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Investigating the Role of Salicylic Acid in Determining the Lifespan of Short-Lived and Long-Lived Flax Species and Lines

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Abstract

Introduction: Reactive oxygen species (ROS) accumulation has been related to aging in both plant and human physiology. Leaked electrons from the electron transport system (ETS) react with molecular oxygen producing ROS, such as hydrogen peroxide (H_2O_2) and superoxide anions. There are numerous antioxidative mechanisms present in plants that aid in redox balance, one of which is the mild uncoupling of mitochondria. Uncoupling agents, such as salicylic acid (SA), can abolish the connection between oxidation and phosphorylation allowing electron transport to take place without the need for parallel ATP synthesis. "Uncoupling to Survive" Hypothesis states that uncoupling activity should be greater in long-lived species since uncoupled mitochondria result in reduced ROS production thereby increasing lifespan. Since ROS accumulation has been linked to lifespan in the Oxidative Stress Theory of Aging, the idea that long-lived species should exhibit greater antioxidative capacity than short-lived species is the foundation upon which this hypothesis is established. We tested this hypothesis in various flax species and lines differing in lifespan.

Methods: Spectrophotometry was used to determine SA levels in plant tissue while respiration rates were measured using a dissolving oxygen electrode.

Results: The "Uncoupling to Survive" Hypothesis does not seem to hold for flax since long-lived and short-lived species did not exhibit significant differences in oxygen consumption rates that would indicate differences in the uncoupling activity of their mitochondria.

Discussion: However, early flowering lines exhibited lower levels of SA suggesting that flax plants do not require elevated SA expression to display the early flowering phenotype as seen in *Arabidopsis* species. In addition, SA significantly increased the oxygen consumption rates in wild-type and early flowering plants which further provides evidence for its role as an uncoupling agent.

Conclusion: Our findings add to our understanding of the many roles that SA plays in plant physiology. Advantages of SA are well established in the cosmetic and medical communities and investigating its effects on plants can be beneficial. We also looked at the "Uncoupling to Survive Hypothesis" in plants, which helped us get a better grasp of the antioxidant mechanisms that may eventually reduce oxidative stress and lead to senescence.

Keywords: reactive oxygen species; uncoupling to survive hypothesis; uncoupling agent; long-lived species; short-lived species; flax; salicylic acid; oxygen consumption rate

Introduction

Plants experiencing abiotic stress are subjected to oxidative stress resulting from an excess of reactive oxygen species (ROS) which can cause extensive damage to the DNA of the cell [1, 2]. ROS production in plants takes place in the mitochondria and is the by-product of aerobic respiration occurring at the electron transport system (ETS) [1]. Respiratory ETS is made up of four distinct oxidoreductase (I – IV) complexes along with two mobile electron transporters, cytochrome C, and ubiquinone, that are involved in the process of transferring electrons to molecular oxygen [3]. As reduced substrates are fed into the ETS, electrons flow down their redox gradients which releases energy that is crucial in generating the protonmotive force that aids ATP production [4]. Electron buildup during low ATP demand causes electron leakage from the ETS which contributes to ROS production, such as superoxide anion and hydrogen peroxide [5]. Using mild uncoupling of mitochondria, plants can break the link between oxidation and ATP synthesis, such that oxidation of the reduced substrates can still take place but is no longer coupled with ATP synthesis preventing electron buildup [4]. Using free fatty acids (FFA), plants stimulate uncoupling mitochondrial protein (PUMP) which aids proton translocation and prevents ROS formation [5]. Inhibiting PUMP can result in increased ROS generation, indicating its direct role in mitochondrial ROS control [6].

Salicylic acid (SA) is a well-studied plant hormone due to its extensive role in development, growth, reproduction, and immunity [7]. Effects of SA on plant physiology tend to be concentration and species-specific [8]. Under conditions of increased oxidative stress, endogenous SA levels tend to increase, suggesting a possible role of SA as an antioxidant [9]. Moreover, SA-deficient rice plants exhibited higher levels of ROS relative to the control species [10]. Despite being a well-studied phytohormone, the involvement of SA in redox homeostasis is still a controversial field of study due to its ambivalent capacity to act as an antioxidant and prooxidant depending on the type of stress the plant is experiencing and the basal quantity of SA present within the plant [9]. Oxidative stress and ROS in both plant and mammalian mitochondria have been linked to aging. The Oxidative Stress Theory of Aging suggests oxidative damage resulting from the buildup of ROS throughout an organism's lifetime results in aging [11]. Derived from this theory is the "Uncoupling to Survive Hypothesis", which posits that organisms will increase the uncoupling of their mitochondria to increase lifespan in mammals. To our knowledge, the "Uncoupling to Survive" Hypothesis has never been tested in plants.

This paper aims to explore this hypothesis in flax species along with the possible role of SA as an uncoupling agent. We will be measuring SA levels along with oxygen consumption rates in short-lived and long-lived flax plants. We speculate that, if the "Uncoupling to Survive" Hypothesis holds in plants, long-lived flax plants would show greater uncoupling of their mitochondria relative to short-lived flax plants. Mitochondria that are experiencing an increase in uncoupling activity tend to also experience higher oxygen consumption rates because doing so will alleviate the build of electrons on mitochondrial ETS proteins [4]. We also predict that, if SA is indeed acting as an uncoupling agent in flax plants, we should observe higher SA levels in long-lived flax plants in comparison to short-lived flax plants. We used flax as the model organism because they are easy to grow with minimal care and their growth time is relatively short. Furthermore, the *Linum* genus has a diverse range of annual and perennial species, consequently eliminating any phylogenetic influences from the findings.

Methods

Experiment #1: Comparison of Annual and Perennial Flax Species

The annual species used in the study were *Linum usitatissimum* and *Linum grandiflorum rubrum*. The perennial species used were *Linum perenne* and *Linum lewisii.* The seeds were purchased from various commercial suppliers. In a separate plastic flowerpot, vermiculite was added along with 10-12 seeds of a particular species. In three identical plastic trays, four flowerpots containing each of the mentioned species were randomly placed such that there were 3 replicates of each species. The trays were filled with 1-2L of tap water and fertilized with 2mL of 4-1-4 Max Grow fertilizer (purchased from Indo, sold by Amazon) weekly. The plants were grown and kept at the University of Toronto, Scarborough (UTSC) Greenhouse. Flax seeds were re-planted multiple times over five months to get sufficient and healthy plant material for the experiments. SA assay was done after approximately 2-3 months of plant growth. 3 identical samples for each of the annual species and 2 identical samples for each perennial species were collected for the SA assay. The sample size was small since perennials did not grow large enough to provide us with sufficient plant material.

Experiment #2: Comparison of Early Flowering and Wild Type Annual Flax

Four lines of *Linum usitatissimum* were used: RC, RE1, RE2, and RE3. These lines were made from seeds of the Royal (R) cultivar of *L. usitatissimum* treated with 5-azacytidine, a DNA demethylating agent, which resulted in an early flowering phenotype that has been heritable through a multitude of generations [12]. RC represents the wildtype and was not treated with 5-azacytidine while RE1, RE2, and RE3 were derived from differently 5-azacytidine induced seeds [12]. These seeds were obtained from the Plant Genes Resources of Canada, which has maintained these lines since they were obtained from the Fieldes Laboratory at Wilfrid Laurier University. In separate plastic flowerpots, vermiculite was added along with 10-12 seeds for a particular species. In the first plastic tray, 2 replicates of each of the 4 lines were randomly placed and the tray was filled with 1-2L of water. The plants were grown and kept at the UTSC Greenhouse, at 23 ºC. The second tray was set up similarly but filled with 30mM of H_2O_2 solution instead. This was done to assess the ability of early flowering lines to grow under conditions of oxidative stress. Both trays were fertilized with 2mL of 4-1-4 Max Grow fertilizer (purchased from Indo, sold by Amazon) weekly, and supplied with water or 30mM H_2O_2 solution as needed. SA assay and whole leaf respiration were measured after 2-3 months of plant growth.

SA Assay

SA reacts with iron (III) chloride ($FeCl_3$) to yield a violet complex that absorbs visible light at the wavelength of 540 nm [13]. SA concentration in flax plants was measured using this colorimetric method combined with a spectrophotometer. An adequate amount (at least 0.2g) of leaf material from each plant was obtained and then ground up in 100% acetone to a final volume of 1.5mL. All samples were centrifuged at 7000 RPM for 10 minutes. $FeCl_3$ solution was prepared via the dilution of 135uL of 38% $FeCl_3$ into 50mL tap water. In a cuvette, 3ml of diluted $FeCl_3$ solution was combined with 300uL of supernatant. Absorbance (Absorbance A) of the mixture in the cuvette was measured using the Spectrophotometer at the wavelength of 540 nm. 300uL of supernatant from the same plant sample was also

combined with 3mL of water in a separate cuvette. Absorbance (Absorbance B) for this mixture was also read at the wavelength of 540 nm. This was done to remove obscurity resulting from any plant material absorbing light at 540 nm besides SA. Net absorbance was calculated by subtracting Absorbance B from Absorbance A. Acetone and water blank absorbance readings were also done which acted as negative controls. Absorbance recordings were obtained for each species. To compute the unknown SA concentration from absorbance data, a standard curve was made by measuring SA absorbance at known SA concentrations. The standard curve was constructed using ten known concentrations of SA, starting with 0.01M diluted down to 0.001M (Figure 1). The absorbance value for each known concentration was read at the wavelength 540 nm using the same method as discussed above. The slope of the line of best fit was retrieved from the standard curve which was used to determine the unknown SA concentrations in the plant samples. Final SA concentration, in micromolar, was calculated per gram unit (µmol/g) of the plant material. This process was done for the annual and perennial species discussed above. It was also repeated four times on a weekly basis for RC, RE1, RE2, and RE3 plants in water and H_2O_2 treatments.

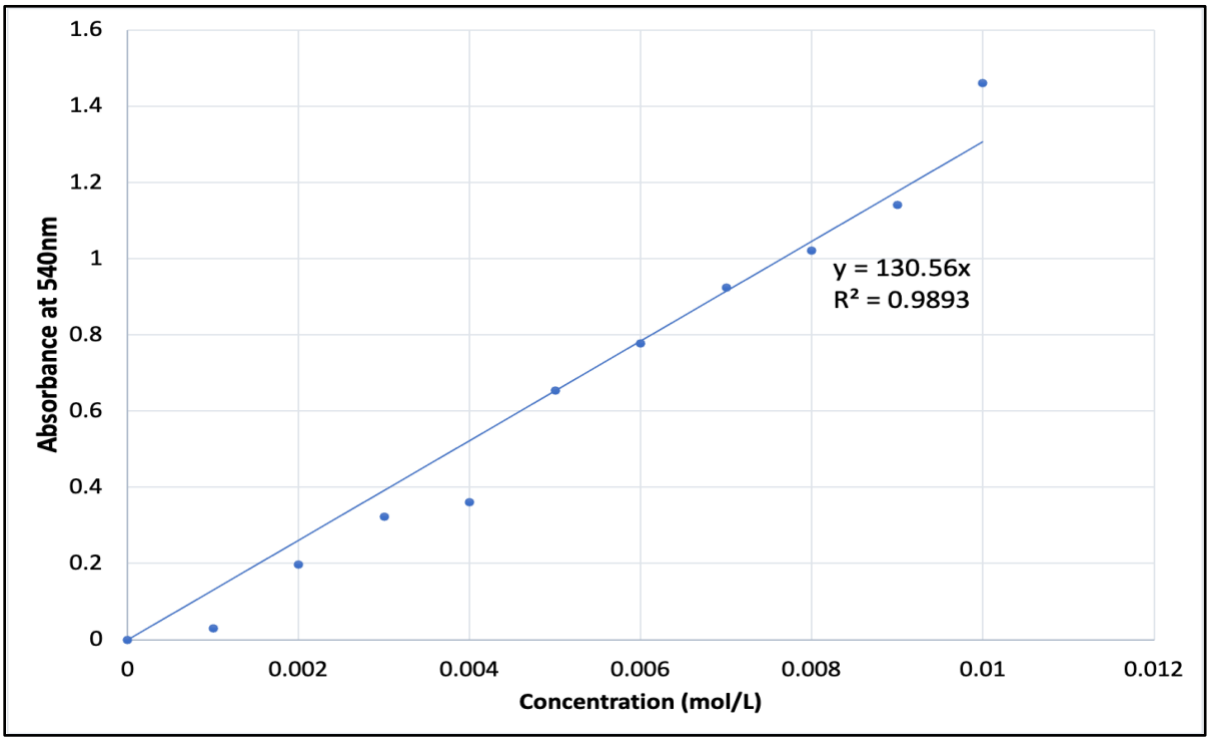

Figure 1. Standard curve for the relationship between SA concentration and absorbance. The X-axis is the known concentration of SA used and the Y-axis is the absorbance recorded at 540 nm using a spectrophotometer. Figure was made using Microsoft Excel.

Intact Leaf Respiration

Respiration rates in plants can be quantified by measuring oxygen consumption rates of intact plant leaves, maintained in complete darkness, using a dissolved oxygen meter [14,15]. The dissolved oxygen meter was first calibrated. Following this, a 40mM phosphate buffer was prepared by dissolving 0.69g of K_2HPO_4 in 100mL of tap water. This buffer was poured into a 50 mL jar along with a stir bar and then placed on a magnetic stirrer. The buffer solution was allowed to saturate with air by stirring it for approximately 1-2 mins. Then, the mass of intact plant tissue, consisting of the stem and leaves, was measured in grams using a weighing scale. The intact plant tissue was placed within the jar and overflowed with the buffer solution. The electrode was carefully submerged into the jar ensuring no air bubbles were trapped in the process. The electrode was penetrated through a rubber stopper that was used to tightly seal the jar. Once the jar was properly sealed with the intact tissue, the magnetic stirrer was turned on and the solution was allowed to stir at the speed of 1,500 RPM, at 23 ºC. The jar was then covered with a black plastic bag to ensure no light was reaching the plant tissue within the sealed jar so the plant cannot carryout photosynthesis which would affect the total oxygen present in the jar. Electrode reading of oxygen in mg/L was read every minute, over 15 minutes, after being completely covered. The solution was allowed to re-saturate with oxygen via air exposure once the 15-minute period was over. In the meantime, a 50mM SA solution was also

prepared using 100% acetone. After the initial 15-minute reading, 1mL of the 50mM SA solution was added to the jar with the same tissue sample as the previous reading. The final concentration of SA in the jar was 1mM. Once again, the jar was sealed via the rubber stopper containing the electrode and covered with a black plastic bag. Another 15-minute reading was obtained for oxygen consumption in mg/L. This procedure was done for RC, RE1, RE2, and RE3 plants in water and H_2O_2 treatments and repeated four times over four weeks. Each time, a blank solution containing only the buffer or buffer with the SA solution was also measured following the same procedure. The final oxygen consumption rate for each species was calculated in micromolar/min per gram of leaf tissue (µmol/g/min). An attempt to measure leaf respiration in annual and perennial species was also made, but due to the lack of plant material, sufficient data could not be gathered.

Statistical Analysis

Statistical analysis for all the SA assay data and intact leaf respiration data was done using the ANOVA model through R software (version 4.1.2). For experiment #1, an analysis of variance model (AOV) was used to look for significant differences in SA expression in annual and perennial species. AOV was first used to see whether *L. usitatissimum*, *L. grandiflorum*, L. *perenne* and *L. lewisii* expressed any significant differences in SA expression in comparison to each other. AOV was then used on annual and perennial species grouped to determine whether SA expression varied based on life-history strategy. For experiment #2, AOV was used similarly to look for differences in wild-type and early flowering lines in terms of their SA levels and oxygen uptake rates. First, we looked at individual differences in SA expression as well as oxygen consumption rates between wild-type, RE1, RE2, and RE3 using AOV. Later, we grouped RE1, RE2 and RE3 to determine if early flowering lines exhibited any significant differences in SA expression or O_2 uptake rates relative to the wild type. This would allow us to see whether lifespan had any influences on uncoupling activity or SA expression. P > 0.05 signifies no significant difference.

Table 1. Example Code for R software (version 4.1.2) used in experiment #2 for SA assay data. Blue text is the code fed into the software and red text explains what the code is testing.

Code for reading file into R software	> SA <- read.csv("//Users//sajanikothari//Desktop//SA1.csv") > print (SA)
Code for applying AOV model	> SA_model <- aov(SA ~ plant + treatment + week + plant:treatment:week + plant:treatment + treatment:week + plant:week, data = SA) > anova(SA_model) *"SA" is the numeric dependent variable tested. "plant", "treatment", and "week" are independent/controlled variables. "plant:treatment:week", " plant:treatment", "treatment:week", and "plant:week" are to look for any interactions that may be present between the variables.*
Code for multiple comparison of means	> TukeyHSD(SA_model, conf.level=0.95)
Code for computing average values for each variable tested	> print(model.tables(SA_model, "means"), digits=3) *"digits" is the number of significant digits desired.*

Results

SA Assay in Annuals and Perennials

There was no significant difference in SA concentrations between annual (*L. usitatissimum* and *L. grandiflorum*) and perennial flax species (*L. perenne* and *L. lewisii*; Figure 2; P = 0.637). However, SA expression was significantly different between the two annual species (P = 0.043; Figure 2). *L. grandiflorum* had higher SA expression than *L. usitatissimum.* Perennial species exhibited insignificant differences in SA expression (P = 0.279). It is worth noting that the sample size for this assay was considerably small which may have influenced the observed results. In the future, these experiments could be repeated with a larger sample size to ensure reliability of the results.

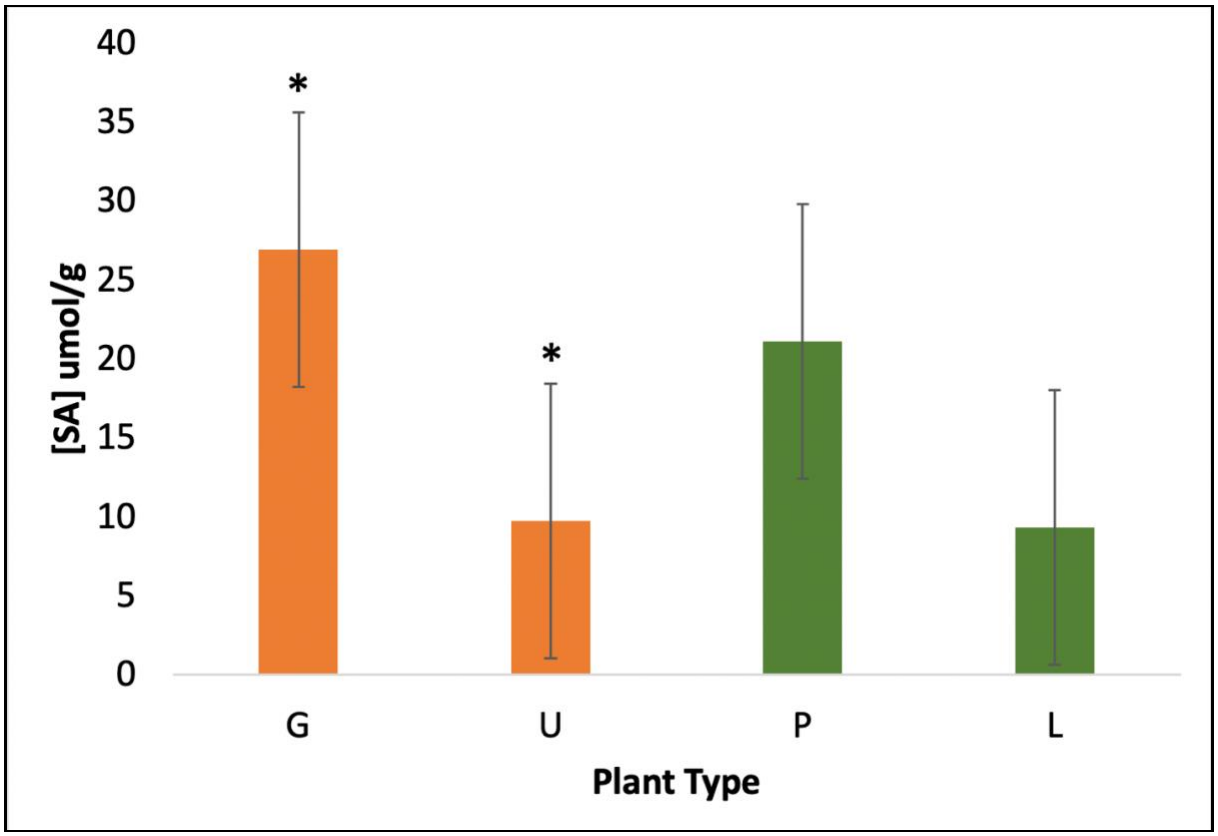

Figure 2. SA concentration for annual and perennial flax species. U is *Linum usitatissimum*, G is *Linum grandiflorum rubrum*, P is *Linum perenne* and L is *Linum lewisii*. Green bars represent perennial species while orange bars represent annual species. Bars with asterisks represent significantly different mean values ($P < 0.05$). Error bars are ± 1 standard deviation from the mean values. Large error bars are due to the use of a small sample size for analysis. Figure was made using Microsoft Excel.

SA Assay in Early Flowering and Wild-Type Plants

There was no significant difference in SA concentration between the plants that were grown in the H_2O_2 treatment in comparison to the ones that were grown in water ($P = 0.079$). RE1, RE2, and RE3 expressed varying amounts of SA, but this difference was insignificant ($P = 0.064$). However, SA concentration significantly differed between wild-type and the early flowering lines (RE1, RE2, RE3 combined; Figure 3). Overall, SA expression was relatively higher in the wildtype, regardless of the treatment ($P = 0.0189$).

Respiration Rates in Early Fowering and Wild-Type Plants

RE3 had a significantly higher oxygen uptake rate than the other early-flowering lines, RE1 and RE2 (Figure 4; $P = 0.033$). However, oxygen consumption rates did not vary between wild-type (RC) and early flowering lines (RE1, RE2, and RE3 combined; $P = 0.427$). Oxygen consumption rates were measured over four weeks. During the fourth week, oxygen consumption rates were significantly lower relative to weeks one and three ($P = 0.028$; Figure 5). Week two data appeared to be outliers and were omitted from the results. The medium that the plants were grown in also did not seem to influence respiration rates. Plants grown in water had O_2 uptake rates that were nearly identical to those treated with H_2O_2 ($P = 0.905$). Although, there was an interaction between the type of treatment and the week at which data was collected which was significant ($P = 0.006$). During week four, the oxygen consumption rate was nearly identical for both treatments. Week one had a higher O_2 uptake rate for H_2O_2 treated plants while week three had a higher O_2 uptake rate for plants grown in water. In all plants, oxygen consumption rate significantly differed when 1mM SA was added to the buffer ($P = 0.002$; Figure 4). All plants experienced an increase in oxygen consumption rate in the presence of SA.

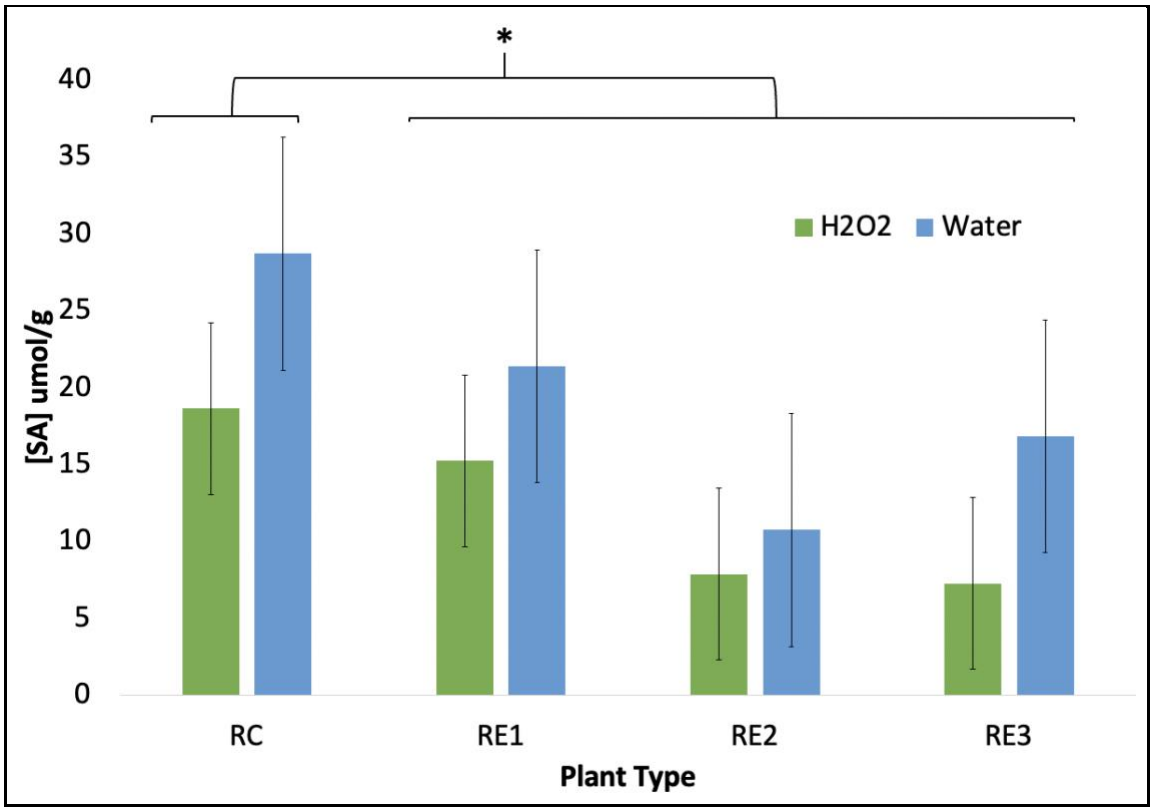

Figure 3. SA concentration for wild-type (RC) and early flowering lines (RE1, RE2 and RE3) grown in water or 30mM H_2O_2. Asterisk represents significantly different mean values ($P < 0.05$). Error bars are ± 1 standard deviation from the mean values. Figure was made using Microsoft Excel.

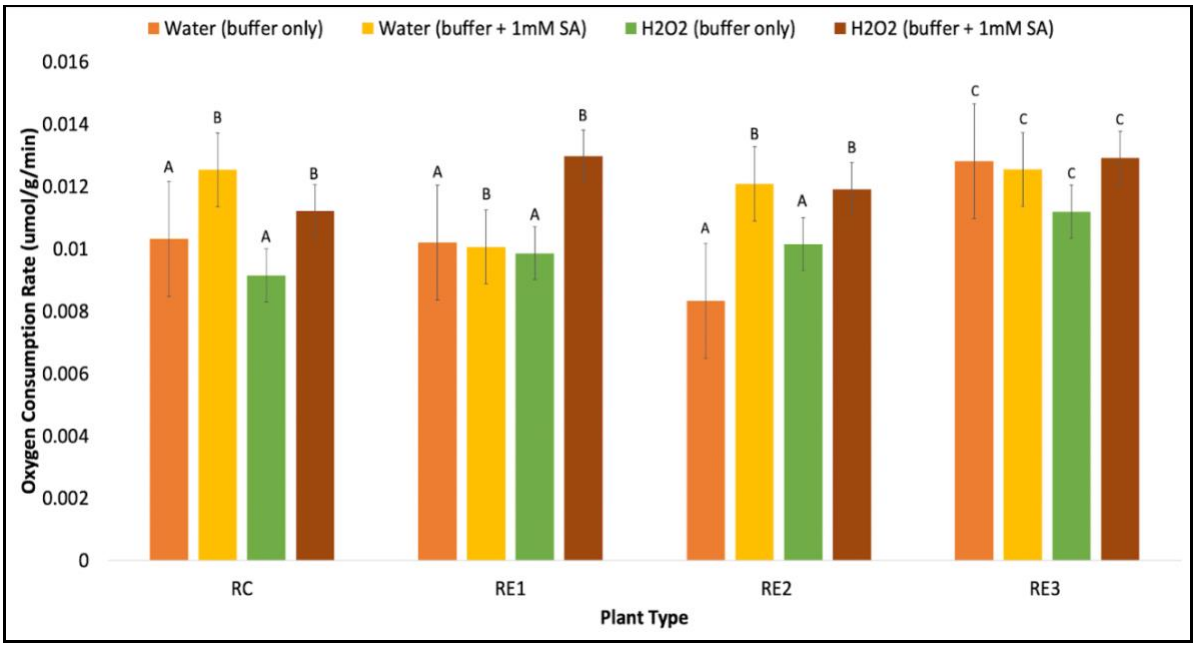

Figure 4. Oxygen consumption rates for RC, RE1, RE2, and RE3 lines. Letters (A, B, and C) represent significantly different oxygen consumption rates ($P < 0.05$). Error bars are ± 1 standard deviation from the mean values. Figure was made using Microsoft Excel.

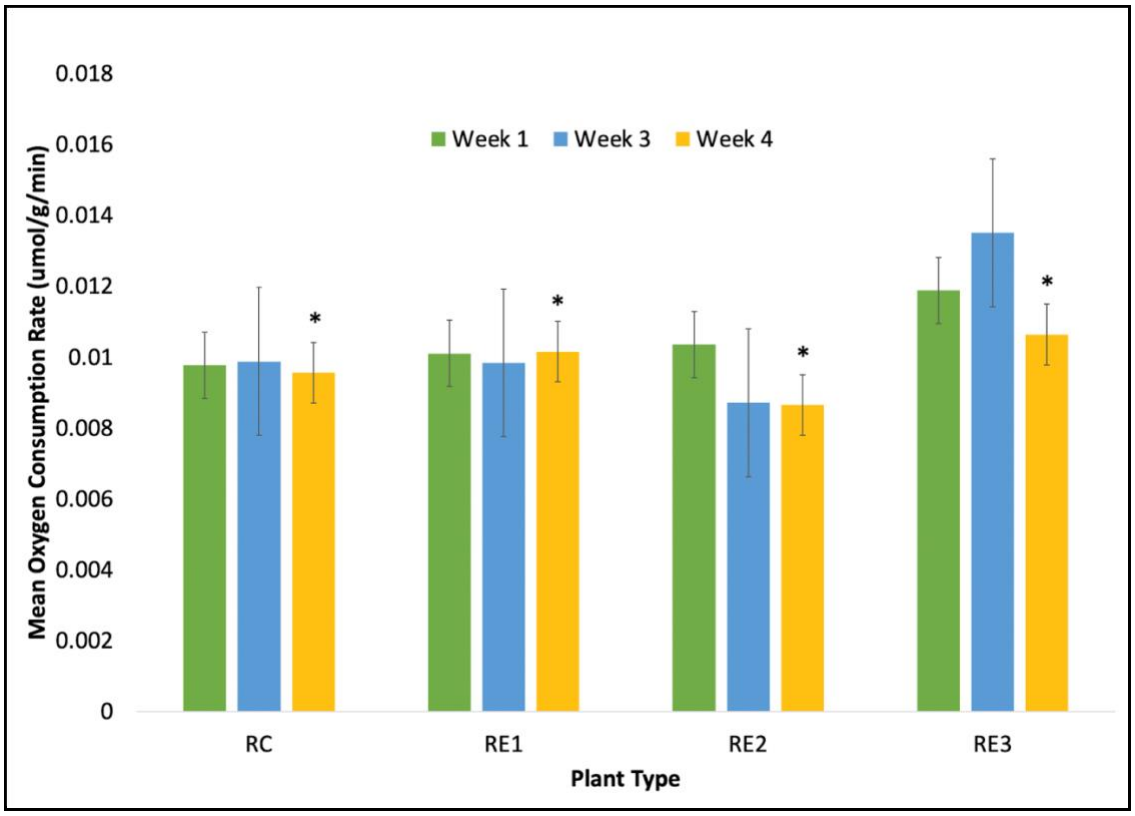

Figure 5. Mean Oxygen consumption rates for RC, RE1, RE2, and RE3 lines retrieved during weeks one, three and four. Asterisks represent significantly different oxygen consumption rates ($P < 0.05$). Error bars are ± 1 standard deviation from the mean values. Figure was made using Microsoft Excel.

Discussion

The central theme behind the "Uncoupling to Survive" Hypothesis is that to increase lifespan, organisms can uncouple oxidative phosphorylation to reduce ROS accumulation. UCP-2 (like PUMP) is a common uncoupling protein found in mammalian mitochondria. Flies overexpressing human UCP-2 exhibited reduced ROS production resulting from greater uncoupling activity, which, in turn, increased lifespan [16]. Comparable results were seen in long-lived mice that also showed greater mitochondrial uncoupling activity while exhibiting higher oxygen consumption rates combined with reduced ROS production [17]. We were able to measure oxygen consumption rates in wild-type and early-flowering lines of *Linum usitatissimum*. Our findings show that oxygen consumption rates in RE1, RE2, and RE3 collectively did not significantly differ from the wild type. Furthermore, AOV revealed that there was no interaction present between the type of plant (wild-type or early-flowering) and oxygen consumption rate in the presence or absence of SA. O_2 uptake rates between wildtype and the early-flowering lines were similar in the absence of 1mM SA suggesting that they did not exhibit any significant differences in the degree to which their mitochondria are uncoupled. Based on these findings, it is possible that perhaps the "Uncoupling to Survive" Hypothesis does not hold for this species, or in plants more generally. Mitochondrial uncoupling activity likely has little effect on longevity in early-flowering flax lines. It has been reported that mitochondrial uncoupling effectively lowers ROS production but at the expense of efficient ATP synthesis [18]. It can be presumed that early-flowering plants may have similar ATP demands as wild-type plants. Although RE1, RE2, and RE3 collectively did not show a significant difference in oxygen consumption rates relative to the wildtype, RE3 specifically exhibited a higher O_2 uptake rate in comparison to RE1 and RE2. We suspect that RE3 likely experienced demethylation of the genes responsible for expressing uncoupling proteins when treated with 5-azacytidine. Overexpression of either uncoupling proteins (such as PUMP) or uncoupling agents (such as FFA) could explain the increased oxygen consumption rates in RE3. Uncoupling activity did not differ substantially between plants grown in water and those growing under stressed conditions. These findings contradict previous research that suggested uncoupling activity in stressed plants is beneficial because it compensates for reduced photosynthetic and respiration rates [19]. Oxygen consumption rates significantly differed based on which week the results were collected. Particularly, week four results exhibited significantly lower respiration rates relative to weeks one and three. We suspect this was likely the result of plant senescence that occurred during week four. Plant

mitochondria were most likely degrading, resulting in reduced oxygen absorption rates.

Upon the addition of 1mM SA, respiration rate significantly increased in both wild-type plants and early-flowering lines. When known uncoupling agents such as 2,4-Dinitrophenol were added to human endothelial cells, the respiration rate similarly increased by 3.5-fold [20]. We postulate that the application of exogenous SA in flax species and the resulting increase in O_2 uptake is due to SA functioning as an uncoupling agent. Similar findings of increased O_2 uptake were also reported in tobacco plants that were subjected to 0.1mM SA in the absence of ADP [21]. It should be mentioned that the optimal concentration at which exogenous SA has the potential to act as an uncoupling agent greatly varies. Tobacco and *Arabidopsis* are low-level SA plants; therefore, higher concentrations (above 0.5mM) of exogenous SA usually led to cell death [22] Meanwhile, for the early-flowering lines in our study, double the amount of SA (1mM) was needed to significantly stimulate the O_2 uptake rate. We cannot be sure whether 1mM is the optimal concentration of SA needed to stimulate respiration in flax species since no other SA concentrations were used while measuring oxygen consumption in the present study and there have been no previous studies on whole-leaf respiration in flax using SA. In general, SA seems to have a dual role. At low doses (<1mM), it can act as an uncoupling agent by breaking the link between oxidation and phosphorylation [8]. At high quantities (1-5mM), it can inhibit respiration through the inhibition of complex I in the ETS [8].

SA is a well-known phytohormone that plays a role in redox homeostasis and protection against oxidative stress [10]. SA assay in annual and perennial species revealed that the degree to which SA is expressed within the four species investigated seemed to be species-specific rather than lifespan specific. Identical results were observed in another study that noted SA expression is not only species-specific but also varies substantially between tissue type, subcellular location, and developmental stage [23]. This is most likely owing to SA's numerous roles in plant physiology, which results in SA having a wide range of functions in any given environment. SA levels did not significantly differ between early-flowering lines, RE1, RE2 and RE3. It was observed that the early-flowering lines collectively exhibited lower levels of SA than the wild-type. This is intriguing because studies suggest elevated endogenous SA positively influences flowering time [24]. SA-depleted plants displayed a late-flowering phenotype in *Arabidopsis thaliana* [25]. Our findings reveal that even when SA concentrations are low, the early-flowering phenotype may still be expressed in flax plants. Furthermore, both wild-type and early-flowering lines showed insignificant differences between H_2O_2 treated plants and those grown in water. In many plant species, it has been noted that exposure to abiotic or biotic stress of any kind usually leads to significant changes in endogenous SA concentration [26]. Whether the SA levels significantly increase, or decrease is usually dependent on the plant species or the type of stress exposure [26].

Conclusions

Our research indicates that the "Uncoupling to Survive" Hypothesis likely does not hold for long-lived and short-lived flax species and lines. Perhaps in flax, mechanisms to counteract the effects of ROS accumulation and aging do not solely involve the use of uncoupling proteins. This data implies that while perennial flax has a higher antioxidant ability to limit ROS buildup, allowing them to live longer, uncoupling their mitochondria may not be a primary source of lowering oxidative stress in these plants. Furthermore, we also did not see any significant differences in endogenous SA expression between long-lived and short-lived flax species. It was concluded that SA expression is highly variable and species-specific. Lastly, our hypothesis regarding the possibility of SA acting as an uncoupling agent was supported by our observation of an increase in O_2 uptake rate in early-flowering flax lines upon exposure to exogenous SA.

List of Abbreviations Used

ROS: reactive oxygen species
ATP: adenosine triphosphate
SA: salicylic acid
H_2O_2: hydrogen peroxide
ETS: electron transport system
FFA: free fatty acids
AOV: analysis of variance

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study did not require ethics approval as it was done in a greenhouse and the subjects used were flax plant species.

Authors' Contributions

JB: made contributions to the design of the study and planning, assisted with the collection and analysis of data, edited the manuscript, and gave final approval of the version to be published.
SSK: made contributions to the design of the study, the collection of data as well as interpretation and analysis of the data, revised and drafted the manuscript critically, and gave final approval of the version to be published.

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