TESTING THE RELATIONSHIP BETWEEN LEAF MITOCHONDRIAL ACTIVITY AND LEAF DENSITY BY USING A TETRAZOLIUM COLORIMETRIC ASSAY

By

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Abstract

The leaf economic spectrum (LES) is a universal pattern describing the intercorrelation of many leaf traits underlying plant structural and physiological function. Dry leaf mass per unit area (LMA) is one of the main descriptor traits for the LES and reflects leaf density, the amount of mass allocated to cellular structure. Despite having a high epistemological impact for plant functional ecology, the underlying cellular trade-offs or coordination for the LES remain unknown. Here, we derive and use a triphenyl-tetrazolium chloride (TCC) colorimetric assay to determine mitochondrial activity through spectroscopic methods to produce empirical evidence at the cellular level. We found a negative relationship between LMA and leaf mitochondrial activity per mass across species. That is, plants that allocate more resources to tissue density had lower metabolic rates per mass. The relationship between dry leaf mass per unit volume (LMV) and leaf mitochondrial activity was also negative, but the models did not fit as strongly as in using LMA. We believe these two negative relationships are caused by the trade-off between cellular space and cellular building structure. This study not only provided empirical evidence, at the cellular level of the negative relationship between mitochondrial activity and leaf density, but also validates the efficacy of the tetrazolium test on leaves to measure leaf mitochondrial activity in an easy, ready, and available way.

I. Introduction

The leaf economic spectrum (LES) drives the intercorrelation of many leaf traits underlying plant structural and physiological function (Wright *et al.,* 2004), including leaf construction cost per unit area, nutrient concentration, rates of carbon fixation, and tissue turnover (Onoda *et. al*., 2017). The main descriptor trait for the LES is leaf dry mass per unit area (LMA). This trait varies substantially among species globally and up to 10-fold between co-occurring species (Wright *et al*., 2004). In general, lower LMA is associated with short-lived, thin, or flimsy leaves, whereas higher LMA is associated with long-lived, hearty, or thick leaves (Wright *et al.,* 2004). Also, high density leaves have lower photosynthesis rates than low density leaves. As photosynthesis is a major factor driving plant growth, the LES helps to predict variation on productivity depending on the type of leaves in terms of LMA living in a given individual up to a given landscape.

The underlying causes for the LES are unknown despite having a high epistemological impact for plant functional ecology. Several hypotheses have been suggested (Kikuzawa & Lechowicz, 2006; Blonder *et al.,* 2010; Michaletz *et al.,* 2016), although there has not been an explicit causal link with plant fitness reflecting either trait trade-off or trait coordination (Reich *et al*., 2003). The closest attempt at addressing this gap of knowledge is the hypothesis posed by Shipley *et al*. (2006). They suggest that the LES is driven by a trade-off between cell lumen space and building structure. As high LMA leaves allocate more resources to structure per tissue volume they have less space for metabolic organelles, such as chloroplast and mitochondria. According to this, the trade-off consists that high LMA leaves grow slower and have lower photosynthesis rates as they have less space for chloroplasts but are stronger and live longer than low LMA leaves. (Wright et al., 2004). These are all important implications for plant functional ecology, but more empirical evidence at the cellular level is needed in order to keep clarifying the causes for the LES.

Here, I continue to evaluate Shipley's hypothesis in terms of leaf respiration directly tied to mitochondrial activity. That is, what is the relationship between leaf mitochondrial activity (or leaf respiration rate) and the trade-off between cell lumen space and building structure? Finding incongruent results of mitochondria from chloroplasts would suggest rejecting Shipley's hypothesis as chloroplast function critically depends on mitochondria, so the number of chloroplasts should be linearly proportional to the number of mitochondria (Yoshida & Noguchi, 2011). On the other hand, congruent results would not only support Shipley's trade-off hypothesis but also implies that chloroplast activity is linearly proportional to mitochondrial activity. Although there are many intuitive factors supporting Shipley's hypothesis, there is strictly no direct empirical evidence to support it, either in terms of chloroplasts or mitochondria.

In order to produce this evidence at the cellular level I applied the tetrazolium colorimetric assay to seven species across campus. Triphenyl-tetrazolium chloride (TCC)

is the most common tetrazolium salt used in botanical research (Ruf & Brunner, 2003). TTC is a colorless substance that replaces oxygen in the electron transport chain in the mitochondria. *Figure 1* shows the electron transport chain and the mechanisms involved in reducing TTC (taken from Ruf & Brunner, 2003). Plants use this process to create energy for cell life in the form of ATP (Schertl & Braun, 2014). TTC is oxidized by this mechanism producing triphenyl-formazan (TF), which is both insoluble in water and red in color. The chemical structures and reduction of TCC to TF is shown in *Figure 2*. This process allows us to measure leaf mitochondrial activity as the concentration of TF of a given leaf sample by using extraction and spectrophotometric methods.

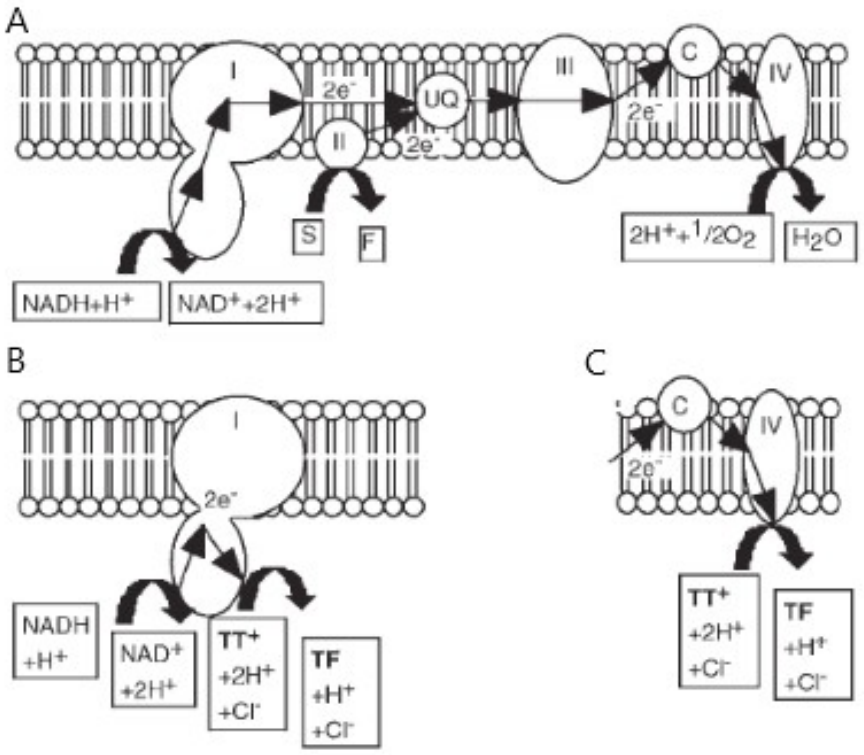

Figure 1 – This shows the (A) flow of electrons through the electron transport chain's enzyme complexes located in the inner mitochondrial membrane. Two postulated coupling points made by Ruf and Brunner (2003) of triphenyltetrazolium (TT^+) are shown (B) with complex I, and (C) with complex IV. Abbreviations: I = NADH dehydrogenase complex; II = succinate dehydrogenase complex; UQ = ubiquinone; III = cytochrome bc_1 complex; C = cytochrome c; IV = cytochrome c oxidase complex; S = succinate; and F = fumarate (taken from Ruf & Brunner, 2003).

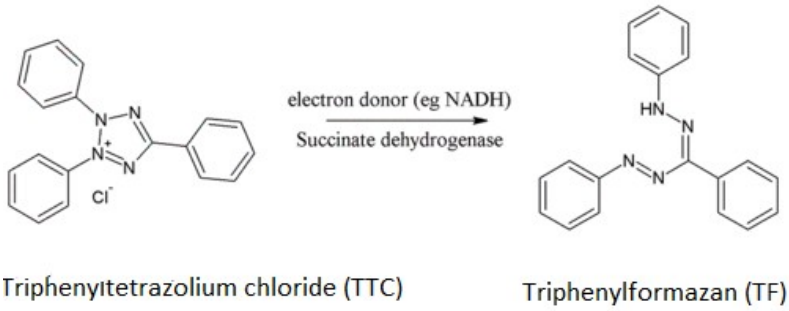

Figure 2 - Triphenyl-tetrazolium chloride (TTC) chemical structure and the reduction to triphenyl-formazan (TF) in the electron transport chain. This reaction is catalyzed by the protein succinate dehydrogenase.

Although tetrazolium assays and their analogs have been used to determine seed viability (Porter, et al., 1947; Wharton, 1955), tree vitality in roots (Ruf & Brunner, 2002) and cancer research (Cory, *et al.,* 1991; Chacon, *et al*., 1997), as far as I know, this is the first time this assay has been adopted to measure leaf mitochondrial activity. In this study, I aim to test the tetrazolium assay in live leaves and use the results produced from this assay to test Shipley's hypothesis for leaf mitochondrial activity in relation to LMA.

II. Materials and Methods

IIA. Plant Sample Collection

I collected a total of 7 trials with different leaf species, with a wide range of LMA, that were used in the tetrazolium assay to determine leaf mitochondrial activity. First, I prepared a cooler with 35 vials of tetrazolium solution and 35 vials of buffered water for the control. A batch of tetrazolium solution was prepared with 8.71g of K2HPO4 (potassium phosphate dibasic anhydrous can be substituted for any phosphate buffer at 0.1M), 3g of TTC, and 250µL of Tween 20 all dissolved in 500mL of distilled water. The solution must be kept in an amber or opaque flask and within a fridge, as the tetrazolium solution is heat and light sensitive (Ruf & Brunner, 2003). This batch can be used for subsequent tetrazolium trials. 4mL of this solution was added to each of the 35 opaque or amber vial. 1mL of buffered water was added to 35 control vials of any material. All the tubes were placed in the cooler for storage. Once in the field, I selected a specific tree, or single species, to start with and chose a leaf. The thickness was measured first. Three measurements were collected per leaf using a millimeter range digital caliper at the distal, medial, and proximal ends of the leaf. Then, I punched 7 holes in the leaf, without cutting the leaf off of the base, using a hand-held hole puncher. This simple tool helps insure uniformity among the leaf disks. I immediately inserted 6 disks into one vial of the TTC solution. The 7^{th} disk was placed in the separate control vial with buffered water. All of the vials were marked for species and leaf number and kept in the cooler. This process was repeated for 5 different leaves per species and 7 species, for a total of 70 vials (control and TTC test).

IIB. Tetrazolium Test and Spectroscopy

Upon arrival to the lab, the flasks were incubated in a bath at 30°C for 24 hours. This allows the TTC to fully react, where it then reaches its asymptotic peak (Ruf & Brunner, 2003). After incubation, the disks were dehydrated with silica. I removed the 6 disks from each vial and placed them in one small paper envelope. The control was removed and placed in a separate envelope. The envelopes were all labeled for species and leaf number. This process was repeated for all 70 vials. I placed all of the envelopes, unsealed, in a plastic bag with fresh silica. The samples were left to dry for at least 48 hours. After all of the disks were completely dry, I removed them from the envelopes and measure all 245 individual disks dry mass using a micro-balance. The 6 disks from each leaf were placed into one lyser tube with 4 lyser beads for each of the 35 trials. The 35 control disks were also placed in a separate lyser tube with 4 lyser beads. All 70 of the tubes were placed in a Tissue Lyser with an equal number of control and sample tubes in each run to ensure the correct balance of the machine. After all the disks were grounded, I tilted the tube and use a magnet to remove all grinding beads from each tube. 1mL of ethanol was added to each tube, TTC and control, and they were vortexed for 20sec to dissolve the TF from the samples. Then, I placed the tubes in a centrifuge at 120,00rpm for 1min. I calibrated the spectrometer with ethanol between each collection. The supernatant was removed from each tube and placed in a spectroscopic quartz cuvette. I measured the absorbance from 400-750nm to observe all peaks and detect any irregularities. The absorbance at the formazan peak around 425nm was recorded for each of the 70 tubes. The cuvette was washed with ethanol and distilled water and dried thoroughly between each collection.

IIC. Formazan and LMA Data Analysis

I created a calibration curve using 10 different known amounts of formazan. I recorded their absorbances at 425nm and plot this against their relative concentrations. The slope of the calibration curve, which should be linear and closely fit to the data, is the epsilon value for the calculation. Using Beer's Law (*See Section V., Equation 1) and the absorbances observe, I calculated the concentration of TF in each sample. To calculate the LMA the dry mass of each disk was divided by the area of the disks, which was 32.17mm^2 for my hole-puncher. To calculate the leaf mass per volume, the thickness values were used from each separate leaf. An average of the three values (proximal, medial, and distal) for each leaf was multiplied by the area of each disk, 32.17mm^2 to get the volume. The dry mass of each disk was divided by the volume to obtain the LMV per disk. A logarithmic manipulation was applied to each data set. Mitochondrial rate values were corrected for the incubation time of 24 hours by dividing all TF concentrations by 24.

III. Results

IIIA. Species Analysis and Collection

The species used in this study were selected based on diversity. The focus was to test a wide array of different species from different families and origins to get the best indication of this test's efficacy to be applied globally. *Table 1* shows the species used with their relative LMA to show the range of species utilized in this study.

Species	Origin	Average LMA (mg/mm^2)
Justicia spicigera	Northeastern Mexico, South to tropical Mexico	0.0242
Dalbergia sissoo	India	0.0540
Cordia boissieri	Texas, Northeastern Mexico	0.0625
Cocculus laurifolius	Japan, Himalayas	0.0740
Pittosporum tobira	Eastern Asia	0.0919
Populus alba	Europe, Central Asia	0.0992
Ceiba insignis	South America	0.1204

Table 1- Represents the species used in this project arranged by ascending LMA values. Their locations of origin are also listed for each species.

IIIB. Tetrazolium Test of Metabolic Activity

The absorbance was read and recorded for all of the samples and manipulated to determine the concentration of TF using Beer's law (*See Section V., Equation 1). A calibration curve was used to find the epsilon value for formazan to be 0.1833 under these conditions. The average value per species was calculated for each dataset from the 5 leaves and 25 disks per species. The resulting data was analyzed logarithmically and with respect to mass, volume and area. Each relationship was fit with a linear regression. The equation of the fit with the subsequent R-squared value are present on each graph. The p-value of each relationship was also calculated to determine the probability of the null hypothesis being a better indicator of the data set. If the p-value is less than 0.05 then the set is statistically significant (Gotelli & Ellison, 2004)

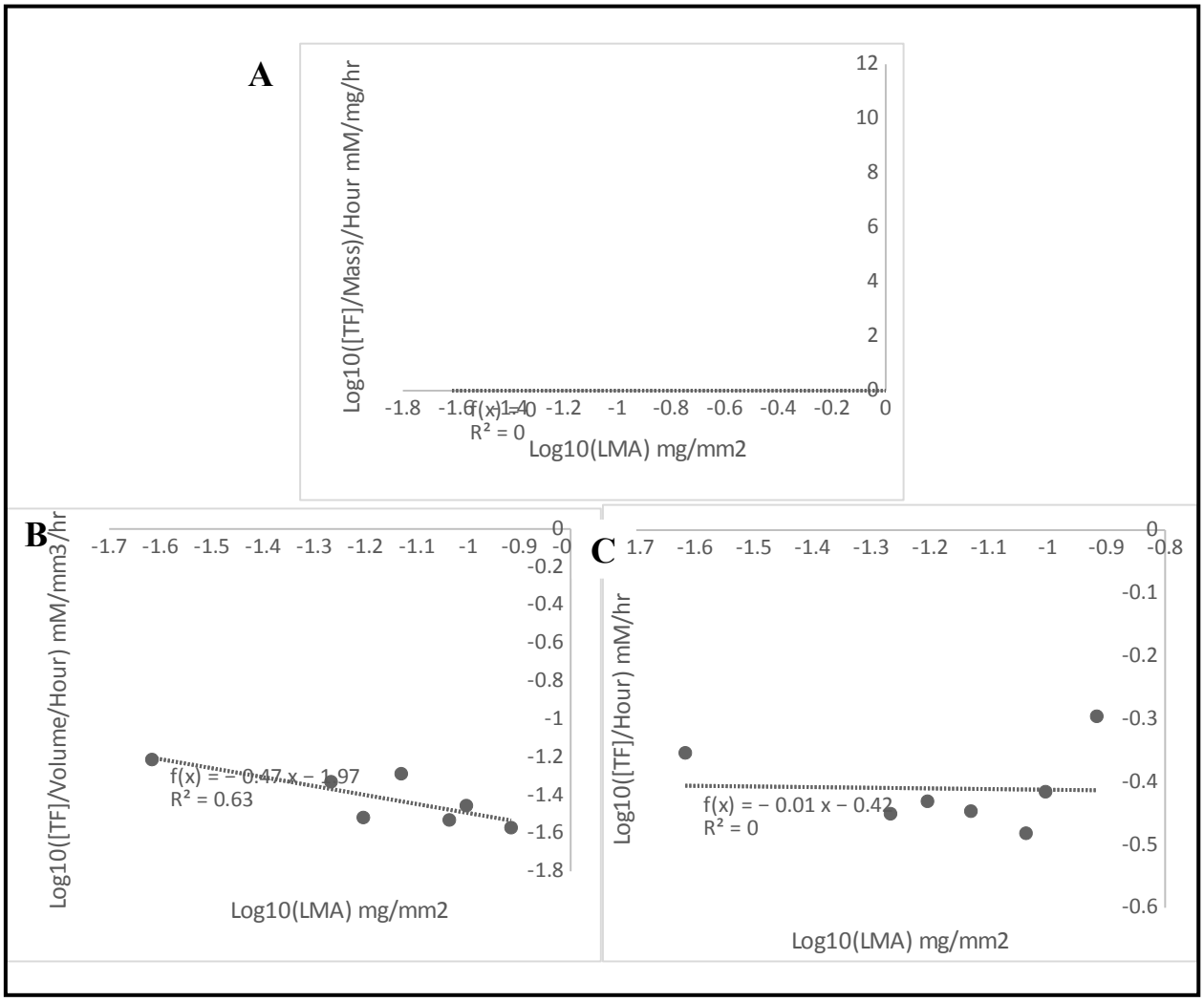

Figure 3 – Graph (A) shows the relationship between the average concentration of TF per mass unit, corrected with respect to time, and their relative average LMA per species. A linear trendline was fit to this data resulting in an $R^2 = 0.9017$, slope = -1.0279, x-intercept = -1.9361. The resulting p-value of this data set is 0.001, therefore the relationship is statistically significant. Graph (B) shows the relationship between the average concentration of TF per volume unit, corrected with respect to time, and their relative average LMA per species. A linear trendline was fit to this data resulting in an $R^2 = 0.6264$, slope = -0.4718, x-intercept = -1.9657. The resulting p-value of this data set is 0.034, therefore the relationship is statistically significant. Graph (C) shows the relationship between the average concentration of TF, corrected with respect to time, and their relative average LMA per species. A linear trendline was fit to this data resulting in an R2 = 0.0014, slope = -0.0103, x-intercept = -0.4226. The resulting p-value from this data set is 0.937, therefore no conclusion can be made from this relationship as it is not statistically significant.

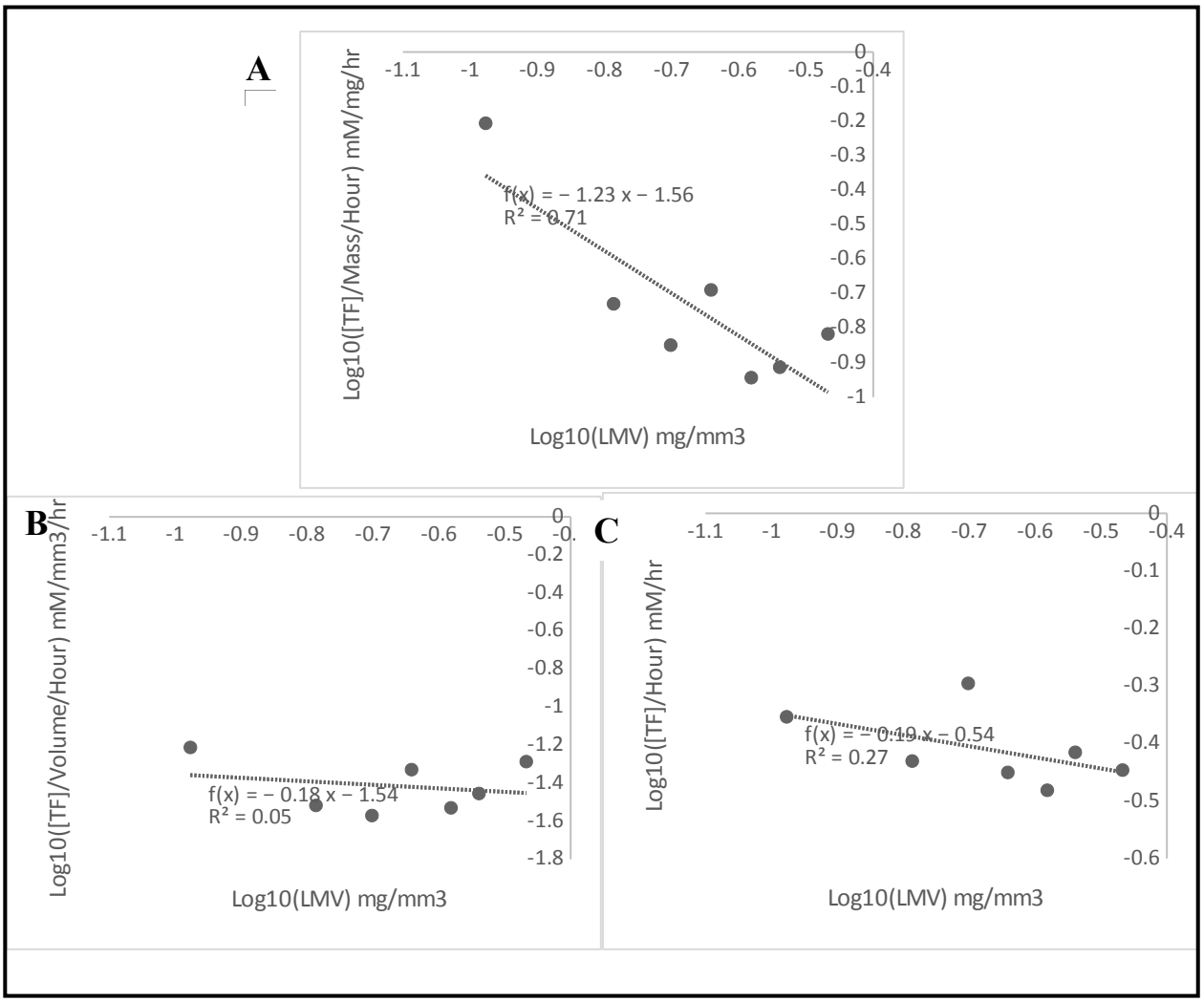

Figure 4 - Graph (A) shows the relationship between the average concentration of TF per mass unit, corrected with respect to time, and their relative average LMV per species. A linear trendline was fit to this data resulting in an $R^2 = 0.7079$, slope = -1.2326, x-intercept = -1.562. The resulting p-value from this data set is 0.018, therefore the relationship is statistically significant. Graph (B) shows the relationship between the average concentration of TF per volume unit, corrected with respect to time, and their relative average LMV per species. A linear trendline was fit to this data resulting in an $R^2 = 0.0523$, slope = -0.1845, x-intercept = -1.5383. The resulting p-value of this data set is 0.622, therefore no conclusion can be made from this relationship as it is not statistically significant. Graph (C) shows the relationship between the average concentration of TF, corrected with respect to time, and their relative average LMA per species. A linear trendline was fit to this data resulting in an $R^2 = 0.2662$, slope = -0.1938, x-intercept = -0.5406. The resulting p-value of this data set is 0.236, therefore no conclusion can be made from this relationship as it is not statistically significant.

To corroborate that the tetrazolium assay works on leaves, I also compared TF production rates between young and old tissue as in Ruf & Brunner (2003) did with roots. For this, samples of *Justicia spicigera* were collected from old and young leaves. This comparison was performed not only to validate the TTC assay on leaves, but to better understand the relationship of leaf aging and mitochondrial activity. Leaves were determined to be 'old' based on a yellow or brown coloration change to a small part of the leaf or if there were pieces missing, or cuts, in the leaf surface. The leaves that were collected as 'young' were very green, small leaves without any damage. Young leaves are expected to have a higher growth rate (Poorter, 1989), requiring more energy from mitochondria. Validation of the tetrazolium assay on leaves is shown in *Figure 5*. Here we see a higher concentration of TF produced per milligram of leaf matter in young leaves than old leaves. Conversely, the old leaves had a lower concentration of TF produced per LMA. This is a trend experienced by many other studies (Geigenberger, 2003; Atkin *et al.,* 2005; Xu *et al.,* 2015) with various plant stressors reducing metabolic activity, such as water availability, air quality, and temperature fluctuations. In general, the metabolic rate decreases with aging, which is reflected in *Figure 5*.

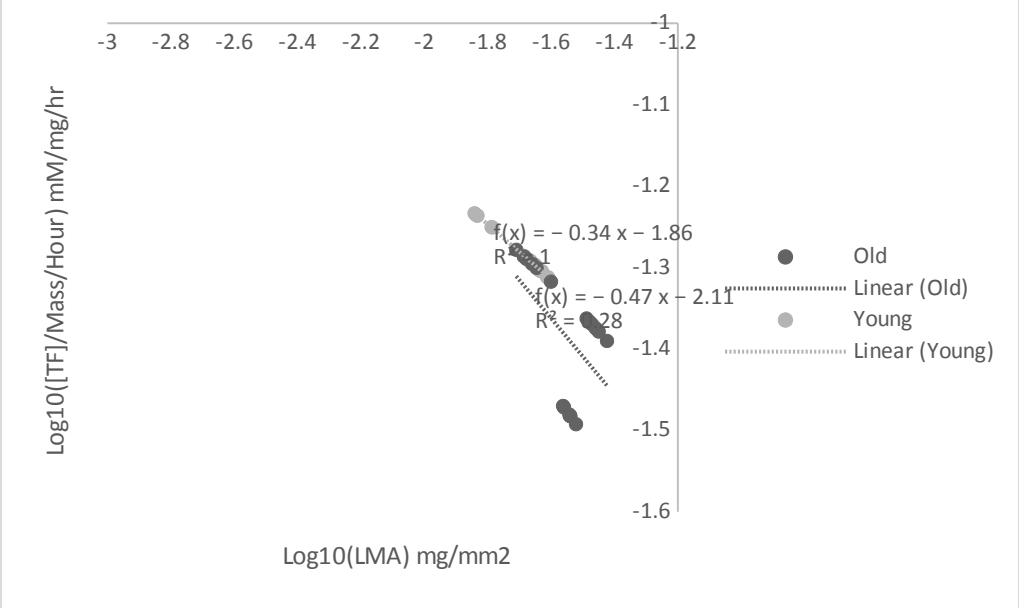

Figure 5 – The difference in the concentration of TF per mass unit, corrected for incubation time, with respect to LMA from damaged and young *Justicia spicigera* samples. A linear regression of the 'young' species yielded an $R^2 = 0.9992$, slope = -0.3385, and x-intercept = -1.8565. The p-value of the 'young' data set is 2.89×10^{-26}, therefore the relationship is statistically significant. A linear regression of the 'old' samples yielded an $R^2 = 0.2794$, slope = -0.4691, and x-intercept = -2.113. The p-value of the 'old' data set is 0.024, therefore the relationship is statistically significant.

IV. Discussion

Understanding the causes of the LES are pivotal to understand plant form and function. Specifically, an explicit causal link of a trade-off or coordination has been left unsolved. Although some, such as Shipley *et al*. (2006), have formed hypothesis into these causes, no empirical evidence currently exists at the cellular level. In this study, we used the tetrazolium colorimetric assay to better understand the direct link between LMA, a key descriptor trait of the LES, and mitochondrial activity. My main result is that leaf mitochondrial activity is negatively related to leaf density supporting Shipley's hypothesis applied on leaf chloroplast activity and leaf density explaining the LES. In other words, species with higher LMA tend to have lower TF production rates per milligram of leaf tissue, and therefore less mitochondrial activity, than species with lower LMA (*Figure 3*). We believe this is due to the trade-off between cellular space and cellular building structure (Shipley *et al.,* 2006). Chloroplast critically depend on mitochondria due to the oxidative species produced by photosynthesis that are reduced by the processes in the mitochondria (Yoshida & Noguchi, 2011). Because of this close relationship, I was able to assume that mitochondrial activity is linearly proportional photosynthesis, which depends on leaf surface area.

Comparing LMA to LMV gives us an additional layer of understanding the driving factor behind these trade-offs. Upon deeper analysis of the TTC data, it was determined that LMA has a closer relationship to TF production than LMV based on the fit of the linear regression models of each graph. In all but comparing graph C from Figure 1 and 2, the R^2 value was closer to 1, indicating a closer fit with less noise, for the relationship between LMA and TF concentration. I believe this is due to the importance of resource allocation in leaf area for more photosynthetic surface to harvest sunlight. Adding leaf thickness (to determine LMV) not only adds another size-dimension variable with its associated error, but also seems to be driven by other mechanisms than maximizing leaf surface such as water and starch storage deviating from the negative relationship (Lambers *et al.,* 2006). Although the relationship between LMV and TF concentration was overall negative in all comparisons, the high p-values of Graph B and C show that the relationship is not statistically significant in order to form concreate conclusions.

The analysis between LMA and LMV also provided a deeper look into the relationship of TF production between species. Although the concentration of TF was standardized in various ways, using the relative mass, area and volume of the disks, there does not seem to be a significant change in the overall production rate of TF. This relationship can be seen in *Figure 3* and *Figure 4* upon comparison of the regressions across LMA and LMV comparisons and between the two. I believe this is showing that across all of the species tested, there is ultimately a similar mitochondrial activity per lifetime under incision stress (from cut to artificial death). All tissue disks were submitted to a drastic homeostatic rupture when being excised from the leaf. My results show a common absolute incision response in terms of mitochondrial net activity across species.

We argue that this connection makes sense as mitochondria across plants are ultimately functioning similarly and are essentially built with the same components.

With the empirical evidence gained from this study using the tetrazolium test, there is a strong indication that there could indeed be a trade-off between cellular space and building structure, as Shipley *et al.* (2006) suggests. This connection is shown here in *Figure 3* and *Figure 4*. Graph A from both figures shows a strong negative correlation between leaf mitochondrial activity and LMA. Space is associated with light harvesting performance whereas building structure is associated with mechanical and thermal performance underlying this trade-off. From this we can clearly see that leaf mitochondrial activity is indeed space limited. Therefore, chloroplast activity is also space-limited as the two are implicitly linked, as stated before. In all, this trade-off may be underlying the LES reflecting low LMA leaves with more space for chloroplast and mitochondria causing a relatively higher metabolic rate. Conversely, high LMA leaves have low metabolic rates, but can endure more stress with time as they are structurally stronger. In conclusion, the negative relationship that I am showing in this work might be driven by a space-structure trade-off at the cellular level explaining the evolutionary origin for the LES.

V. Supplemental Information

1. **Beer's Law:** $Absorbance = \varepsilon\, l\, c$
    a. Absorbance (A) is proportional to the length of the distortion of the wavelength through the solution, or the length of the cuvette (l), the concentration of the solution (c), and some constant for each solution (ε) determined by a calibration curve or found in a table of constants.

VI. References

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