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Research Article

ATLANTIC FOREST MEDICINAL PLANT *Aristolochia triangularis Cham* (ARISTOLOCHIACEAE) AS SOURCE OF FUNGAL ENDOPHYTES CAPABLE OF CONTROLLING STRAWBERRY DISEASES

ANDRESSA KATISKI DA COSTA STUART[1*], RODRIGO MAKOWIECKY STUART[2], IDA CHAPAVAL PIMENTEL[3]
Department of Basic Pathology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, Paraná, Brazil
*Corresponding Author: Email - andressa.katiski@gmail.com

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Abstract- Atlantic Forest is one of the richest biomes of the world. Among their plants, *Aristolochia triangularis* stands out by its uses in Brazilian popular medicine. Endophytic fungi live inside plants producing several bioactive metabolites. They may, therefore, be used in biological control. Strawberry culture is one of the most important cultures worldwide. Its main phytopathogens are *Botrytis cinerea* and *Rhizopus stolonifer*. Biological control, are being used to control this kind of pathogens. In this work, a total of 263 endophytes were isolated from A. triangularis. They were submitted to ARDRA assay, resulting in 26 different haplotypes, representing 9 families and 13 different genera. Dual culture test showed 7 isolates capable to promote biological control of these pathogens in vitro. A total of 66 isolates showed presence of Polyketide synthase (PKS) I, a gene linked to bioactive metabolites production. PKS presence in the isolates, demonstrate their ability to produce bioactive compounds.

Keywords- *Biocontrol, Endophytes, Biodiversity, Polyketides, Bioactive compounds*

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Introduction

Atlantic Forest biome is one of the richest regions of the world in biodiversity. It is formed by a set of forest and ecosystems as restingas, mangroves and altitude fields. It corresponds originally to 1,300,000 km^2 in 17 different states in Brazil. Nowadays, the leftover native vegetation is reduced to about 22% of their original cover and is in different stages of regeneration. Although reduced and very fragmented, it is estimated that there are about 20,000 plant species at Atlantic Forest, including endemic and endangered species [1]. Among these, the vine of *Aristolochiaceae* family, *Aristolochia triangularis*, is endemic of Atlantic forest region and stands out by its wide application in Brazilian folk medicine. The use of its vines, leaves and stem is mainly associated with anti-inflammatory, antiseptic, emmenagogue, antipyretic, antirheumatic [2] and antimicrobial actions [3]. Many of the metabolic compounds produced by plants may also be associated with their endophytic community. These microorganisms are able to produce bioactive metabolites structurally and biologically similar to those produced by their hosts [4]. They colonize the host tissues developing a symbiotic relationship, without causing immediately disease symptoms [5]. Endophytic fungi may help the host defense against phytopathogens and plagues [6] as well promote plant growth through phytohormones production [7]. These endophytes have attracted attention by its ability to produce secondary metabolites with several biological activities. Some examples are Penicillin and Cephalosporin [8], and anticancer drugs as Taxol [9], among others. Besides that, they are effective in biological control, which is defined as the use of one organism to control the population density of other and has shown large efficiency against many pathogens of several cultures [10]. Strawberry (Fragaria x ananassa Dusch.) is one of the most consumed fruits around the world. It is economically important, mainly in USA and Europe, with increase in Latin America in the latest decades [11,12]. In 2016, its production exceeded 7 million tons in the top 10 producing countries [13]. Due to its sensibility, this fruit is highly susceptible to fungal contamination. Grey mold disease, caused by *Botrytis cinerea* and Rhizopus rot, caused by *Rhizopus stolonifer* are two of the most important diseases of this culture. They are responsible for harvest losses of up to 25%, causing important economic impact worldwide [14, 15]. Use of agrochemicals is the first choice to control those diseases. However, health and environmental concern has led to a decrease of chemical applications and the rise of alternative methods of control, as integrated pest management (IPM) [16, 17]. IPM considers the use of all available pest control techniques, mostly biological control, minimizing the risks to environment and human health [17]. There are several pathways involved on fungal bioactive metabolites production, and the detection of PKS I gene is one of the most usual alternatives to evaluate the metabolic pathway which these microorganisms use to produce such metabolites. The PKS I gene is extremely diverse in structure and biological activity [18] and their products, the polyketides, are derived from secondary metabolism. These polyketides have large pharmaceutical and industrial application. In addition, included antimicrobial compounds such as Penicillin and Rifampicin [8], and medicinal compounds as leucinostatins [19], lovastatin, cytochalasins, among others [20]. Despite the relation of biocontrol activity and PKS I gene are still unclear, compounds as leucinostatins, lovastatin and cytochalasyns are also linked to biological control [21, 22, 23]. Due to its huge application, the detection of PKS I gene constitutes an important strategy to select endophytic fungi with potential to bioactive compound production [24, 25]. Despite the wide application of A. triangularis in Brazilian popular medicine, its endophytic community remains unknown. Thus, this work was pioneer to isolate and characterize them, evaluate their capacity to growth control of *B. cinerea* and *R. stolonifer*. Furthermore, seeking to better understand the secondary metabolites production, the presence of PKS I gene was evaluated, through polymerase chain reaction (PCR), on cultivable endophytic fungi isolated from A. triangularis.

Materials and Methods

Sampling and fungal isolation

Healthy vine, stem, fruit, young and mature leaves (Fig. 1) were sampled in March

2015, from Almirante Tamandaré, Paraná state, Brazil - a Atlantic Forest region. Ten fragments of each different tissue were collected from different plants. Plant tissue were surface disinfected in 70% ethanol for 1 min and 2% sodium hypochlorite for 4 min. Sterilized segments were rinsed in sterilized distilled water and then dried at room temperature. Vine were cut in half, exposing its interior, then leaves, vine stem and fruits were cut into 0.5 cm² fragments and plated onto Sabouraud medium (SBR) (Sigma-Aldrich) added with Streptomycin (100 µg / mL-1). Plates were incubated at 28º C with 12 hours period of lighting for 14 days [26]. Two hundred and sixty-three pure cultures were obtained and stored on potato dextrose agar (PDA) (Himedia) tube at 4º C.

Fig-1 Different A. triangularis tissues, used to endophytic fungi isolation. a) Vine b) Leaf c) Fruit

DNA extraction and Polymerase Chain Reaction (PCR)

DNA extraction was performed from all fungal isolates [27]. The PCR amplifications were performed in a total volume of 40 µL of PCR mixture containing 27 µL of distilled water, 1 µL (6 ng) of DNA template, 1 µL (0,50 mM) of each primer and 11 µL of PCR mix (0,03 U/µL of Taq DNA Polimerase, 1X reaction buffer, 2,50 mM of MgCl, 0,31 mM of each dNTP; Invitrogen, California, USA). The ITS gene region was amplified using V9G (5'TTACGTCCCTGCCCTTTGTA3') and LS266 (5'GCATTCCCAAACAACTCGACTC3') primers [28] in Thermal Cycler (BIOER, Hangzhou, ZJ). PCR conditions consisted of 40 cycles (denaturation 94º C for 35 s, primer annealing for 15 s at 50º C and extension for 1 min at 72º C). PCR product was analyzed by electrophoresis on 1.6% (w/v) agarose gels, stained with GelRed (Biotium, Hayward, CA) and visualized under UV light.

ARDRA, ITS Sequencing, Shannon diversity index and statistical analyses

Amplified Ribosomal Restriction Analysis (ARDRA) was performed, using Mbo I and Hae III restriction enzymes in 30 µL and 20 µL of ITS amplified PCR product respectively, according to manufacturer (Fermentas Inc, Hanover, MD) [29]. The fragments generated were separated on 2% (w/v) agarose gels stained with GelRed and visualized under UV light. ARDRA patterns were grouped into haplotypes and the ITS regions of at least one individual of each haplotype were sequenced using ABI 3500 DNA Analyzer (Applied Biosystems, Waltham, MA) allowing the identification of the culturable entophytic community present in A. triangularis. Shannon diversity index (H') was used to estimate the ecological diversity of fungal endophytes isolated from each tissue of plant, based on different genera identified after sequencing. To compare isolation frequency (IF) of each plant tissue, Tukey test was performed [30].

Phylogenetic analyses

Phylogenetic analyses were based on ITS sequence data and carried out to establish the phylogenetic placement of each isolated taxon. Sequences were compared with NCBI sequence database using Basic Local Alignment Search Tool (BLAST) algorithm. The alignment and phylogenetic analysis were further improved with MUSCLE implemented in MEGA 6v [31]. Neighbor-joining method was used to calculate the clustering, with 1,000 bootstrap replicates based on genetic distances.

***In vitro* antagonistic assay**

The antagonistic activity of A. triangularis endophytic fungi against B. cinerea and R. stolonifer, was tested in dual-culture confrontation assay [32]. Agar plugs (0,6 cm), containing pure cultures of the endophytes and phytopathogens, were inoculated into petri dishes containing potato dextrose agar (PDA). The distance between the antagonist and pathogens was 4 cm. Plates were incubated at 28 ºC for seven days. The Antagonism Index was measured according to the following formula: AI = (RM – rm)/RM x 100, where rm = ray of the pathogen colony towards the antagonist and RM = average of the three rays of the colony in the other directions. The significance of the observed inhibition was determined by analysis of variance – ANOVA, using Dunnett's test ($p < 0.05$).

Detection of PKS I gene

To investigate biotechnological potential of the isolates, PKS I gene presence were evaluates through PCR using two degenerate primer pairs. LC1 (5'GAYCCIMGITTYTTYAAYATG3') / LC2c (5'GTICCIGTICCRTGCATYTC3'), which amplifies PKS I WA-type, involved in mycotoxins and pigment production, and LC3 (5'GCIGARCARATGGAYCCICA3') / LC5c (5'GTIGAIGTICRTGIGCYTC3'), which amplifies PKS I MSAS-type, involved in 6-methylsalicylic acid biosynthesis [33]. The amplifications were performed in a total volume of 20 µL of PCR mixture containing 12.4 µL of distilled water, 1 µL (6 ng) of DNA template, 1 µL (0,50 mM) of each primer and 5.6 µL of PCR mix (0,01 U/µL of Platinum Taq DNA Polimerase, 1X reaction buffer, 2 mM of MgCl, 0,25 mM of each dNTP; Invitrogen, California, USA). PCR conditions consisted of 94 ºC for 30 s, 40 cycles of denaturation 94º C for 30 s, primer annealing for 30 s at 56º C and extension for 1 min at 72º C, and final extension at 72 ºC for 3 min. PCR product was analyzed by electrophoresis on 1.6% (w/v) agarose gels, stained with GelRed (Biotium, Hayward, CA) and visualized under UV light.

Nucleotide accession number

All sequences obtained in this work were deposited in Gene Bank: accession numbers MF076578 to MF076627.

Results

Endophytic fungi isolation and Phylogenetic analyses

In this study we isolated and characterized endophytic fungi associated to the medicinal plant A triangularis, from Atlantic forest region. A total of 500 fragments of vine, stem, fruit, young and mature leaves were evaluated for endophytes. At least one fungal colony was isolated from 88% of the samples. The highest IF was recorded from mature leaves (100%), followed by young leaves (97%), fruits (94%), vine (87%) and the lowest IF was from stem (65%) ($p \leq 0,001$) (Fig. 2). Mature and young leaves showed higher IF, compared to other plant tissue. Stem, in turn, showed lowest IF value, statistically different from other plant tissue. A total of 263 endophytic fungi were isolated from healthy tissues of *A. triangularis*. The most representative haplotype was 7, in which 36% of the isolates are inserted. Haplotype 1 corresponded to 20% of the isolates and hap. 5 to 15%. The lowest representative haplotypes corresponded, together, to 17% of the isolates (Fig. 4).

Selected isolates of each haplotypes were further analyzed using ITS sequencing. Based on similarity to sequences in the NCBI sequence database, were found 13 different genera. These are *Colletotrichum, Fusarium, Xylaria, Trichoderma, Nectria, Phomopsis, Stenocarpella, Phylosticta, Ascochyta, Neurospora, Gibberella, Yarrowia and Botryosphaeria*.

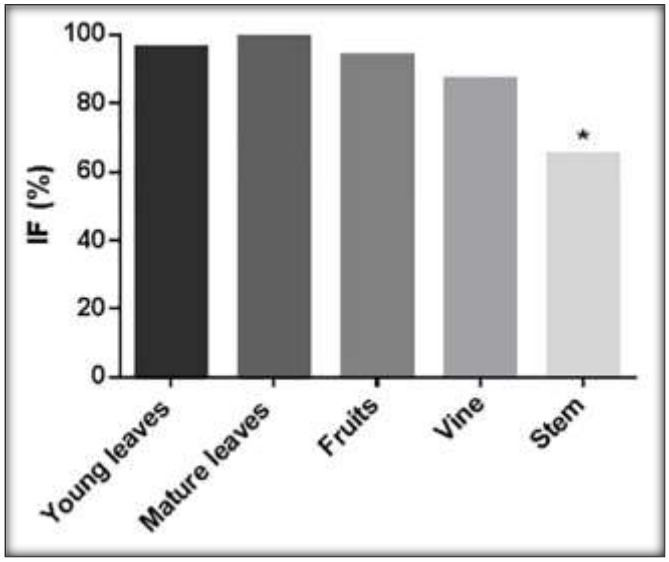

Fig. 2 – Isolation frequency (IF) from different plant tissues, *p<0,001

The initial analysis of the ITS sequences of the 263 isolates revealed 26 different haplotypes, (identified Hap.1 – 26) (Fig. 3).

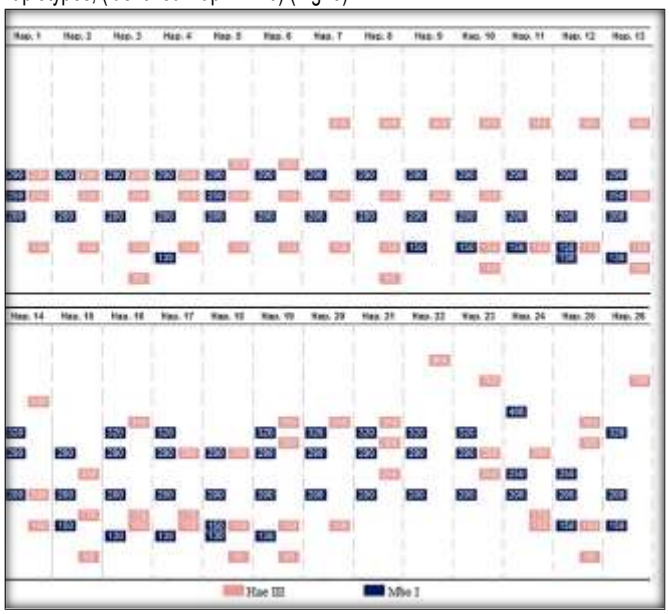

Fig-3 Twenty-six different haplotypes and its fragment sizes (bp) after ITS amplicon digestion with Mbo I and Hae III enzymes

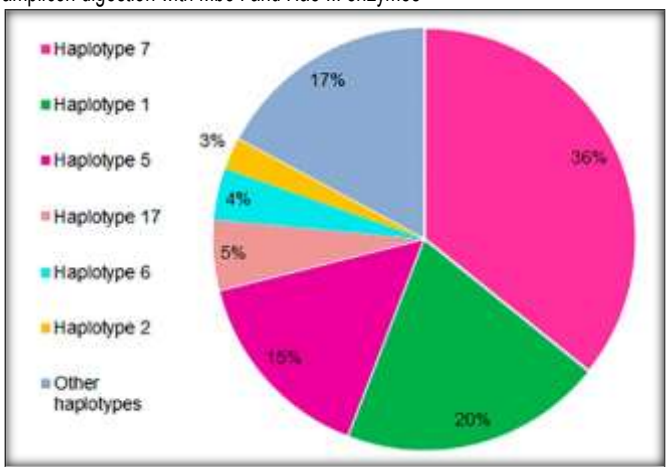

Fig-4 Most representative haplotypes in relation to the number of isolates

These genera belong to 9 distinct families: *Glomerellaceae, Sordariaceae, Nectriaceae, Hypocreaceae, Xylariaceae, Didymelaceae, Botryosphaeriaceae*, *Diaporthaceae* and *Dipodascaceae* (Fig. 5). In each family, isolates of different haplotypes are present, but each haplotype is concentrated within only one family. Based on the genus found, Shannon index showed greater diversity on vine (H': 1.4402), followed by fruit (H': 1.0035), stem (H': 0.7889), mature leaves (H': 0.2378) and young leaves (H': 0.2074). As IF values and the number of haplotypes, differences observed in diversity may also be influenced by natural factors [34, 35]. Although the leaves presented higher IF than the other tissues evaluated, Shannon's index shows that the higher diversity is in vine and lower diversity in leaves. This is because, despite leaves having a higher IF, the number of different genus found on it is lower.

***In vitro* antagonistic assay**

Of the 263 isolated fungi, only 4,9% (thirteen isolates) were able to control *B. cinerea* (p<0,0001). Of these, 3% (seven isolates) completely inhibited phytopathogen growth. On the other hand, 2,7% (seven isolates), were able to control *R. stolonifer*. However, none of those isolates were able to completely inhibit its growth. Controlling *R. stolonifer* is extremely hard due to its high growth rate. We observed that in only few days, phytopathogen have covered the entire surface of control plate. Thus, a control rate of 50%, as observed in this study, show huge potential to new tests with these endophytes against *R. stolonifer*. The isolates which showed biocontrol potential against both phytopathogens belong to *Colletotrichum, Botryosphaeria, Stenocarpella, Nectria, Phomopsis, Gibberella* and mainly to *Trichoderma* genus (Fig. 6).

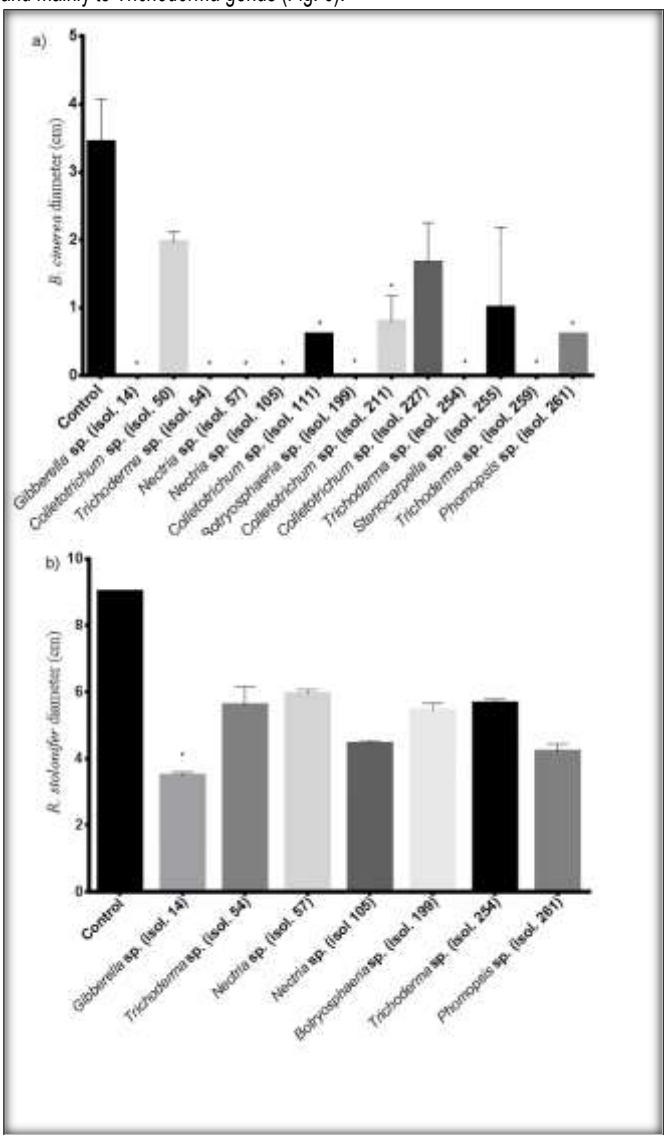

Fig-6 Antagonistic activity of the A. triangularis endophytic fungi against a) *B. cinerea*, b) *R. stolonifera*, * Inhibition rate ≥ 50%, p<0,0001

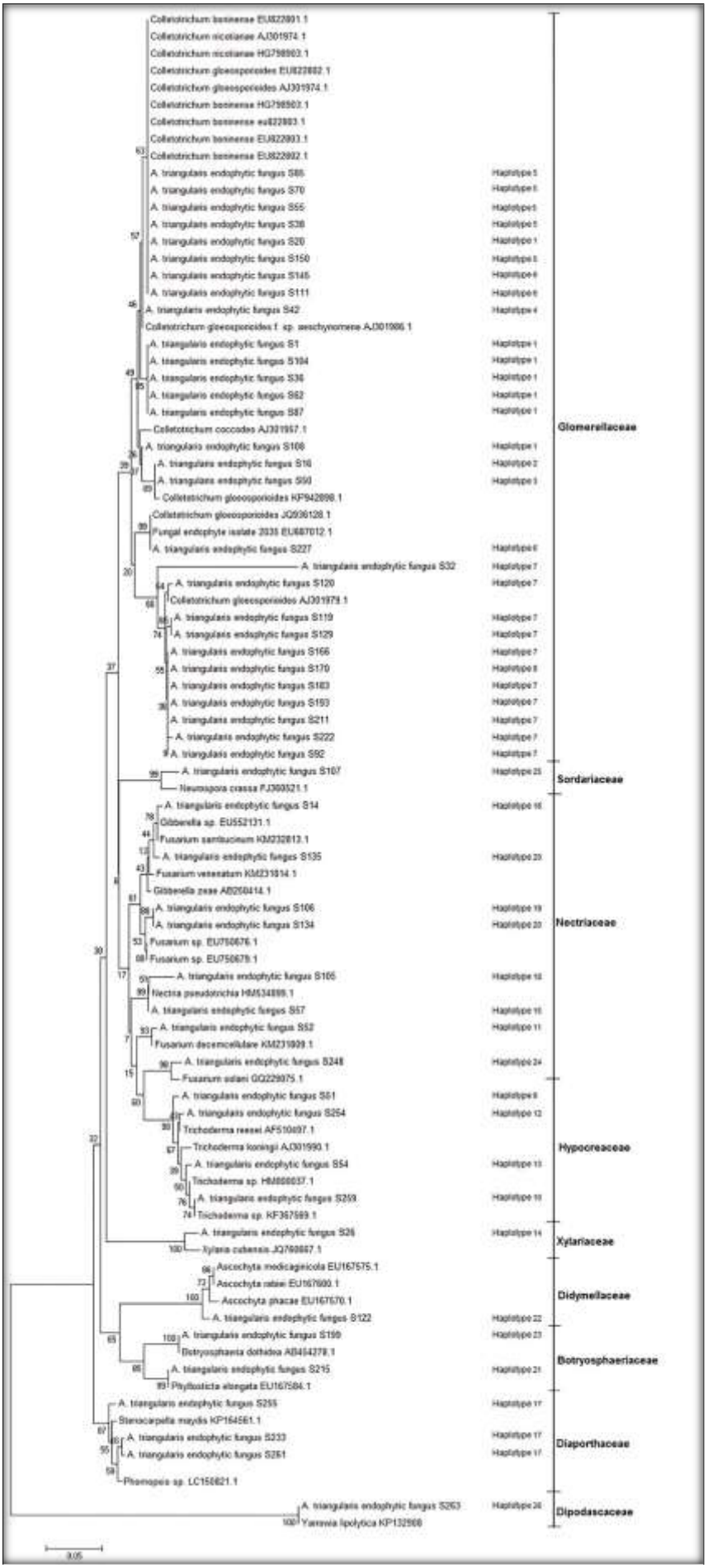

Fig-5 Neighbor-joining phylogenetic tree of 50 isolates related to 26 ITS haplotypes isolated from *A. triangularis*

Detection of PKS gene

Fifty-eight isolates showed LC1/2c amplification only and 1 isolate showed LC3/5c amplification only. Seven isolates showed both LC1/2c and LC3/5c amplification, totalizing 66 isolates with PKS I gene presence (Fig. 7). Of those isolates which showed antagonistic activity against *B. cinerea* and *R. stolonifer*, only six presented PKS gene (Table 1).

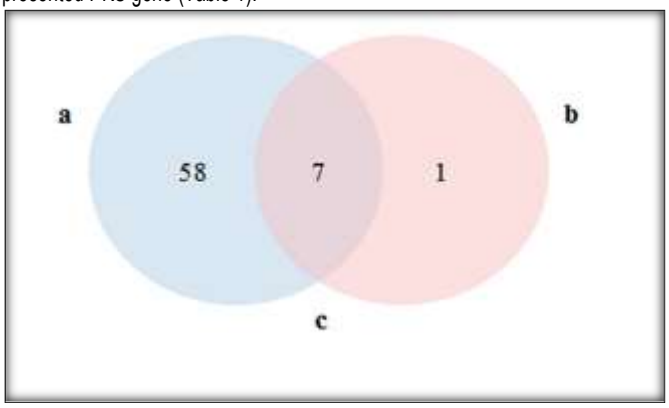

Fig-7 Venn diagram of PKS I gene amplification using two different prime pairs. a) LC1/2c b) LC3/5c c) both LC1/2c and LC3/5c

Table-1 PKS gene presence in the endophytes which showed antagonistic activity against *B. cinerea* and *R. stolonifer*.

		Antagonistic activity		
Isolates	Genus	*B. cinerea*	*R. stolonifer*	PKS
14	*Gibberella* sp.	Y	Y	+
50	*Colletotrichum* sp	Y	N	+
54	*Trichoderma* sp.	Y	Y	-
57	*Nectria* sp.	Y	Y	-
105	*Nectria* sp.	Y	Y	+
111	*Colletotrichum* sp.	Y	N	-
199	*Botryosphaeria* sp.	Y	Y	+
211	*Colletotrichum* sp.	Y	N	-
227	*Colletotrichum* sp.	Y	N	-
254	*Trichoderma* sp.	Y	Y	-
255	*Stenocarpella* sp.	Y	N	+
259	*Trichoderma* sp.	Y	N	-
261	*Phomopsis* sp.	Y	Y	+

Y = antagonistic activity, N = no activity; (+) = presence of PKS; (-) = absence of PKS

Discussion

Differences on IF values, from different plant tissues, as observed in this study, were previously described. Differences between bark and xylem of *Pinus tabulaeformis* was previously reported [36]. This was also observed in different tissues of *Plantago lanceolate* [35]. However, the comparison of IF values between different studies and authors is a problem, due to different protocols used to isolation, surface sterilization, culture media used and size of tissue fragment [37]. Besides that, factors as season, humidity, temperature and soil chemistry are determinant to number of isolates, richness and biological diversity. The nutrients composition also influences colonization, which make some species specifically colonize the leaves, for example [38]. The number of haplotypes is directly influenced by same factors as the number of isolates (season, humidity, temperature, soil chemistry and nutrients composition). In sugarcane, 14 different haplotypes were found in 300 endophytic fungi isolated from this grassy. In this case, the fact that the work was conducted in a monoculture directly influences the lower number of haplotypes found [39]. In our work, we reach 26 different haplotypes in isolated endophytic fungi from a plant collected in Atlantic forest region, which is largely known by its biodiversity, influencing the number of haplotypes [40]. Some studies show that ARDRA's technique is able to separate different families, genera and species [41, 42]. In the isolates from A. triangularis, considering Mbo I and Hae III restriction enzymes, this technique was effective to distinguish among different families. Using ITS sequencing, 13 different genera were identified, belonging to 9 distinct families (Fig. 5). From those genera identified, some are commonly isolated as endophytes, as *Colletotrichum* and *Trichoderma* [43, 44, 31], *Fusarium* [45], and *Xylaria* [46], for example. The genus that showed greater abundance was *Colletotrichum*, representing 58% of the isolates identified. This genus was previously associated to important metabolic activity, as antimicrobial activity [21]. This group described three new compounds produced by a *Colletotrichum* spp. endophytic from *Bruxus sinica* (used in Traditional Chinese Medicine - TCM), to treat syphilis and malaria. The compounds isolated, named colletotrichones A – C, showed inhibitory potential against the pathogenic bacteria *Staphylococcus aureus, Escherichia coli, Bacilus subtilis* and *Pseudomonas aeruginosa*. A group from China, besides isolate and elucidate new compounds from *Colleototrichum* spp., related these metabolites to *in vitro* biological control of *Aspergillus niger, Gaeumannomyces graminis, Rhizoctonia cerealis, Helminthosporium sativum* and *Phytophthora capisici*, five crop pathogenic fungi [47]. Another *Colletotrichum* spp. isolated as endophyte from geranium (*Pelargonium graveolens*) showed potential to produce nanoparticles. The researchers observed that both plant and isolate were able to reduce chloroaurate ions in gold nanoparticles [48]. Endophytes of *Colletotrichum* genus have several applications in the most different areas of medicine and industry. They were already found producing the anticancer drug Taxol [49], volatile metabolites used in cosmetics [50], promoting plant growth [51], biological control [47, 52], among other important activities. *A. triangularis* plant is rich in isolates of *Colletotrichum* genus, showing huge potential to production of bioactive metabolites. Second most representative genus was Fusarium (14%). These genera also possess large biological activity, such as biological control through induction of systemic plant resistance [53], antagonist activity against phytopathogenic fungus *Alternaria brassicae, Penicilium digitatum* and *Verticillium alboatrum* [54], production of different drugs, as anticancer camptothecin [55], antineoplasic and ativiral podophyllotoxin [56], and antimycobacterial javanicin [57], among others. Both of most isolated genera, *Colletotrichum* and *Fusarium*, were previously associated to Atlantic Forest biome. Isolated, for example, from orchids [58, 59, 60], dye plants as *Indigofera suffruticosa* [61], medicinal plants as *Vismia guianensis* [62], soil [63], among others. Demonstrating that, these genera, are endemic on this ecosystem. The other genera found in this work also have previous records of biological activity. Trichoderma spp., which represented 6% of the endophytic isolates, for example, is one of the most studied genera about biological control. There are several reports of its genus producing bioactive metabolites and enzymes [64, 65]. *Stenocarpella* spp. [66], *Phomopsis* spp. [67, 68], *Gibberella* spp. [69] and *Nectria* spp. [70], also show antimicrobial and antifungal activity. Observing the potential of the endophytes isolates in this work to produce bioactive metabolites with antifungal activity, they were tested against strawberry phytopathogens *B. cinerea* and *R. stolonifer*. From those tested, 7 were able to inhibit both the pathogens. All of them belongs to genera previously reported as phytopathogen controllers. Trichoderma is widely, if not the most, known genus by its production of bioactive metabolites, enzymes and uses in biological control. Isolates of this genus showed previous activity against several phytopasthogenic fungi as *Fusarium oxysporum* [65], *Fusarium solani* [71], *Pythium ultimum, Sclerotinia sclerotiorum* [72], among others. Their mode of action is broad, ranging from mycoparasitism, nutrient competition and metabolites production [73]. Among the bioactive metabolites produced by *Trichoderma* spp. are, *anthraquinones, sesquiterpenes, pironas, koninginins, trichodermamides, viridines, viridiofungin, trichodenones* and statins [64], besides lytic enzymes as β-(1-3) glucanases, chitinases and proteases [65], which act on biological control. Isolates of *Colletotrichum* genus were also observed in this study, controlling *B. cinerea* and *R. stolonifer*. *Colletotrichum* spp., are a huge producer of secondary metabolites with biocontrol activity. Phytopathogens as *Aspergillus niger, Phytophthora capsici, Rhizoctonia cereales* [47], *Cladosporium cladosporioides, Cladosporium sphaerospermum* [74], *Helmionthosporum sativum*, besides human pathogens as *Staphyllococcus aureus* [52] and *Candida albicans* [47], was previously controlled by *Colletotrichum* spp. metabolites. In addition to bioactive metabolites, as well *Trichoderma* spp., *Colletotrichum* genus is either able to produce several lytic enzymes [3]. All the other genus of endophytes isolated from *A. triangularis* showed previous antifungal action and potential use in biological control. Antagonist activity of *Botryosphaeria* spp. against *Aspergillus terreus* and *F. oxysporum* was previously described [75].

Stenocarpella spp. isolates was described with antifungal activity against *Aspergillus flavus* and *Fusarium verticilioides* [66]. *Phomopsis* spp. showed activity against *R. cereales, Bipolaris sorokiniana* and *Gaeumannomyces graminis* [68]. The genus Gibberella was active against *A. flavus, R. solani*, *Ceratocystis fimbria* and *Geotrichium candidum* [69]. Finally, *Nectria* spp. showed previous activity against *Penicillium avellaneum* and the human pathogens *C. albicans, S. aureus and Enterococcus faecalis* [70]. We observed that the endophytic community of *A. triangularis*, besides rich are also able to reduce and even inhibit the growth *of B. cinerea* and *R. stolonifera*. In addition, they have a huge potential to produces bioactive compounds. Due to this, the evaluation of PKS gene presence was an important strategy to detect isolates with this potential. Sixty-six isolates showed PKS I gene presence (Fig. 7). Of those isolates which showed antagonistic activity against *B. cinerea* and *R. stolonifer*, only six presented PKS gene (Table 1). Despite of several studies are being performed to understand the relation of PKS I gene with biological control, it remains unclear. Recently the correlation of the expression of two PKS genes from *Trichoderma harzianum*, in response to the presence of three phytopathogenic fungi *R. solani*, *S. sclerotium* and *F. oxysporum* was shown [22]. A PKS gene from *Purpureocillium lilacinum* was also related to their *in vitro* biocontrol activity against the phytopathogen *Phytophthora* sp. [21]. The main hypothesis involving PKS presence evaluation in this study, was that fungi with antagonist activity against *B. cinerea* and *R. stolonifer*, had same PKS pattern. However, no correlation was observed, since not all the isolates with antagonistic activity showed gene presence. It is certain that this gene is related to bioactive metabolites production, but not necessarily with biocontrol observed in this study. Filamentous fungi produce several polyketides PKS-derived, and this gene is involved with production of many bioactive secondary metabolites [33]. Evaluating PKS I gene presence in all A. triangularis isolates, we observed that 66 of them presented positivity to this gene, confirming the isolates potential (Fig. 7). These isolates belong to genera *Colletotrichum, Trichoderma, Nectria, Fusarium, Ascochyta, Phomopsis and Stenocarpella*, which were previously reported as bearers of PKS I gene involved in bioactive metabolites production [20, 23, 25, 76, 77, 78, 79, 80]. Furthermore, PKS I gene presence was previously evaluated in endophytic fungi from medicinal plants. The potential of 23 endophytic fungi isolated from cranberry fruit (Vaccinium macrocarpon), to produce polyketide metabolites, was evaluated. The researchers found 12 distinct PKS genes in 11 isolates. All sequences were phylogenetically related to MSAS-type PKS, and important metabolites as antitumoral and antibiotic molecules [81]. The presence of PKS I gene was also evaluated in endophytic fungi isolated from 8 different medicinal plants. From 18 isolates that showed PKS I presence, 9 of them demonstrated *in vitro* antiproliferative action in human myeloma cells. In addition, 2 isolates were able to inhibit Escherichia coli growth and other 11 isolates showed antifungal action against the opportunistic pathogen *Candida albidus* [25]. Besides the link of PKS I gene to medicinal metabolites, it was also related to biocontrol of phytopathogenic fungi, as mentioned before [21, 22]. Studies like these demonstrate that endophytic fungi isolated from medicinal plants shows important biological activities. In addition, PKS I gene presence evaluation is important as a screening for detection of isolates capable of producing bioactive metabolites. Most of the isolates from A. triangularis showed the presence of subclass WA-type (Fig. 7), which is involved with pigment and mycotoxins biosynthesis. However, there is a lot to understand about such metabolic pathways. A PKS gene, responsible for conidia pigmentation of *Trichoderma reesei*, was also closely related to its defense and mechanical stability. Producing a knockout strain to this gene, the group observed that this knockout strain demonstrated a decrease in biological control against phytopathogenic fungi *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Alternaria alternata*, when compared to normal strain. Besides that, they showed that this gene, was directly related to another PKS gene expression [23]. In a phylogenetic analysis, two PKS I genes from *P. lilacinum*, involved on leucinostatins biosynthesis (secondary metabolites with antibiotic, antiviral, antitumoral and antifungal activity) was strongly related to gene Fum1p, responsible to biosynthesis of fumosin mycotoxin [21]. Much still needs to be understood about full functioning of PKS I and their involvement on secondary metabolites production. However, although phylogenetically distinct, different PKS I genes are closely related and, in many cases, depend on each other to produce biological actions.

Conclusion

This study was pioneer to access the endophytic fungi community of *A. triangularis* and revealed a large and diverse microbiota, living in all the plant tissues studied. This plant is widely used in popular medicine and the fungi isolated from it, may be related to its biological activity. Some of the endophytes isolated, were able to promote *in vitro* biological control of *B. cinerea* and *R. stolonifer*, showing important potential to agricultural applications. Some isolates caused total inhibition of the phytopathogens, indicating metabolites secretion. PKS I gene presence in the isolates, demonstrate that they are able to produce a wide range of bioactive compounds with priceless pharmaceutical, industrial and agricultural applications.

Application of research: These article findings are extremely important, due to the description of a community never studied before and their large potential to produce metabolites ranging from antibiotics to mycotoxins. As well the potential of some isolates in controlling two important phytopathogens of strawberry crops. In addition, this work may assist the understanding of the dynamics between host-endophyte and their behavior towards the metabolites production.

Research Category: Environmental Microbiology, Agricultural Microbiology.

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Research Guide: Ida Chapaval Pimentel
University: Federal University of Paraná, Curitiba, Paraná, Brazil.
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