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Abraam Zakhary
University of Toronto Scarborough

Aashika Nagarajan
University of Toronto Scarborough

Charlotte Ngo
University of Toronto Scarborough

Marwa Saidajan
University of Toronto Scarborough

Supreet Babbar
University of Toronto Scarborough

Jason Brown (✉ nysuloem.brown@utoronto.ca)
University of Toronto Scarborough  https://orcid.org/0000-0001-8244-5633

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The evolution of life history strategies in plants and its relationship to the evolution of protective cellular mechanisms against reactive oxygen species

Abraam Zakhary, Aashika Nagarajan, Charlotte Ngo, Marwa Saidajan, Supreet Babbar, and Jason C. L. Brown

Department of Biological Sciences, University of Toronto Scarborough, Toronto, ON, CANADA

1Author for correspondence; email: nysuloem.brown@utoronto.ca

Author Contributions
Abraam Zakhary conducted the phylogenetic analysis and part of the germination rate experiment. Aashika Nagarajan conducted the electrolyte leakage experiment. Charlotte Ngo conducted the chlorophyll degradation experiment. Marwa Saidajan conducted part of the germination rate experiment. Supreet Babbar conducted part of the germination rate experiment. Jason C. L. Brown conceived the objectives and design of the study and wrote the manuscript. All authors contributed to editing the manuscript.

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Abstract

Purpose. The Oxidative Stress Theory of Aging (OSTA) states that accumulation of oxidative damage is a major contributor to aging; however, until now, no studies have examined whether perennial plants exhibit cellular mechanisms to better protect themselves against oxidative damage than annual plants, nor how these mechanisms may have evolved.

Methods. We undertook three approaches to evaluate the capacity for annual and perennial plants to resist oxidative damage. The first approach involved using an electrolyte leakage assay to assess the rate of cellular damage in leaves exposed to exogenous H$_2$O$_2$. The second approach involved determining the concentration of exogenous H$_2$O$_2$ required to maximize germination rates, which provides insight about the antioxidant levels in seeds. The third approach involved assessing the susceptibility of chlorophyll a and chlorophyll b to exogenous H$_2$O$_2$ and determining chlorophyll a/b ratios. We also conducted an ancestral state reconstruction of life history strategies in order to interpret our results in an evolutionary context.

Results. Leaves from deciduous and evergreen perennials showed a lower rate of cellular damage than leaves from annuals when exposed to exogenous H$_2$O$_2$. Seeds from deciduous perennials—but not biennials or evergreen perennials—required a higher H$_2$O$_2$ concentration to maximize germination rate compared to seeds from annuals, suggesting that seeds from deciduous perennials have higher antioxidant levels. Although chlorophyll b was found to be more susceptible to damage from exogenous H$_2$O$_2$, chlorophyll a/b ratios did not differ among life history strategies. Ancestral state reconstruction revealed that the ancestral plant was most likely an evergreen perennial.

Conclusion. Our results showcase that resistance to oxidative stress is necessary for perennial plants to survive over multiple years. The mechanisms responsible for the increased tolerance of perennial species to oxidative stress has not been fully elucidated by this study, but it does not involve changes to chlorophyll a/b ratios, as such changes could disrupt photosynthesis. The developmental onset of these protective mechanisms was delayed in evergreen perennials compared to deciduous perennials, perhaps because the ancestral evergreens were primarily focused on rapid colonization of the terrestrial environment, which requires faster germination rates induced by higher H$_2$O$_2$ levels.

Keywords: longevity, germination, electrolyte leakage, reactive oxygen species, life history, maximum lifespan
Declarations

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Conflicts of Interest/Competing Interests
On behalf of all authors, the corresponding author states that there is no conflict of interest.

Availability of Data and Material
Not applicable.

Code Availability
The R coding used to construct the phylogenetic tree and perform the ancestral state reconstruction has been provided in an appendix to this paper.

Ethics Approval
Not applicable.

Consent to Participate
Not applicable.

Consent for Publication
Not applicable.
Introduction

Charlesworth (2001) noted that “senescence of multicellular plants and animals is an almost universal phenomenon; it needs to be explained both in terms of cellular and physiological mechanisms, and of evolutionary forces.” While there has been an abundance of research on the ecological forces driving the evolution of annual, biennial, deciduous perennial, and evergreen perennial life histories in plants (e.g., Klinkhamer et al. 1997), and even some recent work on genetic factors regulating those developmental traits that differ among life histories in plants, such as flowering (Friedman 2020), there has been a dearth of research examining the underlying cellular and physiological mechanisms that permit these changes in life history strategies to occur. The present study—and our research program, in general—seeks to rectify this disparity.

According to the Oxidative Stress Theory of Aging (OSTA), the maximum lifespan of an organism is determined by the rate at which it accumulates oxidative damage to its biological molecules (Golden et al. 2002). Such damage is caused by interactions between reactive oxygen species (ROS; such as superoxide, singlet oxygen, $\text{H}_2\text{O}_2$, and hydroxyl radicals) and lipids, proteins, and nucleic acids (Bandyopadhyay et al. 1999). The mitochondrial electron transport system (ETS) is a major source of ROS in all aerobic organisms, but significant amounts of ROS are also produced by peroxisomes, endoplasmic reticulum, and cellular oxidases (Snezhkina et al. 2019). In plants, the photosynthetic ETS in chloroplasts is also a major source of ROS, with smaller amounts of ROS arising from the apoplast, plasma membrane, and cell walls (Das and Roychoudhury 2014).

Based on OSTA, there are four principal ways by which organisms could increase their maximum lifespan: i) reduce the rate at which they produce ROS, esp. from mitochondria and chloroplasts; ii) increase their activity and/or amount of antioxidants, which are compounds or enzymes capable of eliminating ROS after their formation; iii) construct their tissues from biological molecules that are more resistant to oxidative damage; and/or iv) repair their biological molecules after they suffer oxidative damage. While a few researchers have proclaimed the OSTA “dead” (e.g., Doonan et al. 2008; Pérez et al. 2009), we believe that such declarations are premature since they have been entirely based on just one facet of the OSTA: antioxidants. The OSTA can only be rejected when, for a given species, its lifespan cannot be explained by any facets of the OSTA; however, such a comprehensive investigation has never, to our knowledge, been undertaken. Indeed, there are several studies which provide evidence for many of the predictions of the OSTA in animals. For example, Robert et al. (2007) showed that liver mitochondria isolated from long-lived colubrid snakes (e.g., common king snake) produced $\text{H}_2\text{O}_2$ at a slower rate than those isolated from short-lived conforamials, and Herrero and Barja (1998) showed that rates of $\text{H}_2\text{O}_2$ production by heart mitochondria isolated from
parakeets and canaries, which have maximum lifespans of 21 and 24 years, respectively, were significantly lower than that from similar-sized mice, which have a maximum lifespan of 3.5 years. Moreover, methionine residues in proteins are known to be particularly susceptible to oxidative damage (Stadtman et al. 2003), and Pamplona et al. (2005) showed that proteins from parakeets and canaries had lower methionine content that those from mice. Similarly, it is well established that polyunsaturated fatty acids (PUFA) are more susceptible to oxidative attack than either monounsaturated or saturated fatty acids, and there is evidence in mammals that long-lived species frequently have lower levels of PUFA in their cell membranes than short-lived species (Pamplona et al. 1999). Furthermore, MacRae et al. (2015) showed that humans and naked mole rats, with maximum lifespans of 120 and 30 years, respectively, exhibited higher expression of DNA repair genes than mice, and Salway et al. (2011) showed a significant correlation between expression of heat shock proteins (HSPs) and maximum lifespan among 13 mammalian and avian species, reflecting that HSPs have been shown to refold denatured proteins resulting from oxidative damage (Perišić et al. 2007).

By contrast, to our knowledge, studies related to OSTA in plants are far less common and have been largely restricted to intraspecific comparisons. For example, Munné-Bosch and Alegre (2002) showed that 7-year-old individuals of *Cistus clusii* had higher malondialdehyde levels (a marker of lipid oxidation) in their leaves and chloroplasts, and lower chloroplast antioxidant levels, than 1- and 3-year old conspecifics. Similarly, Kurepa et al. (1998) showed that late-flowering *Arabidopsis* mutants, which had an extended lifespan because their vegetative phase was protracted, were more tolerant of paraquat, which stimulates superoxide production in organisms. In contrast to these studies, our laboratory has begun to explore whether interspecific differences in lifespan among plant species can be explained by OSTA. Most commonly, plants are classified as being annuals (lifespan < 1 year) or perennials (lifespan > 1 year). Annuals complete their entire life cycle—including vegetative growth and reproduction—within a single year, propagating among seasons via seed production only (Bazzaz and Morse 1991). Among perennials, there is considerable variation in lifespan, both within and among species. Within species, while a perennial plant may live for thousands of years, its leaves, flowers, and fruits may only live for a few months (Robbins 1957). Among species, biennials live for two years, usually accumulating resources via vegetative growth in their first season and reproducing in their second season (Hart 1977). Deciduous perennials have regions that persist for several years (e.g., roots) but have leaves that only persist for one year, falling each autumn in a synchronous fashion as low temperatures and short days preclude net photosynthetic gain of carbon (Estiarte and Peñuelas 2015). Evergreen perennials have leaves that persist for several years, being shed at a constant, low rate throughout the year so that the plant always
possesses some leaves (Williams-Linera 1997), though they may or may not be able to conduct photosynthesis in winter (Taneda and Tateno 2005). These differences in life history make studying the interspecific differences in plant aging more complex than similar studies in animals, which could explain the paucity of interspecific studies in plant aging to date.

One study, conducted by Brown et al. (2012), found that catalase, an antioxidant enzyme, was significantly higher in both the cotyledons and roots of perennial flax species compared to annual congenerics. We are unaware of any other studies comparing aspects of ROS metabolism and/or oxidative damage between annual and perennial species; therefore, it remains uncertain whether the differences observed between annual and perennial flax in this previous study are broadly applicable within plants. On this basis, for the present study, we decided to investigate the effects of exogenous H$_2$O$_2$ in a wide array of plant species, covering annuals, biennials, deciduous perennials, and evergreen perennials. Moreover, we decided to utilize three independent approaches in order to ensure that any observations made were robust, and we also decided to undertake an ancestral state reconstruction of the species used in our study so that we could gain insight into how ROS handling by plants may have evolved over time. Based on OSTA, we predicted that perennials would have a greater resistance to exogenous H$_2$O$_2$ than annuals. Moreover, given that evergreen leaves have a minimum lifespan that is 3- to 4-fold higher than that of annual and deciduous perennial leaves (Tessier 2008), we also predicted that leaves from evergreen perennials would have greater resistance to exogenous H$_2$O$_2$ than leaves from deciduous perennials.

Our first approach involved assessing the capacity of exogenous H$_2$O$_2$ to cause cellular damage to leaf cells. For this approach, we utilized a protocol that measures the rate of electrolyte leakage in leaf samples subjected to abiotic stressors, which has been used in several previous studies to assess, for example, freezing tolerance (Nunes and Smith 2003) and disease resistance (Scheffer and Livingston 1980). This assay is based on the principle that abiotic stressors cause cellular damage primarily via disruption of cell membrane integrity, which causes the release intracellular contents, including ions, into the external medium; therefore, by measuring the rate at which the conductivity of this external medium increases, we can infer the rate at cellular damage occurs in leaf cells in response to exogenous H$_2$O$_2$.

Our second approach took advantage of the known effects of exogenous H$_2$O$_2$ on germination rate. Exogenous H$_2$O$_2$ has been found to accelerate germination rate in many species (Barba-Epsín and Hernández 2012; Riffle and Springfield 1968), likely via oxidation of germination inhibitors (Ogawa and Iwabuchi 2001). Various antioxidants are expressed in germinating seeds, and their activities are known to increase shortly following imbibition
(Cakmak et al. 1993). These antioxidants would eliminate some proportion of exogenous \( \text{H}_2\text{O}_2 \), thereby preventing its stimulatory effects on germination rate. On this basis, we sought to determine the concentration of exogenous \( \text{H}_2\text{O}_2 \) that would maximally stimulate germination rate as a proxy for seedling antioxidant activity.

Our third approach was inspired by animal studies showing that biomolecules can differ in terms of their susceptibility to oxidative damage, allowing animals to alter their maximal lifespan via changes in body composition and/or diet. While PUFA composition of cellular membranes (Hulbert et al. 2014) and/or methionine composition of cellular proteins (Sun et al. 2009) are the canonical markers for biomolecular susceptibility to oxidative damage, we decided to focus on chlorophyll composition in the present study. While it is known that semi-synthetic versions of chlorophyll have antioxidant properties (Hsu et al. 2005), suggesting that chlorophyll can interact with ROS, it is largely unknown whether chlorophyll a and chlorophyll b differ in their susceptibility to oxidative damage and, therefore, whether alterations of chlorophyll a/b ratio can serve as a mechanism to promote longevity in perennial plants.

While it has been acknowledged that ROS likely play a significant role in shaping the evolution of life history strategies in animals (Dowling and Simmons 2009), there appears to be little understanding of how the physiological mechanisms related to ROS production, consumption, resistance, and repair may have evolved in plants, and whether they could similarly constrain the evolution of life history in this group. For this reason, we sought to evaluate the results of our study from an evolutionary perspective. Most studies examining the evolution of life history strategies in plants have focused on lower taxonomic levels, especially orders (e.g., Saxifragales; Soltis et al. 2013), tribes (Castillejinae; Tank and Olmstead 2008), and genera (e.g., Medicago; Bena et al. 1998; Nemesia; Datson et al. 2008), and have generally demonstrated that the perennial state is ancestral in plants. No studies, to our knowledge, have sought to determine whether evergreen or deciduous perennialism is ancestral in plants, at least at a broad taxonomic scale. Indeed, Kenrick and Crane (1997) discussed the early evolution of land plants but did not at all consider life history strategies. For these reasons, we conducted our study using a wide variety of plant species from numerous different families, covering ferns, gymnosperms, and angiosperms. Additionally, we built a phylogenetic tree using the species examined in this study to envision their evolutionary relationships and completed an ancestral state reconstruction to determine how life history strategies may have evolved in land plants at a broad level.
Material and Methods

To investigate rates of cellular damage in leaves caused by exogenous H\textsubscript{2}O\textsubscript{2} exposure, we used an electrolyte leakage assay (Hatsugai and Katagiri 2018). Leaves were harvested in early autumn from plants growing naturally on the University of Toronto Scarborough campus grounds as well as public and private (with permission) lands in the adjacent neighbourhoods. Only green leaves were harvested, which reduced the likelihood that senescent leaves were included in our study since it has been shown that loss of greenness is one of the earliest markers of leaf senescence (Bertold et al. 2019). When not already known, plants were identified using publicly-available databases (e.g., ontariotrees.com). For plants known to exhibit different life history strategies in different regions, we classified them according to the life history strategy that they most commonly exhibit when grown in southern Ontario. The species used for this experiment are listed in Table 1. Once harvested, leaves were subjected to the electrolyte leakage assay as soon as possible. Multiple species, with different life history strategies, were processed in parallel to minimize variation in the results due to disparity in the concentration of the prepared H\textsubscript{2}O\textsubscript{2} solutions, which were made fresh just prior to each assay being conducted. For each species, two 1 cm\textsuperscript{2} samples of leaf tissue were obtained. For species with large leaves, scissors were used to excise the sample from the leaf, and for species with small leaves, multiple leaves were used together. Surface area is a major factor determining the rate of electrolyte leakage from leaves (Whitlow et al. 1991), which is why we used a standardized leaf surface area for all our species. One leaf sample was placed into 7 mL of distilled water (i.e., control medium), and the other leaf sample was placed in 7 mL of 30 mM H\textsubscript{2}O\textsubscript{2} (i.e., experimental medium). We used this concentration of H\textsubscript{2}O\textsubscript{2} because previous studies in our laboratory have shown that it reduces vegetative growth without preventing flowering (Dave and Brown, unpublished observations), suggesting that it causes mild oxidative stress in plants. The conductivity of the media was immediately measured using a conductivity meter (Extech EC400), and then every 10 minutes thereafter for 60 minutes. Both leaf samples were kept at room temperature. The rate of cellular damage (estimated from the rate of change in the conductivity of the medium, in μS min\textsuperscript{-1}) for both control and experimental leaves was calculated from these measurements and corrected for changes in the conductivity of the external medium without leaves, which were measured simultaneously. The rate of cellular damage due solely to H\textsubscript{2}O\textsubscript{2} was determined as the difference between the experimental and control rates of electrolyte leakage, with the control rate of electrolyte leakage accounting for any differences in electrolyte permeability and/or harvest-induced cellular damage that might occur among different species.

To investigate the effects of exogenous H\textsubscript{2}O\textsubscript{2} on germination rate, we purchased seeds from various commercial suppliers. The species used for this experiment are listed in Table 2. Seeds were germinated in a
temperature- and humidity-controlled greenhouse in plastic Petri dishes lined with filter paper and filled with tap water
or H$_2$O$_2$ (10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, or 80 mM; diluted in tap water). Petri dishes
were refilled as necessary to prevent seeds from dehydrating. Petri dishes were covered with an opaque material so
that germination occurred in the dark. Seeds were examined for evidence of germination at approximately 12-hour
intervals. Germination was scored when the radicle had emerged from seed and was visible, as radicle emergence has
been shown to be a repeatable and reproducible test of seed vigour (Matthews et al. 2011). Germination was scored
until no further germination was observed for at least two days. A magnifying glass was used where necessary to
examine germination in smaller seeds. In order to determine the H$_2$O$_2$ concentration that brought about the fastest time
to 50 % germination in each species (i.e., hereafter called ‘optimal [H$_2$O$_2$]’), we fit the data for time since sowing and
number of germinated seeds to a four-parameter logistic curve and determined the inflection point, which was
interpreted as the time required for 50 % of the seeds to germinate. The curve fitting was accomplished using AAT
Bioquest’s Four Parameter Logistic Curve Calculator, which is available at: https://www.aatbio.com/tools/four-
parameter-logistic-4pl-curve-regression-online-calculator.

To investigate the susceptibility of chlorophyll a and chlorophyll b to exogenous H$_2$O$_2$, as well as chlorophyll
a/b ratios, leaf samples were harvested in early autumn from plants growing naturally on the University of Toronto
Scarborough campus grounds, as well as public and private (with permission) lands in the adjacent neighbourhoods
and nearby commercial buildings. As for the electrolyte leakage assay above, only green leaves were harvested. When
not already known, plants were identified using publicly-available databases (e.g., ontariotrees.com). For plants
known to exhibit different life history strategies in different regions, we classified them according to the life history
strategies that they most commonly exhibit when grown in southern Ontario (or the particular region from which they
were harvested). The species used for this experiment are listed in Table 3. The leaf samples were ground in 5mL of
100% with acetone using a mortar and pestle. Subsequently, the ground leaf samples were filtered (using coffee filters
inserted into funnels) in order to remove any insoluble debris (e.g., unbroken leaves). The resulting chlorophyll extract
was partitioned into two aliquots. To one aliquot, 1mL of tap water was added (as a control); to the other aliquot, 1mL
of 30mM H$_2$O$_2$ was added. Both aliquots were mixed and, immediately, the absorbance of each aliquot was measured
at 662nm and 644nm using a visible spectrophotometer (Vinmax LDC, Model 721), which allowed for the calculation
of both chlorophyll a and b concentrations using previously-published equations (Holm, 1954). The aliquots were then
placed in the dark, and their absorbance at these same two wavelengths was remeasured again after 30 and 60 minutes,
which allowed us to calculate the rate of degradation of both chlorophyll a and chlorophyll b. Chlorophyll a/b ratios were calculated from time 0 measurements made from the aliquots to which water had been added.

Statistical analysis of the data was conducted using the Univariate General Linear Model procedure of SPSS (version 23) with lifespan as the fixed factor and electrolyte leakage rate, optimal [H$_2$O$_2$], chlorophyll degradation rate, or chlorophyll a/b ratio as the dependent variable, as appropriate. The least significant difference post-hoc test was used to determine specific differences among annuals, biennials, deciduous perennials, and evergreen perennials, where appropriate. For chlorophyll degradation rates, a covariate (chlorophyll concentration at time = 0) was included in the analysis since chlorophyll degradation rates were likely to be dependent on the amount of chlorophyll present initially. In addition, for chlorophyll degradation rates, we used the t-test function in Google Sheets to determine the P-value for comparisons between water- and H$_2$O$_2$-induced chlorophyll degradation rates as well as degradation rates between chlorophyll a and b. For all tests, differences were considered significant if P < 0.05.

The construction of a phylogenetic tree and its utilization for ancestral state reconstruction of life history strategy was accomplished using R (R Core Team 2017) and its ‘bbranching’ package, which permits the creation of supertrees using Phylomatic (Webb and Donoghue 2004). A list of the 84 species used in this study, as well as their life history strategies, were input and read by the packages installed. Using the Zanne et. al (2014) phylogeny database as a reference, a circular phylogenetic tree was constructed with each species assigned to its corresponding life history. After ensuring the tree was rooted by having a common ancestor, we used a continuous-time Markov chain model to perform ancestral state reconstruction (Pagel et al. 2004). Scaled likelihoods were measured at each root of the tree. For a visual representation of the tree after the analysis of the ancestral state was determined, each root included the likelihood pie graphs of each life history. The margin of error for these likelihood values is 0.05%. The R code used for this entire procedure is provided in an Appendix for reference.
Results

The rate of electrolyte leakage, which is a common marker of cellular damage, of leaves exposed to distilled water was above zero in all plant species used in this study, but did not differ significantly among life history strategy, being the same in annuals, deciduous perennials, and evergreen perennials (P = 0.892; Fig. 1a). By contrast, the rate of electrolyte leakage of leaves due solely to exogenous H$_2$O$_2$ exposure, which was the difference in electrolyte leakage rate between H$_2$O$_2$- and distilled water-exposed leaves, was 7.5- and 13-fold higher in annuals compared to deciduous perennials (P = 0.043) and evergreen perennials (P = 0.035), respectively (Fig. 1b).

To determine the H$_2$O$_2$ concentration that brought about the fastest time to 50% germination (i.e., optimal [H$_2$O$_2$]), for each species, we measured the number of germinated seeds at roughly 12-hour intervals until either all seeds had germinated or no further germination had occurred for several days. Subsequently, we fit these data using a 4-parameter logistic curve, using the inflection point as the time to 50% germination. Representative data sets for one annual, one biennial, one deciduous perennial, and one evergreen perennial are shown in Fig. 2. Time to 50% germination for seeds sown in water did not differ significantly among life history strategies (P = 0.13; Fig. 3a), though it tended to be fastest in annuals. As expected, exogenous H$_2$O$_2$ reduced the time to 50% germination in all species (except Dianthus barbatus). The overall effect of lifespan on optimal [H$_2$O$_2$] was significant (P = 0.03). Post-hoc analysis revealed that the optimal [H$_2$O$_2$] was 2-fold higher for deciduous perennials than annuals (P = 0.010) and biennials (P = 0.017) but was not higher than evergreen perennials (P = 0.122; Fig. 3b). Optimal [H$_2$O$_2$] did not differ among annuals, biennials, and evergreen perennials (P > 0.05 for all comparisons).

Both chlorophyll a and chlorophyll b showed a significantly greater rate of degradation when exposed to H$_2$O$_2$ compared to water (P = 0.017 and P = 0.005, respectively; data not shown), confirming that H$_2$O$_2$ reacts with and alters the chemical structure of chlorophyll. We calculated the H$_2$O$_2$-specific rate of chlorophyll degradation as the difference between these two chlorophyll degradation rates (i.e., water- and H$_2$O$_2$-exposed). The H$_2$O$_2$-specific degradation rate of chlorophyll b was found to be nearly 2-fold higher than that of chlorophyll a (compare Figs. 4a, 4b; P = 0.007). No significant difference among life history strategy was observed for chlorophyll a/b ratio (P = 0.612; Fig. 4c).

For all three of our experiments combined, we analyzed a total of 84 different species (including variants and subspecies). A phylogenetic tree was constructed to illustrate the evolutionary relationships among these species (Fig. 5). Only 78 species are shown on this tree because i) two species (Abies balsamea and Allium porrum) were not found in the Zanne et al. (2014) database used to construct this tree, and ii) the four variants of Brassica oleracea and two
variants of *Beta vulgaris* were not identified separately in the database. An ancestral state reconstruction for these species revealed that the perennial evergreen condition is most likely ancestral (39.4% likelihood vs. 24.2% for deciduous perennial, 18.8% for annual, and 17.6% for biennial). From this evergreen perennial ancestor, there evolved a fern lineage, an evergreen perennial lineage, and a deciduous perennial lineage. The evergreen perennial lineage gave rise to various evergreen perennial species but also *Ginkgo biloba*, which is a deciduous perennial species. No annuals or biennial species have descended from this evergreen lineage. The deciduous perennial lineage has given rise to species with all four life history strategies, with annual and biennial species having evolved only relatively recently. Notably, within the lineage, we only observed two instances where a suspected annual ancestor gave rise to a perennial descendant (*Iberis sempervirens* and *Mentha spicata*).
Discussion

Our ancestral state reconstruction of the 84 species used in this study yielded two major findings. First, the evergreen perennial condition is the most likely ancestral life history strategy in plants. From this evergreen ancestor, there arose a fern lineage; an evergreen plant lineage, which may have given rise to the gymnosperms; and a deciduous plant lineage, which may have given rise to the flowering plants (Taylor and Hickey 1992). Second, at multiple, independent times, annuals and biennials evolved within this deciduous perennial lineage, suggesting a transition to shorter lifespans in plants over time. Several factors are known to favour the annual strategy in plants, including short growing season length, low habitat reliability, and low storage efficiency (Iwasa and Cohen 1989); however, it is not clear how these factors have changed over time on Earth since plants first arose. Notwithstanding, we will discuss our findings on the effects of exogenous H$_2$O$_2$ on plants in the context of this phylogenetic information as such an approach will permit us to understand how the physiological mechanisms associated with ROS tolerance in plants may have evolved. What is particularly striking about our ancestral state reconstruction is how infrequently perennial species re-evolved from annual ancestors: in our analysis, it happened only twice (with *Iberis sempervirens*, an evergreen perennial, and *Mentha spicata*, a deciduous perennial, both having evolved independently from an annual ancestor). While evidence for annual-to-perennial transitions is not absent (Tank and Olmstead 2008), it does seem to be relatively rare. We ponder whether the physiological changes that occur within plants during the perennial-to-annual transition are difficult to reverse, and we address this possibility as well.

The rate of cellular damage of excised leaves due solely to exogenous H$_2$O$_2$ exposure was significantly lower in both deciduous and evergreen perennials compared to annuals. While we had expected the leaves of evergreen perennials to be more resistant to exogenous H$_2$O$_2$ exposure than those of annuals, since the leaves of evergreen perennials can live as long as 40 years (Aerts 1995), we had not expected the leaves of deciduous perennials to be equally resistant to H$_2$O$_2$ exposure since the leaves of deciduous perennials persist for less than one year. It seems, therefore, that the resistance of leaves to exogenous H$_2$O$_2$ depends on the lifespan of the entire plant, not the lifespan of the leaves themselves. One possible explanation for this observation is that ROS produced within one plant tissue—whether directly or via free radical chain reactions, which can produce secondary ROS that have been shown to be particularly important in aging (Montgomery et al. 2011)—can freely diffuse to other plant tissues, causing oxidative damage at locations distant to the site of ROS production; therefore, even though the leaves of deciduous perennials do not, themselves, survive longer than one year, they may have mechanisms to eliminate any ROS produced within them in order to prevent ROS from diffusing to the perennating tissues, such as buds, roots, and tubers. Indeed, it has
been shown that H$_2$O$_2$, whose chemical structure is similar to that of water, uses certain aquaporins (called ‘peroxiporins’) to cross cell membranes (Henzler and Steudle 2000), though it is unclear how far leaf-derived ROS could travel within a plant. We have routinely grown flax in diluted H$_2$O$_2$ solutions in our greenhouse and have observed that it negatively impacts not only root growth but also shoot growth, suggesting that H$_2$O$_2$ can travel from the roots to the apical meristem (personal observations).

Electrolyte leakage from plant cells in response to abiotic stress (e.g., exogenous H$_2$O$_2$) is thought to reflect the loss of cell membrane integrity, which is a common definition of cell death. Therefore, it is likely that exogenous H$_2$O$_2$ accelerated the rate of electrolyte leakage from the leaves in our study because it induced cell death. Although H$_2$O$_2$ is known to induce apoptosis in plants (Gadjev et al. 2008), at the high concentrations used in this experiment, it is more likely that H$_2$O$_2$ caused cellular death via necrosis (Saito et al. 2006). Brunk et al. (1995) have shown that, when cells are exposed to high levels of H$_2$O$_2$, damage to lysosomal membranes occurs, causing the leakage of destructive enzymes (possibly including phospholipases) that degrade the cell within 30 minutes, which is shorter than the length of time to which our leaves were exposed to exogenous H$_2$O$_2$. Given the short duration that our leaves were exposed to exogenous H$_2$O$_2$, we believe that there are two possible reasons why deciduous and evergreen perennial leaves experienced less H$_2$O$_2$-induced cellular damage than annuals. First, deciduous and evergreen perennial leaves may have higher antioxidant activities than annual leaves, permitting them to better detoxify any exogenous H$_2$O$_2$. Consistent with this hypothesis, Brown et al. (2012) showed that perennial flax (which are deciduous) had higher catalase activity than annual flax. Second, the cell membranes of deciduous and evergreen perennial leaves may have a lower proportion of polyunsaturated fatty acids (PUFA) than those of annual leaves. It is well established that PUFA are more susceptible to oxidative attack via ROS, and there is evidence in animals that long-lived animals frequently have lower levels of PUFA that short-lived animals (Pamplona et al. 1999). We suspect that the former possibility is more likely than the latter because, given that evergreen leaves must be able to endure low temperatures during winter, we would expect that their cell membranes would be enriched with PUFA, as these fatty acids help maintain membrane fluidity during cold exposure (Samala et al. 1998).

Bulk membranes are thought to be relatively impermeable to H$_2$O$_2$ (Bienert et al. 2006), so it seems likely that exogenous H$_2$O$_2$ enters leaves through either stomata or aquaporin channels expressed in the leaf epidermal cells (Bienert and Chaumont 2014; Hachez et al. 2008), in the same manner as water. On this basis, it is possible that the lower rate of H$_2$O$_2$-induced cellular damage in deciduous and evergreen perennials observed in this study resulted from their lower H$_2$O$_2$ permeability compared to annual leaves; however, we believe that this possibility is unlikely.
With regards to stomatal density, Wang et al. (2015) showed that stomatal density was nearly 2-fold higher in broadleaf deciduous leaves than evergreen conifers, and Liu et al. (2019) showed that stomatal density was lowest in C3 annuals, with C4 annuals as well as perennials being higher but not different from each other. With regards to aquaporin expression, Brodribb et al. (2004) showed that the hydraulic conductance of angiosperms (both annuals and perennials) was 3- to 4-fold higher than that of conifers, likely reflecting a significant difference in leaf aquaporin expression. Consequently, it is clear that the patterns of ROS-induced cellular damage observed in this study do not match previously-established patterns in either stomatal density and/or aquaporin expression, leading us to conclude that differential leaf H2O2 permeability cannot likely explain our results.

For all species examined, there was significant electrolyte leakage from leaves soaked in distilled water (i.e., in the absence of H2O2). This electrolyte leakage from control leaves is not likely due to leaves beginning autumnal senescence prior to the time of harvesting because i) only green leaves were used, as discussed in the Methods and Materials, and ii) electrolyte leakage was observed in annuals and deciduous perennials to same extent as in evergreen leaves, even though the latter do not exhibit autumnal senescence (Chapin and Kedrowski 1983). Some previous studies have shown that necrosis in leaf samples does occur even in the absence of perturbing factors (Maxwell et al. 1997), so it is possible that the electrolyte leakage observed for leaves in distilled water in our study reflects the onset of cellular damage following leaf excision. Bar-Dror et al. (2011) have shown that, following leaf abscission, there are changes in nuclear morphology and nuclear fragmentation—hallmarks of apoptosis—that occur in the leaf cells distal to the abscission zone. Separation of a leaf from its mother plant, whether naturally-occurring or forceful, may induce cellular damage, leading to electrolyte leakage. Regardless of the underlying mechanism by which electrolyte leakage occurred in leaves exposed to distilled water in this study, there were no differences in the rate of electrolyte leakage among annuals, deciduous perennials, and evergreen perennials. This reinforces that the differences in rates of cellular damage that we observed in response to H2O2 reflected a lesser capacity for annuals to mitigate the effects of exogenous ROS exposure compared to perennials, rather than simply inherent differences in the invocation of cell death responses.

Given that the evergreen perennial strategy is likely ancestral in plants, our observation that evergreen perennial leaves are better able to resist damage in the face of exogenous H2O2 suggests that cellular mechanisms to protect leaf cells from ROS damage have likely existed since plants first evolved. This finding is consistent with work by Inupakutika et al. (2016) who showed that genes encoding ROS scavenging mechanisms originated as early as 4.1 billion years ago, prior to the first great oxygenation event, suggesting that localized oxygen originating from
photosynthesis is likely sufficient to allow for ROS production, necessitating the endogenous synthesis of antioxidant enzymes. Deciduous perennial leaves would likely have retained this inherited trait, despite their transition to an annual leaf-dropping character, in order to limit their production of ROS and its possible transfer to perennating tissues, as described above. By contrast, annuals may have abandoned this trait due to its cost, allowing them to direct more energy towards reproduction. Indeed, Vaanholt et al. (2008) showed that the daily energy expenditure of mice was positively correlated with antioxidant levels in the liver, suggesting that antioxidant synthesis (and likely other ROS protective measures) may entail a substantial energetic burden for organisms.

Compared to annuals, we observed that deciduous perennials—but not evergreen perennials or biennials—required a higher concentration of exogenous \( \text{H}_2\text{O}_2 \) in order to bring about maximal germination rate, which is consistent with the notion that the seeds of these species have an increased capacity to eliminate ROS, most likely via elevated antioxidant levels, as predicted by OSTA. It is important to note that the seeds used for this experiment were germinated within a few months of being purchased, so their antioxidant levels should have been maximal. This is a relevant consideration because it is known that antioxidant levels in seeds decrease with storage time (Pukacka 1991).

The fact that evergreen perennials did not also require higher \( \text{H}_2\text{O}_2 \) concentrations to maximize their germination rate is a curious observation given their comparable longevity to deciduous perennials, but we believe that our ancestral state reconstruction may offer some insight here. While high seed antioxidant levels may better protect developing plants against oxidative damage, they may also retard germination rate, as \( \text{H}_2\text{O}_2 \) is thought to facilitate several processes necessary for germination. Given that evergreen perennials were likely the first plants to evolve, they would have felt pressure to rapidly colonize the terrestrial environment in order to establish themselves. Consistent with this notion, Wainwright and Cleland (2013) found that invasions by exotic plant species were facilitated by their faster germination rates compared to native species. On this basis, evergreen perennials may only begin to express antioxidants following germination, as their tissues begin to develop and mature, in order to facilitate rapid germination and colonization of their landscape. When deciduous perennials subsequently evolved, they would have found themselves in direct competition with evergreen perennials, which occurs even in modern habitats (Álvarez-Yépez et al. 2017). Deciduous perennials may have advanced the timing of their antioxidant expression so that their seeds were better endowed with protection against ROS. While this would have slowed down their germination rate, it would also have significantly increased their seed viability during storage (and soil bank dormancy) compared to evergreen perennials (Panetta 2008), which, in turn, would have increased their ability to survive periods of climatic variability (Morris et al. 2008).
Biennials were found to be like annuals, from the perspective of exogenous H$_2$O$_2$ concentrations required to maximize germination rate. Although biennials have a longer lifespan than annuals (2 years vs. 1 year), biennials have a much shorter lifespan than deciduous and evergreen perennials, the average lifespan of which is 35 years (Ehrlén and Lehtilä 2002); therefore, from the perspective of OSTA, it is not surprising that biennials would be more similar to annuals than perennials. Moreover, our ancestral state reconstruction revealed that, similar to the annual condition, the biennial condition evolved only recently in the history of plants, further suggesting that biennials are more annual-like than perennial-like.

We did not observe a significant difference in germination time among annuals, biennials, deciduous perennials, and evergreen perennials in the present study, although germination time tended to be lower in annuals, consistent with previous work (Shipley and Parent 1991). Even if we presume that annuals generally do have faster germination rates than other plant species, it remains unlikely that this observation would account for the results of our study for two reasons. First, the optimal [H$_2$O$_2$] for annuals was approximately 25mM in the present study, which is substantially higher than the endogenous [H$_2$O$_2$] reported previously for dry and germinating annual seeds (2-3µM in *Pisum sativum*; Barba-Espín et al. 2011). Given the large concentration of exogenous H$_2$O$_2$ required to maximize germination rate, it is unlikely that endogenous variation in H$_2$O$_2$ levels among seeds from different species would have any significant effect on germination rate and/or optimal [H$_2$O$_2$]. Second, evergreen perennials, despite tending to have longer germination times than annuals, had the same optimal [H$_2$O$_2$] as annuals, again suggesting that differences in endogenous H$_2$O$_2$ levels (or germination rate) did not determine the amount of exogenous H$_2$O$_2$ required to maximize germination rate. The most likely explanation for the differences observed in terms of optimal [H$_2$O$_2$] in the present study is that seeds from deciduous perennials have a greater H$_2$O$_2$ degradation capacity than seeds from other plants, which helps them to keep ROS production under control during early development, thereby permitting increased longevity as predicted by the OSTA.

Neither chlorophyll a nor chlorophyll b, following its extraction from leaves, showed any difference in susceptibility to exogenous H$_2$O$_2$ exposure among species with different life history strategies. Given that the structure of chlorophyll a and chlorophyll b is the same in all the species investigated in this study, this finding is unsurprising. Our study did reveal, however, that isolated chlorophyll b was approximately 2-fold more susceptible to exogenous H$_2$O$_2$ exposure than isolated chlorophyll a, suggesting that the formyl group may be more susceptible to ROS attack than the methyl group, as this is the only structural difference between these two pigments. To our knowledge, little work has been done to assess the ROS susceptibility of the formyl group, though it has been shown that tocopherols
with a higher number of methyl groups serve as better antioxidants, being more capable of interacting with ROS (Sharma et al. 2012), so we suspect that the formyl group must be even more susceptible to ROS attack. Given that chlorophyll b is more susceptible to degradation in the face of exogenous H$_2$O$_2$ than chlorophyll a, it might be expected that perennial plants would minimize the amount of chlorophyll b within their photosystems, resulting in a higher chlorophyll a/b ratio; however, this was not observed in the present study. Reger and Krauss (1970) used illumination intensity to modify chlorophyll a/b ratios in *Chlorella vannielli* and found that higher chlorophyll a/b ratios caused a significant decline in photophosphorylation capacity, requiring supplementation via oxidative phosphorylation to maintain ATP balance. On this basis, perennials may be constrained with regard to their ability to modulate chlorophyll a/b ratios to prevent oxidative stress. Consistent with this notion, Smeets et al. (2005) found that chlorophyll a/b ratio was not altered following Cd exposure in beans, a condition which is known to cause oxidative stress.

Chlorophyll is bound to various light-harvesting proteins in vivo (Büchel 2015), so it is possible that these proteins serve to shield chlorophyll from ROS exposure. It is even possible that the amino acid sequence of these proteins may differ between annuals and perennials in a way that maximizes the ability of proteins from the latter to react with, and thereby neutralize, ROS that would otherwise inflict damage upon chlorophyll. Moreover, there is evidence that certain plant secondary metabolites can function as antioxidants to mitigate the effects of ROS (Franklin et al. 2009), so their presence within chloroplasts could also serve to protect chlorophyll from ROS exposure. Furthermore, Nisbett et al. (submitted) have shown that perennials have a greater capacity to synthesize and repair chlorophyll following exposure to oxidative stress, so it is also possible that perennials rely upon their repair capabilities to replenish their chlorophyll stores in the face of any oxidative damage.

Our ancestral state reconstruction revealed that the evolution of perennial species from annual ancestors is quite rare, having occurred only twice in the evolutionary history of the 84 species that we examined in this study. While recent climatic shifts to conditions, such as aridity and drought, that favour the annual condition have been proposed to explain this observation (Evans et al. 2007), it prompted us to consider whether there may be physiological constraints that prevent a reversion to perennialism from annualism. Most of our results have suggested that the longevity of perennial plants may result from their higher levels of antioxidants. Hasanuzzaman et al. (2014) reported that in rice, which is normally grown as an annual, upregulation of the antioxidant defense system in the face of oxidative stress is limited by the lack of biosynthetic capacity of regulatory molecules, such as proline and glycine betaine, and further demonstrated that exogenous supplementation of these compounds could significantly boost antioxidant expression. It is possible that annual plants have reduced their proline and/or glycine betaine biosynthetic
capacity in order to redirect resources to reproduction. Perhaps, then, transition from the annual to perennial condition is constrained by lack of environmental availability of such molecules or their precursors. Indeed, Hayat et al. (2012) found that both proline and antioxidant levels could be boosted in tomatoes, an annual species, via exogenous application of sodium nitroprusside, a nitrogen donor, consistent with the notion that, in annuals, their ROS resistance may be intimately tied to environmental nutrient supply.

While our study is the first, to our knowledge, to provide evidence that perennial plant species have physiological mechanisms to minimize the accumulation of oxidative damage, consistent with the OSTA, we do recognize that our study has three significant limitations. First, many aging studies conducted in animals treat maximum lifespan as a continuous variable and look for correlations between maximum lifespan and potential aging-related variables. This is possible because there are databases from which the maximum lifespan of most animal species can be easily extracted (e.g., AnAge: https://genomics.senescence.info/species/). To our knowledge, no similar database is available for plants, which requires studies conducted in plants to treat lifespan as a discrete variable (annual, biennial, deciduous perennial, evergreen perennial), as we did. One of the drawbacks to this approach is that some plants straddle the line between different categories, growing as annuals in some regions but as perennials in other regions. Indeed, nearly half of the plants that we classified as annual plants in our study are known to exhibit perennialism under some growing conditions, though not in the region where our study was conducted, which is why we classified them as annuals. Franco and Silvertown (1996) have shown that continuous variables, such as adult mortality rate, can account for the different life histories found in plants, and future aging studies in plants should examine whether there are relationships between these variables and resistance to exogenous H$_2$O$_2$ exposure. The construction of databases for such information would be a helpful first stage in this process. Second, we only made a single measurement of H$_2$O$_2$ resistance for each plant species in our experiments. One of us (JCLB) has experience with conducting aging studies in animals (Brown et al. 2009), and the most common critique of such studies is their lack of phylogenetic coverage; therefore, we sought to maximize our phylogenetic coverage in the present study, and this would not have been feasible if we had made multiple measurements for each plant species. Certainly, we recognize that neglecting intraspecific variation—while common practice in comparative studies—is problematic (Garamszegi and Møller 2010), but we feel that it was justified because our stated objective for this study was to extend our observations from flax to the broadest possible range of species. Third, the plant material used in this study was obtained from various sources, i.e., plants growing naturally or artificially in the local area and/or seeds available for purchase from commercial suppliers. We utilized this approach because it would be impossible for us to grow
plants from the 84 different species investigated in this study under the same conditions in such a manner where not only leaves but also seeds could be harvested and analyzed. We recognize that this approach precludes our ability to account for certain factors on our measurements; for example, electrolyte leakage is known to increase with age for potato tubers (De Weerd et al. 1995), so knowing the age of the leaves used in our electrolyte leakage experiment could have allowed us to control for its effects on our results. Nevertheless, we submit that our inability to control for these factors should have increased the variability in our dataset, thereby making it more difficult for us to obtain statistically significant results (Biau et al. 2008); therefore, the fact that we observed significant differences among life history strategies for both cellular damage rate and optimal H$_2$O$_2$ for germination rate underscores the robustness of these findings.

Overall, our results demonstrate support for OSTA in plants as we have shown that both deciduous and evergreen perennials exhibit greater resistance to the deleterious effects of exogenous H$_2$O$_2$ compared to annuals during at least some point in their life. Furthermore, our ancestral state reconstruction has shown that the evergreen perennial state is ancestral in plants, suggesting that mechanisms to resist ROS-induced biomolecular damage are ancient. Future studies in our laboratory will focus on investigating the underlying mechanisms responsible for this increased ROS tolerance. In particular, we plan to measure cellular antioxidant levels in annuals, deciduous perennials, and evergreen perennials, especially since our data have suggested that antioxidant levels may be higher in these latter two groups.
References

Aerts R (1995) The advantages of being evergreen. Trends Ecol Evol 10:402-407

Álvarez-Yépez JC, A Búrquez, A Martinez-Yrizar, M Teece, EA Yépez, M Dovciak (2017) Resource partitioning by evergreen and deciduous species in a tropical dry forest. Oecologia 183:607-618

Bandyopadhyay U, Das D, Banerjee RK (1999) Reactive oxygen species: oxidative damage and pathogenesis. Curr Sci 77:658-666

Bar-Dror T, Dermastia M, Kladnik A, Žnidaric MT, Novak MP, Meir S, Burd S, Philosoph-Hadas S, Ori N, Sonego L, Dickman MB, Lers A (2011) Programmed cell death occurs asymmetrically during abscission in tomato. The Plant Cell 23:4146-4163

Barba-Epsín G, Hernández JA (2012) Role of H$_2$O$_2$ in pea seed germination. Plant Signal Behav 7:193-195

Barba-Epsín G, Diaz-Vivancos P, Job D, Belghazi M, Job C, Hernández JA (2011) Understanding the role of H$_2$O$_2$ during pea seed germination: a combined proteomic and hormone profiling approach. Plant Cell Env 34:1907-1919.

Bazzaz FA, Morse SR (1991) Annual plants: potential responses to multiple stresses. In: Mooney HA, Winner WE, Pell EJ (eds) Integrated response of plants to stress. Academic Press, Orlando, pp. 283-306

Bena G, Lejeune B, Prosperi J-M, Olivieri I (1998) Molecular phylogenetic approach for studying life-history evolution: the ambiguous example of the genus Medicago L. Proc R Soc Lond 265:1141-1151

Bertold M, Manuela B, Inge D, Sebastien L, Lorène MJ, Charly G, Miguel P-E, Hamada A, Han A, Matteo C (2019) Detecting the onset of autumn leaf senescence in deciduous forest trees of the temperate zone. New Phytol 224:166-176.

Biau DJ, Kernéis S, Porcher R (2008) Statistics in brief: the importance of sample size in the planning and interpretation of medical research. Clin Orthop Relat Res 466:2282-2288.

Bienert GP, Chaumont F (2014) Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim Biophys Acta 1840:1596-1604

Bienert GP, Schjoerring JK, Jahn TP (2006) Membrane transport of hydrogen peroxide. Biochim Biophys Acta 1758:994-1003

Brodribb TJ, Holbrook NM, Zwieniecki MA, Palma B (2004) Leaf hydraulic capacity in ferns, conifers, and angiosperms: impacts on photosynthetic maxima. New Phytologist 165:839-846

Brown JCL, Marshall KE, Staples JF (2012) Differences in tissue concentrations of hydrogen peroxide in the roots and cotyledons of annual and perennial species of flax (Linum). Bot 90:1015-1027
Brown JCL, McClelland GB, Faure PA, Klaiman JM, Staples JF (2009) Examining the mechanisms responsible for lower ROS release rates in liver mitochondria from long-lived house sparrow (Passer domesticus) and big brown bat (Eptesicus fuscus) compared to the short-lived mouse (Mus musculus). Mech Ageing Dev 130:467-476

Brunk UT, Zhang H, Dalen H, Öllnger K (1995) Exposure of cells to nonlethal concentrations of hydrogen peroxide induces degeneration-repair mechanisms involving lysosomal destabilization. Free Radic Biol Med 19:813-822

Büchel C (2015) Evolution and function of light harvesting proteins. J Plant Physiol 172: 62-75

Cakmak I, Strbac D, Marschner H (1993) Activities of hydrogen peroxide-scavenging enzymes in germination wheat seeds. J Exp Bot 44:127-132

Chapin III FS, Kedrowski RA (1983) Seasonal changes in nitrogen and phosphorus fractions and autumn retranslocation in evergreen and deciduous Taiga trees. Ecology 64:376-391.

Charlesworth B (2001) Patterns of age-specific means and genetic variances of mortality rates predicted by the mutation-accumulation theory of aging. J Theor Biol 210:47-65

Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers Environ Sci 2 doi:10.3389/fenvs.2014.00053

Datson PM, Murray BG, Steiner KE (2008) Climate and the evolution of annual/perennial life-histories in Nemesia (Scrophulariaceae). Plant Systematics Evol 270:39-57

De Weerd JW, Hiller LK, Thornton RE (1995) Electrolyte leakage of aging potato tubers and its relationship with sprouting capacity. Potato Res 38:257-270.

Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR, Gems D (2008) Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans. Genes Dev 22:3232-3241

Dowling DK, Simmons LW (2009) Reactive oxygen species as universal constraints in life-history evolution. Proc Royal Soc B 276: 1737-1745

Ehrlén J, Lehtilä K (2002) How perennial are perennial plants? OIKOS 98:308-322

Estiarte M, Peñuelas J (2015) Alteration of the phenology of leaf senescence and fall in winter deciduous species by climate change: effects on nutrient proficiency. Glob Change Biol 21:1005-1017

Evans MEK, Hearn DJ, Hahn WJ, Spangle JM, Venable DL (2007) Climate and life-history evolution in evening primroses (Oenothera, Onagraceae): a phylogenetic comparative analysis. Evol 59:1914-1927
Franco M, Silvertown J (1996) Life history variation in plants: an exploration of fast-slow continuum hypothesis. Phil Trans R Soc Lond B 351:1341-1348

Franklin G, Conceição LFR, Kombrink E, Dias ACP (2009) Xanthone biosynthesis in Hypericum perforatum cells provides antioxidant and antimicrobial protection upon biotic stress. Phytochemistry 70: 60-68

Friedman J (2020) The evolution of annual and perennial plant life histories: ecological correlates and genetic mechanisms. Ann Rev Ecol Evol Syst 51:461-481

Gadjev I, Stone JM, Gechev TS (2008) Programmed cell death in plants: new insights into redox regulation and the role of hydrogen peroxide. Int Rev Cell Mol Biol 270:84-144

Garamszegi LZ, Møller AP (2010) Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. Biol Rev 85:797-805

Golden TR, Hinerfeld DA, Melov S (2002) Oxidative stress and aging: beyond correlation. Aging Cell 1:117-123

Hachez C, Heinen RB, Draye X, Chaumont F (2008) The expression pattern of plasma membrane aquaporins in maize leaf highlights their role in hydraulic regulation. Plant Mol Biol 68:337-353

Hart R (1977) Why are biennials so few? Am Nat 111:792-799

Hasanuzzaman M, Alam MM, Rahman A, Hasanuzzaman M, Nahar K, Fujita M (2014) Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxylase systems provides better protection against salt-induced oxidative stress in two rice (Oryza sativa L.) varieties. BioMed Res Int https://doi.org/10.1155/2014/757219

Hatsugai N, Katagiri F (2018) Quantification of plant cell death by electrolyte leakage assay. BioProtocol 8:1-7

Hayat S, Yadav S, Wani AS, Irfan M, Alyemini MN, Ahmad A (2012) Impact of sodium nitroprusside on nitrate reductase, proline content, and antioxidant system in tomato under salinity stress. Hort Environ Biotechnol 53:362-367

Henzler T, Steudle E (2000) Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H₂O₂ across water channels. J Exp Bot 51:2053-2066

Herrero A, Barja G (1998) H₂O₂ production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. Mech Ageing Dev 103:133-146

Holm G (1954) Chlorophyll mutations in barley. Acta Agriculturae Scandinavica 4:457-471
Hsu C-Y, Yang C-M, Chen C-M, Chao P-Y, Hu S-P (2005) Effects of chlorophyll-related compounds on hydrogen peroxide induced DNA damage within human lymphocytes. J Agric Food Chem 53:2746-2750.

Hulbert AJ, Kelly MA, Abbott SK (2014) Polyunsaturated fats, membrane lipids and animal longevity. J Comp Physiol B 184:149-166

Inupakutika MA, Sengupta S, Devireddy AR, Azad RK, Mittler R (2016) The evolution of reactive oxygen species metabolism. J Exp Bot 2016: 5993-5943

Iwasa Y, Cohen D (1989) Optimal growth schedule of a perennial plant. Am Nat 133:480-505.

Kenrick P, Crane PR (1997) The origin and early evolution of plants on land. Nature 389:33-39

Klinkhamer PGL, Kubo T, Iwasa Y (1997) Herbivores and the evolution of the semelparous perennial life-history of plants. J Evol Biol 10:529-550

Kurepa J, Smalle J, Van Montagu M, Inzé D (1998) Oxidative stress tolerance and longevity in Arabidopsis: the late-flowering mutant gigantea is tolerant to paraquat. Plant J 14:756-764

Liu H, Taylor SH, Xu Q, Lin Y, Hou H, Wu G, Ye Q (2019) Life history is a key factor explaining functional trait diversity among subtropical grasses, and its influence differences between C3 and C4 species. J Exp Bot 70:1567-1580

MacRae SL, Croken MM, Calder RB, Aliper A, Milholland B, White RR, Zhavoronkov A, Gladyshev VN, Seluanov A, Gorbunova V, Zhang ZD, Vijg J (2015) DNA repair in species with extreme lifespan differences. Aging 7:1171-1182

Matthews S, Wagner M-H, Ratzenboeck A, Khajeh Hosseini M, Casarini E, El-Khadem R, Yakhlfifi M, Powell AA (2011) Early counts of radicle emergence during germination as a repeatable and reproducible vigour test for maize. Seed Test Int 141:39-45

Maxwell DL, Kruger EL, Stanosz GR (1997) Effects of water stress on colonization of poplar stems and excised leaf disks by Septoria musiva. Phytopathol 85:381-388

Montgomery MK, Hulbert AJ, Buttemer WA (2011) The long life of birds: the rat-pigeon comparison. PLoS ONE 6:e24138

Morris WF, Pfister CA, Tuljapurkar S, Haridas CV, Boggs CL, Boyce MS, Bruna EM, Church DR, Coulson T, Doak DF, Forsyth S, Gaillard J-M, Horvitz CC, Kalisz S, Kendall BE, Knight TM, Lee CT, Menges ES (2008) Longevity can buffer plant and animal populations against changing climatic variability. Ecology 89: 19-25

Munné-Bosch S, Alegre L (2002) Plant aging increases oxidative stress in chloroplasts. Planta 214:608-615
Nunes MES, Smith GR (2003) Electrolyte leakage assay capable of quantifying freezing resistance in rose clover. Crop Sci 43:1349-1357

Ogawa K, Iwabuchi M (2001) A mechanism for promoting the germination of Zinnia elegans seeds by hydrogen peroxide. Plant Cell Physiol 42:286-291

Pagel M, Meade A, Barker D (2004) Bayesian estimation of ancestral character states on phylogenies. Systematic Biol 53:673-684

Pamplona R, Portero-Otín M, Ruiz C, Gredilla R, Herrero A, Barja G (1999) Double bond content of phospholipids and lipid peroxidation negatively correlate with maximum longevity in the heart of mammals. Mech Ageing Dev 112:169-183

Pamplona R, Portero-Otín M, Sanz A, Ayala V, Vasileva E, Barja G (2005) Protein and lipid oxidative damage and complex I content are lower in the brain of budgerigar and canaries than mice: relation to aging rate. AGE 27:276-280

Panetta FD (2008) Seedling emergence and seed longevity of the tree weeds Celtis sinensis and Cinnamomum camphora. Weed Res 41:83-95

Pérez VI, Bokov A, Van Remmen H, Mele J, Ran Q, Ilkeno Y, Richardson A (2009) Is the oxidative stress theory of aging dead? Biochim Biophys Acta 1790:1005-1014.

Périšić T, Srećković M, Matić G (2007) Changes of antioxidant enzyme activity and heat shock protein content in lymphocytes of children with asthma. Arch Bio Sci 59:257-266

Pukacka S (1991) Changes in membrane lipid components and antioxidant levels during natural ageing of seeds of Acer platanoides. Physiol Plant 82:306-310

R Core Team (2017) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org

Reger BJ, Krauss RW (1970) The photosynthetic response to a shirt in the chlorophyll a to chlorophyll b ratio of Chlorella. Plant Physiol 46: 568-575

Riffle JW, Springfield HW (1968) Hydrogen peroxide increases germination and reduces microflora on seed of several southwestern woody species. For Sci 14:96-101

Robbins WJ (1957) Physiological aspects of aging in plants. Am J Bot 44:289-294

Robert KA, Brunet-Rossini A, Bronikowski AM (2007) Testing the ‘free radical theory of aging’ hypothesis: physiological differences in long-lived and short-lived colubrid snakes. Aging Cell 6:395-404
Samala S, Yan J, Baird WV (1998) Changes in polar lipid fatty acid composition during cold acclimation in ‘Midiron’ and ‘U3’ bermudagrass. Crop Sci 38:188-195

Saito Y, Nishio K, Ogawa Y, Kimata J, Kinumi T, Yoshida Y, Noguchi N, Niki E (2006) Turning point in apoptosis/necrosis induced by hydrogen peroxide. Free Rad Res 40:619-630

Salway KD, Gallagher EJ, Page MM, Stuart JA (2011) Higher levels of heat shock proteins in longer-lived mammals and birds. Mech Ageing Dev 132:285-297

Scheffer RP, Livingston RS (1980) Sensitivity of sugarcane clones to toxin from Helminthosporium sacchari as determined by electrolyte leakage. Phytopathol 70:400-404

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidant defense mechanism in plants under stressful conditions. J Bot 2012: 217037

Shipley B, Parent M (1991) Germination responses of 64 wetland species in relation to seed size, minimum time to reproduction and seedling relative growth. Funct Ecol 5:111-118

Smeets K, Cuypers A, Lambrecht A, Semane B, Hoet P, Van Laere A, Vangronsveld J (2005) Induction of oxidative stress and antioxidant mechanisms in Phaseolus vulgaris after Cd application. Plant Physiol Biochem 43: 437-444

Snezhkina AV, Kudryavtseva AV, Kardymon OL, Savvateeva MV, Melnikova NV, Krasnov GS, Dmitriev AA (2019) ROS generation and antioxidant defense systems in normal and malignant cells. Oxid Med Cell Longev https://doi:10.1155/2019/6175804

Soltis DE, Mort ME, Latvis M, Mavrodiev EV, O’Meara BC, Soltis PS, Burleigh JG, Rubio de Casis R (2013) Phylogenetic relationships and character evolution analysis of Saxifragales using a supermatrix approach. Am J Bot 100:916-923

Stadtman ER, Berlett BS (1998) Reactive oxygen-mediated protein oxidation in aging and disease. Drug Metab Rev 30:225-243

Sun L, Akha AAS, Miller RA, Harper JM (2009) Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age. J Gerontol 64A:711-722

Taneda H, Tateno M (2005) Hydraulic conductivity, photosynthesis and leaf water balance in six evergreen woody species from fall to winter. Tree Physiol 25:299-306

Tank DC, Olmstead RG (2008) From annuals to perennials: phylogeny of the subtribe Castillejinae (Orobanchaceae). Am J Bot 95:608-625
Taylor DW, Hickey LJ (1992) Phylogenetic evidence for the herbaceous origin of angiosperms. Plant Syst Evol 180:137-156

Tessier JT (2008) Leaf habitat, phenology, and longevity of 11 forest understory plant species in Algonquin State Forest, northwest Connecticut, USA. Bot 86:457-465

Vaanholt LM, Speakman JR, Garland Jr T, Lobley GE, Visser GH (2008) Protein synthesis and antioxidant capacity in aging mice: effects of long-term voluntary exercise. Physiol Biochem Zool 81:148-157

Wainwright CE, Cleland EE (2013) Exotic species display greater germination plasticity and higher germination rates than native species across multiple cues. Biol Invasions 15: 2253-2264

Wang R, Yu G, He N, Wang Q, Zhao N, Xu Z, Ge J (2015) Latitudinal variation of stomatal traits from species to community level in forests: linkage with ecosystem productivity. Sci Rep 5:14454

Webb CO, Donoghue MJ (2004) Phylomatic: tree assembly for applied phylogenetics. Mol Ecol Notes 5:181-183

Whitlow TH, Bassuk NL, Ranney TG, Reichert DL. (1992) An improved method for using electrolyte leakage to assess membrane competence in plant tissues. Plant Physiol 98:198-205

Williams-Linera G (1997) Phenology of deciduous and broadleaved-evergreen tree species in a Mexican tropical lower montane forest. Glob Ecol Biogeogr Lett 6:115-127

Zanne AE, Tank DC, Cornwell WK et al. (2014) Three keys to the radiation of angiosperms into freezing environments. Nature 506: 89-92
Table 1. List of species used for the electrolyte leakage experiment.

| Scientific Name                                      | Common Name       |
|------------------------------------------------------|-------------------|
| **Annuals (16 species)**                             |                   |
| *Abelmoschus esculentus*                             | Okra              |
| *Alliaria petiolata*                                 | Garlic Mustard    |
| *Ambrosia artemisiifolia*                            | Ragweed           |
| *Brassica nigra*                                     | Black Mustard     |
| *Brassica oleracea var. sabellica*                   | Green Kale        |
| *Capsicum annuum*                                    | Green Bell Pepper |
| *Capsicum annuum*                                    | Green Chili Pepper|
| *Erigeron canadensis*                                | Canadian Horseweed|
| *Helianthus annuus*                                  | Sunflower         |
| *Ipomoea purpurea*                                   | Morning Glory     |
| *Ocimum basilicum*                                   | Basil             |
| *Pelargonium sp.*                                    | Geranium          |
| *Salvia officinalis*                                 | Sage              |
| *Solanum lycopersicum*                               | Tomato            |
| *Solanum melongena*                                  | Eggplant          |
| *Vigna radiata*                                      | Mung Bean         |
| **Deciduous Perennials (11 species)**                 |                   |
| *Acer saccharum*                                     | Sugar Maple       |
| *Aegopodium podagraria*                              | Bishop’s Goutweed |
| *Cornus florida*                                     | Flowering Dogwood |
| *Dianthus caryophyllus*                              | Carnation         |
| *Melissa officinalis*                                | Lemon Balm        |
| *Mentha piperita*                                    | Peppermint        |
| *Mentha spicata*                                     | Spearmint         |
| Plant Name                              | Common Name                |
|----------------------------------------|----------------------------|
| *Rosa sp.*                             | Rose                       |
| *Syringa vulgaris*                     | Common Lilac               |
| *Taraxacum officinale*                 | Common Dandelion           |
| *Toxicodendron diversilobum*           | Poison Oak                 |

**Evergreen Perennials (12 species)**

| Plant Name                              | Common Name                |
|----------------------------------------|----------------------------|
| *Abies balsamea*                       | Dwarf Balsam Fir           |
| *Abies concolor*                       | White Fir                  |
| *Hibiscus rosa-sinensis*               | Rose Mallow Hibiscus       |
| *Juniperus horizontalis*               | Creeping Juniper           |
| *Picea glauca*                         | White Spruce               |
| *Picea pungens*                        | Blue Spruce                |
| *Pinus halepensis*                     | Aleppo Pine               |
| *Pinus strobus*                        | White Pine                 |
| *Podocarpus macrophyllus*              | Buddhist Pine              |
| *Taxus baccata*                        | English Yew                |
| *Taxus brevifolia*                     | Pacific Yew                |
| *Thuja occidentalis*                   | Eastern White Cedar        |
Table 2. List of species used for the germination rate experiment.

| Scientific Name | Common Name           |
|-----------------|-----------------------|
| **Annuals (11 species)** |                       |
| *Beta vulgaris*  | Beet                  |
| *Capsicum annuum* | Green Bell Pepper     |
| *Celosia argentea* | Plumed Cockscomb     |
| *Helianthus annuus* | Sunflower            |
| *Impatiens balsamina* | Touch-Me-Not        |
| *Lathyrus odoratus* | Sweet Pea             |
| *Linum grandiflorum* | Scarlet Flax          |
| *Linum usitatissimum* | Grain Flax           |
| *Nigella damascena* | Love-in-a-Mist       |
| *Papaver rhoeas*  | Flanders Field Poppy  |
| *Phaseolus vulgaris* | Pole Bean             |
| **Biennials (3 species)** |                      |
| *Campanula medium* | Canterbury Bells      |
| *Daucus carota*   | Carrot                |
| *Dianthus barbatus* | Chabauds Giant Carnation |
| **Deciduous Perennials (9 species)** |                   |
| *Asclepias tuberosa* | Butterfly Weed       |
| *Catananche caerulea* | Cupid’s Dart        |
| *Dianthus caryophyllus* | Carnation            |
| *Gypsophila paniculata* | Baby’s Breath      |
| *Lavandula officinalis* | Lavender            |
| *Linum compactum*  | Golden Flax           |
| *Linum perenne*    | Blue Flax             |
| Plant Name                        | Common Name       |
|----------------------------------|-------------------|
| *Malva sylvestris*               | Common Mallow     |
| *Nepeta mussinii*                | Catnip            |

**Evergreen Perennials (5 species)**

| Plant Name                        | Common Name       |
|----------------------------------|-------------------|
| *Iberis sempervirens*             | Evergreen Candytuft|
| *Picea glauca*                    | White Spruce      |
| *Picea pungens*                   | Blue Spruce       |
| *Pinus strobus*                   | White Pine        |
| *Pseudotsuga menziesii*           | Douglas Fir       |
Table 3. List of species used for the chlorophyll degradation experiment.

| Scientific Name                     | Common Name     |
|-------------------------------------|-----------------|
| **Annuals (12 species)**            |                 |
| Brassica oleracea var. palmifolia   | Black Kale      |
| Brassica oleracea var. sabellica   | Green Kale      |
| Beta vulgaris                       | Beet            |
| Beta vulgaris subsp. vulgaris       | Swiss Chard     |
| Capsicum annuum                     | Cayenne Pepper  |
| Capsicum annuum                     | Gong Bao Pepper |
| Dahlia pinnata                      | Dahlia          |
| Glebionis coronaria                 | Tong Ho         |
| Solanum lycopersicum                | Tomato          |
| Solanum melongena                   | Eggplant        |
| Spinacia oleracea                   | Spinach         |
| Zea mays                            | Corn            |
| **Biennials (4 species)**           |                 |
| Apium graveolens                    | Celery          |
| Brassica oleracea var. italica      | Broccoli        |
| Brassica oleracea var. viridis      | Collard Green   |
| Daucus carota                       | Carrot          |
| **Deciduous Perennials (10 species)**|                |
| Acer saccharum                      | Sugar Maple     |
| Allium fistulosum                   | Green Onion     |
| Allium porrum                       | Leek            |
| Asparagus officinalis               | Asparagus       |
| Euonymus fortunei                  | Fortune’s Spindle|
| Plant Name                         | Common Name               |
|-----------------------------------|---------------------------|
| Ginkgo biloba                     | Maidenhair Tree           |
| Hydrangea paniculate              | Panicled Hydrangea        |
| Rhus typhina                      | Staghorn Sumac            |
| Salix integra                     | Brocade Willow            |
| Symphyotrichum cordifolium        | Blue Wood-Aster           |

**Evergreen Perennials (10 species)**

| Plant Name                         | Common Name               |
|-----------------------------------|---------------------------|
| Artemisia schmidtiana             | Silver Mound Artemisia    |
| Gerbera jamesonii                 | Gerbera Daisy             |
| Hibiscus rosa-sinensis            | Rose Mallow and Orange Hibiscus |
| Phegopteris hexagonoptera         | Broad Beech Fern          |
| Picea pungens                     | Blue Spruce               |
| Pinus strobus                     | White Pine                |
| Saintpaulia ionantha              | African Violet            |
| Taxus cuspidata                   | Golden Japanese Yew       |
| Thuja occidentalis                | Eastern White Cedar       |
Figure Captions

**Fig. 1** Rate of electrolyte leakage from 1 cm² leaf samples from annual, deciduous perennial, and evergreen perennial plants. Rate of electrolyte leakage, which was assessed by measuring changes in the conductivity of the external medium in which leaf samples were placed over a period of 60 minutes, measured every 10 minutes, was interpreted to reflect the rate of cellular damage in response to exogenous H₂O₂. a. Rate of electrolyte leakage from leaves exposed to distilled water. b. Rate of electrolyte leakage from leaves due solely to H₂O₂ exposure (calculated as the difference between rate of electrolyte leakage from leaves exposed to H₂O₂ and rate of electrolyte leakage from leaves exposed to distilled water). Values are mean ± SEM. Means with different letters were significantly different (P < 0.05). All values were corrected for the rate of change in conductivity of the external medium in the absence of any leaf samples, which was 0.025µS min⁻¹ for distilled water and 0.005µS min⁻¹ for H₂O₂. See Table 1 for list of species sampled for each life history strategy. Each species was sampled once.

**Fig. 2** Cumulative germination of four representative species used in this experiment at their optimal [H₂O₂]. Optimal [H₂O₂] is the concentration of H₂O₂ that caused the fastest time to 50% germination. Cumulative germination data were modeled using AAT Bioquest’s Four Parameter Logistic Curve Calculator (https://www.aatbio.com/tools/four-parameter-logistic-4pl-curve-regression-online-calculator), with the inflection point (indicated by the dashed lines) being interpreted as the time to 50% germination. a. *Nigella damascena* (annual). b. *Duacus carota* (biennial). c. *Malva sylvestris* (deciduous perennial). d. *Picea glauca* (evergreen perennial). For each species, 10 seeds were sown, but not all seeds germinated.

**Fig. 3** Effect of H₂O₂ on the germination rate of seeds from annual, biennial, deciduous perennial, and evergreen perennial species. a. Time to 50% germination of seeds sown in water only. b. Concentration of exogenous H₂O₂ that caused the fastest time to 50% germination (called optimal [H₂O₂]). Germination rates excluded seeds for which no radicle emergence was ever observed. Values are mean ± SEM. Means with different letters were significantly different (P < 0.05). See Table 2 for list of species sampled for each life history. Each species was examined once at each of 8 different H₂O₂ concentrations (10, 20, 30, 40, 50, 60, 70, 80 mM) and in water.
Fig. 4 Rate of chlorophyll degradation and chlorophyll a/b ratio of annuals, biennials, deciduous perennials, and evergreen perennials. Total chlorophyll was isolated from leaves using 100% acetone and subjected to 30mM exogenous \( \text{H}_2\text{O}_2 \) (or water, as a control) to induce oxidative damage. Chlorophyll concentration was measured spectrophotometrically at 0, 30, and 60 minutes using absorbance measurements and previously published equations (Holm 1954). Chlorophyll degradation rate was calculated as the difference in the rate of change of chlorophyll concentration in 30mM \( \text{H}_2\text{O}_2 \) samples and water samples over 60 minutes. All chlorophyll degradation rates were corrected for the initial chlorophyll concentration at time = 0 as higher initial chlorophyll concentrations led to higher rates of chlorophyll degradation rates. a. Degradation rate for chlorophyll a. b. Degradation rate for chlorophyll b. c. Chlorophyll a/b ratio for chlorophyll samples subjected to water only at time = 0. Values are mean ± SEM. No significant differences among mean values were observed within any parameter, but the degradation rate for chlorophyll b was found to be significantly higher than that for chlorophyll a. See Table 3 for list of species sampled for each life history. Each species was sampled once.

Fig. 5 Ancestral state reconstruction of life history strategy for the 84 different species used in this study. Shown is maximum likelihood phylogeny inferred using seven gene sequences (Zanne et al. 2014). Ancestral state reconstruction of life history strategy was computed using a continuous-time Markov chain model. The pie chart at each node in the tree shows the scaled likelihood for the life history strategy of the ancestor, with a margin of error of 0.05%.
Appendix: R Code used for Phylogenetic Analysis and Ancestral State Reconstruction

These analyses were performed using the data analysis software R i386 4.0.2.

```r
require(brranching)
require(ape)
require(phytools)
library("diversitree")
taxa <- read.csv('taxa_list_x.csv', header=T, sep='\', stringsAsFactors=F)
taxa_list <- c()
for (i in 1:nrow(taxa)) {
  family_name <- tolower(taxa$Family[i])
split_binomial_name <- strsplit(taxa$Species[i], split='\ ')
genus_name <- tolower(split_binomial_name[[1]][1])
species_name <- paste(split_binomial_name[[1]][1],
                      split_binomial_name[[1]][2], sep='_')
phylomatic_syntax <- paste(family_name, genus_name, species_name, sep='/')
taxa_list <- c(taxa_list, phylomatic_syntax)
}
taxa_subset <- sample(taxa_list, size=84)
tree <- phylomatic(taxa=taxa_subset, storedtree='zanne2014')
taxa <- read.csv('taxa_list_order.csv', header=T, sep='\',
stringsAsFactors=F)
X<-(taxa[,3],taxa[,2])
cols<-(palette()[1:length(unique(x))],sort(unique(x)))
tiplabels(pie=to.matrix(x,sort(unique(x))),piecol=cols,cex=0.3)
add.simmap.legend(colors=cols,prompt=FALSE,x=0.9*par()
$usr[1],
y=-max(nodeHeights(tree)),fsize=0.8)
```
is.rooted(tree)

tree<-multi2di(tree)
is.binary.tree(tree)

dst<-tree
dst$edge.length[dst$edge.length==0]<-max(nodeHeights(tree))*1e-6
fitER<-ace(x,dst,type="discrete",model="ER")

fitER

plotTree(tree,type="fan",fsize=0.8,ftype="i")
nodelabels(node=1:tree$Nnode+Ntip(tree),
  pie=fitER$lik.anc,piecol=cols,cex=0.5)
tiplabels(pie=to.matrix(x,sort(unique(x))),piecol=cols,cex=0.3)
add.simmap.legend(colors=cols,prompt=FALSE,x=0.9*par()$usr[1],
  y=-max(nodeHeights(tree)),fsize=0.8)
Rate of electrolyte leakage from 1 cm² leaf samples from annual, deciduous perennial, and evergreen perennial plants. Rate of electrolyte leakage, which was assessed by measuring changes in the conductivity of the external medium in which leaf samples were placed over a period of 60 minutes, measured every 10 minutes, was interpreted to reflect the rate of cellular damage in response to exogenous H₂O₂. a. Rate of electrolyte leakage from leaves exposed to distilled water. b. Rate of electrolyte leakage from leaves due solely to H₂O₂ exposure (calculated as the difference between rate of
electrolyte leakage from leaves exposed to H2O2 and rate of electrolyte leakage from leaves exposed to distilled water). Values are mean ± SEM. Means with different letters were significantly different (P < 0.05). All values were corrected for the rate of change in conductivity of the external medium in the absence of any leaf samples, which was 0.025µS min⁻¹ for distilled water and 0.005µS min⁻¹ for H2O2. See Table 1 for list of species sampled for each life history strategy. Each species was sampled once

Figure 2

Cumulative germination of four representative species used in this experiment at their optimal [H2O2]. Optimal [H2O2] is the concentration of H2O2 that caused the fastest time to 50% germination. Cumulative germination data were modeled using AAT Bioquest’s Four Parameter Logistic Curve Calculator (https://www.aatbio.com/tools/fourparameter-logistic-4pl-curve-regression-online-calculator), with the inflection point (indicated by the dashed lines) being interpreted as the time to 50% germination. a. Nigella damascena (annual). b. Duacus carota (biennial). c. Malva sylvestris (deciduous perennial). d. Picea glauca (evergreen perennial). For each species, 10 seeds were sown, but not all seeds germinated
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**Figure 4**

Rate of chlorophyll degradation and chlorophyll a/b ratio of annuals, biennials, deciduous perennials, and evergreen perennials. Total chlorophyll was isolated from leaves using 100% acetone and subjected to 30mM exogenous H2O2 (or water, as a control) to induce oxidative damage. Chlorophyll concentration
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