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Cephaleuros virescens in Brazilian Mahogany: Algae Parasitic Disease Threatening an Important Reforestation Tree

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Abstract

The forest species are frequent targets of diseases. In this context, the plant-parasitic algae comprise a separate group in this process, including the algae genus *Cephaleuros*, which attacks mahogany cultivars. The objective of this work was to accomplish the molecular and morphological characterization of the causal agent of algae spots occurring in Brazilian mahogany (*Swietenia macrophylla*). Somatic and reproductive algae structures contained in lesions on the leaves had the DNA extracted and amplified by primers 18SHf and 18SLr of the rRNA nuclear gene 18S. Then, the amplicons were purified and sequenced. The algae structures too were characterized under light microscope. In addition, a total of 12 injured leaves located in the lower third of each attacked tree were randomly extracted for lesion characterization according to its size and number. After algae structures measurements, sporangiophores and sporangia had 260.2 x 12.9 μm and 25 x 18.8 μm. These measurements, combined with the molecular identification, indicated that the algae found in Brazilian mahogany leaves is *C. virescens*. Attacked leaves showed an average of 33 lesions, 60% of which were smaller than 2 mm in diameter, which shows great capacity of the pathogen to reach different parts of the foliar limb. These measurements, combined with molecular identification, confirmed the algae found in Brazilian mahogany leaves to be *C. virescens*.

Keywords: *Swietenia macrophylla* King, algae, DNA

1. Introduction

The Brazilian mahogany (*Swietenia macrophylla* G. King) tree can be cultivated in tropical climate in most of Brazil, except in the South, where there are frosts. The culture of this species is considered low risk, providing that all stages of planning and execution are executed and complied with. Its wood is highly valued in the international market, as it is used to make luxury furniture, besides being an ornamental tree used in afforestation and reforestation (Carvalho, 2007; Silva et al., 2013). The forest species are a frequent target of diseases caused mainly by fungi and bacteria (Kharwar et al., 2010; Verzignassi et al., 2009), whereas the plant-parasitic algae comprise a separate group in this process, lacking research and further information.

Among such organisms, the algae of the *Cephaleuros* genus has higher reputation with the scientific community, particularly because it is widely distributed throughout tropical and

subtropical regions worldwide (Gokhale & Shaikh, 2012). According to Malagi et al. (2011), in Brazil, incidence of algae spots has already been reported in avocado, black pepper, acerola (*Malpighia emarginata*), annatto, cashew, black tea, yerba mate, mango (Vasconcelos et al., 2019), coffee, soursop, apple, cassava, basil, guava, olive and citrus cultivars. However, molecular studies with this genus are incipient, making the morphological and symptomatic characterization crucial for the species identification. In this respect, circular spots can be observed distributed on the adaxial surface of the leaf limb, containing protruding colonies of orangish or rusty chlorophyll pigments (Duarte et al., 2005). Over time, the spots can expand, showing smooth surface in grayish-brown color and taking a large leaf area (Han et al., 2011).

The damage caused by *Cephaleuros* comes from the reduction in the leaf photosynthetic area, which may represent economic loss, especially in times of high humidity and temperature (Malagi et al., 2011; Piccinin et al., 2005). In this context, knowledge of plant diseases is important when considering the adoption of measures for integrated management and productivity increase. Therefore, correct identification of the pathogen is necessary, constituting the first step towards subsequent disease control. Regarding the *Cephaleuros* morphological characterization, there is no standardization as to which characters should be measured and, although measures of the most known characters have already been published, some need to be reevaluated (Rindi & López-Bautista, 2008). Additionally, some isolates of *Cephaleuros* have been identified through the sequencing of conserved genes, occurring in mango trees (Vasconcelos et al., 2018). As a result, this study was designed to carry out the morphological characterization and molecular identification of the causal agent of algae spots in Brazilian mahogany.

2. Methods

2.1 Morphological Characterization

Brazilian mahogany leaves (10-year-old trees) presenting symptoms of algal spot disease were collected in November 2012 in the Campus of the Goias State University (UEG), Ipameri, Goias, Brazil (17º43’00.38’’S, 48º08’40.96’’W, 796 m). The leaves were exhibiting lesions grew larger and protruding. Then, microscope slides were made using SDW as a mounting means and removing body structures with a platinum needle and lesion scraping. Images of the morphological structures were rendered in a Leica DM500 light microscope with an ICC 50 HD digital camera attached. The algae structure measurements were performed using the LAS EZ 2.0 (100x) software, with an average of 30 measurements for each structure (length, width, coloring and shape of the sporangiophore and sporangium). In addition, a total of 12 injured leaves located in the lower third of the injured tree were randomly extracted for determination of lesion size, into three classes: (Class 1: lesions up to 0.1 to 2.0 mm in diameter; Class 2: 2.1 to 4.9 mm and Class 3: lesions larger than 5.0 mm in diameter) and number of lesions per leaf. A total of five trees were sampled.

2.2 Molecular Characterization

Leaves from the same batch of the previous item were recovered aiming to the molecular characterization. The material was sent to the Laboratory of Genetics and Molecular Biology of the Federal University of the Reconcavo of Bahia (UFRB), where molecular analysis was carried out. The leaf damaged areas were excised and the algae vegetative and reproductive tissue obtained was used for DNA extraction using UltraClean® Microbial DNA Isolation kit (Mobio, USA), following the manufacturer's recommendations. The DNA integrity and quantity were verified by using agarose gel electrophoresis at 0.8% and Qubit® 2.0 Fluorometer (Invitrogen), respectively. PCR amplifications were performed with universal primers of the rRNA 18S nuclear gene (Hamby et al., 1988), designated as 18SHf and 18SLr, using the following reagents and concentrations: 60 ng DNA from each sample; 1x Taq DNA polymerase enzyme buffer; 3.7 mM $MgCl_2$; 0.6 pmol dNTPs; 0.4 pmol of each primer; 5 U Taq DNA polymerase, adjusted to a final volume of 50 µl with ultrapure water. The amplification cycles were performed in a Veriti Thermal Cycler PCR (Applied Biosystems) at initial denaturation temperature of 95 °C for 3 minutes; 30 amplification cycles at 95 °C for 1 minute; 52 °C for 1 minute; 72 °C for 2 minutes, and final extension at 72°C for 10 minutes. The amplified products were visualized in agarose gel at 1% (wt vol^{-1}), stained with EtBr and visualized under UV light. Then, the amplicons were purified using the PureLink™ PCR Purification kit (Invitrogen) for subsequent nucleotide identification using the automatic sequencer ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). The editing and assembling of sequences were performed with the Sequencher 4.1.4 software (Gene Code Corporation). The taxonomic identity of the isolates was verified through the GenBank database, using the Basic Local Alignment Search Tool (BLAST) of NCBI (http://www.ncbi.nlm.nih.gov).The sequence for the *Cephaleuros virecens* form Brazilian mahogany leaves was deposited in GenBank database with accession number KR535994. Data on the algae structure measures were subjected to variance analysis and the number of leaf injuries data were submitted to Scott-Knott test ($P \leq 0.05$) using the SISVAR 5.3 software (Ferreira, 2011).

3. Results and Discussion

The mahogany leaves showed round, velvety, greenish-yellow lesions on the limb spread throughout the adaxial surface (Figures 1a and 1b). The lesion average diameters obtained were 1.3; 2.9 and 5.5 mm with variation coefficients 15.17; 10.66 and 16.53% for Classes 1, 2 and 3, respectively (Table 1). As for the number of leaf lesions, they were found to be statistically different: 19.4, 11.4 and2.6 lesions per leaf for Classes 1, 2 and 3, respectively.

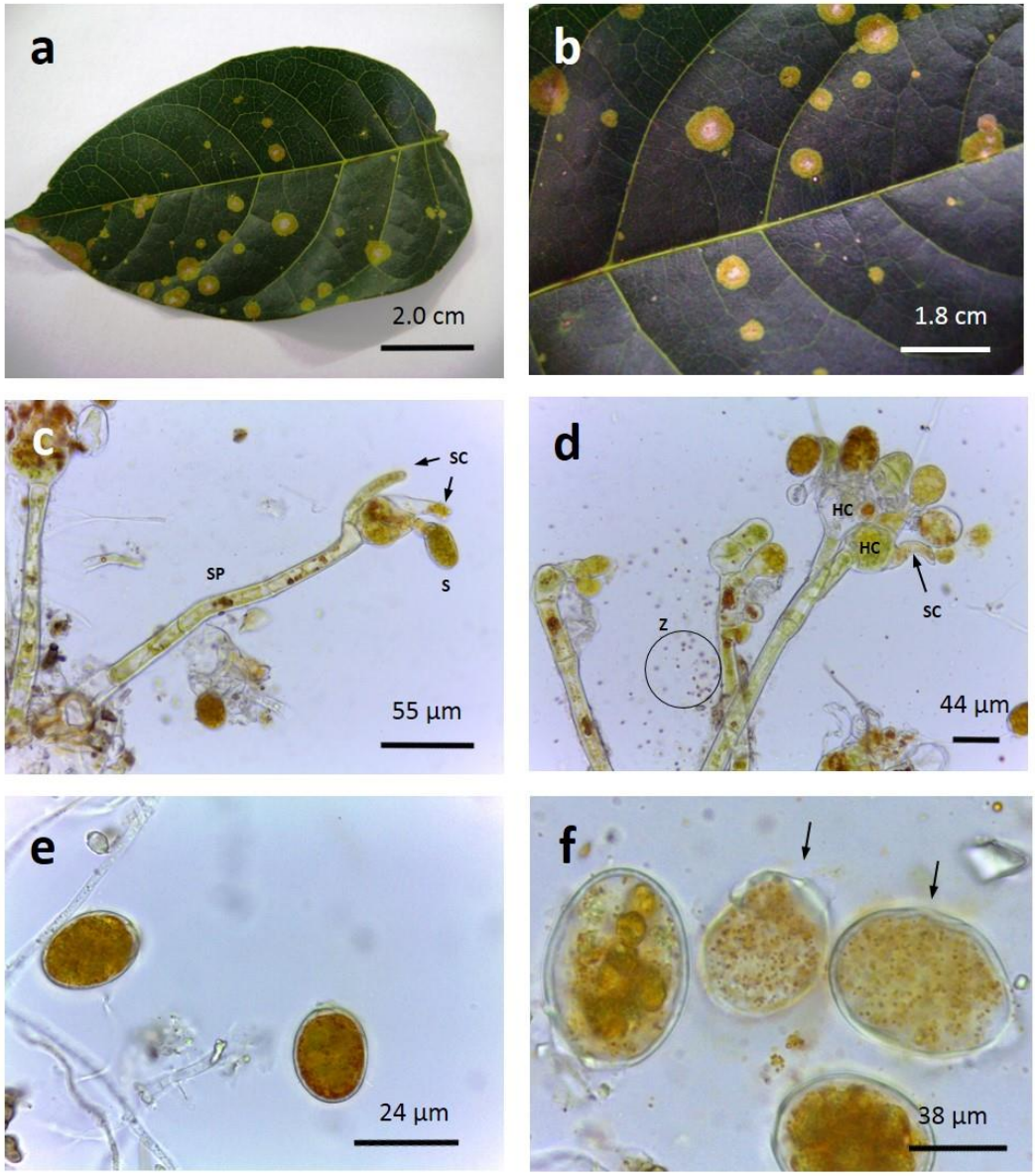

Figure 1. (a) Brazilian mahogany leaf showing symptoms of algal spots; (b) Close-up of symptoms on mahogany leaf; (c) SP – sporangiophore, SC – suspensor cell, S – sporangium; (d)Z – zoospores, HC – head cell, SC – suspensor cell(e)sporangia; (f) Close-up of sporangia in containing mature zoospores

Table 1. Average diameter (Ømm) and number of lesions of algae spot per leaf of Brazilian mahogany. Ipameri, Goias, Brazil

Classes	Lesion average diameter		Number of lesions per leaf[(1)]
	Ø(mm)	Coefficient of variation (%)	
1	1.3	15.17	19.4 a
2	2.9	10.66	11.4 b
3	5.5	16.53	2.6 c
Coefficient of variation (%)	–	–	44.0

[(1)]Values followed by the same letter in each column do not differ significantly by Scott-Knott test (P≤0.05)

After algae structure measurement, the sporangiophores were found to have from 150.0 – 407.3 x 8.0 – 18.8 µm (260.2 x 12.9 µm) (Figure 1c), whereas the sporangia measured 18.2 – 31.6 x 16.5 – 21.3 µm (25.1 x 18.8 µm) (Figures 1e and 1f). The number of sporangiophore septa ranged from 2 to 5 (Figure 1c) with an average of 3.1 septa per sporangiophore. The total number of sporangia produced by each sporangiophore was 2 to 5 (Figures 1c and 1d), reaching an average of 3.2, 1.4 of which attached to the sporangiophore suspensor cell and 1.8 detached from it. Table 2 summarizes the morphological characteristics obtained from the algae occurring in Brazilian mahogany compared to similar reports on other economically important crops.

Table 2. Characteristics of *Cephaleuros* in different hosts, including *C. virescens* found on Brazilian mahogany, Ipameri, Goias, Brazil

Algal species	Host	Sporangiophore (µm)		Sporangium (µm)	
		Length	Width	Length	Width
Cephaleuros virescens[(1)]	*Swietenia macrophylla*	150.0 - 407.3	8.0 - 18.8	18.2 - 31.6	16.5 -21.3
Cephaleuros virescens[(2)]	*Ficus benghalensis*	500 - 1000	12.0 – 25.0	30.0	22.0
Cephaleuros virescens[(3)]	*Citrus sinensis, C. reticulata, C. limetta*	200.4	15.9	20.0	19.6
Cephaleuros parasiticus[(4)]	*Camellia sinensis*	880 - 1256	22.5 - 32.2	17.4 - 27.5	17.4 - 20.8

[(1)]*C. virescens* found in Brazilian mahogany leaves; [(2)]Malagi et al. (2011); [(3)]Han et al. (2011);

[4]Ponmurugan et al. (2010).

A sequence of 538 base pairs was obtained from primers 18SHf and 18SLr of the rRNA 18S nuclear gene. The fragment aligned with 13 *Cephaleuros* sequences, 10 of which being *C. virescens* sequences and 3 being *C. parasiticus* sequences deposited with GenBank. However, the greatest similarity coefficients were found for *C. virescens* (Table 3). Symptomatic leaves, used for the molecular and morphological analysis were deposited and stored in the UEG's Herbarium of Plant Diseases under the registration number H-29-01.

Table 3. Molecular identification based on similarity coefficients found in the DNA of *C. virescens* deposited at GenBank

Access n° in Herbarium and NCBI	Query size (bp)[1]	Identity[2]	Access n° references[3]
H-29-01 KR535994	538	95%	AY052564[4]
		94%	KM020142[5]
		93%	AY220984[6]
		93%	DQ399585[6]
		93%	DQ399584[6]

[1]Amplicons were sequenced in both orientations and the consultation fragments presented correspond to the obtained sequence;[2]E-values were equal to zero for isolates (100% coverage);[3]Access numbers references corresponding to the descriptive sequences indicated in the previous column; [4]López-Bautista & Chapman (2003); [5]Friedl et al. (2019); [6]López-Bautista et al. (2006).

Besides the morphological differences, *C. virescens* and *C. parasiticus* differ as to host symptoms. According to Nelson (2008), *C. virescens* algae species appear on the leaf adaxial surface, with orange to brown or rusty color lesions with circular areas of up to 2 cm diameter, being a subcuticular parasite wherein *C. virescens* is considered to be relatively harmless to plants. As for the *C. parasiticus* algae species, it causes full thickness necrosis to the plant leaf abaxial surface and to all intervening tissues. Moreover, *C. parasiticus* is an intercellular parasite, where more tissue damage occurs than in subcuticular ones caused by *C. virescens*.

Based on the morphological characteristics presented in Table 2, the measurements obtained for *Cepahleuros* structures in Brazilian mahogany plants were found to be close to those obtained for *C. virescens* in citrus trees in Brazil (Malagi et al., 2011). Another important feature of *C. virescens* species is the sporangiophore length, which, when occurring in Brazilian mahogany, was 150.0 – 407.3 μm (Table 2). Conversely, the *C. parasiticus* algae

have larger sporangiophore length and width: 880 – 1256 µm x 22.5 - 32.2 µm (Ponmurugan et al., 2010). Therefore, there is great difference mainly as to width, since for *C. virescens* in Brazilian citrus and mahogany the average was 15.9 and 12.9 µm, respectively. As a result, the characteristics presented in Table 2, together with the molecular characterization, indicated that the algae found in Brazilian mahogany (*S. macrophylla*) was *C. virescens*. A hindrance point for the distinction between *C. parasiticus* and *C. virescens* based on morphological characters would be the small overlapping areas in sporangiophore dimensions obtained by some authors. However, these overlapping areas are very limited and do not compromise the morphological taxonomy. As an example, the minimum sporangiophore dimensions obtained by Han et al. (2011) can be mentioned, which are do not happen in *C. parasiticus*. Furthermore, the starting point for sporangiophores *C. parasiticus* dimensions corresponds to the maximum found for *C. virescens* (Ponmurugan et al., 2010).

Malagi et al. (2011) claimed that there is higher algae spot incidence on the shaded leaves of the plant lower third, and this motivated the collection of leaves in the lower third of Brazilian mahogany trees to obtain the data shown in Table 2. Interestingly, attacked leaves showed on average 33 lesions, which can be considered a low value when compared with *Mycosphaerella citri*, which showed on average 131 injuries per leaf (Silva et al., 2009). Moreover, 60% of the algae spot lesions were smaller than 2 mm in diameter (Class 1), which demonstrates greater capacity of the pathogen to reach different points of the leaf limb. Within the same injury class, there is little variation among sizes, suggesting symmetry typical of algae spots. According to Keller et al. (2000), the number of lesions is an important fact because it is a variable highly correlated with the disease severity.

The occurrence of algae spots is favored by average monthly temperatures around 23°C and average monthly rainfall of 127 mm (Malagi et al., 2011). During the month of November 2012, when collection of damaged leaves was carried out to obtain the data in Table 2, the temperature in Ipameri town ranged from 18.3 to 38.0°C, a condition associated with precipitation of 133.9 mm (Inmet, 2015) that favors the disease development. This occurs because rainy periods with temperature ranging from 28 to 32°C are ideal for the envelope membrane of sporangia to break, which facilitates wind dispersion of zoospores (Duarte et al., 2005).

Another object of this research was the variability of measurements obtained from *C. virescens* sporangiophores and sporangia, where variation coefficients of 31.28 and 22.06% for sporangiophore length and width and 11.94 and 6.68% for sporangia length and width, respectively, were observed. There are no reports on the measurement variability found in *C. virescens*. However, it can be inferred that the variability found for the sporangia is close to those found in studies in the mycology area (Carvalho et al., 2008). These values are important because they show that the sporangiophores length is more variable when compared with other structures measured. Finally, it is worth noting that the homogeneity obtained in the measurement of micromorphological structures helps the characterization and standardization of *C. virescens* structure measurement.

Hamby et al. (1988) report that the information from the rRNA 18SHf and 18SLr gene is important for the differentiation of *Cephaleuros* pathogenic species. Similarly, this study enabled the identification of the *C. virescens* algae from the excision of symptomatic leaf lesions, despite the reduced number of sequences of this kind deposited at GenBank. On this platform, sequences of *Trentepohlia* and *Phycopeltis* species included in the same *Cepahleuros* monophyletic group occur in greater amount (Boedeker et al., 2013; López-Bautista et al., 2006; Rindi et al., 2009; Suutari et al., 2010).

The phylogenetic proximity between *C. virescens* and *C. parasiticus* was present in previous studies (López-Bautista & Chapman, 2003; López-Bautista et al., 2006; Zhu et al., 2015). Therefore, the alignment of H-29-01 sequence with 93% similarity to *C. parasiticus* not invalidate the morphological results that refer to *C. virescens*. Similarity equal to and greater than 93% was assigned to *C. virescens* sequences deposited in GenBank (Table 3). Although both C. *virescens* and *C. parasiticus* typically occur in leaves, the lack of genetic information caused by very short length or contamination by other microorganisms Trentepohliaceae can explain this phenomenon.

4. Conclusion

The conclusions of this research are: symptomatology-based descriptions associated to morphologic and molecular characterization indicated that *C. virescens*is the algae found on leaves of Brazilian mahogany (*S. macrophylla*) in the state of Goias, Brazilian attacked leaves showed on average 33 lesions, 60% of which were smaller than 2 mm in diameter, evidencing greater capacity of the pathogen to injure different parts of the foliar limb.

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