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Antioxidant and chlorophyll dynamics differ between perennial flax (Linum perenne and Linum lewisii) and annual flax (Linum usitatissimum and Linum grandiflorum).

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Abstract

Among plants, there is considerable variation in maximum lifespan, with annual species living less than one year and perennial species living as long as 43 600 years. According to the Oxidative Stress Theory of Aging, the maximum lifespan of an organism is partly determined by the rate at which it accumulates oxidative damage to its biomolecules. Consequently, perennial species have likely evolved mechanisms to slow down the accumulation of biomolecular oxidative damage relative to annual species. The present study aimed to evaluate this prediction using annual and perennial flax (*Linum*). While there are several mechanisms by which the accumulation of oxidative damage to biomolecules can be reduced, we focused on the role of antioxidants and biomolecular damage repair. Epicotyl growth and leaf production of annual—but not perennial—species were significantly affected by supplementation with exogenous ascorbic acid or glutathione, suggesting that annuals may have lower levels of endogenous antioxidants than perennials. Furthermore, while rates of chlorophyll degradation in response to exogenous H_2O_2 did not differ between annual and perennial species, rates of chlorophyll resynthesis following such exposure were 2-fold faster in perennial species, whether assessed via image colour or spectrophotometric analysis, reflecting their greater capacity to repair oxidative damage to chlorophyll compared to annuals. Similarly, when plants were exposed to complete darkness for several days, chlorophyll degradation rates were significantly lower in perennials than annuals, perhaps because annuals quickly redistributed resources from leaves to flowers. When subsequently reintroduced to natural light cycles, chlorophyll resynthesis occurred 2-fold more quickly in perennials than annuals. Overall, our study illuminates that the evolutionary transitions between life history strategies in plants have been accompanied by physiological modifications to antioxidant and chlorophyll dynamics that reflect the need for perennial species to delay the aging process in order to survive for multiple growing seasons.

Keywords: ageing, ascorbic acid, glutathione, life history, maximum lifespan, oxidative stress

Introduction

While there is a continuum of variation in maximum lifespan among plant species, they are commonly classified into two large groups: annual species live for less than one year, completing all stages of their life cycle in that time, whereas perennial species live for multiple years, as long as 43 600 years (Lynch et al. 1998), either reproducing each year, in multi-year cycles, or once at the end of their lifespan (Friedman 2020). While considerable effort has been devoted to gaining an understanding of the ecological and/or evolutionary pressures that favour annual and perennial life history strategies in plants (e.g., Hasting and Caswell 1979; Lindborg 2007), only meager effort has so far been devoted to understanding the underlying physiological differences that exist between these types of plants (Adler et al. 2014), especially with regards to longevity-associated traits. Indeed, Pérez-Llorca and Munné-Bosch (2021) recently lamented that little attention has been paid to understanding the lessons that perennial species can teach us about delaying or preventing aging in humans.

The maximum lifespan of an organism is determined by the rate at which it accumulates damage to its biological molecules. In turn, this rate of biomolecular damage is determined by the balance between damage-promoting and damage-preventing/repairing mechanisms (Liochev 2015). There are several different ways by which biomolecular damage can occur within organisms, as well as an ongoing effort to unify these various mechanisms into a single theory of aging (Barja 2019). One particular damage-promoting mechanism that has gained considerable attention for several decades is reactive oxygen species (ROS), whose role in determining maximum lifespan has been encapsulated by the Oxidative Stress Theory of Aging (OSTA; Bokov et al. 2004). ROS (e.g., H_2O_2) are predominantly derived from the respiratory and photosynthetic electron transport systems in plants, though they can also be derived from peroxisomal metabolism. They are known to cause oxidative damage to proteins, fatty acids, nucleic acids, and chlorophyll (Saed-Mouchesi et al. 2014; Sharma et al. 2012; Tripathy and Olemüller 2012). Plants can prevent oxidative damage to these biomolecules through their use of antioxidants, both enzymatic (e.g., catalase) and non-enzymatic (e.g., ascorbic acid [AsA] and glutathione [GSH]; Beligni and Lamattina 1999). Additionally, oxidative damage can be repaired, either by excision of the damaged portion of the compound or by degradation and resynthesis. OSTA would predict that perennial species should have higher levels of antioxidants and/or greater capacity for

biomolecular repair than annual species; however, to our knowledge, no studies to date have compared the antioxidant levels and/or biomolecular repair capacity of annual and perennial species. Consequently, the objective of the present study was to compare antioxidant and chlorophyll dynamics between annual and perennial species.

While several studies have conducted broad surveys of antioxidant levels among plant species (e.g., McCune and Johns 2007), these studies have never focused on the relationship between antioxidant levels and maximum lifespan or life history strategy. While there are published assays to measure the activity or concentration of several antioxidants found in plants, it has recently been suggested that hundreds of antioxidants may operate synergistically to prevent plants from experiencing oxidative damage due to ROS (Halvorsen et al. 2002), in which case measurements of a handful of selected antioxidants may not necessarily reveal any differences between annuals and perennials despite total antioxidant capacity being significantly different. Therefore, in the present study, we inferred antioxidant levels in plants by assessing how exogenous supplementation with antioxidants impacted their growth. Several studies have documented how oxidative stress reduces growth rate in both shoots and roots (Singh et al. 2009), and several studies have also demonstrated how exogenous antioxidants can mitigate oxidative stress caused by various environmental conditions (e.g., salt exposure; Wang et al. 2014). On this basis, the degree to which exogenous supplementation with antioxidants increases the growth rate of plants should be a reliable indicator of total endogenous antioxidant capacity. Since OSTA predicts that annuals should have lower levels of endogenous antioxidants than perennials, we predicted that annuals would experience a significantly greater increase in growth rate when supplemented with exogenous antioxidants than perennials.

Two different exogenous antioxidant supplements were used in this study to increase the robustness of our results: AsA and GSH. These antioxidants were selected because i) they are the two major low molecular weight antioxidants found in plant cells (Foyer 2001) and ii) they are water-soluble and are known to be taken up via roots (Mozafar and Oertli 1993), which means they could be administered to our plants via daily watering. Moreover, it is well established that AsA and GSH—together with several enzymes—are components of an antioxidant cycle that is localized within both mitochondria and chloroplasts and is upregulated in response to oxidative stress (Chew et al. 2003).

Investigations of whether biomolecular repair capacity differs between annual and perennial species have only recently been undertaken. For example, Blue et al. (2021) showed that perennial herbs and trees had a higher copy number of certain DNA repair enzymes than annuals, consistent with OSTA. In the present study, we examined whether perennials could repair and/or replenish their chlorophyll levels at a faster rate than annuals following chlorophyll damage or degradation. We focused on chlorophyll because its concentration within intact leaves can be easily assessed via image colour and spectrophotometric analysis (Liang et al. 2017; Sánchez-Sastre et al. 2020) and because it is commonly used as a marker of plant health (Agarwal and Gupta 2018). In order to induce oxidative damage to chlorophyll, we subjected plants to exogenous application of H_2O_2 via foliar spray. This approach has been previously shown to increase endogenous H_2O_2 in other species (e.g., tomato, Işeri et al. 2013). The mechanism by which H_2O_2 damages chlorophyll is not well established, but, in a previous study (Zahkary et al., 2022), we showed that H_2O_2 could degrade chlorophyll (particularly chlorophyll b) following its extraction from leaves. This finding is consistent with the work of Zhang et al. (2009), who showed that sweet potato subjected to artificial oxidative stress exhibited a 50% decline in chlorophyll levels, and Shimazaki et al. (1980), who showed that SO_2 fumigation of spinach leaves significantly decreased their chlorophyll content in oxygenated environments via production of ROS.

In order to increase the robustness of our study's findings, and to determine whether any differences in rates of chlorophyll repair between annuals and perennials were specific to oxidative stress, we utilized a second approach to induce chlorophyll degradation: complete darkness. Yan et al. (2007) employed a similar dual approach in order to investigate the effect of the gene *DWARF3* on chlorophyll degradation rates in rice leaves. Darkness causes a decline in chlorophyll levels for two reasons: i) chlorophyll synthesis requires light exposure (Suzuki and Bauer 1995), and ii) darkness triggers the upregulation of proteins involved in chlorophyll degradation, such as SGR1 (Li et al. 2016).

For our study, we elected to focus on one particular genus of plants, *Linum* L. (flax), for several reasons. First, within this genus, there are both annual and perennial species whose evolutionary relationships have been previously established (Brown et al. 2012). In this way, we could be more confident that any differences in antioxidant and/or chlorophyll dynamics observed among annual and perennial species in our study were due to differences in maximum lifespan

rather than phylogenetic differences. Second, given that our previous ancestral state reconstruction of life history strategies in flax revealed that the perennial state is most likely ancestral, with the annual state having evolved independently several times, by focusing our current study on flax, we were able to gain some understanding of how the differences we observed between annual and perennial flax species in this study may have evolved. Third, the flax genus is thought to have evolved 46 million years ago, making it a relatively old lineage within plants (McDill et al. 2009), meaning that there has been sufficient time for physiological adaptation among species with different life histories to have evolved within this genus.

Materials and Methods

Plants and Growing Conditions

Five species of flax were used in this study: *Linum usitatissiumum* L. (annual), *L. grandiflorum* Desf. (annual), *L. perenne* L. *(*perennial), *L. lewisii* Pursh (perennial), and *L. flavum* L. (perennial). *L. flavum* was only used in the antioxidant supplementation experiments since seeds for this species were unavailable from commercial suppliers when the chlorophyll repair experiments were conducted. These five species were selected among all available species of flax (~180) because they are known to germinate and grow well under the climatic conditions of our region (i.e., southern Ontario). Seeds were obtained from various commercial suppliers (Everwilde Farms, Sand Creek, WI; Baker Creek Heirloom Seeds, Mansfield, MO; Salt Spring Seeds, Salt Spring Island, BC). Seeds were grown in a rooftop greenhouse on the university campus grounds. Plants were exposed to natural photoperiod, but the greenhouse was also equipped with an artificial lighting system that activated whenever ambient light levels were below 50,000 lux during the day (8:00h-20:00h). Plants were also exposed to natural variations in temperature and humidity, though the greenhouse is equipped with heaters and misters that prevent it from getting too cold or dry. For each species, seeds were sown in 3-inch pots, lined with paper towel and filled with vermiculite, which were placed into shallow, white rectangular tubs filled 1/3 full with tap water. Approximately 10 seeds were sown per pot, spread out equally over the surface. A small amount of vermiculite was used to cover the seeds as flax seeds germinate more successfully in darkness (Kirmizi et al. 2018). Plants were watered as required, approximately 2-3 times per week. Once

the seeds had germinated, 2mL of fertilizer solution (FloraGro and FloraMicro, General Hydroponics, Santa Rosa, CA) was added weekly, alternating between the two solutions.

Experiments #1 and #2: Exogenous AsA or GSH supplementation

Experiment #1 was conducted in Summer 2018, whereas Experiment #2 was conducted in Fall 2018. In order to provide AsA or GSH supplementation for some of the plants, during each routine watering, 0.5mM AsA (Pure Source Inc., Guelph, ON; diluted in tap water) or 0.5mM GSH (Belle Chemical, Billings, MT; diluted in tap water) was added to the tubs in place of tap water. Once germination had been observed in all species (except *L. flavum*, which takes up to 14 days to germinate), weekly measurements of epicotyl height and leaf number were conducted. Using a ruler, epicotyl height was measured as the length of the stem from the point of attachment of the cotyledons to the tip of the terminal leaf cluster (to the nearest millimeter). When lateral branches were present, which emerged from lateral meristems in the axils of the cotyledons, their length was measured in the same manner and added to the epicotyl length. Leaf number included only true leaves (i.e., not the cotyledons) that were not part of the terminal leaf cluster, including leaves on lateral branches, when they occurred.

Experiments #3 and #4: Chlorophyll degradation and repair in response to foliar H_2O_2 spraying

Experiment #3, which examined rates of chlorophyll degradation and repair using color image analysis, was conducted in Fall 2019, whereas Experiment #4, which examined rates of chlorophyll degradation and repair using spectrophotometric analysis, was conducted in Fall 2020. Experiment #4 was undertaken to confirm the results of Experiment #3 using a more traditional method for assessing chlorophyll levels in leaves.

For both experiments, five weeks after sowing, all plants had reached a size where they had at least six true leaves, and the experiment began. Photographs of representative leaves (Experiment #3) or chlorophyll extracts (Experiment #4) were taken in order to determine baseline chlorophyll levels (see below). Subsequently, foliar spraying was used to subject the leaves to H_2O_2 and cause oxidative stress-induced chlorophyll degradation. The concentration of H_2O_2 used

was 30mM as lower concentrations (≤ 20mM) have been shown to promote chlorophyll synthesis in other studies (Jamaludin et al. 2020). Surfactants are necessary when conducting foliar sprays (Fernández et al. 2006), so Triton X-100 was added to the H_2O_2 solution (0.1% v/v). While it has been demonstrated that Triton X-100 can replace natural esterifying groups during the *in vitro* synthesis of chlorophyll (Michalski et al. 1987), it is not clear whether such reaction would occur *in vivo* and, regardless, the Triton X-100 version of chlorophyll has the same absorbance characteristics as wild-type chlorophyll, so should not have impacted our study in any significant way. Since H_2O_2 largely enters the leaves via their stomata, we covered the plants with a transparent plastic dome for 15 minutes prior to spraying so that the stomata would open to a greater degree in response to an increase in humidity (Lange *et al.* 1971). This was especially important since exogenous H_2O_2 has been shown to induce stomatal closure in other species (e.g., *Plumeria rubra;* ZhouYu and YongFei 2010). Plants were sprayed with H_2O_2 twice daily for three days, and photographs or chlorophyll extracts of representative leaves were taken approximately 12 hours following the final spray, which permitted calculation of the rate of chlorophyll degradation in response to H_2O_2 exposure. Subsequently, plants were allowed to recover for eight days, with photographs and/or chlorophyll extracts of representative leaves taken at the midpoint and end of this recovery period in order to measure the rate at which chlorophyll resynthesis and/or repair occurred.

Experiment #5: Chlorophyll degradation and repair in response to complete darkness

Experiment #5 was conducted in Summer 2019. Five weeks after sowing, all plants had reached a size where they had at least six true leaves, and the experiment began. Photographs of representative leaves were taken each day for three days in order to determine baseline chlorophyll levels (see below). Subsequently, the rectangular tubs in which the plants were growing were entirely covered with cardboard boxes so that the plants were subjected to complete darkness for five days. Each day, the cardboard boxes were removed only long enough (approx. 10-15 minutes) to take photographs of representative leaves (so that rates of darkness-induced chlorophyll degradation could be determined) and to water the plants. After five days, the plants were noticeably yellowed, and the cardboard boxes were removed to allow the plants to return to natural

photoperiod. Photographs of representative leaves were taken each day for five days in order to determine the rate at which chlorophyll was resynthesized upon re-exposure to light.

Measurement of Chlorophyll Concentration

Chlorophyll concentration was measured using either image colour analysis of photographed leaves (Experiments #3 and #5) or spectrophotometric analysis of chlorophyll extracts (Experiment #4).

For image colour analysis of photographed leaves, leaves were photographed using either a Canon SL1 (equipped with Canon Speedlite 430EX flash and a Canon EFS 18-55mm lens with image stabilization; Experiment #3) or an iPhone X Max (which has a 12 megapixel back camera with a smart HDR option; Experiment #5). Although different cameras were used for these two experiments, due to their availability, this had no impact on the validity of our findings since we were only comparing values within—not between—experiments. ImageJ software was used to obtain RGB values for the leaf photographs. Care was taken to minimize variation in light conditions among photographs as much as possible, but it has been previously shown that normalizing the values for total light intensity of the images (i.e., R+B+G) gives good correlation with chlorophyll concentration (Yadav et al. 2010). Subsequently, normalized green index (NGI: [G/(R+G+B)]), which was used as the measure of chlorophyll concentration, was calculated. Several previous studies have shown that NGI is positively correlated with other measures of chlorophyll concentration, such as spectrophotometry and SPAD, in several species (e.g., corn, Vesali et al. 2015; pomegranate, Özreçberoglu and Kahramanoglu 2020). Consequently, color image analysis is emerging as a cost-effective technique for the non-destructive determination of chlorophyll concentrations in plants subjected to varying conditions.

For spectrophotometric analysis of chlorophyll extracts, leaves were removed from plants, weighed, and then homogenized in 1.5mL of 100% acetone with a mortar and pestle. The resulting extract was pipetted into an Eppendorf tube and centrifuged at 10,000 RPM for 10 minutes. The samples were then stored at 4˚C until all samples had been collected. Ward et al. (1994) have shown that chlorophyll extracts can be stored for prolonged periods without risk of statistically significant chlorophyll degradation. To measure chlorophyll concentration, 200 µL of supernatant

was diluted into 3 mL of 100% acetone inside a glass cuvette. Samples were then placed inside of a spectrophotometer (Vinmax UV Visible Spectrophotometer 721) and their absorbance was measured at 662nm and 644nm, which allowed for determination of chlorophyll a and b concentrations using previously published equations (Holm 1954).

Statistical Analysis

Data were analyzed using R (version 4.0.2). An analysis of variance (aov) model was constructed to determine whether life history strategy (i.e., annual vs. perennial) could significantly explain variation in epicotyl height, leaf number, chlorophyll concentration, chlorophyll degradation rate, or chlorophyll repair rate. For Experiments #1 and #2, week of measurement was also included in the model, since measurements of epicotyl height and leaf number were made over several weeks. For Experiments #3, #4, and #5, baseline chlorophyll concentration (i.e., before foliar spraying or dark exposure) was used as a covariate in the analysis of chlorophyll degradation rates because the rate at which chlorophyll degraded would likely be influenced by the amount of chlorophyll initially present. Similarly, chlorophyll degradation rates were used as a covariate in the analysis of chlorophyll repair rates because the rate at which chlorophyll was repaired would likely be influenced by the amount of chlorophyll that was degraded in the first place.

Results

Experiment #1: Exogenous AsA supplementation

The epicotyl height (Fig. 1) and leaf number (Fig. 2) of all five species increased significantly over the 6-week duration of the experiment ($P < 0.001$ for week for both parameters), especially for annuals ($P<0.001$ for week x lifespan for both parameters). AsA supplementation increased the rate of epicotyl growth and leaf production in annuals but not perennials ($P < 0.01$ for treatment x lifespan for both parameters); however, these differences were only observed during the last 3 weeks of the experiment ($P < 0.01$ for lifespan x treatment x week for both parameters).

Experiment #2: Exogenous GSH supplementation

The epicotyl height (Fig. 3) and leaf number (Fig. 4) of all five species increased significantly over the 6-week duration of the experiment ($P < 0.001$ for week for both parameters), though to a lesser extent than was observed in Experiment #1, likely due to seeds being planted in the fall, rather than summer, for this experiment. Beyond the first week, annual species grew and produced leaves at a faster rate than perennial species ($P = < 0.001$ for week x lifespan for both parameters). GSH-supplemented annuals had a slower rate of epicotyl growth ($P < 0.001$) and leaf production ($P < 0.001$) than perennials over the entire 6-week duration of the experiment. Post-hoc analysis of the data for annuals (using Tukey's HSD) revealed that only *L. usitatissimum* grew more slowly when exposed to GSH.

Experiment #3: Chlorophyll degradation and repair in response to foliar H_2O_2 spraying – image colour analysis

Just prior to spraying entire shoots with H_2O_2, we measured chlorophyll concentration (using NGI values from photographs) in the leaves of our plants in order to determine baseline levels, which were found to be similar between annuals and perennials ($P = 0.712$; Fig. 5a). Subsequently, the shoots of the plants were sprayed with H_2O_2 twice daily for three days, after which we measured chlorophyll concentration in the leaves again. Chlorophyll concentration was significantly lower in all plants, reflecting that H_2O_2 had caused oxidative damage to chlorophyll and/or inhibited enzymes involved in chlorophyll synthesis, but the rate of decline in chlorophyll concentration over these three days of H_2O_2 exposure did not differ between annuals and perennials ($P = 0.443$; Fig. 5b). The plants were subsequently permitted to recover from H_2O_2 exposure for eight days, during which chlorophyll concentration was measured twice, once at the midway point and once at the end. At the midway point, we found that chlorophyll levels in most plants had continued to decline since the end of the H_2O_2 exposure period. Moreover, we observed that there was no difference in the rate of change in chlorophyll levels between annuals and perennials ($P = 0.102$; Fig. 5c). Between the midway point and end of the recovery period, there was a considerable increase in chlorophyll concentration in the leaves of all species as plants began to repair and/or replace their damaged chlorophyll. We found that the rate of increase in chlorophyll concentration

was nearly significantly higher during this period in perennials compared to annuals (P = 0.068; Fig. 5d).

Experiment #4: Chlorophyll degradation and repair in response to foliar H_2O_2 spraying – spectrophotometric analysis

Just prior to spraying entire shoots with H_2O_2, leaf samples were taken to prepare chlorophyll extracts from which baseline levels of chlorophyll a and b could be determined. Both chlorophyll a (P = 0.968; Fig. 6a) and chlorophyll b (P = 0.598; Fig. 6b) concentrations were found to be similar between annuals and perennials. Subsequently, the shoots of the plants were sprayed with H_2O_2 twice daily for three days, after which we measured chlorophyll concentration in the leaves again. Chlorophyll concentration was generally lower in all plants, especially for chlorophyll b, reflecting that H_2O_2 had caused oxidative damage to and/or inhibited enzymatic synthesis of chlorophyll, but the rate of decline in chlorophyll concentration over these three days of H_2O_2 exposure did not differ between annuals and perennials for either chlorophyll a (P = 0.492; Fig. 6c) or chlorophyll b (P = 0.411; Fig. 6d). The plants were subsequently permitted to recover from H_2O_2 exposure for eight days, during which chlorophyll concentration was measured twice, once at the midway point and once at the end. At the midway point, we found that chlorophyll levels in most plants had continued to decline since the end of the H_2O_2 exposure period. Moreover, we observed that there was no difference in the rate of change in chlorophyll levels between annuals and perennials for either chlorophyll a (P = 0.28, Fig. 6e) or chlorophyll b (P = 0.38, Fig. 6f). Between the midway point and end of the recovery period, there was a considerable increase in chlorophyll concentration in the leaves of all species. We found that the rate of increase in chlorophyll a concentration was (nearly) significantly higher during this period in perennials compared to annuals (P = 0.061; Fig. 6g). The rate of increase in chlorophyll b concentration was significantly higher during this period in perennials compared to annuals (P = 0.012, Fig. 6h). It is noteworthy that these observations match perfectly the patterns observed using image colour analysis in Experiment #3, reflecting that image colour analysis is an acceptable method for the non-destructive monitoring changes in chlorophyll levels within plants.

Experiment #5: Chlorophyll degradation and repair in response to darkness – image colour analysis

For three consecutive days prior to darkness exposure, we measured chlorophyll concentration (using NGI values from photographs) in the leaves of our plants in order to determine baseline levels, which were found to be significantly higher in annuals than perennials ($P = 0.001$; Fig. 7a). Subsequently, all plants were covered with large cardboard boxes for five days in order to completely block exposure to light. Chlorophyll concentration decreased in all plants, but the rate at which chlorophyll concentration decreased was found to be significantly higher in annuals than perennials ($P = 0.002$; Fig. 7b). After five days of darkness exposure, the cardboard boxes were removed in order to re-expose plants to a natural photoperiod, thereby allowing for chlorophyll resynthesis to occur. The rate at which chlorophyll concentration increased following re-exposure to the light was nearly significantly higher in perennials than annuals ($P = 0.057$; Fig 7c).

Discussion

Annual species are known to grow faster than perennial species, which has been attributed to the higher specific leaf area of the former (Garnier 1992). Consistent with this notion, the annual flax species used in the present study grew faster than the perennial flax species, which we have documented previously (Brown et al. 2012). Although annual flax species grew faster than their perennial congenerics, exogenous AsA supplementation further increased the growth rate and leaf production rate of annual—but not perennial—flax species, consistent with our predictions. This observation may reflect that annual flax species have lower levels of endogenous antioxidants than perennial flax species, thereby permitting the former to benefit more greatly from exogenous AsA supplementation. Oxidative stress is known to have a profound negative impact on the growth of plants (Kaciene et al. 2015), and AsA is known to reduce oxidative stress through the scavenging of ROS (Xiao et al. 2021); therefore, supplying plants with exogenous AsA should increase plant growth, especially when endogenous antioxidant levels are low. Interestingly, exogenous AsA has been shown to decrease growth rates in *Arabidopsis* by increasing ROS production (Qian et al. 2014), but much higher concentrations were used (2 and 8mM) than in the present study (0.5mM). It has been established that, at low concentrations, AsA serves predominantly as an antioxidant, whereas, at higher concentrations, it serves predominantly as a pro-oxidant (Podmore et al. 1998).

Antioxidants are believed to be costly to produce. The AsA biosynthesis pathway in plants is well characterized (Bulley and Laing 2016), and it has been shown that overexpression of dehydroascorbate reductase (DHAR) can increase AsA levels within plants; however, a 100-fold increase in DHAR expression was necessary to increase AsA levels by 4-fold, (Chen et al. 2003), which may underscore the significant cost required to achieve a modest boost in AsA levels in perennials and help explain why annuals would have lower endogenous AsA levels. The cost of antioxidant synthesis in long-lived species is thought to reduce the organism's energetic investment in other tasks, especially reproduction, though few studies have addressed this idea directly (e.g., Sawecki et al. 2019, Weirsma et al. 2004). Given that annual plants reproduce only once, they should be expected to maximize their reproductive investment at the expense of antioxidant production. Perennial species, on the other hand, which reproduce several times over many growing seasons, must invest more energy in antioxidant synthesis and bodily maintenance to ensure that at least some part of their body (usually their roots) can persist for several years. Consistent with this notion, Vico et al. (2016) recently showed that annuals produce more seeds each year than congeneric perennials, though, after a few years, a perennial plant, if it survives, will have outproduced an annual plant, suggesting that perennial plants may have higher lifetime reproductive output in stable environments.

An alternative explanation for our results is that annual species have a higher capacity for AsA uptake in their roots compared to perennials species, which would allow them to better take advantage of exogenous AsA supplementation. AsA uptake by roots is known to be an active process involving a poorly-characterized transport protein (Mozafar and Oertli 1993), and it is possible that annual plants express this transporter at a higher level than perennial plants. Indeed, Roumet et al. (2006) showed that annual roots do exhibit several properties that enhance their resource acquisition capacity compared to perennials. While increased AsA uptake by annual plants could explain the results of our study, it is unlikely that it would have any significant application in the field for two reasons. First, there is no evidence, to our knowledge, that AsA is present in any significant amount in natural soils. Second, it has been shown that AsA applied to young leaves is preferentially transported to the roots rather than to the mature leaves, suggesting that roots are a sink—rather than a source—of AsA within plants (Franceschi and Tarlyn 2002). Another possible explanation for our results is that the AsA degradation pathway, which has only been recently investigated in much detail (e.g., Truffault et al. 2017), may have higher activity in

perennial species than annual species, thereby rendering the exogenous application of AsA to perennial plants useless. Even if these alternative explanations are true, our study's conclusion remains the same: some aspect of antioxidant dynamics—whether endogenous concentration, soil uptake capacity, and/or degradation rate—must differ between annuals and perennials.

After having obtained results with AsA that were consistent with our predictions, we decided to repeat our experiment with another antioxidant—GSH—in order to assess the robustness of our findings. We chose GSH because, like AsA, it is water-soluble, so it could be easily administered to our plants by its addition to the water supply, and is known to be a major low molecular weight antioxidant in plant cells (Foyer 2001). Contrary to our observations with AsA, GSH was found to hinder the growth and leaf production of annuals species, particularly *L. usitatissimum*, while having no effect on perennial species. There are two possible explanations for these contradictory results. First, GSH may have acted as a pro-oxidant in our plants, generating—rather than eliminating—ROS. Indeed, it has been previously shown that GSH can either protect or promote oxidative DNA damage depending on circumstances (Milne et al. 1993). If GSH acted as a pro-oxidant in our study, then the decreased growth of annual plants treated with glutathione is consistent with OSTA because annual species would have less capacity to deal with the ROS generated by excess GSH within their tissues. Second, elevated levels of GSH may interfere with redox state monitoring in plants, as suggested by Creissen et al. (1999) when they found that transgenic plants with a higher biosynthetic capacity for GSH paradoxically experienced increased—not decreased—oxidative stress. If so, our findings may suggest that perennials constitutively maintain elevated antioxidant levels regardless of their redox state in order to eliminate a large proportion of continually-produced ROS and better protect themselves from oxidative stress, whereas annuals may only increase their expression of antioxidants under conditions where they detect intolerably high rates of ROS production since they prefer to expend their energy on rapid growth and reproduction. In this case, exogenous GSH may have reduced the growth of annual plants because they failed to detect excessive ROS production, leading to increased oxidative stress. Again, regardless of the particular underlying mechanisms that explains our results, we have clearly demonstrated that annual and perennial species respond differently to exogenous antioxidant supplementation, suggesting that antioxidant dynamics differ among annual and perennials species, as predicted by OSTA.

Foliar spray with H_2O_2 caused a significant decrease in chlorophyll concentration in the leaves of our plants, but the underlying mechanism responsible is not known. While it seems plausible that H_2O_2 (or its free radical derivatives) can react with chlorophyll directly, thereby causing its degradation, we are unaware of any published studies that have detailed this reaction. In a previous study (Zakhary et al. in press), we exposed chlorophyll extracts directly to H_2O_2 and observed that it caused the degradation of chlorophyll, especially chlorophyll b; therefore, a direct reaction between H_2O_2 and chlorophyll is a likely mechanism. Alternatively, it has been shown that LHPs can be degraded by ROS (Zolla and Rinalducci 2002), and that oxidative stress can induce protein loss from chloroplasts (Kwon et al. 2013), so H_2O_2 exposure *in vivo* may cause chlorophyll degradation indirectly by separating chlorophyll from the light-harvesting proteins (LHPs), increasing its susceptibility to intracellular degradation. Alternatively, oxidative stress has been shown to inhibit some of the enzymes involved in the chlorophyll biosynthesis pathway in cucumbers (Aarti et al. 2006), so it is possible that H_2O_2 causes a decrease in chlorophyll concentration through blocking the production of chlorophyll, thereby leading to net degradation over time.

Contrary to our predictions, there was no significant difference between annuals and perennials in terms of the rate of H_2O_2-induced chlorophyll degradation in response to foliar H_2O_2 spraying. In light of the results of our antioxidant experiments, we had anticipated that perennial plants would have greater capacity to eliminate exogenous H_2O_2, thereby reducing the damaging effects of H_2O_2 on their chlorophyll. H_2O_2 concentration used in this experiment was 30mM, which is a concentration that impedes growth in flax plants without killing them (JCLB, personal observations); however, this concentration is much higher than endogenous H_2O_2 concentrations found in other plants (e.g., ~1mM in wheat, Chen et al. 2009). On this basis, it is possible that the high concentration of exogenous H_2O_2 used in this study overwhelmed the antioxidant defences of the plants—even the bolstered antioxidant defences of perennials—leading to widespread damage in both annuals and perennials. Indeed, previous work in our laboratory (Zakhary et al. 2022) has shown that leaves from several perennial species, including deciduous perennials, were better protected from exogenous H_2O_2 in terms of cell death, so the fact that they were not better protected from H_2O_2 in terms of chlorophyll degradation is intriguing. Perhaps the greater resistance to cell death observed in H_2O_2-treated leaves in our previous study, where the same H_2O_2 concentration was used (30mM), was due to enhanced resistance to oxidative stress by cell

membranes, which would preserve membrane integrity, the loss of which is a hallmark characteristic of necrotic cell death (van Doorn et al. 2011), rather than increased antioxidant levels.

Prolonged darkness brings about a reduction in chlorophyll content in many plant species, the underlying causes of which are likely two-fold. First, chlorophyll is constantly being degraded and resynthesized (Beisel et al. 2010), and light is essential for chlorophyll synthesis, particularly in flowering plants (Suzuki and Bauer, 1995), such as flax. Consequently, in darkness, chlorophyll degradation continues while chlorophyll synthesis cannot occur, leading to a decrease in overall chlorophyll concentration. While the presence of LHPs can help prevent chlorophyll degradation (Park et al. 2007), these proteins are more expensive to maintain than chlorophyll itself (de Vries 1975); therefore, it seems likely that annual plants, which seek to minimize investment in somatic maintenance, would have lower levels of LHPs than perennial plants, leading to higher baseline rates of chlorophyll degradation. Second, chlorophyll cannot be utilized by plants during darkness, making it sensible for plants to actively degrade chlorophyll under these conditions, perhaps in order to remobilize the nitrogen found in light-harvesting proteins (Thomas et al. 2002), though not the nitrogen found in chlorophyll (Hörtensteiner and Feller 2002). Chlorophyll harbours up to 75% of the nitrogen contained within leaves (Christ and Hörtensteiner 2014). Nitrogen has been shown to be a limiting nutrient for seed production in many species (Sinclair and de Wit 1975), and flower production predominantly utilizes remobilized nitrogen rather than newly absorbed nitrate (Tagliavini et al. 1997). On this basis, in the absence of light, which prevents new vegetative growth, annuals may hastily remobilize their nitrogen reserves for flower and seed production in an effort to maximize reproductive output in their one and only growing season, leading to chlorophyll degradation within their leaves. By contrast, perennials, which will have additional opportunities to reproduce in subsequent growing seasons, may be under less pressure to degrade their chlorophyll to remobilize its nitrogen content, especially since chlorophyll, once separated from LHPs, becomes a phototoxic compound, requiring a complex metabolic pathway for its breakdown (Kräutler 2008) that yields many ROS-producing intermediates (Tripathy and Oelmüller 2012).

Consistent with our predictions, chlorophyll levels rebounded more quickly following both H_2O_2- and darkness-induced degradation in perennials compared to annuals, suggesting that

perennials have a greater capacity to synthesize and/or repair chlorophyll than annuals. This increased capacity would permit perennials to more readily maintain their photosynthetic apparatus in the face of various stressors. Indeed, we believe it is important to emphasize that increased capacity for perennials to resynthesize chlorophyll was an incredibly robust observation, having been observed in response to two different stressors (darkness and oxidative stress) and using two different methods of measuring chlorophyll levels (image colour and spectrophotometric analysis). Our finding that long-lived perennials have an increased capacity for chlorophyll resynthesis and repair nicely complements recent work (reviewed by Zhou et al. 2020) showing that seed longevity is largely determined by the capacity for nucleic acid and protein repair and points to the notion that repair processes are key to plant longevity at all stages of development. It is also noteworthy that chlorophyll b exhibited a significantly higher repair rate than chlorophyll a, especially in perennials. Our previous work (Zakhary et al. 2022) has shown that chlorophyll b is more susceptible to H_2O_2-induced damage, so it makes sense that the capacity to resynthesize this version of chlorophyll should be prioritized. It is known that chlorophyll b is synthesized from the oxidation of a methyl group within chlorophyll a, a process which involves the enzyme chlorophyll a oxygenase (Tanaka et al. 1998), so differences in the activity and/or expression of this enzyme may be a key factor that distinguishes annuals from perennials in terms of their lifespan.

The enzymes necessary for chlorophyll synthesis have been well-characterized (Beale 1999), with aminolevulinate synthase (ALAS) having been found to play a prominent role in the process (Schneider 1976). Zavgorodnyaya et al. (1997) showed that transgenic expression of ALAS in tobacco increased chlorophyll levels by up to 30%, suggesting that ALAS is not necessarily expressed at maximal activity in plants. Given that protein synthesis is one of the major maintenance costs in plants (de Vries 1975), it is not surprising that some plants maintain the activity of chlorophyll synthesis enzymes below maximal levels. This should be especially true for annual plants, which should prefer to invest energy into reproduction rather than somatic maintenance. Moreover, following its degradation, chlorophyll can be resynthesized using precursors such as aminolevulinic acid (ALA) or chlorophyllide (Lin et al. 2014). ALA has been shown to accumulate within leaves following their transition to darkness (Fluhr et al. 1975), suggesting that a readily-available pool of ALA may be accessible to leaves upon re-exposure to light in order to support rapid chlorophyll synthesis. Interestingly, ALA has been shown to

promote the formation of ROS in soybean plants (Noriega et al. 2007); therefore, the size of the ALA pool that is stored in leaves should be expected to correlate with the capacity for protection against ROS. On this basis, given that perennial species likely have higher antioxidant levels than annual species, for the reasons discussed above, it seems reasonable that perennial could afford to maintain a higher ALA pool during periods of darkness, thereby permitting them to more rapidly resynthesize their chlorophyll upon re-exposure to light. Of course, it also remains possible that the re-accumulation of chlorophyll following darkness exposure is controlled by the rate of resynthesis of LHPs, which have been shown to undergo significant degradation during darkness (Bennett 1981), and that annuals maintain lower rates of protein synthesis in order minimize baseline energy expenditure and devote more resources towards reproduction.

While chlorophyll resynthesis was observed over the five-day period following re-exposure to light in Experiment #5, chlorophyll resynthesis was not observed over the four-day period following cessation of foliar H_2O_2 spraying. One way to reconcile these contradictory observations is to consider the action of secondary ROS. H_2O_2 exposure can yield free radicals within organisms, e.g., via Fenton reaction; in turn, these free radicals can react with biological compounds, quenching the original free radicals but producing additional ones (called "secondary ROS") in a chain-reaction mechanism (Buonocore et al. 2010). Indeed, studies in birds have shown that secondary ROS may be a more important contributor to aging than primary ROS (Montgomery et al. 2011). Therefore, even after H_2O_2 spraying was ceased, ROS production within these plants may have persisted, leading to continued degradation of chlorophyll and accounting for the lack of increase in chlorophyll levels in the first four days after foliar H_2O_2 spraying was stopped. Once these secondary ROS reactions finally terminated, the process of repairing chlorophyll could begin, explaining the increase in chlorophyll levels that were observed in the second four days after foliar H_2O_2 spraying ceased. Alternatively, or additionally, H_2O_2 spraying may also have caused damage to the chlorophyll biosynthesis machinery which needed to be repaired before chlorophyll resynthesis could commence. Interestingly, our findings are reminiscent of work by Kudoh and Sonoike (2002) who showed that chilling-induced loss of chlorophyll in cucumber leaves did not manifest until the plants were returned to normal growing temperatures. These authors suggested that irreversible damage to photosystem I (PSI) may occur during exposure to low temperature, leading to greater ROS production as chlorophyll is unable to effectively dissipate light energy. Plants may actively degrade these damaged PSI and/or

associated chlorophyll upon return to normal growth temperature to mitigate deleterious ROS effects. Given that foliar H_2O_2 spraying could also damage PSI, perhaps a similar response upon the cessation of ROS treatment occurred in our plants.

Annual and perennial species differ in terms of their maximum lifespan. Most research comparing these two life history strategies has been focused on the ecological and/or evolutionary pressures that favour one strategy over the other. There have been comparatively few studies comparing physiological traits between annuals and perennials, with emphasis on such characteristics as water balance (Vaughn et al. 2011) rather than attributes that can explain differences in the longevity of these plants. Our results shed light on how antioxidant and chlorophyll dynamics differ between annuals and perennials in a way that permits perennials to maintain the integrity of their leaves and the resources they contain for a longer period of time, which is key to their multi-year survival and higher lifetime reproductive output. Given that the perennial condition is thought to be ancestral in flax, with the annual condition having evolved independently several times (Brown et al. 2012), our study also provides insight in terms of how the perennial to annual transition may have occurred, with decreased expression of antioxidant- and chlorophyll-synthesis genes.

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Figure Legends

Fig. 1 Effect of ascorbic acid (AsA) supplementation on epicotyl height. Data are mean ± SEM. Data for control plants are joined together by a solid line, whereas data for AsA-supplemented plants are joined together by a dashed line. (a) *Linum usitassiumum*, annual, N = 15 for both control and AsA-supplemented. (b) *Linum grandiflorum*, annual, N = 15 for both control and AsA-supplemented. (c) *Linum lewisii*, perennial, N = 6 for control, N = 12 for AsA-supplemented. (d) *Linum perenne*, perennial, N = 9 for both control and AsA-supplemented. (e) *Linum flavum*, perennial, N = 7 for control, N = 8 for AsA-supplemented. Asterisks indicate a significant difference ($P < 0.05$) control and AsA-supplemented plants.

Fig. 2 Effect of ascorbic acid (AsA) supplementation on leaf number. See Fig. 1 for details.

Fig. 3 Effect of glutathione (GSH) supplementation on epicotyl height. Data are mean ± SEM. Data for control plants are joined together by a solid line, whereas data for GSH-supplemented plants are joined together by a dashed line. (a) *Linum usitassiumum*, annual, N = 9 for control, N = 10 for GSH-supplemented. (b) *Linum grandiflorum*, annual, N = 8 for control, N = 4 for GSH-supplemented. (c) *Linum lewisii*, perennial, N = 7 for control, N = 8 for GSH-supplemented. (d) *Linum perenne*, perennial, N = 7 for control, N = 6 for GSH-supplemented. (e) *Linum flavum*, perennial, N = 4 for control, N = 3 for GSH-supplemented.

Fig. 4 Effect of glutathione supplementation on leaf number. See Fig. 3 for details.

Fig. 5. Reactive oxygen species (ROS)-induced chlorophyll degradation and repair measured via image colour analysis of representative leaf photographs. (a) Total chlorophyll concentration, measured as normalized green index (NGI) of RBG-analyzed leaf photographs, of five-week old plants measured just prior to foliar spraying with H_2O_2. (b) Degradation rate of total chlorophyll, measured as daily rate of change in NGI (ΔNGI/day), during the three days in which plants were subjected to twice-daily foliar spraying with H_2O_2. Values were standardized to account for

variation in total chlorophyll concentration. (c) Repair rate of total chlorophyll, measured as ΔNGI/day, during the first four days following foliar spraying with H_2O_2. (d) Repair rate of total chlorophyll, measured as ΔNGI/day, during the second four days following foliar spraying with H_2O_2. Values for both repair rates were standardized to account for variation in chlorophyll degradation rates. Data are mean ± SEM. Bars with different letters above have significantly different means ($P < 0.05$). N = 16 for annuals and N = 9 for perennials. U, *Linum usitatissimum* G, *Linum grandiflorum* P, *Linum perenne* L, *Linum lewisii*

Fig. 6. Reactive oxygen species (ROS)-induced chlorophyll degradation and repair measured via chlorophyll extracts from leaf samples. *(a)* Chlorophyll a concentration of five-week-old plants measured just prior to foliar spraying with H_2O_2. *(b)* Chlorophyll b concentration of the same plants. *(c)* Chlorophyll a degradation rate, measured as daily rate of change of chlorophyll a concentration over three consecutive days during which plants were subjected to twice-daily foliar spraying with H_2O_2. *(d)* Chlorophyll b degradation rate of the same plants. Chlorophyll degradation rates were standardized to account for variation in chlorophyll concentrations. *(e)* Chlorophyll a repair rate, measured as daily rate of change of chlorophyll a concentration over the first four days following foliar spraying with H_2O_2, i.e., plants were no longer being sprayed with H_2O_2. *(f)* Chlorophyll b repair rate of the same plants. *(g)* Chlorophyll a repair rate, measured as daily rate of change of chlorophyll a concentration over the second four days following foliar spraying with H_2O_2. *(h)* Chlorophyll b repair rate of the same plants. All chlorophyll repair rates were standardized to account for variation in chlorophyll degradation rates. Data are mean ± SEM. Bars with different letters above have significantly different means ($P < 0.05$). Chlorophyll a repair rate during the second four days following foliar spraying with H_2O_2 was nearly significantly higher in perennials than annuals ($P = 0.061$). N = 45 for annuals and N = 14 for perennials. U, *Linum usitatissimum* G, *Linum grandiflorum* P, *Linum perenne* L, *Linum lewisii*

Fig. 7 Darkness-induced chlorophyll degradation and repair measured via image colour analysis of representative leaf photographs. (a) Total chlorophyll concentration, measured as normalized green index (NGI) of RBG-analyzed leaf photographs, of five-week-old plants measured for three days prior to darkness exposure. (b) Degradation rate of total chlorophyll, measured as daily rate

of change in NGI (ΔNGI/day), during five days of complete darkness exposure. Values were standardized to account for variation in total chlorophyll concentration. (c) Repair rate of total chlorophyll, measured as ΔNGI/day, during five days of re-exposure to natural light cycle. Values were standardized to account for variation in chlorophyll degradation rates. Data are mean ± SEM. Bars with different letters above have significantly different means ($P < 0.05$). N = 12 for both annuals and perennials. U, *Linum usitatissimum* G, *Linum grandiflorum* P, *Linum perenne* L, *Linum lewisii*