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Modification of anthracnose severity in açaí seedlings by the endophytic fungus *Hypoxylon anthochroum* strain 2.4996

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Abstract

The cultivation of açai palm, which yields tasteful and nutritional fruits, has been stimulated by the high commercial values of açai pulps; however, the occurrence of anthracnose (a disease caused by the fungus *Colletotrichum gloeosporioides*) on açaí nursery and orchards has been a challenge for the shift from açaí extractivism to its cultivation. The interaction among endophytic fungi and host can change the plant disease severity, facilitating infection by phytopathogens and increasing its damage to plants. Therefore, this study aimed to investigate the antagonistic activity of five endophytic fungi against *C. gloeosporioides*, as well as to evaluate the anthracnose severity in açaí seedlings in response to the inoculation of the most promising of the tested endophytic fungus. First, in vitro evaluation showed that most of the fungi were able to grow over *C. gloeosporioides* mycelia. The endophytic fungi *Graphium* sp. 2.4765 and *Hypoxylon anthochroum* 2.4996 recorded the highest rates of growth inhibition, 79.3% and 77.0%, respectively. Because *H. anthochroum* 2.4996 presented better in vitro growth and spore production, it was chosen for subsequent evaluations. Second, *in planta* evaluation showed that the inoculation of *H. anthochroum* 2.4996 in diseased plants enhanced both the necrotic area in leaflets and disease symptoms. Third, comparative analyses based on the cultural, micromorphological and molecular characteristics have shown that such strain is related to *H. anthochroum*. Overall, this study highlights the complexity of the plant–microbe interactions.

KEYWORDS
Amazon, biological control, *Colletotrichum gloeosporioides*, *Euterpe precatoria*, plant–microbe interactions

1 | INTRODUCTION

The hemibiotrophic fungus *Colletotrichum gloeosporioides* causes anthracnose in açaí palm (*Euterpe precatoria* Mart.). This fungus affects mainly nursery seedlings, damaging their leaflets (Costa et al., 2019; Nogueira et al., 2018). The initial anthracnose visual symptoms in açaí seedlings are tiny circular-to-elliptical brown spots bearing a light central area and chlorotic halo (Castro et al., 2017). Synthetic fungicides have been traditionally used to manage anthracnose; however, natural strategies are required to reduce environmental pollution that arises from the continuous usage of chemical compounds (Zhang et al., 2020).

Endophytic fungi are emerging as a promising biological alternative to manage plant diseases (Fontana et al., 2021). These fungi can provide plant growth through phytohormone production and improve the uptake of nitrogen and phosphate (El-Din Hassan, 2017; Stefanoni-Rubio et al., 2021; Suebrasri et al., 2020). Also, endophytic fungi can enhance plant defence by stimulating host resistance (Rodriguez et al., 2009). Furthermore, these microorganisms can control pathogens through competition, mycoparasitism or antibiosis (Fadiji & Babalola, 2020; Rajani et al., 2020). For instance, previous assays showed that endophytic fungi from açaí can control *C. gloeosporioides*, exhibiting different mechanisms of antagonism (Peters et al., 2020).

Some endophytic fungi, however, can present contrary effects and enable the infection of plants by suppressing plant defences (Arnold et al., 2003; Ridout & Newcombe, 2015) and, consequently, increasing the disease severity (Houterman et al., 2008). For example, *Penicillium* sp. acted as an enabler in the presence of the pathogen *Drepanopeziza populi* in *Populus angustifolia* (Busby et al., 2013). Additionally, pathogens can use endophyte metabolites to enable their own virulence (Busby, Peay, et al., 2016).

The plant–pathogen interactions that involve fungal antagonists are complex, and the effect of endophytic fungi as disease enablers in plants has been poorly investigated. Therefore, this study aimed to evaluate the antagonistic activity of five endophytic fungi against *C. gloeosporioides* and the anthracnose severity in açaí seedlings in response to inoculation with endophytic fungus *Hypoxylon anthochroum* strain 2.4996 through in vitro and *in planta* assessments.

2 | MATERIALS AND METHODS

2.1 | Endophytic fungi and phytopathogen

Endophytic fungi were isolated from leaves of *E. precatoria* grown in the southwestern Brazilian Amazon, and *C. gloeosporioides* was isolated from açaí plants with anthracnose symptoms in Rio Branco, Acre, Brazil (Costa et al., 2019; Peters et al., 2020). The *C. gloeosporioides* identification was confirmed by the conventional approach, using macro- and micromorphological characteristics (Costa et al., 2019; Rufino, 2019). Based on initial screening (Peters et al., 2020), five endophytic fungi (strain *Graphium* sp. 2.4765; *Paecilomyces* sp. 2.5110; *H. anthochroum* 2.4996, *Xylaria* sp. 2.4749 and *Xylaria* sp. 2.4769) were selected for the antagonistic activity. This screening was based on a methodology that tested three endophytic fungi and the pathogen per Petri dish (Guevara-Avendaño et al., 2018). The specimens of these endophytic isolates were stored in the Microbiology Laboratory at UFAC.

2.2 | Dual culture assay

Fungi were cultivated in a medium made of potato dextrose agar (PDA), for 7 days at 28°C. Mycelium (5 mm-diameter discs) of phytopathogen and endophytic were taken from the colony margins and inoculated in 9 cm-diameter Petri dishes containing PDA medium. Plates were incubated for 7 days at 28°C and each treatment (endophytic fungi and pathogen) had five replications ($n = 5$). The radial growth of the *C. gloeosporioides* colony was assessed on the 7th day. The growth inhibition (GI%) was estimated according to Hajieghrari et al. (2008). Macroscopic analysis of the interaction between pathogen and endophytic fungi was based on the categories: (1) contact inhibition; (2) inhibition at distance; (3) overgrowth of a mycelium over the other and (4) replacement, where mycelia of a fungus were replaced by its opponent (Boddy, 2000; Preto et al., 2017).

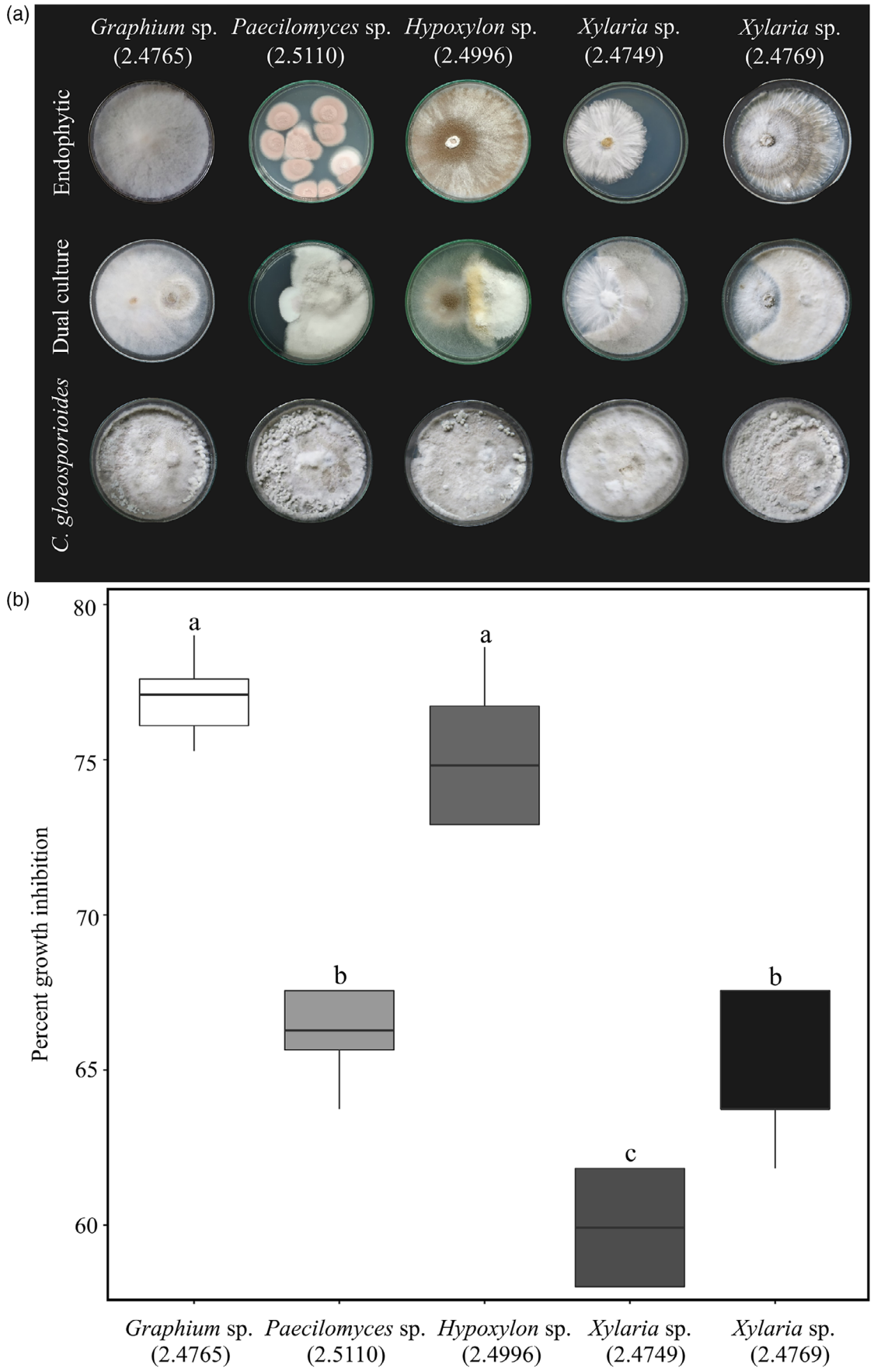
2.3 | *H. anthochroum* 2.4996 identification

Among the fungi evaluated, *H. anthochroum* 2.4996 was selected for molecular/morphological identification and the *in planta* assay because it was easily grown in a PDA medium and exhibited a good spore production, in addition to its good performance in the in vitro assay for antagonism activity. The taxonomic identification was based on both macro- and micromorphology, as well as on the sequencing of the ITS1-5.8S-ITS2 region. The DNA from *H. anthochroum* 2.4996 was extracted by using the Quick-DNA Fungal/Bacterial miniprep kit (ZymoResearch). The rDNA amplification of the ITS region was performed in a 25 µL reaction mixture based on 2 µL DNA template (15 ng) and 0.4 µM of each primer ITS and ITS4 (White et al., 1990) and other components according to Peters et al. (2020). For the PCR amplification, a cycler PCR machine (Bio-Rad) was adjusted to denaturation at 95°C for 2 min; next, 35 cycles of amplification (95°C for 30 s, 55°C for 30 s and 72°C for 1 min) and one extension step of 72°C for 7 min were run. For the quantification of the PCR product (600 bps), a QIAquick PCR Purification Kit (Qiagen) and agarose gel were used.

After the generation of DNA sequences on a 7330xl DNA Analyser (Applied Biosystems), they were blasted through the BLASTn in the National Center for Biotechnology Information (Peters et al., 2020). A phylogenetic analysis was used to further support the identity of *H. anthochroum* 2.4996 by using MEGA (v.11) (Tamura et al., 2021), the Neighbour-joining method (1000 repetitions) and a sequence of *Nemania serpens* as an outgroup. Also, the colour, appearance of mycelium and micromorphological structures of *H. anthochroum* 2.4996 were evaluated according to the key of Ellis (1971), and recorded.

2.4 | Anthracnose severity on *E. precatoria* in response to *H. anthochroum* 2.4996 inoculation (*in planta* assay)

An *in planta* assay was conducted to evaluate the effectiveness of *H. anthochroum* 2.4996 against *C. gloeosporioides*. Açaí seeds (*E. precatoria*) were collected in the municipality of Brasiléia (Acre, Brazil) (19°70′29.83″ S; 88°58′2.67″ W). After surface sterilization of



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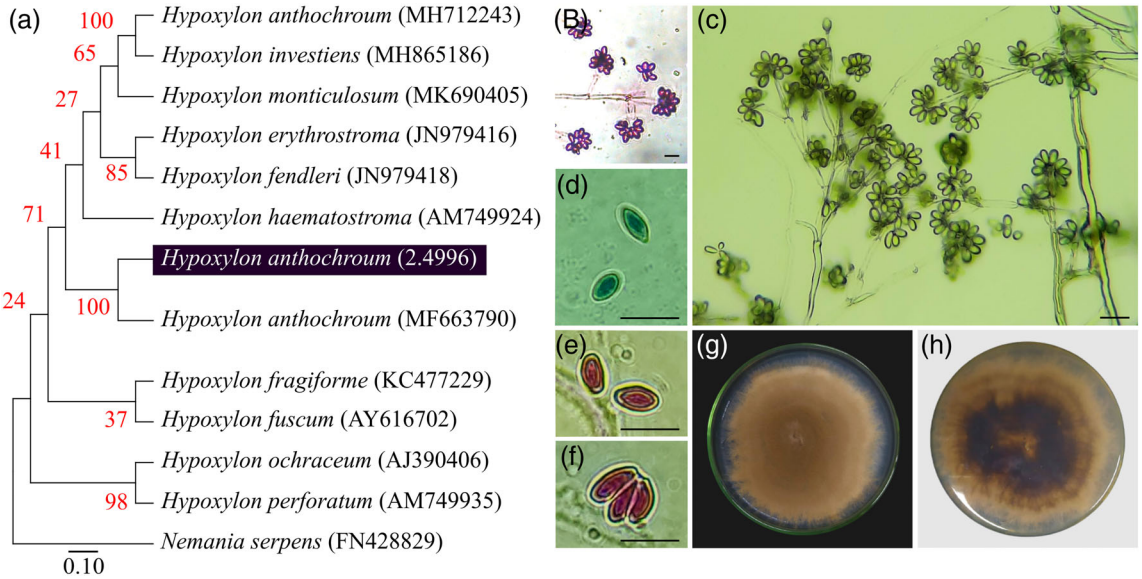

FIGURE 2 Phylogenetic and morphological characterization of the endophytic fungus *Hypoxylon anthochroum* strain 2.4996. (a) Phylogenetic relationship inferred from nucleotide sequence analysis of ITS regions. A neighbour-joining tree was constructed using the MEGA (v.11) software. Numbers at nodes represent bootstrap confidence values or percentage of clade occurrence in 1000 bootstrap replicates. (b) Conidiophores with conidiogenous cells, stained with 1% phloxine dye. (c) Conidiophores with conidiogenous cells from hyaline to light brown (without dye). (d) Ellipsoidal or ovoid conidia, stained with 1% phloxine dye. (e, f) ellipsoidal or ovoid conidia, stained with lactophenol cotton blue. (g,h) Colony on potato dextrose agar (PDA) after 7 days growth at 28°C, in darkness, with sporulation over the entire surface, (front and reverse of the colony). Bar = 6 μM.

açaí seeds with sodium hypochlorite solution (Castro et al., 2017), they were sown in 250 mL plastic cups with expanded vermiculite (Refratil). After 100 days, the seedlings were transplanted to 2 L plastic bags containing a sterile substrate composed of 50% soil (argisol), 25% cow manure and 25% sawdust. The plants were grown in a greenhouse and irrigated daily to replace water lost by evapotranspiration.

On the 130th after the seed sowing, *H. anthochroum* 2.4996 was concurrently grown on PDA for 14–21 days. Then, spores were harvested by adding 5 mL of deionized water to each Petri dish, and their concentration was adjusted to 1×10^6 spores/mL. By using a hand sprayer, the suspension was sprayed on both the abaxial and adaxial leaf surfaces until run-off. After 7 days, *C. gloeosporioides* was inoculated at the same condition as *H. anthochroum* 2.4996. A moisture chamber was used to simulate the inoculation process to provide the best conditions for fungal infection in plants.

Seedlings were divided into four treatments (each treatment contained 25 plants) as follows: (1) negative control [sterile saline solution at 0.85% containing 0.01% (v/v) Tween-20]; (2) plants infected with *C. gloeosporioides* (infected control); (3) healthy plants treated with *H. anthochroum* 2.4996 and (4) infected plants with *C. gloeosporioides* and treated with *H. anthochroum* 2.4996. After 8 weeks, the shoot and root dry masses of the açaí seedlings were evaluated. In addition, the disease severity was estimated by measuring damaged areas (%) in açai leaflets as a result *C. gloeosporioides*, by using the software Image Pro™ Plus (Media Cybernetics Inc.).

2.5 | Statistical analysis

The experiment was carried out in a completely randomized design. There were five treatments with five replications per treatment in the in vitro study and four treatments with 25 replications per treatment in the *in planta* study. Before analysis of variance (ANOVA), data normality and homoscedasticity were checked through Shapiro–Wilk and Bartlett tests, respectively. The one-way ANOVA was used to evaluate data that were originated from antagonism assays and disease severity evaluation and, when a significant difference among treatments was detected by ANOVA ($p < .05$), the least significant difference (LSD) test ($p < .05$) was employed. Root and shoot dry masses were compared by the Kruskal–Wallis test, followed by Conover's test of pairwise multiple comparisons. All statistical analysis was performed using the R software.

FIGURE 1 Dual culture plate assay between endophytic fungi tested against the pathogen *Colletotrichum gloeosporioides*. (a) Interactions on potato dextrose agar between *C. gloeosporioides* and fungal isolates. (b) Percentage of growth inhibition (GI%) of endophytic fungal isolates against *C. gloeosporioides*. Error bars represent mean values ± SEM of five replicates. Means followed by distinct letters are significantly different according to the LSD test ($p < .05$).

3 | RESULTS

3.1 | Endophytic fungi antagonism to *C. gloeosporioides*

The tested endophytic fungi inhibited the *C. gloeosporioides* growth (Figure 1). Macroscopic analysis showed that most of the endophytic fungi were able to grow over pathogen mycelia (Figure 1a). In contrast, only *Paecilomyces* sp. 2.5110 exhibited contact inhibition (Figure 1a). *Graphium* sp. 2.4765 and *H. anthochroum* 2.4996 recorded the best performances (79.3% and 77.0%, respectively), followed by *Paecilomyces* sp. 2.5110 (66.15%) and *Xylaria* sp. 2.4769 (64.8%), and *Xylaria* sp. 2.4749 (59.9%) (Figure 1b). Because *Graphium* sp. 2.4765 did not sporulate enough to be tested in the greenhouse experiment, only *H. anthochroum* 2.4996 was selected for further evaluation.

3.2 | Identification of *H. anthochroum* 2.4996

Based on the cultural, micro-morphological and molecular characteristics of *Hypoxylon* sp. 2.4996, it was identified as *H. anthochroum* (Figure 2). Nucleotide BLAST analysis of sequences (internal transcribed spacer region ITS1-5.8S-ITS2) revealed *H. anthochroum* as the closest match, with 99.81% identity and 99% coverage. Its identification in GenBank is OP247557. In addition, this fungal isolate is phylogenetically related to *H. anthochroum* strain Haeg2 (GenBank accession: MF663790) (Figure 2a).

Microscopic analysis showed conidiophores branched towards the apex, from hyaline to light brown; conidiogenous cells turned hyaline to light brown and ellipsoidal or ovoid conidia, from light brown to brown (Figure 2b–f). Colony on PDA had fast growth in 7 days; at first whitish, becoming taupe with sporulation; Petri plate reverse: dark brown in the centre and cream on the edge (Figure 2g,h).

3.3 | Anthracnose severity in açaí plants in response to *H. anthochroum* 2.4996

The effects of *H. anthochroum* strain 2.4996 on anthracnose severity in açaí seedlings were also evaluated (Figure 3 and Table 1). Plants without *C. gloeosporioides* (negative control) and plants solely inoculated with *H. anthochroum* strain 2.4996 did not present disease symptoms and had similar shoot and root biomasses (Figures 3 and 4). In contrast, açaí seedlings infected with *C. gloeosporioides* showed decreased shoot and root biomasses, in comparison to the control plants (Figure 4). However, *H. anthochroum* 2.4996 had a significant effect on the disease severity (Table 1), enhancing the necrotic areas of the leaflets (Figure 3). The disease symptoms reached 100% in plants inoculated with *H. anthochroum* 2.4996, while the plants inoculated only with the pathogen were affected by anthracnose at 73.17% (Table 1).

4 | DISCUSSION

The purposes of this study were (i) to test the antagonism of endophytes fungi isolated from açaí leaflets towards *C. gloeosporioides* and

TABLE 1 Disease severity (%) caused by *Colletotrichum gloeosporioides* in açaí leaflets inoculated with *Hypoxylon anthochroum* strain 2.4996.

Treatment	Disease severity (%)
Control	0.00 ± 0.00 c
Endophytic	0.00 ± 0.00 c
Endophytic + pathogen	100.00 ± 0.00 a
Pathogen	73.17 ± 0.35 b

Note: Means ± standard errors. Means followed by distinct letters are significantly different according to the LSD test ($p < .05$).

FIGURE 3 Development of *Colletotrichum gloeosporioides* in açaí seedlings inoculated with endophytic fungi *Hypoxylon anthochroum* strain 2.4996. (a) Control. (b) Endophytic + pathogen. (c) Endophytic and (d) pathogen.

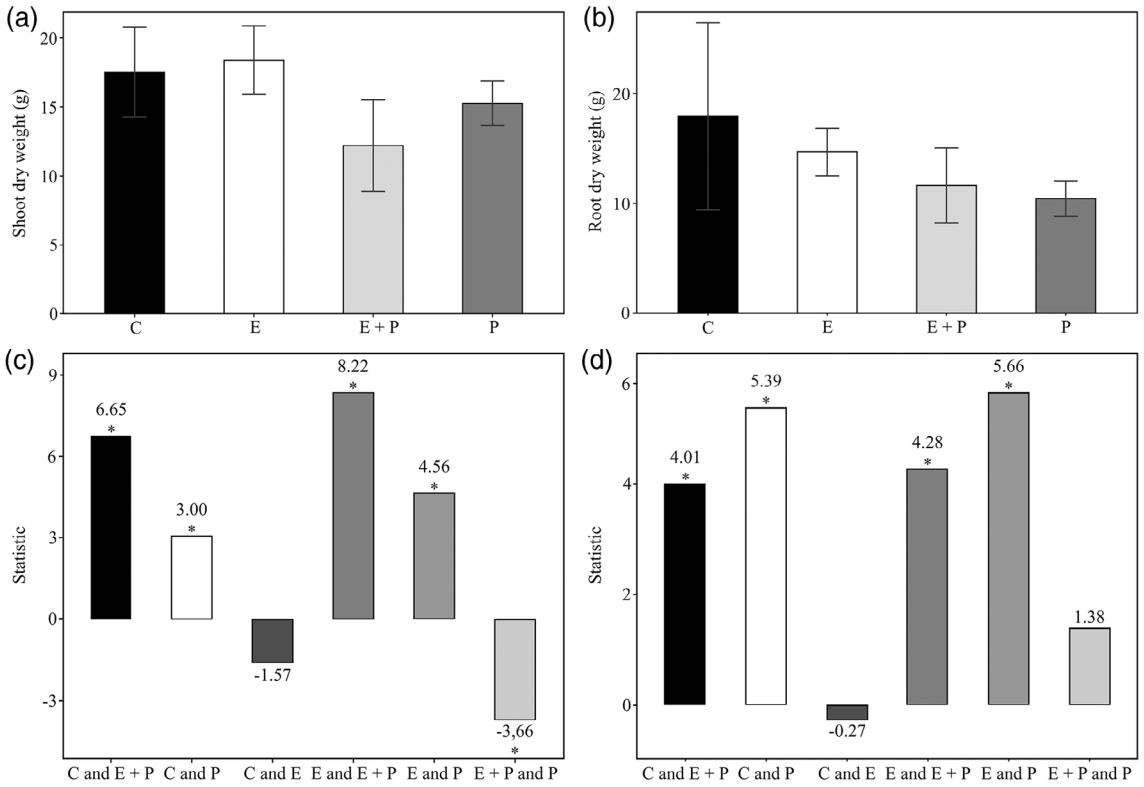

FIGURE 4 Shoot and root dry weight of açaí seedlings inoculated with the endophytic fungus *Hypoxylon anthochroum* strain 2.4996 and the pathogen *Colletotrichum gloeosporioides*. (a) Shoot dry weight. (b) Root dry weight. Error bars represent means ± SEM of 25 biological replicates. (c) Statistical analysis of tested treatments for shoot dry weight (nonparametric data). (d) Statistical analysis of tested treatments for root dry weight (nonparametric data). The letter C represents control plants; P represents plants inoculated with pathogen and E represents plants infected with the endophytic fungus. '*' represents significant results.

(ii) to available the anthracnose severity in açaí seedlings in response to inoculation of the endophytic fungus *H. anthochroum* 2.4996. Açaí endophytes and *C. gloeosporioides* interacted in various ways as revealed by the in vitro antagonism analysis. The fungi *Graphium* sp. 2.4765 and *H. anthochroum* 2.4996 exhibited the highest growth inhibition and the behaviour of growing over pathogen mycelia. This same behaviour was exhibited by fungi of the genus *Xylaria*. The faster growth and better ability to utilize nutrients are important strategies during competition among fungi (Mgbeahuruike et al., 2011), as reported for the antagonistic interaction between the endophytes from Scots pine and pathogen *Sphaeropsis sapinea* (Blumenstein et al., 2021).

Additionally, the ability of *Paecilomyces* sp. 2.5110 for inhibiting the growth of *C. gloeosporioides* indicates its capability to excrete secondary metabolites that have an antagonist effect on the anthracnose causal agent. The genus *Paecilomyces* is best known for its potential in the control of plant diseases (Moreno-Gavíra et al., 2020; Zhang et al., 2022). For example, *P. variotii* showed strong control effects on *Mycosphaerella melonis* (melon plants) and *Podosphaera xanthii* (zucchini plants), reducing the disease severity index by 78% and 76%, respectively (Moreno-Gavíra et al., 2021). Regarding the beneficial effects of endophytes from the genus *Graphium*, in vitro and *in planta* assays revealed that *G. putredinis* was effective against damping-off (*Pythium ultimum*) in cucumber (Carisse et al., 2003). The metabolites from genus *Xylaria*, have been more effective as biofungicides (Huang et al., 2015) and bioinsecticides (Zhang et al., 2014).

In this study, *Hypoxylon* sp. 2.4996 was identified as *H. anthochroum* and it is phylogenetically related to *H. anthochroum* strain Haeg2. According to Macías-Rubalcava et al. (2018), volatile organic compounds from *H. anthochroum* endophytic (strain Haeg2) can be used as a postharvest mycofumigation alternative for cherry tomatoes. In addition, some endophytic strains of *H. anthochroum* produced volatile organic compounds that inhibit the growth and respiration of the phytopathogen *Fusarium oxysporum* (Macías-Rubalcava et al., 2018).

In controlled experiments, nevertheless, foliar endophytic fungi differently modulate the plant disease level (Busby, Peay, et al., 2016; Busby, Ridout, et al., 2016), generating results that did not support those observed in the nature (Martín et al., 2015). In this study, in vitro analysis showed that *H. anthochroum* 2.4996 exhibited antagonistic effects to *C. gloeosporioides*. However, this fungus increased anthracnose symptoms in açaí seedlings that were infected by the pathogen (Figure 3 and Table 1). This result suggests that *H. anthochroum* 2.4996 may act as a potential enabler of *C. gloeosporioides* infection in açaí seedlings. Similar results were observed in other managed ecosystems (Busby et al., 2013; Kurose

et al., 2012). For instance, *Sydowia polyspora*, *Bionectria ochroleuca*, *Penicillium raistrickii* and *Elytroderma* sp. acted as enablers fungi, increasing the severity of *Dothistroma needle* blight in *Pinus ponderosa* (Ridout & Newcombe, 2015).

Experiments with *Populus trichocarpa* demonstrated that a foliar fungus of genus *Epicoccum* acted as a pathogen facilitator, and species of *Alternaria* and *Cladosporium* showed a trend towards facilitation rust pathogen *Melampsora x columbiana* (Busby, Peay, et al., 2016). Previous studies indicate that the production of compounds (metabolites) by endophytic fungi may facilitate pathogen growth and enhance the disease severity (Busby et al., 2013; Busby, Ridout, et al., 2016). Additionally, plant–microbiome interactions play essential roles in many aspects of host functionality (Lyu et al., 2021; Vandenkoornhuyse et al., 2015), including disease suppression (Carrión et al., 2019). However, the inoculation of endophytic fungi in plants can lead to an imbalance in the microbiome and favour the development of pathogens. Busby, Peay, et al. (2016) provided evidence that the abundance and distribution of the foliar fungi from *P. trichocarpa* influence the disease severity level caused by *Melampsora x columbiana* (Busby, Peay, et al., 2016). Another explanation could be associated with endophytes suppressing host defences to allow their own infection, consequently facilitating infection by pathogens (Houterman et al., 2008).

Although *H. anthochroum* 2.4996 increased the severity of anthracnose in açaí seedlings, it did not cause any symptoms when inoculated alone in açaí plants, indicating that this fungus is not phytopathogenic under the tested conditions. In conclusion, it was found that the five endophytic fungi evaluated in vitro assays were effective against *C. gloeosporioides*. However, açaí plants inoculated with both the endophyte *H. anthochroum* strain 2.4996 and *C. gloeosporioides* presented greater pathogen symptom severity than plants inoculated with the pathogen only. These results also showed that, when endophytic fungi are tested in plants, their effects on diseases may be inconsistent with in vitro assays.

AUTHOR CONTRIBUTIONS

Laryssa dos Santos Prado, Bruna Alice Feitosa Mendes, Fábio Ítalo Nascimento da Silva and Leila Priscila Peters contributed to the research design. Experimental design, material preparation and data collection were performed by Laryssa dos Santos Prado, Bruna Alice Feitosa Mendes, Fábio Ítalo Nascimento da Silva and Leila Priscila Peters. Statistical analyses of the data were interpreted by Leila Priscila Peters and José Genivaldo do Vale Moreira. The original manuscript draft was written by Laryssa dos Santos Prado, Leila Priscila Peters and Antonia Jerlene Martins de Lima. The manuscript was reviewed and edited by Clarice Maia Carvalho, Marcia Eugenia Amaral Carvalho and Leila Priscila Peters.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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