Different Leaf Vein Length Per Area of Mistletoe and Eucalyptus Suggests Difference in Their Internal Metabolism

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Abstract

Mistletoe is well-known for its parasitic relationship with its hosts and host-resemblance characteristics such as physical mimicry, camouflage, shape change, and so on. And in recent years, mistletoe is subjected to the problem of ecological disturbance is a major concern that might be related to the local and global ecosphere. This paper serves to determine the relationship between leaf vein per unit leaf area for parasite mistletoe and its host eucalyptus grown in Australia. According to the literature review of the previous studies about mistletoe-eucalyptus mimicry, it is hypothesized that the mean vein length per area of eucalyptus and mistletoe are equal (Ue = Um), while the alternative hypothesis is that the mean VLA of mistletoe and eucalyptus being significantly different (Ue ≠ Um). The experiment in this study is conducted through a chain of chemical treatments to make the leaf vein visible under the digital microscope and measuring the leaf vein length per area (LVA) using the ImageJ program and testing the hypothesis using T-test assuming unequal variance single factor test. ANOVA tests show that the VLA of mistletoe differs significantly from that of the eucalyptus which suggests that mistletoe and eucalyptus might have different internal metabolism.

Keywords: Australia forest preservation, Eucalyptus, Mistletoe, Leaf Vein Length Per Area (LVA), plant metabolism, botanical processing

1. Introduction

The research into the relationship between eucalyptus and mistletoe can be explained by its importance in understanding the ecological disturbance, which is a biological or non-biological event that brings destruction to organisms in the ecosphere. Ecological disturbance not only affects a large number of tree species but also leads to an increased rate of tree mortality around the globe. Particularly, Mistletoes, a parasite that attaches to a host tree and extracts its water and nutrients for living, has been determined to establish a long-lasting parasite-host relationship with a variety of tree species. They extract water from the host plants by having a lower leaf water potential so they can readily pull water from the host xylem. Mistletoes also have an extraordinarily high water use but low photosynthetic rates.

With the impact of climate change, ecological equilibrium is at stake and trees become more susceptible to be infected by mistletoe, which results in higher forest mortality rates. Reid, Yan, Fittler (2003) agree with the statement, and they claimed that mistletoe has a damaging effect on eucalyptus. They used an experiment disinfection approach to determine the effect of mistletoe on its two main hosts, Eucalyptus blakelyi and eucalyptus melliodora (Reid et al., 2003). Reid et al. stated that after 33 months, all disinfected trees were still alive, yet the 7 of 29 untreated Eucalyptus blakelyi and 1 of 20 eucalyptus melliodora had died (2003). Furthermore, Reid et al., (2003) indicated that the disinfected trees experienced significantly significant diameter increment and significantly more foliage than the untreated trees.

Leaf vein length per unit leaf area (VLA) is a crucial determinant of water and sugar transport, photosynthetic function, and biomechanical support (Sack et al., 2014). In order to determine the effect of mistletoe on its host, the measurement of VLA is needed to estimate the leaf development, physiology, metabolism, and other bio-physiological properties between the two. Unlike non-biological disturbance such as wildfire, flood, or drought that will only pose immediate destruction to the local tree community, the parasitic disturbance is globally distributed and may cause a long-term modification on the global ecosphere.

The parasite Mistletoe will be very likely to have a similar VLA with its host Eucalyptus. According to Griebel, Watson, and Pendall (2017), parasitic plants are globally distributed and serve as an integral component of most of the ecosystems, and their relationship with the host species is often mutualism (Griebel et al., 2017). Griebel et al. also believe that parasite mistletoes can make a vast range of adaptation and mimic various morphological traits specific to their local hosts (Griebel et al., 2017). Barlow (2012) also points out that the parasite mistletoe and its host resemble in shape, size, and presentation of the leaves (Barlow, 2012). Sack et al. (2014) also claim that LVA is a critical determinant of water and sugar transport, photosynthetic function, and biomechanical support (Sack et al., 2014).

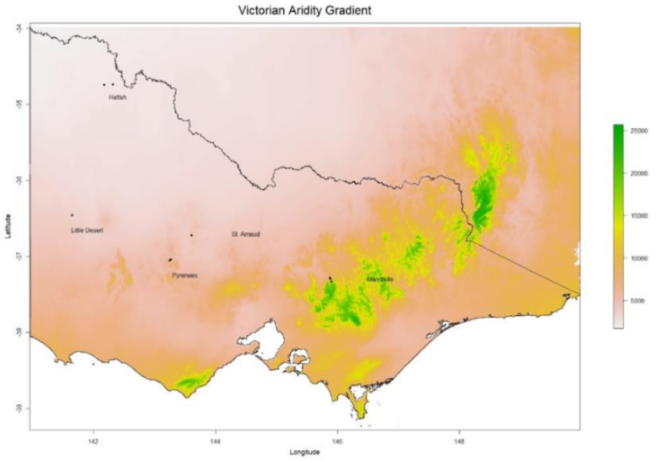

Figure 1: Victorian aridity Gradient (Source: Damschen's lab)

Site	Vegetation	Coordinates	Elevation (m)	MAT (° C)	MAP (mm)	PET (mm)	P/E_p
Marysville - upper	temperate rainforest	37°19'S 145°54'E	731	110	1565	1102	1.421
Marysville - mid	temperate rainforest	37°18'S 145°53'E	495	117	1377	1153	1.194
Marysville - lower	woodland	37°17'S 145°52'E	368	131	1018	1153	0.883
Pyrenees - upper	woodland	37°03'S 143°15'E	500	126	739	1411	0.524
Pyrenees - lower	woodland	37°02'S 143°16'E	363	132	662	1411	0.469
St. Arnaud	woodland	36°43'S 143°36'E	293	140	557	1462	0.381
Little Desert	mallee scrub	36°27'S 141°39'E	156	143	461	1604	0.287
Hattah - east	mallee scrub	34°44'S 142°19'E	56	166	332	1967	0.169
Hattah – west	mallee scrub	34°44'S 142°10'E	43	167	326	1967	0.166

Figure 2: Sampling locations and its corresponding climatic and ecological conditions. Source: UW-Madison Damschen Lab

2. Methods

2.1 Sampling Locations

The sampling procedure was done entirely by Damschen's lab during its visit to Australia in August 2019. Fifty-five eucalyptus-mistletoe pairs were collected at eight sites clustered within five locations
(Marysville, St. Arnaud, Pyrenees, Little Desert, and Hat tah) following an aridity gradient Victoria, Australia (see Figure 1). The sampling locations vary in Mean Annual Precipitation (MAP), Potential Evapotranspiration (PET), Mean Annual Temperature (MAT), Elevation, and Biome (See Figure 2). Among them, MAP, PET, and local biome differ most significantly. For instance, In the location of upper elevation in Marysville, it has a high MAP of 1565 mm, relatively low PET of 1102 mm, and a biome of the temperate rainforest at the upper elevation. Nevertheless, in the western location of Hattah, it has a low MAP of 326 mm, relatively high PET of 1967 mm, and a local biome that consists mostly of mallee scrubs. And mistletoe and eucalyptus samples were collected from mistletoe-infected trees on the roadside of each sampling location.

2.2 Fresh Leaves Processing

The experiment will calculate the vein length per area of four times magnified bleached and dyed mistletoe and eucalyptus vein photos and compare the average of the two. The experiment will use 42 mistletoe vein photos and 57 eucalyptus vein photos.

All of the mistletoe and eucalyptus samples were collected throughout Australia in the past summer. The experiment contains two major processes: 1) Processing of the raw leaves using a series of chemical treatments 2) measuring the vein length of properly processed leaves using the ImageJ program. The purpose of processing the leaves is to make both kinds of leaves visible under the digital binocular compound microscope.

The first step of processing is to digest the thick waxy cuticle and mesophyll until the leaf turns transparent. 5% of NaOH is used for the digestion of eucalyptus, and an average duration of the process is around 2 hours. 20% of NaOH is used for the digestion of mistletoe the process commonly lasts for 2-7 days depending on the thickness of the leaves. The difference in concentration and duration is due to the fact that

the waxy cuticle of mistletoe is much thicker than that of eucalyptus, making it very difficult to digest. Once digestion is complete, it is followed by water treatment.

The second step is a bleaching process in which the leaves are bleached using Sodium Hypochlorite to get rid of the dark-brown color caused by the digestion, the average duration of bleaching is 20 minutes. Upon the completion of bleaching, leaves will have a yellowish or white color. A water treatment follows.

The third step is the dehydration process; 50 % of the ethanol is used to extract all the water inside the leaves to minimize interference. This process takes about 10 minutes.

The fourth step is further dehydration, 100% ethanol is used to extract the remaining water inside the leaves to ensure minimal water interference. This process takes no longer than 5 minutes.

The fifth step is the dying of the leaves using the red safranin dye. Safranin will give the mesophilic region of the leaves a red color which forms a contrast to the dark veins, and the dying process takes no longer than 5 minutes.

The final step is the conserving of the properly processed samples using glycerol for photo capture. Vein photos will be taken using the 4X magnifying lens of the digital compound microscope.

2.3 Measuring LVA

Measuring the vein length per area takes place using the ImageJ program. The scale is set to 1 pixel = 1.7 um and global, the SquareAOI macro is used to draw a 2mm^2 square area of interest (AOI), the segmented line tool is used to trace the vein network within the AOI, and all of the segmented lines will be integrated by ROI manager. Veinstats will calculate the length and AOI and tabulate them in a spreadsheet.

2.Statistical tests

Once the data collection is complete, a series of statistical tests need to be carried out to determine if the main leaf vein length per area (LVA) of mistletoe and eucalyptus is significantly different.

First of all, it is vital to determine the three fundamental assumptions of deciding what test to choose: Normality, independence, and variance. First, normality for the two sampling distributions will be checked by using the Quantile-quantile plot generated by R programming. Second, the independence assumption is checked by using the fact that the samples were collected from different trees in different locations. Third, The equality of variance is checked by performing a descriptive statistics and using the fact that if the variance is equal in the two populations, then one sample variance is no larger than twice the size of the other.

After all the assumptions are appropriately determined, the T-test assuming unequal variance is chosen as the test statistics, and 95% is chosen as the confidence level for this particular test.

3. Results

Descriptive statistics (See Table 1) show that mistletoe samples have a mean LVA of 0.0100181 with a sample variance of 1.026E-05 and a standard error of 0.0005. The eucalyptus sample has a mean LVA of 0.0084, with a sample variance of 3.79E-06 and a standard error of 0.00026. The sample variance of the mistletoe sample is approximately three times larger than that of the eucalyptus. Therefore unequal variance is assumed.

Table 1: A descriptive statistics of mistletoe and eucalyptus VLA

LVA (Eucalyp)		LVA (Mistlet)	
Mean	0.008381943	Mean	0.010018081
Standard Error	0.000257896	Standard Error	0.000494279
Standard Deviation	0.001947073	Standard Deviation	0.003203292
Sample Variance	3.79109E-06	Sample Variance	1.02611E-05
Count	57	Count	42

The normality of the two sampling distributions is checked using the quantile-quantile plot. The VLA for each sample is input into the R studio, and qqnorm(data) and qqline(data) functions are used to produce a qq-plot with a reference line. And both the sampling distributions are approximately normal due to their distribution of a straight linear line (See Figure 2 and Figure 3). The assumption of independence is also assumed since all of the samples are collected from different trees in different trees in environmentally and climatically different locations.

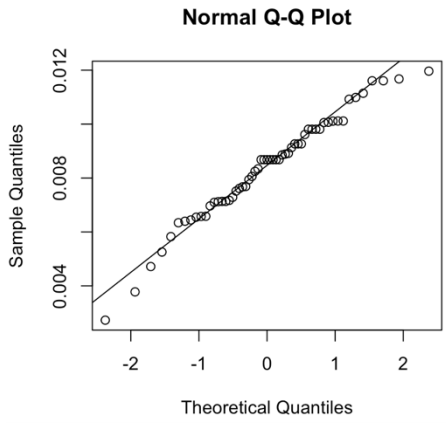

Figure 1: The QQ-plot for the eucalyptus VLA

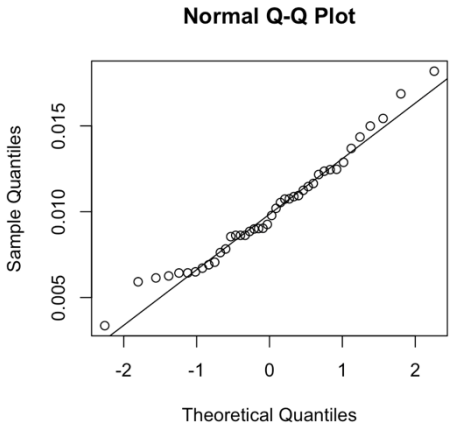

Figure 2Figure 3: QQ-plot for the mistletoe VLA

After the determination of the above assumptions, T-test assuming unequal variance is used to test if the mean LVA of mistletoe is significantly different from that of eucalyptus, with a significance level of 0.05. The T-test produced a p-value of 0.00465 which is less than the significance level of 0.05. Therefore, the null hypothesis is rejected, and it is concluded that there mean VLA of mistletoe and eucalyptus samples collected from varying locations in Victoria Australia does not equal to each other.

The conclusion drawn by the T-test is reinforced by graphing the mean VLA of the two with their corresponding standard error as the error bars and the line graph showing every single sample of the two. In the bar graph, there exists a noticeable difference in the mean VLA between the two species, in addition, the error bars of the two do not overlap each other (along with the p-value). Furthermore, the line graph shows the data points for every single sample collected, and there is a significant difference between the VLA for most of the data point matches (See Figures 4 and 5). Therefore, we can conclude that the difference in the mean is statistically significant.

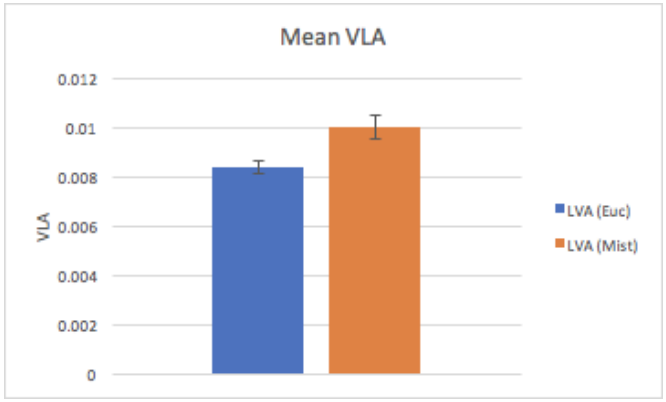

Figure 3: The mean VLA of mistletoe and eucalyptus with error bar

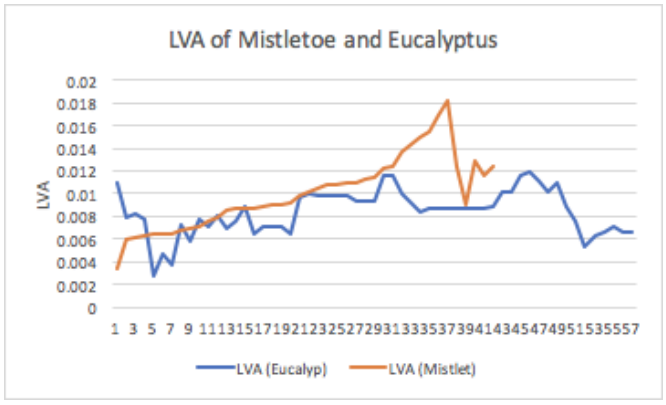

Figure 4: The VLA of each sample of mistletoe and eucalyptus

4. Discussion

The T-test test rejects the null hypothesis that mistletoe and eucalyptus have the same LVA which suggests that there is a difference between eucalyptus' and mistletoe's water and sugar transport, photosynthetic function, and biomechanical support. This might further imply that the significant difference between a mistletoe and eucalyptus LVA is probably caused by different internal metabolism and structures even though the two share some physical similarities.

While most mistletoe-eucalyptus related studies focus on the physical mimicry or the morphological similarities between the two, few had analyzed the internal or metabolic aspects. Canyon and Hill (2006) conducted an experiment to assess the nitrogen content, moisture content, and toughness of mistletoes (Amyema biniflora barlow and Dendrophthoe glabresens barlow) and their host trees (Eucalyptus tessellaris F. Muell. and Eucalyptus platyphylla F. Muell). Canyon and Hill (2006) not only found a difference in mistletoe leaf shape but also greater levels of herbivory and lower nitrogen levels

of the non-cryptic mistletoes compared with their hosts. Canyon and Hill (2006) also indicated that the moisture content and toughness of mistletoes are greater than those of eucalyptus which implies a potentially different internal structure or metabolism.

The implication for different internal metabolism is further reinforced by Scalon and Wright (2015) who argued that even though mistletoe exhibit similar morphological mimicry response to precipitation and moisture index gradient to its hosts, but it has higher nitrogen concentration compared to its host. This finding suggests that the evolution of mistletoe mimicry could be associated with higher nitrogen availability in the host (Scalon and Wright, 2015). Also, they argued that mistletoe uses much more water per unit carbon fixed during photosynthesis than its hosts suggesting a lower water use efficiency and faster transpiration rate (Scalon and Wright, 2015). The implication that the mistletoe mimicry with its hosts could be related to host nitrogen availability could indicate a direction for future research about mistletoe disinfection and conservation of biomass of the local ecosystem. In addition, Scalon and Wright (2015) also argued that mistletoe and its hosts respond differently in terms of a temperature gradient suggesting a difference in the internal mechanism for responding to temperature change.

The assumption of different internal metabolism is further reinforced by the recent discovery by Damschen's lab at the University of Wisconsin-Madison (Richards, Henn, Sorenson, et al.,). They find that in medium to high precipitation conditions, eucalyptus has higher specific leaf area than mistletoe. They also claim that mistletoe tends to maintain a constant leaf thickness while that of eucalyptus tends to decrease as precipitation increases. They further argue that as precipitation level increases, both mistletoe and eucalyptus nitrogen levels tend to increase; however, eucalyptus has a much higher nitrogen level compared to mistletoe. Furthermore, they argue that when precipitation level increases, the leaf dry matter content for both species tends to decrease, but eucalyptus contains significantly more leaf dry matter contents than mistletoe does (Damschen's lab, Richards, Henn, Sorenson, et al.,).

5. Limitation

A major limitation of the experiment arises from the limited number of samples and the highly variable nature of the samples. According to Damschen's Lab in University of Wisconsin Madison, samples collected in Australia are highly variable in terms of specific leaf area (ratio of leaf area to dry mass), leaf thickness, leaf dry matter content, and leaf nitrogen content due to the fact that they are collected from various locations ranging from extremely arid and humid environment. As a result, according to Damschen's Lab, both species collected from area with high precipitation tend to have larger specific leaf area (See Figure 10), and eucalyptus from area high precipitation area tend to have thinner leaves, but mistletoe leaf thickness remains about the same in both environment (See Figure 11).

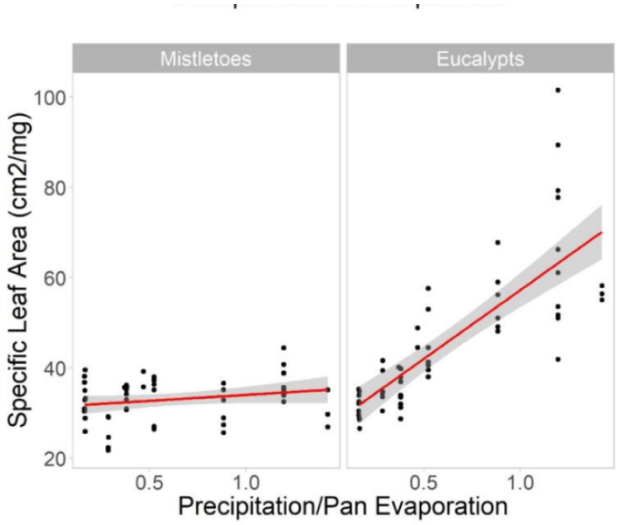

Figure 10: The specific leaf area of mistletoe and eucalyptus following a precipitation gradient. Source: Damschen's lab

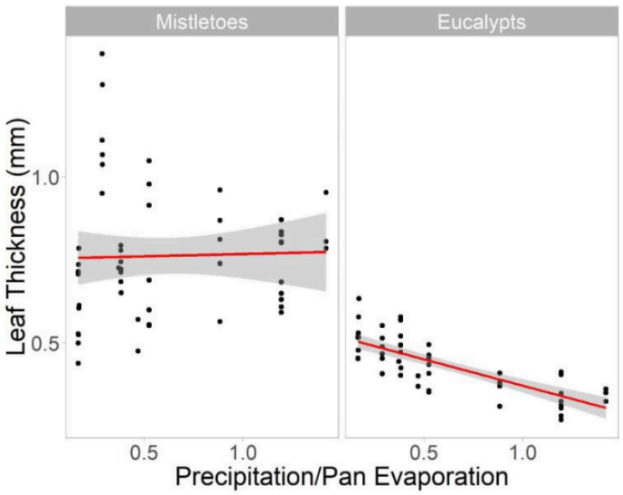

Figure 5Figure 11: The leaf thickness of mistletoe and eucalyptus following a precipitation gradient. Source: Damschen's lab

Both species tend to have lower leaf dry matter content leaves at the area with higher precipitation (See Figure 13), and higher leaf nitrogen content at the area with high precipitation (See Figure 12). Both species exhibit adaptations to their living habitats, as a result, species from an area with

lower precipitation rates may possess different intrinsic properties with their counterparts under high precipitation rates. Due to such variability in the intrinsic trait, the experiment might face various confounding variables that might impact the precision of the experiment.

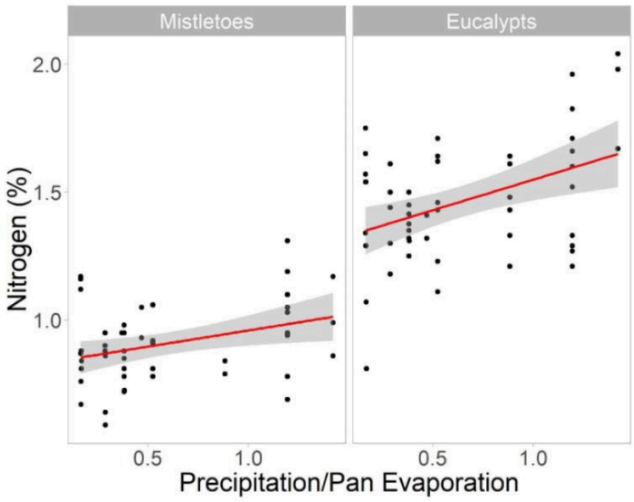

Figure 12: The nitrogen level of Mistletoe and eucalyptus following a precipitation gradient. Source: Damschen's lab

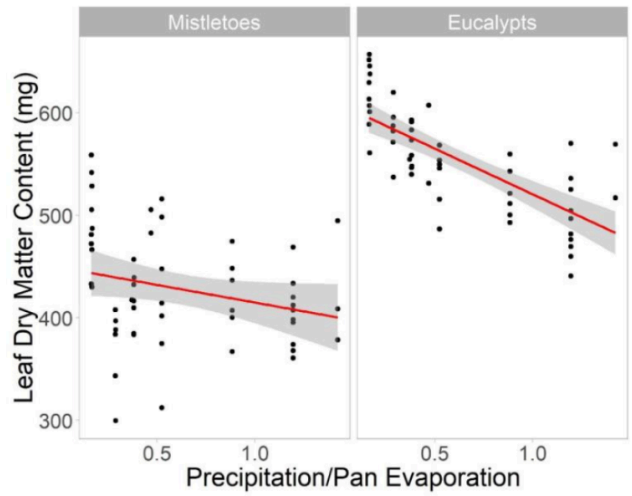

Figure 13: The leaf dry matter content of mistletoe and eucalyptus following a precipitation gradient. Source: Damschen's lab

The second major limitation is due to the inevitable error generated during the processing of fresh leaves. The standard for the digestion process is highly variable due to the different levels of thickness of each leaf. As a result, many leaves may face longer digestion duration such as the mistletoe leaves and mature eucalyptus leaves. The duration ranges from merely 20 minutes to 7 days with the use of different concentrations of NaOH (5% and 20% respectively). Both Under-digestion and over-digestion might decrease the LVA due to either not counting the veins covered by thick cuticle and mesophyll or damaging the veins (See Figure 6, Figure 7, and Figure 8). Therefore, the digestion process requires careful control and the real mean LVA for both eucalyptus and mistletoe might be underestimated. In addition, the dyeing process might also face errors. Over-dyeing would cause the sample to be overwhelmed by the red color of the safranin dye, and the veins would be obscure as a result. Under-dyeing faces the same problem; it will not successfully highlight the network of veins and a decrease in precision might result (See Figure 9).

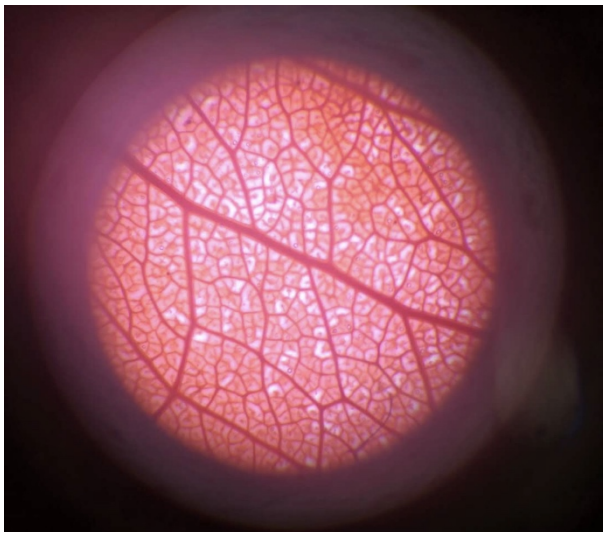

Figure 6. A properly processed eucalyptus vasculature

Figure 7. A over-digested eucalyptus vein vasculature

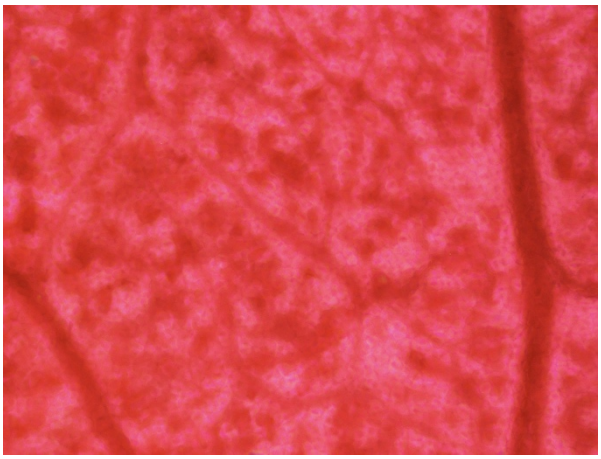

Figure 8. A under-digested mistletoe vein vasculature

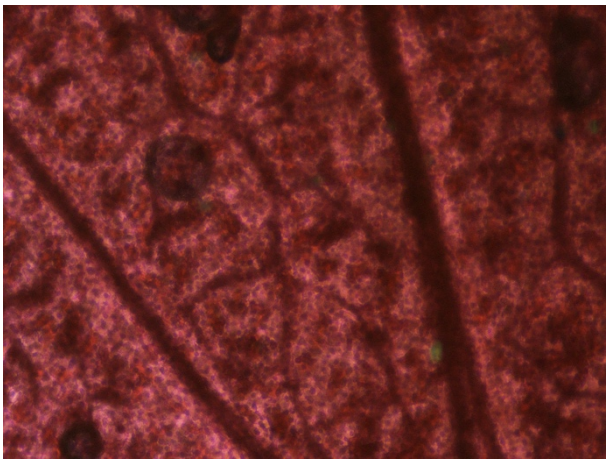

Figure 9. A over-dyed mistletoe vasculature

6. Conclusion

The result of the T-test rejects the null hypothesis that mistletoe and eucalyptus collected in Victoria Australia exhibit similar LVA, and such a significantly different LVA between the two might be explained by different internal structure and metabolism of the two species. Future biological investigations are necessary to compare the cellular functions between mistletoes and eucalyptus such as the xylem water transport, phloem sugar transport, the reproduction cycle, plant hormones, and so on in order to establish an affirmative relationship between the two species. Furthermore, ecological investigations should also be conducted to determine the long-term effect of mistletoe infection on the local biodiversity, biome, or even the ecosphere as a whole.

Acknowledgements

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