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Survey of endophytic Alternaria species isolated from plants in the Brazilian restinga biome

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Abstract: *Saprophytic, endophytic and pathogenic Alternaria have been isolated from a wide range of environments. Endophytic fungi from Brazilian "restingas" have not been studies to date. Here endophytic Alternaria isolates from a range of restinga plants were investigated. One hundred and fifty seven plant species were screened, from which 71 endophytic Alternaria isolates were provisionally identified. Nineteen isolates were selected for DNA sequencing and phylogenetic analysis. All 19 isolates were confirmed as Alternaria with a division into two distinct clades. Seventeen of these isolates were screened for anti-fungal activity against 4 species of plant pathogenic fungi: Colletotrichum gloeosporioides, Thielaviopsis paradoxa, Ceratocystis paradoxa and Myrothecium roridum.Alternaria isolates 1B, 5B1 and 7A3 inhibited T. paradoxa mycelial growth by 50, 40 and 10% respectively. The non-volatile metabolites produced by 1B were fungistatic against T. paradoxa, reducing mycelial development by 89% and 99% after incubation for 4 and 11 days respectively. This isolate is a promising candidate for use in biological control of this phyto-pathogen, demonstrated the potential of the restinga biome as a source of interesting endophytic fungi.*

Keywords: *Antagonism, bioactive compounds, coastal ecosystem, diversity, tropical plants.*

I. Introduction

The Brazilian Atlantic rainforest is considered to be one of the worlds thirty-four most important biodiversity hotspots, containing high species diversity and high levels of endemism [1; 2; 3]. There are a total of 512,434.83 hectares of protected areas in the central corridors of the Atlantic Forest that encompass the "Restinga" ecosystem, which is composed of coastal areas covered with herbaceous and shrubby vegetation [4; 5].

Restingas are defined as coastal sandy plains of marine origin, including beaches, sand bars, dunes, depressions between sand banks and depressions between dunes with bogs, marshes, swamps and lake flora and fauna adapted to the local environmental conditions [6]. Variations in the water table depth, proximity to the ocean and land topography are responsible for the existence of a diverse set of plant formations in restinga areas [7; 8]. Unlike homogeneous areas, the restingas are represented by a mosaic of communities with different levels of environmental complexity [9; 10]. In the state of Rio de Janeiro more than 1,200 species of higher plants belonging to 120 botanical families, especially legumes, bromeliads, mirtaceas, rubiaceas and orchids have been identified [6]. In 2009, the Brazilian National Environmental Council (CONAMA) established the basic parameters for analysis and definition of primary vegetation and the distinct Restinga successional stage vegetation in the Atlantic Forest and approved a list indicating the species that are found in each stage [11; 12].
Located in the northern part of Rio de Janeiro state, the municipalities of São Francisco de Itabapoana, Campos dos Goytacazes, Quissamã, Carapebus and Macaé have a diversified phyto-physiognomy, being designated as important forest transition areas of the Brazilian Southeast coast, where diversification of ecosystems can be found, such as the restingas [13]. The restingas of northern Rio de Janeiro form a unique environment with a high diversity of species, both vegetal and microbial. The native or exotic plants that develop in this ecosystem are adapted to the salinity conditions, depending on the phyto-physiognomy to which they are incorporated. Within this region there are federal, state and privately conserved areas of restinga [3].

The most studied reserve, due to its high diversity, is the restinga in the Jurubatiba National Park (RJNP) at 22º17'S; 41º41'O, with a coastal area of 14,860 hectares, covering the municipalities of Quissamã (65%), Carapebus (34%) and Macaé (1%), being considered a Biosphere Reserve by UNESCO [14; 15]. The

creation of the Caruara Natural Patrimony Private Reserve (NPPR) in 2012 (21º47'S; 41º01'O) was a milestone for conservation of restingas with 3,845 hectares, which became the largest private conservation area of Brazilian restinga, housing one of the largest remaining preserved restingas of the North Fluminense region [16]. The other two restingas, in different stages of degradation are found in Grussaí (21º40'S; 41º01'O) [17] and Gargaú (21º36'S; 41º04'O) [18], the latter influenced by the sea on one side and fresh water on the other.

Jurubatiba National Park despite being one of the most thoroughly studied restingas in the country, its microbiota is poorly-understood, especially in relation to endophytic microorganisms. These microorganisms are regarded as an important source of bioactive compounds [19; 20]. For example Taxol, Vincristine and Podofilotoxin are produced by the endophytic fungi *Taxomyces andreanae*, *Fusarium oxysporum*, *Trametes hirsuta* and *Phialocephala fortinii* [21]. The occurrence of compounds of plant origin that are also produced by fungi that colonize plants suggests a transposition of genes between plants and fungi, a type of genetic engineering in vivo [22].

In addition to presenting several important roles in the host plant, these microorganisms are potentially useful in agriculture and industry, especially for the production of pesticides and pharmaceuticals. Bioactive compounds from fungi are potential substitutes for synthetic chemicals, biocontrol agents and/or plant growth promoters, preserving the environment. They have been identified as viable alternatives for agricultural/ecological production systems and are economically sustainable [23].

The amount of scientific information concerning the restingas is restricted when compared to other ecosystems [24], and as a result of a set of disturbances continuously imposed on these habitats, most of their original area has been degraded and a large portion of it lost [25]. Until this time no studies on endophytic fungi from the restinga have been published.

Endophytic fungi are ubiquitous organisms found in the plants, in the intercellular or intracellular regions, at least for a portion of their life cycle, without causing apparent symptoms of infection. Almost all plants are known to harbor endophytes [26]. This includes the fungal genus *Alternaria*, with saprophytic, endophytic and pathogenic species associated with a wide variety of substrates [27], although some species are well known as destructive plant pathogens [28].

In recent years, DNA based studies revealed multiple non-monophyletic genera within the *Alternaria* complex, and *Alternaria* species clades that do not always correlate to species-groups based on morphological characteristics. The *Alternaria* complex currently comprises nine genera and eight *Alternaria* sections [27; 29; 30].

Our interest in endophytic fungi is due to the fact that *Alternaria* was isolated at a high frequency during this study from a variety of restinga plant species and the importance of this fungus as a producer of toxins in fruits and vegetables [31; 32], besides being a prolific source of new pharmacologically active metabolites [33; 34]. The objective of the present study was to identify and quantify endophytic *Alternaria* isolates in the northern restingas of Rio de Janeiro state, as well as carry out molecular analysis of these isolates to establish possible comparisons with previously described species and screen isolates for production of bio-active compounds.

II. Materials And Methods

2.1 Study sites and plant species

The studied plant material was collected in an area of restinga vegetation in northern Fluminense, in the municipalities of São Francisco de Itabapoana, São João da Barra and Macaé, Rio de Janeiro State, Brazil. The climate is classified as tropical sub-humid to semi-arid [35], with average annual rainfall ranging from 800 to 1,200 mm, with the highest rates of rainfall during the summer and lowest in winter. Leaf samples were collected from 157 host species from June 2013 to June 2014 at four restinga sites (Gargaú, Grussaí, Açu and Jurubatiba; coordinates already stated in the introduction). These plants are representative of three physiognomic unities: Beach Grass, Shrub Clusia and Restinga Forest Formation [17]. Each species from which endophytic fungi were isolated were compared and identified on the basis of specimens already deposited in the Herbarium of the Botanical Garden in Rio de Janeiro.

At the Jurubatiba National Park restinga 35 plant species were obtained for the vegetation zone psamophyte and creeping halophyte (PCH), which starts next to the beach escarpment, in a strip of approximately 5 - 10 m [36]. From the PCH zone a transect was made perpendicular to the sea towards the interior of the restinga until reaching the closed areas of clumps [37; 38], thus forming a rectangle with 300 m x 150 m, with one side bordering the Jurubatiba lagoon.

In the Grussaí restinga 49 plant species were obtained, collected at random, in the remaining fragments of the biome as a result of its advanced stage of degradation always from the ocean to the interior until reaching higher vegetation.

In the Açu restinga (Caruara NPPR), 33 plant species were obtained from a transect in a rectangle with 200 m parallel to the sea x 500 m perpendicular to that line in the direction of the most enclosed forest.

Finally, in the Gargaú restinga, 40 plant species were obtained collected in distinct points, interspersed by the mangrove, maintaining the same direction as the coastal strip.

2.2 Isolation of endophytes

Fresh leaves were collected from healthy plants, stored in a clean plastic bag and transported to the lab. The leaves were washed with running tap water and cut into 0.6 cm diameter pieces, which were surface-sterilized with sequential washes of 70% EtOH (2 min.), 10% bleach (1% NaOCl; 2 min.), 70% EtOH (30 s) and sterile distilled water [39]. Fifteen segments per plant species (2,355 total segments) were selected at random and plated onto 2% potato dextrose agar (PDA), which promotes growth of diverse endophytes [40]. Plates were sealed with parafilm and incubated at room temperature (aproximadamente 28 ^{o}C) with approximately 12 h light/dark cycles. Emergent hyphae were isolated immediately onto PDA media to obtain pure cultures. Following 7 days, whole-colony morphology was documented and photographed.

The morphological identification of the isolates of *Alternaria* was carried out by observations of the structures of the axenic colonies [41] mounted on slides containing lactic acid. The observations were performed using a light microscope with x20 and x40 objectives.

2.3 DNA extraction, PCR amplification and sequencing

Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions with the following modifications: mycelia were grown on PDA media in a Petri dish for approximately 2 weeks at 28 °C. The mycelia were homogenized using a mortar and pestle under liquid nitrogen before the addition of 600 µL of Nuclei Lysis Solution and mixed with polyvinylpyrrolidone to a final concentration of approximately 5%. Homogenized material was heated for 15 min at 65 °C and then processed following the remainder of the Promega protocol. A NanoDrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) was used to check the quality and concentration of the genomic DNA.

PCR was performed using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'TCCTCCGCTTATTGATATGC3'). The PCR conditions (Touchdown) were as follows: initial denaturation at 95 °C for 15 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, elongation -1 °C per cycle and a final extension at 72 ºC for 2 min. Products used were REDTaq® and ReadyMix™ (Sigma-Aldrich®). PCR was successfully performed with 22.5 µL of each primer and 1 µL of template. The sequencing was carried out by Eurofins Ltd. (Germany) using the Sanger method [42].

2.4 Phylogenetic analysis

This methodology was based on [43] with minor modifications. All alignments were checked manually and the nucleotide positions were adjustment when necessary. New sequences were deposited in the database GenBank (http://www.ncbi.nlm.nih.gov).

Additional ITS sequences were obtained from the GenBank database using the Mega BLAST program for sequence identification. Consensus sequences were compared against GenBank's database using Mega BLAST program. New sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE [44], which is built into MEGA v. 6 software [45]. Gaps were treated as missing data. Bayesian inference (BI) analysis was employing using a Markov Chain Monte Carlo (MCMC) method performed with every sequence. MrMODELTEST 2.3 [46] was used to determine the best nucleotide substitution model for each gene. The likelihood scores were calculated and the model selected was SYM+I+G according to the Akaike Information Criterion (AIC). BI analyses were completed using MrBayes v. 3.2.3 [47]. Four MCMC chains were run simultaneously, starting from random trees, for 10,000,000 generations. The trees were sampled every 1,000th generation for a total of 10,000 trees. The first 2,500 trees were discarded as the burn-in phase of each analysis. The posterior probabilities [48] were determined from a majority-rule consensus tree that was generated from the remaining 7,500 trees. The log likelihood convergence was analyzed using the TRACER software v. 1.4.1 [49]. The tree was visualized in FigTree [50] and exported to a graphics program. The tree was rooted with *Exserohilum pedicellatum* EEB.1336.

2.5 In vitro antagonism tests

Antibiose assessments were made using all *Alternaria* isolates against pathogenic fungi. Agar diffusion method was assayed to test the inhibitory effects of *Alternaria* isolates on the four phytopathogenic fungi important on post-harvest (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.; *Thielaviopsis paradoxa* (De Seynes) Höhn.; *Ceratocystis paradoxa* (Dade) C. Moreau and *Myrothecium roridum* Tode), the causal agents of disease on banana, pineapple, coconut and melon, respectively) which were used for screening antifungal activity of the isolates. Potato dextrose agar (PDA) plates were prepared aseptically and one mycelial disk (6 mm diameter) of each phytopathogenic fungi were peaked around the different *Alternaria* which was placed 7 days before [51].

The efficiency of growth inhibition of other fungi was assessed by the presence of the growth inhibition zone between the colonies. Each treatment consisted of three replicates and the experiment was repeated three times [52; 53].

2.6 Dual culture assays

The *Alternaria* isolates, which tested positive, were selected to continue the in vitro tests. Nine centimeters Petri plates containing PDA were placed with 6 mm mycelial disks of *Alternaria* isolates selected by previous test, 10 mm away from the edge of the plate opposite to each other. Plates with each *Alternaria* isolates alone served as control. Plates were incubated at 26 ± 1 °C for seven days (12 h light/12 h darkness). The linear growth was measured. The percentage inhibition was obtained following equation proposed by [54], and now modified: I% = ((Ac – At)/Ac) x 100, where: Ac is the *Alternaria* colony in the control treatment area (without plant pathogenic fungus) and At is the pathogen colony area against the antagonist. Each treatment consisted of three replicates and the experiment was repeated twice.

2.7 Production of non-volatile compounds

A 8 mm disc of autoclaved cellophane was placed on the surface of a PDA plate then a 6 mm *Alternaria* spp. isolate disc was placed on the cellophane. The plates were incubated at 28 ± 1 °C for 4 to 11 days. After the incubation period, the cellophane and the adhering *Alternaria* mycelia were aseptically removed and in the center centre of each plate a 6 mm diameter disc of *T. paradoxa* was inoculated, which had been previously removed from an actively growing colony.

To verify the possible presence of nutrients in the medium, after the removal of *Alternaria* colonies, new disks of the same isolates were added and the growth evaluated. The plates were incubated at 28 ± 1 °C for a further five days. The control treatment consisted of monitoring *T. paradoxa* development on PDA plates after removal of the cellophane, where the same fungus had been growing, for 4 to 11 days. Three replicate plates were used for each treatment [55].

III. Results

A total of 157 species of plants were randomly sampled within the perimeters previously defined in the four restingas studied in the north of the state of Rio: Grussaí, Açu, Jurubatiba and Gargaú (Fig. 1). The number of *Alternaria* isolated from the various plant species was 28, 24, 18 and 1, for the restingas respectively.

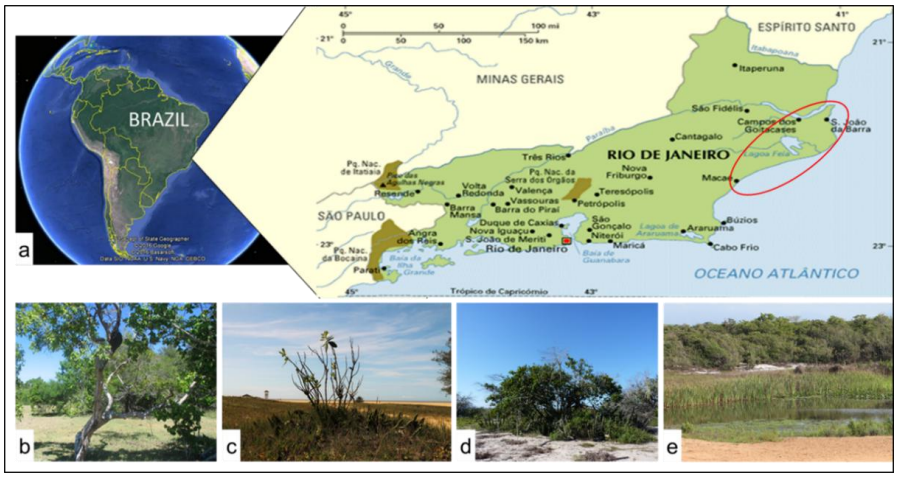

Figure 1 Geographical location of the four restingas where plant species were collected and used for isolation of endophytic *Alternaria*. a) Northern Rio de Janeiro State with the restingas circled in red; b) Gargaú restinga in the municipality of São Francisco do Itabapoana; c) Grussaí restinga and d) Açú restinga both in the municipality of São João da Barra; e) Jurubatiba restinga municipality of Macaé.

From the isolations performed to detect endophytic fungi, 71 isolates of the genus *Alternaria* were obtained from 24 different plant species (Fig. 2). There were extreme variations between 1 and 10 isolates/plant species and *Capparis flexuosa* (L.) L.,*Blutaparon portulacoides* (A. St.-Hil.) Mears, *Canavalia rosea* (Sw.) DC., *Eugenia uniflora* L. and *Ipomoea imperati* (Vahl) Griseb.Had the highest frequency of *Alternaria* isolates. *Capparis. Flexuosa* and *I. imperati* were the only species that presented endophytic *Alternaria* obtained from three of the four restingas sampled. In the Gargaú restinga, only one isolate of *Alternaria* was obtained, originated from *Syzygium cumini* (L.) Skeels.

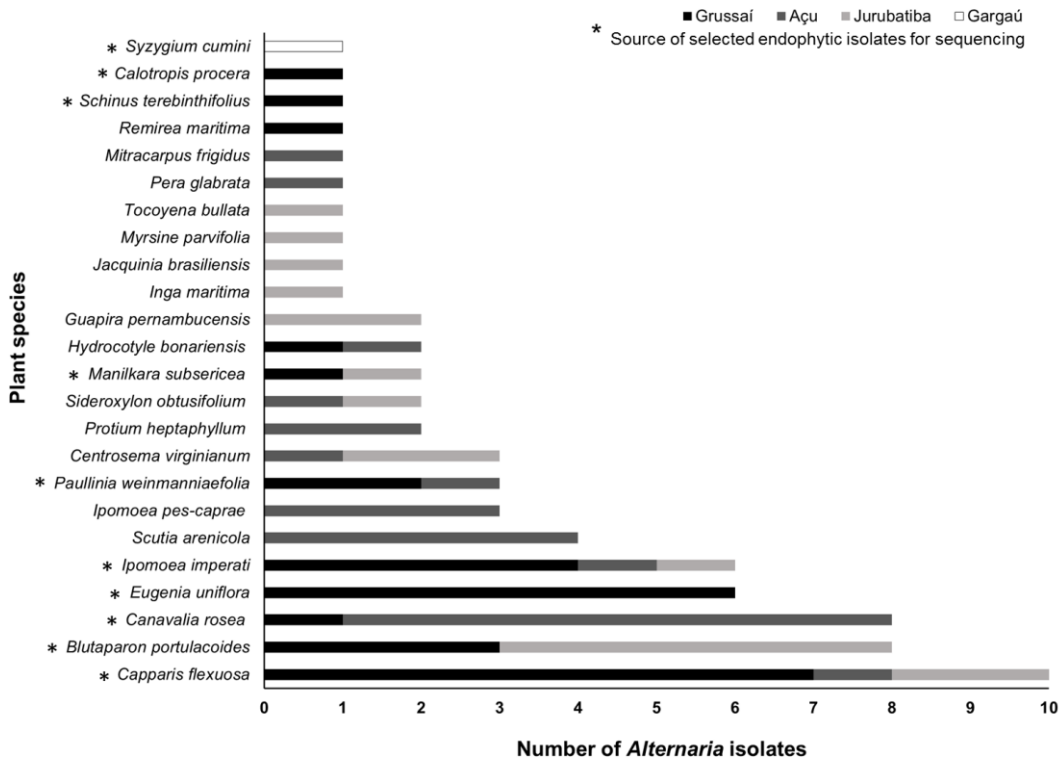

Figure 2 Number of *Alternaria* isolates from different plant species sampled in four restingas found in the northern of Rio de Janeiro state, Brazil.

From the total number of *Alternaria* isolates, from 24 plant species, all colonies were grouped by morphological similarities, reducing from 71 to 46 the number of isolates which were obtained from 10 plant species, as indicated by asterisks (Fig. 2). These isolates were well adapted to restinga plants in function of the phytophysiognomy in which each one is inserted in its respective restinga of origin. We selected 19 isolates *Alternaria* (Table 1) for antibiosis assessments and DNA sequencing. The Genbank accession numbers are also shown in Table 1.

Parts of the plants were placed in a herbarium and compared with the species deposited in the Rio de Janeiro Botanical Garden Herbarium. Vouchers of all fungal isolates were deposited in the culture collection in the Laboratory of Chemistry and Biomolecules (LAQUIBIO/ISECENSA), Brazil, which are kept under sterile distilled water [56] and tubes containing inclined media.

The interactions between *Alternaria* and phytopathogenic fungi were evaluated by the presence or absence of antibiosis. Antagonistic effects were detected for three *Alternaria* isolates against *T. paradoxa* and for two isolates against *C. paradoxa*, with one isolate causing antagonistic effect against both fungi (Table 1). For *C. gloeosporioides* and *M. roridum*, no effect was observed. The *Alternaria* isolates 7A3, 5B1 and 1B were selected for pair testing with *T. paradoxa*. The results showed that 1B was more effective at inhibiting phytopathogen growth, followed by 5B1 and 7A3, respectively (Fig. 3 and Fig. 4).

Alternaria 1B secreted non-volatile compounds that diffused through the cellophane membrane onto the agar medium, resulting in inhibition of pathogen growth. A high level of reduction in mycelial growth was recorded after 4 days (89%) while after 11 days 99% inhibition was observed (Fig. 5). Control fungal growth was normal and *T. paradoxa* sporulated at the end of the experiment.

Table 1 Relation of the endophytic *Alternaria* spp. obtained from restinga plants in Rio de Janeiro State, Brazil

***ALTERNARIA* ISOLATES**	**FAMILY**	**PLANT SPECIES (VULGAR NAME)**	**TYPE OF VEGETATION AND SUCESSION STAGE**	**GENBANK ACESSION**
1B	Apocynaceae	*Calotropis procera* (Bombardeira, algodoeiro de seda)	Invasive species originating from Africa and Asia but it fits into the type of restinga herbaceous vegetation	LE201505
5B1	Capparaceae	*Capparis flexuosa* (Feijão bravo)	Restinga arboreal vegetation in the primary stage of regeneration and restinga forest transition vegetation in primary stage of regeneration	LE201510
5B1.1				LE201517
5C2				LE201502
6A1	Myrtaceae	*Eugenia uniflora* (Pitanga)	Restinga shrubby vegetation in primary and advanced stages of regeneration	LE201514
6A3				LE201518
6A4				LE201509
7A3	Sapindaceae	*Paullinia weinmanniifolia* (Cipó sangue)	Restinga shrubby vegetation in primary and advanced stages of regeneration	LE201516
46C1	Myrtaceae	*Syzygium cumini* (Jamelão do mato, azeitona)	Invasive species originating from India and Sri Lanka but it fits into the type of restinga arboreal vegetation	LE201515
54 A1	Convolvulaceae	*Ipomoea imperati* (Salsa da praia)	Restinga herbaceous and subshrub vegetation on the climax stage of regeneration.	LE201501
54B1.1				LE201500
54C2				LE201512
54D				LE201507
57A1	Amaranthaceae	*Blutaparon* portulacoides (Pirrixiu)	Restinga herbaceous and subshrub vegetation on the climax stage of regeneration.	LE201506
57B1				LE201503
57C2				LE201508
60B3.2	Fabaceae	*Canavalia rosea* (Feijão de porco)	Restinga herbaceous and subshrub vegetation on the climax stage of regeneration.	LE201504
70C1.2	Anacardiacea	*Schinus terebinthifolia* (Aroeira)	Restinga arboreal vegetation in primary and advanced stages of regeneration and restinga forest transition vegetation in the middle stage of regeneration	LE201511
82B2.3	Sapotaceae	*Manilkara subsericea* (Maçaranduba)	It fits into the type of restinga arboreal vegetation in primary and advanced stages of regeneration	LE201513

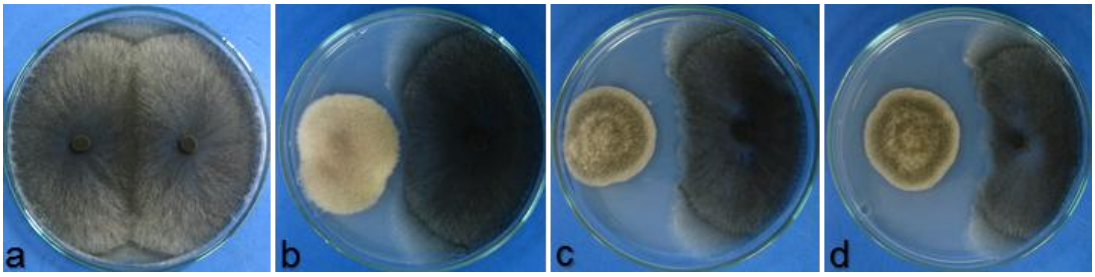

Figure 3 Pairing challenges of fungal colonies. (a) *Thielaviopsis paradoxa* v *T. paradoxa* negative control; (b) antibiosis of the *Alternaria* isolate 7A3 v *T. paradoxa*; (c) isolate 5B1; (d) isolate 1B.

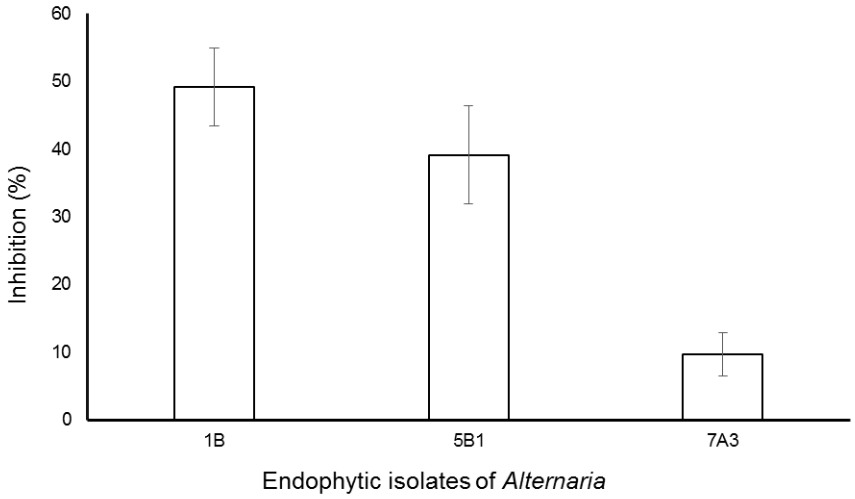

Figure 4 Mean percentage of growth inhibition of *Thielaviopsis paradoxa* when confronted with three endophytic *Alternaria* isolates. Error bars = Confidence Interval with a significance level of 0.05.

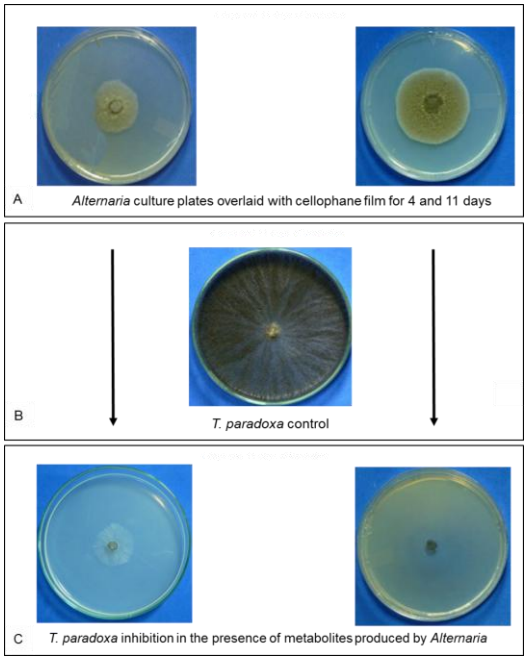

Figure 5 Inhibition of *Thielaviopsis paradoxa* by non-volatile secretions from endophytic *Alternaria*. (**A**) *Alternaria* overlaid with cellophane film cultivated onto PDA; (**B**) *T. paradoxa* control on PDA; (**C**) Inhibition of *T. paradoxa* following a 4 and 11 day incubation in the presence of metabolites produced by *Alternaria*.

Phylogenetic analysis based on the ITS gene included 48 taxa and the sequence alignment resulted in a total of 457 characters, of which 51 were informative for parsimony, 83 were variable and 370 conserved. Phylogenetic analysis shows that the isolates were grouped into two distinct clades (Fig. 6), one with 17 isolates and the other with 2 isolates.

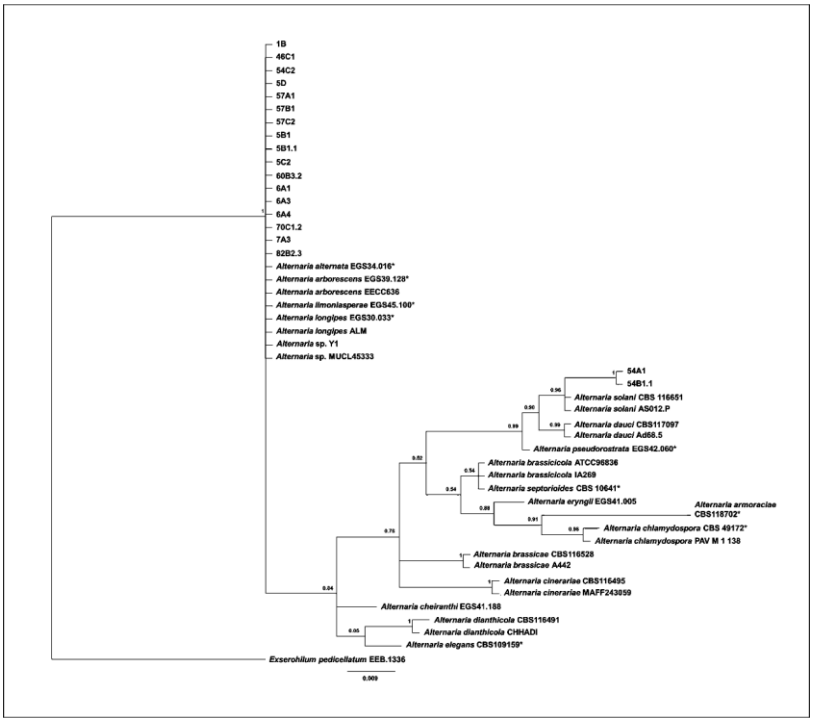

Figure 6 ITS sequence based phylogenetic tree constructed using Bayesian analysis showing the relationship of *Alternaria* spp. endophytic fungal isolates from Brazilian restinga when compared to known *Alternaria* sequences from GenBank (NCBI). The Bayesian posterior probabilities (PP) are indicated next to the nodes. The tree was rooted with *Exserohilum pedicellatum* (EEB1336). The type species are indicated by (*) and the isolates obtained during this study are highlighted in bold.

IV. Discussion

The presence of different *Alternaria* isolates in one host plant species (Table 1) was shown from observations of differences in fungal morphological (data not shown) and confirmed by molecular characterization. It is possible that different endophytic species can colonize the same host, in a non-specific manner, since different isolates from the same plant species have different behaviors. Many factors affect the structure and species composition of the microbial communities that colonize roots, stems, branches and leaves. Endophytic communities vary spatially within the plant or may be dependent on interactions with other endophytic or pathogenic microorganisms [57]. For example, the fungus *Fusarium verticillioides* has a dual role both as a pathogen and as a beneficial endophyte in maize and the equilibrium between these two states is dependent on locally occurring abiotic stress factors that reduce host fitness, resulting in alterations in this delicate balance [58; 59]. Some endophytes are host-specific while others are able to colonize multiple host species [60; 61].

The highest frequency of *Alternaria* isolations was related to certain hosts, as in the case of *C. flexuosa*, *B. portulacoides*, *C. rosea*, *E. uniflora* and *I. imperati* (Fig. 2) which suggests that these plants are natural repositories of this fungus, especially *C. flexuosa* and *I. imperati*, from which isolates were obtained in three of the four restingas prospected.

The species of plants along restinga differ in terms of abundance, richness and dominance, and these differences can be translated in habitat types in this environment [10]. The most preserved restinga is Jurubatiba, followed by Açu (represented by NPPR Caruara), Grussaí and Gargaú, respectively, the latter being in an advanced state of anthropic degradation. The lack of preservation of plant species has causes a great loss of biodiversity [62]. This condition can be correlated to the number of *Alternaria* isolates bioprospected, since the greatest number of isolates were obtained from the most preserved restinga and only one isolate was found in the Gargaú restinga, where agricultural activity has practically destroyed this environment.

In preserved biomes, usually there is an environmental gradient which increases the spatial heterogeneity in the restinga from the beach habitats (close to the sea) towards the more shaded environments with vegetation, which could reflect on the fungal diversity present in plant species found in areas with less severe environmental conditions such as those farther from the sea being more protected from the wind and presence of lower soil salinity levels [8].

Most of the isolates (17) formed a larger clade, grouping with previously described *Alternaria* species (*Alternaria alternate* EGS 34-016 and *Alternaria arborescens* EGS 39-128) with 1.0 posterior probability of Bayesian inference (PP). This value should be considered as these isolates only group to genus level, requiring further analysis with a less conserved gene, such as TEF1-α, to further classify the species [43]. Genetic variability was observed among these isolates, which may show that some of them may be novel *Alternaria* species. However trees using the ITS region do not give strong support, as these regions are ribosomal and highly conserved [63]. The coding regions of proteins (TEF1-α) may confirm distinct and new species of *Alternaria* sp. [64]. By grouping 2 isolates (54A1 and 54B1.1) in dichotomous clades with PP = 1:00 values, shows that more attention should be given to the study of these two isolates that are genetically diverse from the other isolates of *Alternaria* isolated from the same plant (54C2 and 54D), occupying different positions in the phylogenetic tree presented here (Fig. 4).

The presence of different *Alternaria* isolates in the same host plant species (Table 1) was demonstrated not only from fungal morphological variation, but also by the antagonistic effect of certain isolates on plant pathogenic fungi (data not shown).

Alternaria is considered to have potential for toxin production [65]. The fact that a majority of the isolates tested here did not cause antibiosis when challenged with plant pathogenic fungi used in this study, does not mean that they do not produce toxic compounds. Only four plant pathogenic fungi were used here and two of them were sensitive to compounds produced by three of the *Alternaria* isolates. Similar results were obtained by [66], which confirmed the high frequency of endophytic *Alternaria* over other genres revealing its potential antagonist by only some of them against *Botrytis cinerea*.

When using a paired cultures method against *T. paradoxa*, the inhibition levels varied, with for example, isolate 7A3 did not inhibit the growth of *T. paradoxa*, whereas isolate 1B inhibited *T. paradoxa* (Fig. 3). There is a consensus on the diversity of chemical compounds produced by different *Alternaria* isolates such as steroids, terpenoids, pyrones, quinones, phenolics and nitrogen-containing compounds [33], dibenzopyranones with polyketide origin, such as alternariols and altenuenes [67] among other compounds [68; 31]. Although many tests have already been performed demonstrating antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* [69; 67], the bioactive metabolites are promising against pathogenic fungi which cause plant disease, such as those applied for the control of *Fusarium solani* [70].The production of non-volatile metabolites by one of the antagonists here caused a reduction in the mycelial development of the phytopathogen *T. paradoxa*, confirming that the production of antibiotics may be more important in the inhibition of other organisms than the competition for nutrients [71]. Among the effects caused by antibiotics, reduction or paralysis of growth and sporulation, reduction in spore germination, hyphal distortion and endolysis may be observed [55].

The fungus *T. paradoxa* is an important soil-borne plant pathogen in Brazil attacking crops like pineapple (*Ananas comosus*), coconut (*Cocos nucifera*), banana (*Musa* spp.), sugarcane (*Saccharum officinarum*) and pupunha (*Bactris gasipaes*) [72; 73; 74]. The use of biological control agents to control soil-born pathogens should be an important alternative to the use of chemical pesticides that are known to be harmful to the environmental [75]. Some works have explored the use of other fungi like: *Trichoderma* sp. and *Chaetomium* sp. to control *T. paradoxa* showing its potencial to suppress plants diseases [76; 77; 78]. However, the search of alternative biocontrol agents should be an important tool considering different environments and climate around the world. The recurrent presence of *Alternaria* as an endophyte of Restinga plants may suggest the genera success in this type of environment. Therefore, the use of *Alternaria* or its nonvolatile compounds should be a potential substitute for *T. paradoxa* control and its plant diseases in tropical regions. Nevertheless, field and *in vivo* assays are necessary to confirm the use of *Alternaria* and its metabolites to suppress *T. paradoxa* diseases.

V. Conclusion

These results demonstrate the potential of endophytes for the production of bioactive compounds and show the urgent need to study the microbiota associated with plant species of the Brazilian flora, in view of the risk of losing endophytic biodiversity as a consequence of the rapid degradation of the Brazilian restingas.

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