# Endophyte Diversity Mediates Leaf Optical Properties

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**Abstract -A single tropical plant species can harbour hundreds of endophyte species within its tissues. Otherwise, little is known about the relationship between endophyte colonization, leaf traits, and spectral properties of leaves. We explore those relationships on *Coccoloba cereifera*, a plant well known for its symbiotic properties. Endophyte richness in *C. cereifera* was statistically correlated with leaf traits such as water content, the ratio of fresh weight/dry weight, and polyphenol/leaf specific weight. Endophyte diversity was also related to spectral vegetation indices of chlorophyll content. The association between endophyte diversity, leaf traits and spectral reflectance pose new questions about our understanding of plant-fungal symbioses and related leaf optical properties.**

**Key words:** Leaf optics, Rupestrian Field, plant-endophyte interaction, spectral reflectance, photosynthesis

## 1. INTRODUCTION

Endophytic fungi, organisms that colonize internal plant tissues without causing disease to its host, have been regarded as crucially relevant in many to plant physiology and ecosystem dynamics (Malinowski and Belesky, 2000). However, the mechanisms that drive endophyte diversity as well as the magnitude of their role in a given plant are still poorly known.

Tropical plant species and ecosystems support a wide community of endophytic fungi. A single tropical plant species can harbour hundreds of fungal species from 60 in *Baccharis dracunculifolia* to 500 in *Palicourea longiflora* (Oki et al., 2009; Souza et al. 2004).

The richness of endophytes is higher in leaves and increases during leaf development (Arnold et al., 2003), probably due to the changes in the structural, and chemical properties that accompany the leaf cycle (Arnold 2005; Malinowski and Belesky 2000).

The presence of endophytes alters the traits and metabolism of many plant species. Some endophytes produce enzymes such as celluloses and ligninases that assist in the degradation of leaves (Carroll and Carroll, 1978), plant hormones (gibberellins) (Hamayun et al., 2009), secondary compounds (Strobel and Daisy, 2003), or even enhance photosynthesis (Marks and Clay, 1996).

Given the range of endophyte effects on leaves, it seems likely that this plant-fungal symbiosis would affect leaf optical properties. Biochemical and structural differences between leaves have significant effects on their spectral reflectance (Sims and Gamon, 2002). Light that penetrates a leaf follows a complex and unpredictable path due to internal reflection and scattering (Sims and Gamon, 2002). The mean path length of light through a leaf can be two to four times the leaf thickness (Fukshansky et al., 1993). Variations in leaf pigment concentration detectable by spectral reflectance have also been shown to be related to leaf development and senescence (Gamon and Surfus, 1999). Otherwise, little is known about how endophytes influence the optical properties of leaves.

This work explores two hypotheses: i) that changes in endophyte richness at the leaf level is influenced by specific leaf traits such as specific leaf weight, water content, polyphenols, chlorophyll and carotenoid concentration which, in turn, can be observed via hyperspectral reflectance as leaves age; and ii) endophytes richness affects reflectance in the visible and near infrared wavelengths, and spectral vegetation indices - derived from these two wavelength regions- can be use as tools to estimate endophyte richness. We postulate that a feed-back mechanism may operate in the interaction between endophyte and host plant, mediating leaf optical properties. Leaf features influence the endophyte community which in turn modifies the leaf traits, therefore having a feedback influence on the endophytes.

To address these two hypotheses, we studied the relationship between endophyte community and hyperspectral reflectance in the Brazilian shrub *Coccoloba cereifera* (Polygonaceae) (Moreira et al., 2008). We chose to address the effects of endophytes on this species' leaf optical properties owing to the ease of evaluating leaf age (ontogeny) by leaf colour. The leaves of *C. cereifera* are short-petiolate, bluish-purple, strongly coriaceous and present a thick silver waxy layer on the lamina.

## 2. MAIN BODY

### 2.1 Material and Methods

We randomly chose 20 individuals of *C. cereifera* in the Reserva Particular Vellozia, Serra do Cipó, Minas Gerais, Brazil in June 2008. On each individual, one single stem was randomly selected from which one leaf belonging to four different ages (just unfolded (hereafter called unfolded), young, mature, and old) were selected (n = 80 leaves total). As *C. cereifera* leaves age, sclerophylly increases and the colour shifts from deep purple (unfolded) through bluish purple (young), turquoise green (mature), to eventually to green (old) (Moreira et al., 2008) Spectral reflectance and UV-excitable chlorophyll fluorescence were measured from each leaf in the field. For leaf spectra assessments, the leaves were measured with the use of a portable spectrometer (Unispec SC, Analytical Spectral Devices, U.S.A.) in the range of 400 to 1100 nm. All spectra were converted to bidirectional reflectance by dividing the data by the radiance from a barium sulphate standard and the internal halogen light source. Spectral sampling protocols followed those presented by Castro-Esau et al. (2006). A UV-excitable chlorophyll fluorescence index was formulated from measurements collected by a dual excitation fluorimeter (Dualex® , ForceOne, France). The Dualex was used for the non-destructive assessment of phenolics present in leaf epidermis (Castro-Esau et al., 2006). Dualex reading were taken from both adaxial and abaxial sides of the leaf. All leaf reflectance data were gathered at three points per leaf on June 22, 2008 at midday sun. Measurements were proximal to the medial axis while avoiding innervations, damaged tissue or areas where the wax coating had been removed. The polyphenolic content (EPhena) of the leaf for each position was estimated according to Meyer et al. (2007).

The leaves sampled were removed and digitally photographed accompanied by a ruler reference. The program Image Tool 3.0 (ImageTool) was used to measure length and area of the leaves. The leaves collected were covered with aluminum foil and kept

away in a thermal box with ice for transportation to the laboratory (4ºC). In the laboratory, water content, specific leaf weight (SLW, dry mass/leaf area), pigment concentration (total chlorophyll, carotenoid) and endophytic assessments for the sampled leaves were determined.

Leaves collected were stored for no longer than 24h before endophyte analyses. For endophyte analysis, we used one circular area (1.54 cm$^2$) from top (proximal) and other from the basal (distal) region of each leaf, once the distribution of endophytic fungi in the leaves is typically not homogenous and spatially structured (Gamboa and Bayman, 2001). Disc surface was sterilized according to Cannon and Simmons (2002) and then were cut in sections of 1mm². Then the fragments of each leaf region were placed onto PDA (Potato-Dextrose-Agar supplemented with cloranphenicol 100ppm to inhibit the growth of bacteria) Petri plates and incubated at 25ºC for 5-10 days. The cultured fungi were separated according to their logical traits, including aerial mycelium form, colony and medium color, surface texture, and margin characters. After hyphae proliferation (about 7-15 days) colonies were submitted to microcultive on glass slides for examination of reproductive structures. The number of species found in different positions (top or base) and leaf ages were recorded.

Chlorophyll analysis was performed from two circular sections (area=1.54 cm$^2$) from each leaf sample, one from the proximal region and other from the distal region. Due to the high degree of leaf sclerophylly, leaf circular sections were thoroughly ground with an electric grinder whilst immersed in 10mL 80% acetone as the solvent. Solutions were refrigerated (at -4ºC) in the dark for 24 hours. After that, they were filtered and centrifuged (14000 rpm for 22 minutes, - 4ºC), then measured at wavelengths 470, 645 and 663nm using a spectrophotometer (Femto 80MB). Calculations of total chlorophyll and carotenoid concentration follow those presented in Holden (1976).

For leaf specific weight and water content one square of leaf tissue (0.5x0.5cm from unfolded and young leaves, 1x1cm from mature and old leaves) was weighed fresh as sampled, and then oven-dried at 75°C until constant weight. The final dry weight was used to calculate the dry weight in relation to the leaf area (specific leaf weight, SLW). The percentage water content was calculated as the difference between the dry and fresh weight divided by fresh weight of each leaf sampled.

As the data did not fit a normal distribution, we used non-parametric test of Wilcoxon Signed Rank to compare the variation of endophyte richness, leaf water, chlorophyll and carotenoid concentration, specific leaf weight (SLW) between leaf regions (proximal and distal region) within each leaf age (Conover, 1980). As no statistically significant difference in endophyte richness, leaf water, chlorophyll and carotenoid concentration, and specific leaf weight (SLW) between proximal and distal region, data were pooled and their average used to observe variations among leaf age using the non-parametric test of Kruskal-Wallis. The average ordinations (rank mean) were compared by the Tukey's test.

Variations of the polyphenol concentration found among three positions within each leaf age were also compared using the non-parametric test of Kruskal-Wallis (Conover, 1980). As we did not detect a statistical difference among the three positions, data pooled and their average used to verify variations among leaf age using the non-parametric test of Kruskal-Wallis. The average ordinations (rank mean) were compared by the Tukey's test.

To evaluate the relationship between endophyte richness and each leaf trait (water content, SLW, chlorophyll and carotenoid concentration, polyphenolics) we used Pearson correlation (Conover, 1980).

To evaluate the influence of the endophytes (richness) in the leaf characteristics studied (water, SLW, the ratio between fresh weight per dry weight, chlorophyll and carotenoid concentration polyphenolics) and spectral index we carried out regression analysis. For this analysis, we excluded the leaves that did not present endophytes. Curve fitting was performed in Sigmaplot (SPSS).

The spectral vegetation indexes used in this study were the Photochemical Reflectance Index (PRI, reference spectra located at 531, 550 and 570 nm), Normalized Difference Vegetation Index (NDVI, at 774 and 800 nm), Simple Ratio (SR at 750, 774 and 800 nm), G and the Water Band Index (WBI at 970 nm) (see Castro-Esau et al., 2006).

## 2.2 Results

Leaf endophytes were diverse (104 species, Table 1) in *C. cereifera*. Endophyte community and leaf ater content, SLW, pigment concentration and polyphenol concentration changed with leaf age. The total number of species was higher in old leaves (87 species) than in mature leaves (11 species) and young leaves (06 species) (Table 1) while no endophytes were isolated from unfolded leaves. We found more species of endophytes per leaf on older leaves (4.35±1.20) when compared to any other leaf age category (Table 1; P < 0.01). The *Coccoloba cereifera* SLW (P < 0.001), water content (P < 0.001), total chlorophyll (P < 0.001), and carotenoid concentration (P < 0.001) were also higher in mature and old leaves compared to young and unfolded leaves (Table 1). On the other hand, unfolded leaves presented the highest concentrations of polyphenols, while no difference in the concentration of polyphenols between young and old leaves was found (Table 1). The ratio polyphenols/SLW found in unfolded leaves was twice higher than in young, mature and old leaves (Table 1; P < 0.001).

Table 1. Endophyte community and leaf traits.

| Parameters | Leaf age | | | |
|---|---|---|---|---|
| | Unfolded | Young | Mature | Old |
| N | 20 | 20 | 20 | 20 |
| TNE | 0 | 6 | 11 | 87 |
| Means | | | | |
| NE | 0a | 0.3a | 0.55a | 4.35b |
| SLW (g/cm²) | 0.016a | 0.030b | 0.031b | 0.031b |
| WC (%) | 0.85a | 0.74a | 0.95b | 0.95b |
| Cl (mMol/m²) | 0.17a | 0.26a | 0.46b | 0.51b |
| Car (mMol/m²) | 0.0007a | 0.0009a | 0.0013b | 0.0016b |
| Pol (µmol.cm$^{-2}$) | 0.172a | 0.159b | 0.148c | 0.159b |
| Pol/SLW (µmol/g) | 10.8a | 5.3b | 4.8b | 5.1b |

*N= number of leaves per leaf age; TE= Total number of endophytic species; NE= Number of endophyte species; WC= Water Content; SLW= Specific Leaf Weight; Cl= Chlorophyll; Car= Carotenoid; Pol= Polyphenols. The means values (±Standard Error) followed by different letters in the same line are statistically different (*P* < 0.05).

*Endophyte community and leaf traits*
We did not detect a statistically significant correlation between the number of species of endophytic fungi and specific leaf weight, carotenoid concentration, and polyphenols (Table 2). We found a low correlation between the number of endophytic species and total chlorophyll concentration (r = 0.2; Table 2). On the other hand, a positive correlation between the number of species and water content (%) (r = 0.30; P < 0.0004), and between the number of endophytic fungi species and ratio polyphenols/SLW (r = 0.40; P < 0.001) was found (Table 2). We also observed that the endophytes found in each leaf were related to water content (%) (r² = 0.43; Table 1), to the ratio

fresh weight/dry weight (r² = 0.54; Table 2) and to the ratio of polyphenols to specific leaf weight (r² = 0.44; Table 2).

Table 2. Summary of correlation coefficient between endophytes richness and leaf traits.

| Person Correlation | | |
|---|---|---|
| Leaf treat | r | P |
| Water content | 0.30 | P < 0.0004 |
| Total chlorophyll | 0.20 | P < 0.05 |
| Polyphenols/Specific Leaf Weight | 0.40 | P < 0.001 |
| Total carotenoid | | NS |
| Specific Leaf Weight | | NS |
| Polyphenols | | NS |
| Regression | | |
| | $R^2$ | P |
| Fresh weight/dry weight | 0.5452 | P < 0.01 |
| Water content | 0.4321 | P < 0.01 |
| Polyphenol/specific leaf weight | 0.44 | P<0.01 |
| Total clorophyll | 0.26 | NS |
| Polyphenols | 0.27 | NS |
| Total carotenoid | 0.20 | NS |
| Specific leaf weight | 0.1 | NS |
| Spectral index | $R^2$ | |
| $SR_{750/705}$ | 0.41 | P < 0.05 |
| $SR_{774/677}$ | 0.38 | P < 0.01 |
| $SR_{800-680}$ | 0.33 | P < 0.01 |
| $NDVI_{(774-677)(774+677)}$ | 0.32 | P < 0.01 |
| $NDVI_{(800-680)(800+680)}$ | 0.30 | P< 0.01 |
| G | 0.57 | P < 0.01 |
| $PRI_{(531-570)/(531+570)}$ | 0.02 | NS |
| $PRI_{(550-531)/(550+531)}$ | 0.07 | NS |
| $PRI_{(570-539)/(570+539)}$ | 0.10 | NS |
| Water Band $_{(970)}$ | 0.05 | NS |

* Not Statistically Significant relationships (P>0.05) are indicated with NS.

*Endophyte influence on leaf optical properties*
The number of endophyte species was correlated to several spectral vegetation indices serving as chlorophyll measures: SR750/705 (r² = 0.41; Fig. 1), SR 774/677 (r² = 0.38), SR800-680 (r = 0.33), NDVI (774-677) (774+677) (r² = 0.32) and NDVI (800-680) (800+680) (r² = 0.30) (see Table 2). We did not find any support for the contention that endophyte richness affected PRI indices (r² < 0.1; P > 0.05) and Water band (r² = 0.01; P > 0.05) (Table 2).
Although a relationship between the endophytes and total chlorophyll was found, it was rather weak (r = 0.20; Table 2), which also may have reflected on the lack of correlation with any of the PRI indices. This study indicates that endophyte diversity may influence total chlorophyll concentration.

**2.3 Discussion**

The differences in the diversity of endophytic species found among leaf ages in *Coccoloba cereifera* may be associated with the nutritional/defense properties of each developmental stage of the plant. During their young stages, tropical leaves often show high levels of anthocyanins, which may function as antifungal defences (Coley and Barone, 1996). *Coccoloba cereifera* leaves may have less chemical defence against fungal colonization when compared to younger ones.
The link between water content and richness of endophytes in *C. cereifera* leaves may have several reasons. The moisture content of plant's tissues can affect growth, frequency of endophyte emergence (Bissegger and Sieber, 1994) and the interaction with fungal symbionts (Abe et al., 1990). Additionally, water content near saturation can also restrict the

entrance of fungi and their growth (Rayner and Boddy, 1986). The endophyte infection may markedly influence minimum leaf conductance, and consequently affect the water relation of the host, therefore functions as a feedback mechanism (Arnold and Engrelbrecht, 2007).
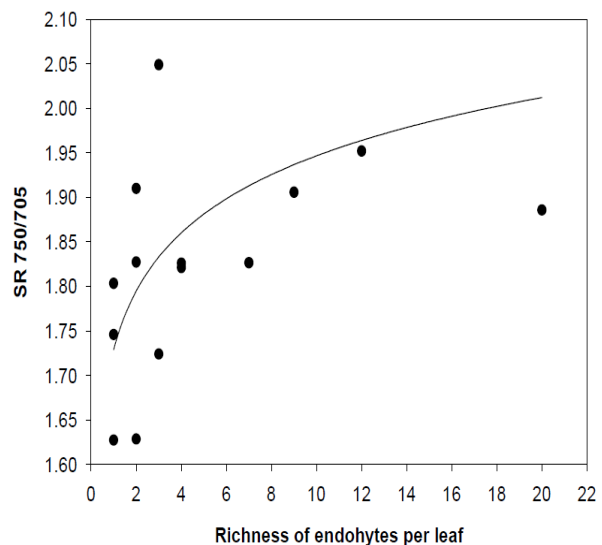


Figure 1. Relationship between the number of morphospecies and SR $_{750/705)}$.

The influence of the endophyte richness on the fresh/dry weight ratio means that the endophytes can increase the water content per unit leaf mass. Thus, it is likely that plant tissues (such as parenchyma) that store water may be involved with endophyte richness. Some evidence points that these fungi generally exist in parenchyma, as well as the xylem and phloem (Vega et al., 2007).
Our results are consistent with the hypothesis that young leaves are generally more chemically protected against natural enemies than mature leaves, and such defences may limit endophytic colonization. In the leaves of *C. cereifera,* a clear correlation between defences (polyphenols per specific leaf weight) and endophytes were found. This correlation was even higher than the correlation found between water content and endophytes (Table 2). These findings suggest that the high polyphenol concentration per specific leaf weight found in unfolded leaves has blocked or limited the growth of the microorganisms.
Other spectral indices (as SR and NDVI) appear to have a strong relationship with endophyte richness. Pigment concentration varies with ontogeny and, hence, may play an important role in visible reflectance (Sims and Gamon, 2002). The Simple Ratio (SR), and Normalized Difference Vegetation Index (NDVI) have been used to estimate chlorophyll concentration among many different applications, but it has also been used to observe intrinsic variations on leaf age, which in fact could be the main reason for this strong correlation. However, we did not detect any relationship between endophytes and the photochemical reflectance index (PRI). Nevertheless, reflectance measurements associated to chlorophyll are sensitive to leaf traits (as waxes, thickness) and the structure of canopy (Castro et al., 2006) and it can interfere in the results found here.
Although a relationship between endophyte richness and total chlorophyll was found, it was rather weak. Some endophyte species can increase the total chlorophyll (Hunt et al., 2005) and (or) affect the photosynthetic rate (Costa-Pinto et al., 2000), suggesting a further link between endophytes and pigment levels.

Although endophytes were related to the water content in the leaves of *C. cereifera*, no correlation with the optical metrics of water content was found. The greater amount of wax in older leaves, which also contain more endophytes, may have influenced this result. The waxes can increase reflectance throughout the visible region of the spectrum but the highest effect is on the shorter wavelengths (Reicosky and Hanover 1978). On the other hand, the clear relationship between endophytes and chlorophyll indices may be intrinsically associated with the water content found in the leaves.

## 3. CONCLUSION

We propose that phenolic content mediates endophyte colonization which, in turn, alters leaf structure and physiology in ways detectable with spectral reflectance. Phenolic content declines as leaves mature, supporting increasing levels of endophytes. Reciprocally, the colonization of endophytes can also modify the leaf structure and pigment content and affect leaf physiology. Therefore, it is possible that several factors, associated with developmental stage, have a direct relationship with the diversity of endophytes.

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