Perennial flax are much more resistant to oxidative damage compared to annuals.

Dhruv Vyas

1University of Toronto Scarborough, Toronto, Ontario, Canada

Abstract: According to the oxidative theory of aging, aging is the result of damage accumulated over time by reactive oxygen species (ROS). ROS are produced by regular cell metabolism, and they damage lipids, carbohydrates, amino acids, and DNA. One way by which organisms could extend their lifespan is to build biological molecules that are resistant to ROS. We studied induced oxidative damage to chlorophyll in flax (Linum) because this genus consists of annuals (L. usitatissimum and L. grandiflorum) and perennials (L. lewisii, L. flavum, L. perenne) which are closely related. This allowed us to minimize other phylogenetic effects on our data. Oxidative damage to chlorophyll was measured by looking at the colour change in the leaves and in isolated chlorophyll using a cell phone camera and image analysis. As chlorophyll degrades, the relative red and blue in the image should increase. When subjected to exogenous hydrogen peroxide, we found that annual flax accumulated 2.48 times more chlorophyll damage compared to perennial flax (p < 0.001) in six days. Since perennials live longer than annuals, they should have a more ROS-resistant chlorophyll. Therefore our results are consistent with the oxidative theory of aging.

Introduction

Metabolic processes often produce reactive oxygen species (ROS) as a byproduct. These highly reactive chemicals (also called free radicals) can cause damage to proteins, lipids, carbohydrate, and DNA (Jacob, et al. 2013) According to the oxidative stress theory of aging, the accumulation of damage caused by ROS leads to aging and other age-related diseases.

There are several ways for cells to deal with the damage. Low metabolic rate which leads to reduced ROS production, has been linked to longer life spans according to the rate-of-living theory (Pearl, 1928). Cells also have protective enzymes (e.g., catalase, superoxide dismutase) that degrade ROS into less harmful products very effectively. NADPH oxidase is the main source of the radical superoxide anion (O2•) This is a byproduct of the redox reactions that occur during cellular respiration. Most of the O2• is processed into hydrogen peroxide (H2O2) by superoxide dismutase (Genestra, 2007). Catalase is one of the most efficient enzymes; one catalase enzyme can process millions of hydrogen peroxide molecules into water and oxygen each second (Chelikani, Fita & Loewen, 2004). Long-lived species can have more ROS-resistant biomolecules (lipids and proteins) (Pamplona, 2008), and crucial elements like DNA can be repaired after oxidative damage (Houten, Santa-Gonzalez & Camargo, 2018).

It has been shown in previous research that within Flax (Linum), perennial species have higher levels of catalase in roots compared to
their annual congener (Brown, Marshall & Staples, 2012). Annual species in the Flax (Linum) genus are dispersed throughout the phylogeny tree rather than being nested in a subset (McDill, Repplinger, Simpson & Kadereit, 2009). That is why I chose to work with these Flax (Linum) because the way that the Flax (Linum) genus has evolved gives an excellent model to study aging, because the other environmental factors in which species evolved should be controlled.

Oxygen free radicals sometimes react with amino acids and change the structure and function of a protein or molecule. Degradation caused by oxidative damage can be measured if the chosen protein or its function can be quantified in some way. Measuring the chlorophyll content of a plant leaf is easy and reliable because as chlorophyll degrades, the pigmentation of the leaf changes color. We can quantitatively assess the degradation of chlorophyll caused by added oxidizing agents like hydrogen peroxide (H2O2). The hypothesis of this paper is that when exposed to oxidizing agents, perennials will exhibit lower degradation of chlorophyll. Perennials species of Linum showed higher catalase activity than annual species (Brown et al., 2012), the higher catalase activity should break down the H2O2. Therefore my hypothesis is a logical extension of previous research. If the hypothesis is correct, it would indicate that organisms evolve to invest just enough in protective mechanisms that their lifespan requires. This would mean that an organism such as humans would not be equipped to handle the amount of oxidative damage that a significantly longer lifespan would cause.

**Methods**

Plants were grown in a greenhouse, so temperature and amount of light would be consistent across multiple growing periods. The soil was substituted with vermiculite to control for nutrient variability. Plants were grown in an aquaponics where half of the pot was submerged in nutrient-rich water. Fertilizer and micronutrients were added to the water bi-weekly in an alternative manner. First leaves were harvested 6 weeks after seeds were planted and the experiment continued for 4 consecutive weeks.

Chlorophyll extraction - Leaves were added to a mortar along with 15 ml of 90% ethanol. Leaves were ground up until no visible piece of leaf existed. The solution was then filtered. 4 mL of extracted chlorophyll solution was added to 1 mL of water or H2O2. Whole leaf experiment - Whole leaves were placed in a small well plate and directly exposed to water and H2O2. Pictures were taken using a smartphone camera. Pictures were analyzed with ImageJ software. Each sample was broken down into its red, blue and green components. IBM SPSS software was used to analyses the data measured from the experiment. Univariate Analysis of Variance was used for analysis of two conditions and POSTHOC tests were performed for significance during the 6 days.

**Results**

The relative blue light reflected is negatively correlated with chlorophyll concentration as chlorophyll absorbs blue light and oxidative damage is positively correlated with blue light. The addition of hydrogen peroxide leads to more oxidative damage (Fig. 1) validates our methods because more oxidative damage was observed in the hydrogen peroxide treatment. The results are
highly significant after day 4 (p < 0.001). Now that we know that blue light is a good measure of oxidative damage, we can use it to examine the difference between annuals and perennials (Fig. 2). Results are highly significant after day 4 (p < 0.001). There is also a significant two-way interaction of treatment and lifespan conditions (p = 0.001). On day 6, annuals had accumulated 2.48 times more damage compared to perennials (185.03% & 134.35% respectively). Even when breaking the data down to individual species, a clear difference between annuals (yellow) and perennials (green) can be seen. Fig. 3 shows that there is no single outlier species among the groups. There is no significant difference between relative oxidative damage in the leaves and extracted chlorophyll solution. This indicates that the determining factor responsible for most of the difference between annuals and perennial oxidative resistance is present in both annuals and perennials.

Discussion

These findings are consistent with the oxidative theory of aging. As expected, perennials exhibited less chlorophyll damage when subjected to oxidative stress. This makes sense because perennials must last for a longer period compared to annuals. Therefore, they should invest more in protective mechanisms to mitigate the damage from ROS compared to annuals. Feasibility of using smartphones to measure chlorophyll concentration was established with red light (R² = 0.94) by a studied conducted by Vesali, Omid, Mobli & Kaleita (2016). They showed that although red light is an excellent measure of chlorophyll concentration, blue (R² = 0.00) and red (R² = 0.21) are not. Each pixel in an image is given a colour value of 0-255. Values close to the minimum indicate an underexposed image and values near the maximum indicate an overexposed image. Vesali et al. (2016) severely underexposed blue light (0.2-1.6) compared to red and green (83-165) which were properly exposed, therefore their data for blue was not ideal. Conceptually it makes more sense that blue light would yield better results because chlorophyll absorbance in blue region is much higher compared to red.

Since these results are from a chlorophyll extract, no more ROS is being produced by the cell metabolism. Damage cannot be repaired by the cell because enzymes and energy molecules (such as ATP) are no longer present. This leaves only two possibilities: 1) perennial chlorophyll is more ROS resistant, or 2) perennial tissue has more antioxidants. Although the extracted solution should not have had any catalase, bubbles were observed only when H₂O₂ was added to the chlorophyll solution. This indicated that the solution did have small amounts of catalase. The results obtained cannot be explained by catalase as previous research has shown that catalase activity is not different in leaves (Brown et al., 2012). There are two types of antioxidants, hydrophilic and hydrophobic. Hydrophilic antioxidant protects soluble enzymes whereas hydrophobic antioxidants protect lipid membranes and membrane proteins (Nordberg & Arner, 2001). It is possible that hydrophobic antioxidants may have been present in the extracted chlorophyll solution as it was dissolved in isopropyl alcohol. As there was no difference between the leaves and the extracted solution, the data could be explained if chlorophyll in the perennials is more resistant compared to chlorophyll in annuals.
Evolutionary analysis shows that perennial is the ancestral state (McDill et al., 2009). The *Linum* genus has evolved to become annuals so annuals lost their oxidative resistance because it probably had significant costs. Annual species that spend energy in reproduction should be favored by natural selection instead of species that invest in oxidative protection, as annuals do not live long enough to benefit from oxidative resistance. To conserve energy, cells would invest the minimum amount necessary in protective mechanisms, according to their life span.

Species are not equipped for added oxidative damage. If their life span changes, their ROS resistance must adjust accordingly. Evolutionary adjustment period could take a long time especially as this would most likely occur in an natural selection shadow (Haldane, 1942). As human lifespan has increased dramatically in the last few centuries, the increase in age-related diseases could be explained if we have yet to adapt to our longer lifespan (Stadtman, 2012).

Previous studies have shown greater catalase activity in roots but not in leaves (Brown et al., 2012). Flax Leaves are shed in fall and new leaves emerge in the spring therefore perennial leaves might behave more like annuals than other tissue like shoots. Leaves are disposable for the plants, so they might behave more like short lived tissue; the results obtained by these experiments are more interesting because of this reason. In the future, if a similar experiment should compare evergreen leaves to deciduous leaves, the results should show an even greater difference. Evergreen leaves last a lot longer compared to deciduous leaves, so they should have even higher levels of oxidative resistance. Roots should have similar differences in all types of plants, but oxidative damage is harder to measure because it does not contain chlorophyll. Future studies can also look at the effect of aging on oxidative resistance within the same species of perennials.

There are several ways cells can mitigate ROS damage. Some studies look at one aspect of it and claim that oxidative theory of aging is incorrect. This is not fair because it does not look at the big picture. This theory has been studied for decades and most of the evidence supports it. Although oxidative stress still accounts for most of the damage, nitrogen reactive species and other molecules have been shown to cause damage in a similar manner. For this reason, oxidative stress theory of aging has been modified to be called the free radical theory or molecular damage theory in order to be more inclusive (Gladyshev, 2014).

ROS can damage DNA, leading to mutations (Genestra, 2007). This could lead to cancer and it is therefore important to understand how oxidative stress and lifespan are related. Oxidative damage to lipids, proteins and carbohydrates have minor energetic cost because they can be degraded and recycled but damage to DNA might be the determining factor of aging in the long term. Since the lifespan of humans has increased drastically in the last century, evolutionarily our bodies would not be ready for this added oxidative stress. This could partially explain the increase in cancer rates as people get older.
Fig 1. Extracted chlorophyll solution was subjected to H$_2$O$_2$ or water. The amount of blue light reflected was measured (Indirect measure of chlorophyll concentration). The percentage of blue light was normalized at day 0.
Fig 2. Extracted chlorophyll solution from annual and perennial species subjected to H₂O₂. The amount of blue light reflected was measured (Indirect measure of chlorophyll concentration). The percentage of blue light was normalized at day 0.
Fig 3. Extracted chlorophyll solution from annual (yellow) and perennial (green) species subjected to H$_2$O$_2$. Day 6 data is plotted, normalized at day 0 of the same sample.
Fig 4. Extracted chlorophyll solution and whole leaves are treated with H$_2$O$_2$. The amount of blue light reflected was measured (Indirect measure of chlorophyll concentration). The percentage of blue light was normalized at day 0.
References


