quantify observable leaf shrinkage during desiccation cycles. Stomatal densities were obtained on abaxial and adaxial surfaces as well as at different locations of the leaf (tip, middle, bottom, and petiole).

Histochemistry was used to compare internal biochemical makeup of hydrated and desiccated samples. Leaf hydrophobicity measurements were additionally recorded to characterize physical leaf/water interactions. This parameter adds to the full body of literature on resurrection plants, as no prior research has documented leaf hydrophobicity measurements. Scanning electron microscopy was performed on desiccated samples to visualize ultramicroscopic leaf surface details relating to water adhesion or re-entry.

**Leaf Measurements during Desiccation**

Five plants were stored in a VWR plant growth chamber to control for light and temperature. The plants received no water and two leaves per plant were selected and marked. Calipers were used to measure length and width of each leaf over a period of 72 days. A one-way analysis of variance compared measurements over time. Visual observations were made into purple pigmentation development and general leaf structure throughout desiccation processes.

**Stomatal Densities**

The abaxial and adaxial sides of harvested leaves were painted with acrylic polish. After a period of drying, the polish was peeled off and viewed under a light microscope. Sections from the tip, middle, bottom, and petiole were analyzed to determine if there were differences in stomatal distribution across the leaf surface that may relate to water absorption. Visualized
stomatal imprints were counted and divided by total number of epidermal cells, yielding stomatal indices. A one-way analysis of variance and two-way analysis of variance were used to determine statistical significance among the different surface locations as well as the abaxial and adaxial sides.

**Leaf Hydrophobicity**

Leaf hydrophobicity was measured at the tip, middle, and bottom of both desiccated and hydrated leaves. Ten microliters of deionized water were dispensed at each location on a harvested leaf anchored to a flat surface. Pictures were taken with a mounted iPad at 90 degrees and the angle of the tangent line of the water was measured using protractors, according to Holder 2011. Multi-factor analysis of variance was used to determine statistical significance.

**Embedding**

Two methods of embedding were performed. Samples from the petiole and middle portion of desiccated and hydrated leaves were embedded. For histochemical staining, the JB-4 Embedding Kit® by Sigma-Aldrich was used. Hydrated samples were fixed with FAA overnight. Due to the importance of minimizing potential for rehydration and the general dormancy of desiccated leaves, desiccated samples were not fixed (Hallam and Gaff 1978). Dehydration was done step-wise beginning with 30% ethanol for an hour, 50% ethanol for 2 hours, 75% ethanol for two hours, 95% for two hours, and then 100% overnight. The dehydration process with one group of desiccated samples began at 75% and another group began at 100%. Again, this was to control rehydration throughout the embedding process.
Sections started at 75% and at 100% could be compared to determine if rehydration occurred at the lower ethanol concentration.

Infiltration solution was created by adding 1.25 g of provided benzoyl peroxide to JB-4 Solution A. Samples were submerged in infiltrate for two hours at a time with two changes. They were covered in foil to minimize light exposure. After infiltration, 25.0 mL of freshly prepared infiltration solution was mixed with 1.0 mL of JB-4 Solution B and the samples were immediately imbedded in beem capsules to minimize oxygen exposure.

Additional embedding was performed to obtain clearer pictures of general anatomy using Toluidine Blue staining. A Low Viscosity “Spurr” Test Kit was used from Ted Pella Inc. (Spurr 1969). The fixing, dehydration, and infiltration procedure was taken from previous literature. Four percent glutaraldehyde fixative was created with a 0.4M cacodylate buffer. Hydrated samples cut to approximately 1 mm² were submerged in fixative overnight. The following day, fresh buffer was changed every 15 minutes for an hour to remove remaining glutaraldehyde fixative. Hydrated cuttings were submerged in freshly made 2% osmium tetroxide and desiccated sections also cut to approximately 1 mm² were placed on cheesecloth at the top of the beem capsules used for the procedure. The lid was closed to ensure fume build up. This was done to minimize the potential for rehydration while still partially fixing the samples. The hydrated samples were rinsed three times with fresh buffer for 15 minutes each. A 10%, 25%, and 50% ethanol series was performed with 15 minutes per concentration. A freshly prepared solution of 1% uranyl acetate in 75% ethanol was applied to the cuttings for 1 hour and then the samples were subjected to 75% ethanol, 100% ethanol (2x), and propylene oxide (2x) for 20 minutes each. Propylene oxide was then diluted 1:1 with epoxy resin, made with materials in the Spurr’s Test Kit, and the samples were submerged in this for 4 hours. After this was completed,
100% epoxy resin was applied and samples sat overnight. The following day fresh epoxy resin was poured into molds and the samples were placed in the molds into an oven at 70 °C overnight.

**Cross Section Production and Staining**

A Reichert-Jung Ultracut E Ultra Microtome was used to produce 2um cross sections of each material. Glass knives were created on a Reichert-Jung Knife Maker. Sections were mounted on glass slides, heat fixed, and stained. After staining, mounting oil was applied, and the sections were viewed under a light microscope.

**Staining**

Ruthenium red solution was made by mixing 0.3 g of ruthenium red powder in 100mL of distilled water and filtering the liquid after 15 minutes. It specifically targets aldehyde sugars and mucopolysaccharides (Johansen, 1940; Retamales and Scharaschkin 2014). Polysaccarides such as mucilages, glycogen, glycolipids, and glycoproteins were targeted with the Periodic Acid-Schiff’s reaction by applying 1% periodic acid solution for 30 minutes followed by washing and 30 minutes of Schiff’s reagent (McManus 1948). A 1% sudan black solution was created with 75% ethanol to detect lipids (Benes 1964).

**Scanning Electron Microscopy**

Abaxial and adaxial hydrated leaf sections from the middle and base were cut to approximately 5mm² and placed on metal stubs with adhesive paper. Samples were sputter-coated with a gold/palladium alloy for conductance using a sputter coater. Pictures were taken at 30kv at different magnifications.
Results

**Structural and Visual Leaf Changes During Desiccation**

During desiccation, leaf width folds in half along the midrib, exposing the abaxial side to the surrounding environment. In addition to folding, the leaf width dramatically shrunk to approximately half the original measurements (p-value: 0.0000) (Figure 6a). The average width of a hydrated leaf was approximately 15.49 cm compared to the desiccated average of 7.37 cm. Lengths were unaffected during desiccation cycles (Figure 6b), although many leaves displayed a slight inward curving toward the stalk of the plant. The average length was 101.30 cm. When this occurred measurements were obtained by carefully extending the leaf and measuring the full length. As desiccation continued the leaves folded so dramatically only half measurements could be taken, so as not to damage the leaf.

Most leaves exhibited a dark purple color when desiccated. This often first developed in the tips and made its way along the edges and into the middle of the lamina. The abaxial side develops purple pigmentation prior to the adaxial side that can remain green for weeks after the first purple pigmentation is seen. Although timing differed based on the leaf and the plant, the purple color intensity decreased with passing time, with leaves predominantly dull brown after approximately 2 months of desiccation. The petiole in hydrated and desiccated states has a white color. This white color remains even during desiccation. It is softer to the touch than other parts of the leaf that feel waxier.
Figure 6: a. The average widths (left) and lengths (right) (mm) of a total of ten leaves from five different plants of *X. elegans* over a period of 72 days without water. As desiccation occurred, the widths shrank significantly (p-value=0.0000) by about 50% of the original measurements. There was no significant difference between the lengths (p-value=0.9236) although some curving did occur.

**Stomatal Density**

Like many plants, there are more stomata on the abaxial side than the adaxial side (p-value=0.0499) of the leaves. Stomatal indices for the abaxial surface tip, middle, bottom, and petiole did not differ significantly. Stomatal lengths were also measured with no significant differences among adaxial and abaxial sides nor along leaf location.

**Leaf Hydrophobicity**

Holder 2011 identifies highly wettable surfaces as those with a water droplet tangent angle between 40 and 90 degrees. Average measured angles ranged from 68 to 72 degrees from the vertical, considered highly wettable (Figure 7). There was no statistical significance between leaf location, abaxial and adaxial sides, nor hydrated or desiccated states.
Figure 7: The average widths (left) and lengths (right) (mm) of a total of ten leaves from five different plants of *X. elegans* over a period of 72 days without water. As desiccation occurred, the widths shrank significantly (p-value=0.0000) by about 50% of the original measurements. There was no significant difference between the lengths (p-value=0.9236) although some curving did occur.

**Scanning Electron Microscopy**

The petiole and middle portions of the leaves were imaged for the abaxial and adaxial sides of desiccated samples (Figure 8). Each image shows grooves between the sclerenchymous ribs. The middle sections seem to display ragged and disrupted epidermal cells in the inter-rib regions. The adaxial middle section shows less pronounced ridging in comparison to the abaxial side, indicating that the ribs pronounce abaxially. There were no ultrasscopic glandular or trichoumous structures on the lamina.
In addition to the general lamina and ribbing as seen above, *X. elegans* also displays teeth that project from the sides of the leaves that run reach about a third of the way down the full leaf length (Figure 10). Visually, these projections seem to alternate in size from larger to smaller back to larger. Not every leaf visually displays this morphology to visual acuity.
Internal Anatomy

Leaves are amphistomatic with the amount of vascular bundles ranging between leaves from 12 to 18 per leaf. The sections displayed are dorsiventral with larger, tightly associated cells near the adaxial side and smaller, loosely packed cells nearer to the abaxial side. Hydrated sections display plastids around the circumference of most cells. This visualization of these structures through light microscopy is lost in all desiccated sections. The vascular bundles in the petiole seem closer together than those in the middle, suggesting a fan-shaped type of leaf structure. Many of the cells in the space between lateral bundles couldn’t be captured in cross sections. Damage to these cells may have occurred in the embedding process or the physical cutting from the plant prior to any chemical applications (Figure 11a).

In each set of desiccated section, cells are shrunken, with partially collapsed cell walls. Epidermal cells are broken creating invaginations along the leaf surfaces. Histochemical staining shows visual differences in the middle sections stained with the PAS reagent and with
Ruthenium Red (Figure 11b, Figure 11c). For both, desiccated samples display more intense staining, indicating a build up of polysaccharides and pectins, respectively. The petiole sections under Ruthenium Red also show some differences, with pinker staining around the cell membranes than in the hydrated section. Little differences are apparent between sections stained with Sudan Black (Figure 11d).
Figure 11: Histochemical staining for desiccated and hydrated leaf sections with Toluidine Blue (a), periodic acid/Schiff’s reagent (b), Ruthenium Red cross stained with Toluidine Blue (c), and Sudan Black (d), visualized with light microscopy. In each set of images, the top set of image are from petiole sections and the bottom set of the images are from the middle sections. The left images are the desiccated samples while the right are the hydrated samples.

a. Toluidine Blue
b. PAS staining
c. Ruthenium Red with Toluidine Blue Cross Stain
d. Sudan Black

Discussion

Visualizations during the desiccation cycle were in accordance with previous literature on *X. elegans*. Similar to findings by Gaff in the 70s, the dramatic leaf folding and shrinkage were documented. Tissue shrinkage may be related to internal cell wall collapse as this shrinkage impacts the full leaf structure. This phenomenon is seen throughout desiccation tolerant species and may relate to protection from excessive sun damage and therefore free radical formation (Mitra et al. 2013; Alpert and Oliver 2002).
The purple pigmentation seen during desiccation is related to anthocyanin build up during the desiccation process (Hallam and Gaff 1978). This is also a characteristic of many desiccation tolerant plant species to reduce sun damage and free radical build up (Bewley 1979; Koonjul et al. 2000). This has additionally been documented in other members of Xerophyta specifically (Sherwin 1996). The locational patterns of anthocyanin build up that were observed in X. elegans have not been previously documented for this plant. Anthocyanin production occurs at different rates throughout the plant, beginning in the flowers, followed by the abaxial sides of the leaves, and finally the adaxial surface. Due to the energy needed to produce anthocyanin pigments, this phenomenon may be related to energy conservation during the period of stress (Farrant et al. 2003; Gaff and Ziegler 1989). Future research may look into this distribution by analyzing anthocyanin concentrations or the transcriptome and proteome throughout desiccation (Dace 2014; Leprince and Buitink 2015).

Although many articles discuss anatomical and morphological characteristics of X. elegans rarely were the petiole region and trichomes discussed. The petiole, with a distinct color and morphology compared to other portions of the leaf, may play a different functional role in the plant’s survival through water stress. The teeth on the leaf edges will project upwards during the desiccated state and may be involved in capturing water.

Potential Impact of the Leaves in Rehydration

The leaves exhibited dramatic folding throughout desiccation, with the abaxial side facing the environment. In comparison to the adaxial side, the abaxial side displays a higher stomatal density. The position of the abaxial side in the dormant state may relate to rehydration,
as water has been noted to enter through stomatal pores (Liang 2009). This is especially interesting in conjunction with hydrophobicity measurements.

Hydrophobicity measurements displayed highly wettable leaves (Holder 2011). There was no significant difference between either the abaxial and adaxial sides nor leaf location. When wettable leaves come into contact with water, water droplets easily sit on the leaf surface for long periods of time (Holder 2012). This may decrease rates of photosynthesis (Smith and McClean 1989), which is beneficial during periods of little water availability; and it may directly relate to leaf water absorption (Liang 2009; Fernandez 2014). Stagnant water has the ability to seep through stomata or epidermal cells and enter into the mesophyll below (Fernandez 2014).

As water readily stays on the leaves of *X. elegans*, it has the potential for absorption that can be readily influenced by the cells’ internal biochemistry. Similar to other desiccation tolerant plants, sugars dramatically increased in desiccated samples (Alpert 2005). With minimal changes in lipid concentrations that may impede water entry through the epidermis, this sugar build up will increase osmotic pressure potentially increasing the potential for water entry. The folded epidermis noted in the cross sections may also increase the likelihood of water entry.

The SEM imaging also provides insight into leaf water absorption. With microscopic abaxial ridging and rough, jagged epidermal tissue between these ridges, water adherence is likely. These features may account for the wettability documented in hydrophobicity measurements.

Gaps in Current Knowledge and Future Considerations

Length of desiccation seems to be of importance to its physical recovery, as plants that were desiccated for longer periods of time often did not rehydrate their leaves. Instead, the
plants grew new leaves from the stalks. Future resurrection plant studies could compare desiccation recoveries after differing lengths of dormancy to determine if rehydration mechanics differ as time without water increases. This observation also grants validity in the examination of stalk water absorption, that has been documented in other plants as a legitimate area of water entry (Oliveira et al. 2005).

This study did not directly measure leaf water absorption. This initial research into leaf surface structure and internal biochemistry warrants direct leaf water absorption measurements in further scholarship. Additionally, hydrated leaf samples could be examined utilizing critical point drying prior to the SEM. This will allow for productive structural comparisons between hydrated and desiccated states.

Most of the studies focusing on desiccation tolerant angiosperms do research in labs. X. elegans, specifically, hasn’t been explored in the field since the 1970s (Gaff 1977). Understanding of the plant’s ecology and its normal water availability may grant further insight into methods of rehydration.

Finally, the literature on most desiccation tolerant angiosperms ignores the possible impact of root and stem structures and biochemistry. Although analyzing these structures is not the norm, it may grant additional insight into necessary plant morphology undergoing desiccation tolerance and rehydration. Future research should analyze the impact that water loss will have on root growth, size, and biochemistry as well as general anatomy and morphology of root systems within these plants.
Conclusion

Leaf water absorption is an exciting new consideration when examining desiccated rehydration in higher level plants. Although research has gone into the biochemical processes of desiccated dormancies, gaps in scientific knowledge still exist when it comes to physical rehydration. Revisiting resurrection plants to examine leaf/water interactions may be beneficial in establishing knowledge into potential desiccation tolerant leaf characteristics necessary for survival. Techniques utilized within this study including SEM, histochemistry, and hydrophobicity along with visual observations can be used to establish knowledge about the leaves in other desiccation tolerant species.

*X. elegans* displays highly wettable leaves that fold during desiccation. Cellular biochemical changes and surface composition may positively influence leaf water absorption. The results found within this study warrant further analysis of leaf water absorption and physical rehydration in this plant as well as other resurrection plants.

Works Cited


