Article

Impact of the temperature in endophytic Ascomycota isolated from Antarctic hair-grass

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Simple Summary: As Antarctica is a continent that reverberates a lot of climate change at a planetary level, it is up to us scientists to seek to understand how the diversity of this polar environment can be affected in the event of major climate changes happening soon. We were able to recover 4 species of fungi that live within the native grass of Antarctica and found that some of these fungi respond better to growth at temperatures higher than the average of polar regions, suggesting that potential increases in global temperature, may favor dormant fungi to spread. Thus, other species of fungi have more harmful behavior to plants and may become collateral damage from eventual climate changes.

Abstract: Antarctica is one of the most inhospitable continents on the planet, with lichens and mosses being the most common terrestrial organisms in ice-free areas. Antarctica is represented by only two species of Angiosperms, *Deschampsia antarctica* Desv. (Poaceae) and *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae). In this study, we characterized fungi isolated from the leaves of this grass species. The fungi were isolated from 4 individual plants from Half Moon Island (246 leave fragments investigated), and 7 from King George Island - Keller Peninsula (with 111 leave fragments investigated) Antarctica. *Neoascochyta paspali*, *Phaeosphaeria elongata*, *Pyrenophora* cf. *chaetomioides* and *Alternaria* sp. were associated with the plant and identified through analysis of the sequences of the internal transcribed spacer region (ITS) of the rDNA and nuclear ribosomal large subunit rRNA gene (LSU) as well as macro and micro-morphological characteristics. The isolates showed a better growth rate ranging from 10–20°C. An interesting result was that the fungi are already recognize as both plant pathogens and endophytic fungi. The results demonstrate that D. antarctica is an interesting fungal source. Those species might provide important information about the relationship on the endemic Antarctic biota.

Keywords: Endophitic Fungi Phylogeny, Biodiversity, Molecular biology, ITS, nLSU.

1. Introduction

The Antarctic region plays a key role in the balance of atmospheric and climatic dynamics [1]. However, the Antarctic continent is considered one of the most inhospitable ecosystems, being the coldest, windiest, and driest, with a high incidence of radiation, all of which restrict the development of many life forms [2].

The Antarctic terrestrial diversity is predominantly composed of lichens and bryophytes (mosses and liverworts) species and includes only two species of native vascular plants: *Deschampsia antarctica* Desv. (the Antarctic hair-grass - Poaceae) and *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) [3,4]. *D. antarctica* is a pioneer species that colonises exposed environments after retraction of the glaciers. It can grow over death mosses or directly on live moss carpets [5-7].

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Several studies to date have demonstrated the ability of this Antarctic hairgrass to survive extreme conditions or the mechanisms involved in this resistance [8-10]. However, few studies have investigated its interaction with the associated fungi. Plant pathogens within the Antarctic region as well known and are mostly identified in lichens and mosses, more so than in angiosperms [4]. For other substrates, fungi have been reported such as soil, woody components, rocks and macroalgae [11].

For *D. antarctica*, associated fungi have been reported in some studies, indicating a wide range of mycorrhizae [12] as well as endophytes, parasites, and predatory fungi [13-16].

The fungi associated with Antarctica plants typically include yeasts and filamentous fungi consisting of species from Chytridiomycota, Zygomycota, Glomeromycota, Basidiomycota, and Ascomycota [17]. Ascomycota represents the major group of endophytic fungi isolated to date [18]. The association can be endophytic, such as with pathogens or decomposers [19,20].

In this study, were evaluated the growth of three endophitic fungi associated with D. antarctica, collected in the Antarctic summer of 2014 and 2016 from Half Moon and King George Islands. The isolated fungi were identified by molecular taxonomy and macro- and micro-morphological characterization and represented the genus *Pyrenophora*, *Phaeosphaeria*, *Neoascochyta*, and *Alternaria*. In addition, mycelial growth was assessed at different temperatures and in different culture media.

2. Materials and Methods

2.1 Study area

The South Shetlands Archipelago is in the Maritime Antarctic, lying in the Northwest of the Antarctic Peninsula. It is composed of 8 large islands and many other smaller ones. The small Half Moon Island is located at 62°36'S; 59°53'W and King George Island at 62º01'21"S, 58º15'05"W [21], where specimens of *D. antarctica* were collected.

Plant material and isolation of the fungi

Fresh *D. antarctica* leaves were collected from plants growing under natural conditions in Half Moon Island during the austral Summer of 2014, and from King George Island (Keller Peninsula) during the austral Summer of 2016. The plant material was stored in sterile plastic bags, frozen, and transported to Brazil. The leaves were sterilized by successive immersion in 70% ethanol (1 min) and 2% sodium hypochlorite (3 min), followed by a sterile distilled water rinse (2 min) [14] (Rosa et al. 2009). The fragments were then plated on Petri dishes containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 µg mL-¹). The plates were incubated up to 60 days at 20 °C, and individual colonies were transferred to PDA, and stored at 20 °C. The long-term preservation of mycelial pieces was performed using the Castellani and Mineral Oil methods [22]. The fungi were isolated from four individual plants in Half Moon Island (246 leaves fragments investigated), and seven from King George Island - Keller Peninsula (with 111 leaves fragments investigated) – Antarctica. The fungal isolates used in this study were deposited in the Bruno Edgar Irgang Herbarium (HBEI) of the Universidade Federal do Pampa - São Gabriel (UNIPAMPA).

2..2 Morphology

Fungal macroscopic parameters (colony color, texture, reverse color, border type) and colony diameters were observed in different media. Colors follow the specification proposed by the OACC (www.worldcat.org). All isolates were inoculated in the following media: PDA, Sabouraud Agar, and Grass Extract Dextrose Agar (GE). All media were incubated at 5, 10, 20, and 23.5 ± 1 °C. The morphological and microscopic characteristics were evaluated from 15-30 days. Media under the same conditions were used to determine the microscopic parameters (hyphae, conidiophores, and conidia), and measures were obtained by determining the length/width of individual chlamydospores.

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2.3 Molecular analysis

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified with universal primers for ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [23]. For ribosomal large subunit (nLSU) analysis, rDNA primers for NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used [24]. The PCR procedure for ITS was as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 54 °C for 45 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. In addition, the PCR procedure for nLSU was as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 45 s, 57 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were purified using the Wizard® Plus SV Miniprep DNA Purification System (Promega, EUA), and sequenced using a ABI-Prism 3500 Genetic Analyzer (Applied Biosystems) with the same primers. The sequences obtained were adjusted using Bioedit software v. 7.0.5.3., and a consensus sequence was obtained using Lasergene SeqMan software (DNASTAR/Inc.). Representative consensus sequences were deposited into GenBank under the accession numbers: nLSU – MF628023, MF628257, MF628108, MF629819 and ITS – MF629817, MF629818.

2.3.1 Molecular identification analysis

To identify species by rDNA sequencing based on ITS and nLSU, the consensus sequences were aligned with sequences from related species retrieved from the NCBI GenBank database using BLAST [25]. The closest matched sequences with query cover and maximum identity ≥ 96% and 90% for ITS and LSU sequences respectively, with an e-value ≥ 0, were included in the phylogenetic analysis. The dataset used as the out group included *Preussia minima* (Auersw.) Arx for ITS and nLSU. Sequences were aligned with ClustalW as implemented in MEGA v. 6.06 [26]. Prior to phylogenetic analysis, ambiguous sequences at the start and end were deleted to optimise the alignment. Bayesian inference (BI) was employed to perform phylogenetic analyses of the two aligned datasets. Bayesian analyses were conducted on the aligned data set using BEAST v. 1.8.3 software [27]. The Hasegawa-Kishino-Yano model of equal base frequencies was used for ITS and the Tamura-Nei model for nLSU. In order to identify the posterior probability tree were set 10 million Markov Chains Monte Carlo (MCMC) were run, and trees were sampled every 1000 generations. Tracer v1.6 [28] was used to evaluate the effective population size (ESS >100), and TreeAnnotator v1.8.3 (from the BEAST package) was used to condense the information from the trees sampled by MCMC. The information about fungal classification followed Onofri et al. [2], MycoBank (http://www.mycobank.org), and the Index Fungorum (http://www.indexfungorum.org).

2.4 Growth experiments

Mycelia disks of 4 mm diameter for the four isolates studied were inoculated in three different culture media: PDA, Sabouraud Dextrose (SAB), and GE. The plates were incubated at 5, 10, 20, and 23.5 ± 1 °C in the dark. Plates containing the mycelium for each of the species in each culture medium and temperature tested were performed in triplicate [29].

Radial mycelial growth was measured using a digital caliper from the back of the plate in four-line directions at 45° of each other in the 2 sectors (0, 45, 90, 135, 180, 225, 270, 315°) at 24-h intervals for each measurement. This was the first reading performed after the 4th day of incubation. The mean length was calculated for each treatment and isolated fungus obtained from the leaves of D. antarctica. The last growth measure was performed when the first isolate reached the border of one of the Petri dishes. This occurred thirteen days after the first length measurement. For the UNIPAMPA 006 isolate, it was not possible to perform statistical tests, since no growth was observed at 5, 10, and 23.5 °C, possibly due to the methodology used for measurement.

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The experiment was conducted in a completely randomized manner. The data were analyzed by analysis of variance (ANOVA) [30], and the means were compared using the Tukey test ($p < 0.05$) of probability, assuming that the data are normal. Verification of the normality of the data was performed as proposed by Shapiro-Wilks [31-33]. When the data were not normal, they were transformed using the Tukey's Ladder of Powers transformation method [34]. All statistical analyses were performed in the R computational environment (R Core Team 2017) with RStudio software [35].

3. Results

Four distinct fungi were isolated directly from the leaf fragments of four individual plants in Half Moon Island (246 leaves investigated), and seven from King George Island - Keller Peninsula (111 leaves investigated). The leaves of *D. antarctica* revealed four fungi morphospecies, as some thus isolated from both sample sites demonstrated the same morphology. These isolates were labelled as follow: UNIPAMPA 004, UNIPAMPA 005, UNIPAMPA 006 for Half Moon Island, and UNIPAMPA 007 for King George Island.

3.1 Macro- and micro-morphological analyses

After detection of the preliminary genetic and morphological characteristics, we identified the fungus UNIPAMPA 004 as belonging to the *Pyrenophora genus*, UNIPAMPA 005 to the *Phaeosphaeria genus*, UNIPAMPA 006 to the *Neoascochyta*, and UNIPAMPA 007 to the *Alternaria* sp. For the Alternaria isolate, we preferred not to continue with the other analyses, as it was impossible to identify which species of the genus our isolate belongs correctly. However, we still left this OTU in the phylogenetic trees to contribute to the positioning of the other species studied. The macro- and micro-morphological characteristics of the other isolated fungi were evaluated on three media as described below.

3.1.1 Pyrenophora cf. *chaetomioides* (UNIPAMPA 004)

The colonies grew at all temperatures, were cottony, and had white (oac909) or orange (oac649) edges, with a grey centre [(oac906) to (oac761) or (oac739-oac746 / oac764-oac765), reverse darker -oac761)] (Fig. 1). Hyphae transformed into chlamydospores (Fig. 3k). Chlamydospores were terminal at 10.5–22.2 × 3.8–10.9 μm in size or catenulate and larger (15–29 × 9–24.5 μm in size) at 10 °C (Fig. 2b–i). The pigment was evident at higher temperatures and dissolved in 5% potassium hydroxide (KOH) (Fig. 3a and j).

Examined material: ANTARCTICA, South Shetland Archipelago, Half Moon Island, Austral Summer of 2014, A. B. Pereira (UNIPAMPA 004).

3.1.2 Phaeosphaeria elongata (UNIPAMPA 005)

Phaeosphaeria elongata demonstrated colonies with borders presenting a hyaline margin (oac857) of up to 1 cm large that was plain and complete, with a white centre (oac909) surrounded by yellow (oac853) or grey (oac908) mycelium. The reverse had colours from oac908 to oac763-oac637 or oac794-oac763 and all growing colonies are cottony (Fig. 3). There were also hyphae hyaline to melleous of 4-11 μm in diameter, with sinuose walls in the terminal (Fig. 4d). There were also initials of conidiophores and conidia at terminal branches, with immature long ellipsoid to cylindrical conidia like those of *Stagonospora* sp. (Fig. 4a-c). Only one nematode capture loop was found at 10°C in GE (Fig. 4e).

Examined material: ANTARCTICA, South Shetland Archipelago, Half Moon Island, Austral Summer of 2014, A. B. Pereira (UNIPAMPA 005).

3.1.3 Neoascochyta paspali (UNIPAMPA 006)

Growth was observed only at 20 °C in PDA and SAB culture media. Colonies generating cottony whitish tufts were formed through radially disposed hyphae, with grey and

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white (oac866/oac903) and reverse (oac908). There were also hyphae of 2-8 μm in diameter. Tufts were also identified in the mycelium in PDA (Fig. 5a). The oldest mycelium had a higher proportion of pigmented hyphae (Fig. 6d) and rare chlamydospores, which were 6–15 × 5.5–8 μm in size (Fig. 5b-c).

Examined material: ANTARCTICA, South Shetland Archipelago, Half Moon Island, Austral Summer of 2014, A. B. Pereira (UNIPAMPA 006).

3.2 Phylogenetic Analysis

To clarify the taxonomic position of the species, we performed a phylogenetic study based on the sequences of the ITS and nLSU regions. The sequences obtained from fungi cultures resulted in BLASTn hits for endophytic and pathogenic fungi. The isolates were considered as belonging to the *Pyrenophora*, *Phaeosphaeria*, *Neoascochyta* and *Alternaria* genus after a comparison of their nucleotide sequences revealed an identity above 90% for ITS regions of rDNA and nLSU.

Detailed phylogenetic analysis of the ITS region of the UNIPAMPA 006 and UNIPAMPA 005 sequences with the nearest taxa obtained from GenBank showed that UNIPAMPA 006 forms a distinct cluster close to species *Neoascochyta paspali* (NR135970) and *Phoma paspali* (KT309957). In addition, the ITS sequence of UNIPAMPA 005 grouped with the *Phaeosphaeria elongata* (KM491546) species.

The fungal isolates of UNIPAMPA 004 and UNIPAMPA 007 were not included in the ITS phylogenetic analysis because the sequence presented low quality and a smaller size than another homologous find in Genbank. Moreover, in the initial Blast survey, these sequences showed an e-value > 0 in the BLASTn query, which could have generated conflicts during the alignment with other sequences of fungi.

The total number of sequences of the ITS rDNA regions compared to sequences associated with Antarctic grass leaf fungi was 31. Based on our results, the Bayesian Inference (Fig. 7) tree in this dataset with two distinct clusters are supported (A and B). Cluster A (Fig. 7) is comprised of 16 endophytic and pathogenic fungi sequences. Within this cluster were grouped sequences of fungi principally reported as endophytic. Of these taxa, all are identified as belonging to the *Phaeosphaeria* genus. These species were close to the UNIPAMPA 005 isolate. *Phaeosphaeria elongata* (KM491546) was collected from dead wood in Italy [36,37]. The analysis of this clade is supported by a posterior probability of 0.99, indicating that our species is *Phaeosphaeria elongata*.

The species that comprised cluster B (Fig. 7) included 14 species of fungi that corresponded to endophytes and pathogens. These species were close to the UNIPAMPA 006 isolate and *Phoma paspali* (KT309957) phytopathogenic fungi and *Neoascochyta paspali* (NR135970) type species. In addition, most of the taxa are continuously present in the environment as saprobic soil organisms [38], *Neoascochyta* europaea (KT389510), and *Neoascochyta graminicola* (KT389518) fungus associated with plants and soil [39]. This clade was heavily supported (PP = 0.99).

Isolated UNIPAMPA 006 was inferred together with a fungus identified as *Neoascochyta paspali*. The genus *Neoascochyta* is ubiquitous and species-rich, with species occurring on a diverse range of substrates, including soil, air, plants, animals, and humans. The posterior probability supports that the isolated belongs to the *Neoacochyta paspali* species complex.

A total of 47 sequences from the LSU region were compared to the sequences obtained in this study (UNIPAMPA 004, UNIPAMPA 005, UNIPAMPA 006 and UNIPAMPA 007). The sequences were the result of the BLASTn search. The tree generated by BI analysis based on nLSU dataset were similar in topology with the ITS region. The phylogenetic tree inferred clearly showed the formation of four large clusters (A, B, C and D) (Fig. 8).

The first cluster (Fig 8 – A) included 11 species that corresponded to most pathogens. Our samples groups with sequences close to *Phaeosphaeria elongata* (KM491548) [40]. This clade was heavily supported (PP = 0.99) (Fig. 8). These data corroborate the analysis

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carried out for the ITS region. The cluster B comprises 11 species that corresponded to pathogens and endophytes of Poaceae. The isolated UNIPAMPA 006 was grouped with sequences of the *Neoascochyta paspali* (GU238124), which are relevant phytopathogenic fungi, including a series of pathogens with quarantine status [41] (Fig. 8). Although most taxa are continuously present in the environment in saprobic soil organisms, many species switch to a pathogenic lifestyle when a suitable host is encountered [38]. This clade was heavily supported (PP = 0.99). These data corroborate the analysis carried out for the ITS region. Other 13 species were grouped at cluster C. These species were close to the UNIPAMPA 007 isolate. The sequence of the isolated fungi related to *Alternaria chlamydospora* (KC584264) are known as severe plant pathogens that cause significant losses on a wide range of crops [43] and *Alternaria oregonensis* (KC584292). This clade was hardly supported (PP = 0.48). The genetic distances obtained not allow us to determine that our species has sufficient similarity with the other species of *Alternari*a mentioned above, suggesting being a new species, further analysis are necessary to the correct diagnosis for this isolate. The last cluster (Fig 8 -D) included 12 species of fungi classified as endophytes and pathogens (Fig. 8). Our isolate grouped closer to *Pyrenophora chaetomioides* (JN940075) which was isolated mainly from the Poaceae species. This finding has posterior probability support (0.63). It is insufficient to consider our sample as belonging to this species, so we treated it as *Pyrenophora* cf. *chaetomioides* because we do not have sufficient morphological and molecular features data to determine this hypothesis of identification definitively. For this reason of the indefinite taxa, no result or discussion of this isolate is presented here. Complementary molecular analyses are being performed, such as hole genomic sequencing, and marks on the effect of temperature on this isolate will be presented in due course when the authors are confident of the taxonomic determination of this isolate.

3.2 Effect of temperature on growth

3.2.1 Pyrenophora cf. *chaetomioides*

The mean radial growth of *Pyrenophora* cf. *chaetomioides* in SAB medium at 5 °C was the lowest among the three media tested (mean = 0.165 mm). The other two media showed a higher growth in this same temperature, with averages of 0.905 mm and 0.537 mm for PDA and GE, respectively. The statistical test indicated a significant difference between these averages at this temperature. Furthermore, GE was identified as the best culture medium for the in vitro growth of this isolate at 20 °C, since it showed an average growth of 2.361 mm. At 23.5 °C, the fungus presented a lower growth in the three media (mean PDA = 0.419 mm, mean SAB = 0.281 mm, and mean GE = 0.703 mm), indicating that this species is more sensitive at this temperature (Fig. 9). The isolate with the selected culture medium (GE), at all temperatures tested, demonstrated significant differences between temperatures (Table 1). The temperature with the highest mycelial growth was 20 °C, considering this criterion, its mean differed statistically from other temperatures. However, the other three temperatures showed no significant differences (Table 1) in their average growth, for the GE medium.

3.2.2 Phaeosphaeria elongata

In the in vitro experiment, at a temperature of 23.5 °C, no growth was identified for the *Phaeosphaeria elongata* isolate. No significant differences were detected in the average growth of the colonies of the isolate (Fig. 10) between the different culture media at temperatures 5 and 10 °C. The isolate at 20 °C showed significant differences between the media used, and the PDA medium demonstrated the highest growth. Samples of the isolate in PDA medium showed the highest mycelial growth at 10 °C, but statistically there were no significant differences between the lowest temperatures tested in the present study. The analysis of the ideal culture medium (PDA) for this species between different temperatures resulted in a significant difference (Table 1). The temperature for the selected medium with the highest growth was 10 °C (mean = 2.295 mm), followed by 20 °C (mean = 1.642 mm), and the lowest growth was observed at 5 °C (mean = 1.111 mm).

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(**a**) (**b**)

Figure 2. This is a figure. Schemes follow another format. If there are multiple panels, they should be listed as: (**a**) Description of what is contained in the first panel; (**b**) Description of what is contained in the second panel. Figures should be placed in the main text near to the first time they are cited. A caption on a single line should be centered.

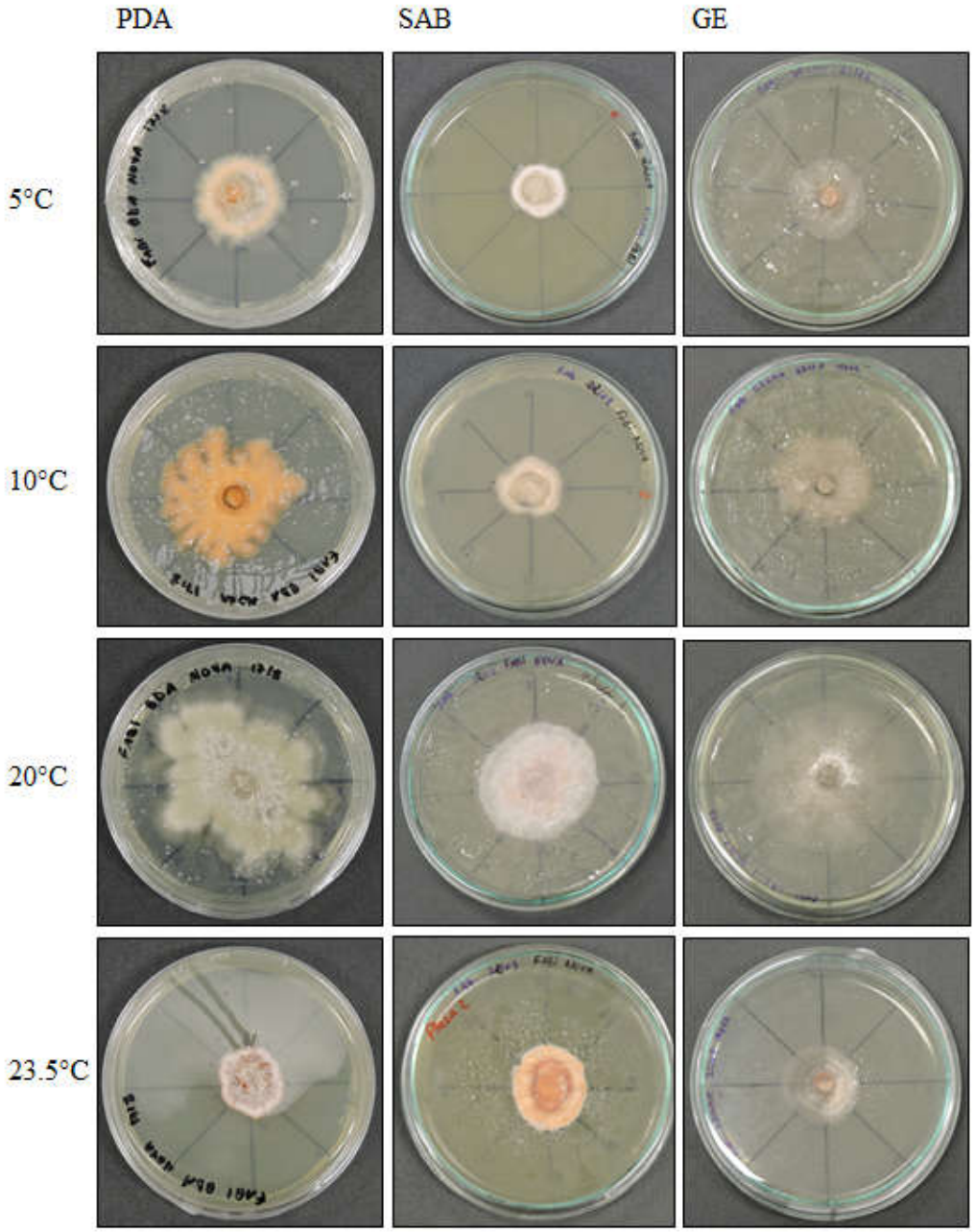

Fig. 1 *Pyrenophora* cf. *chaetomioides* colony morphology on three media (Potato Dextrose Agar - PDA, Sabouraud Agar - SAB, Grass Extract Dextrose Agar - GE) and growth at diferente temperatures (5º, 10º, 20º and 23.5º)

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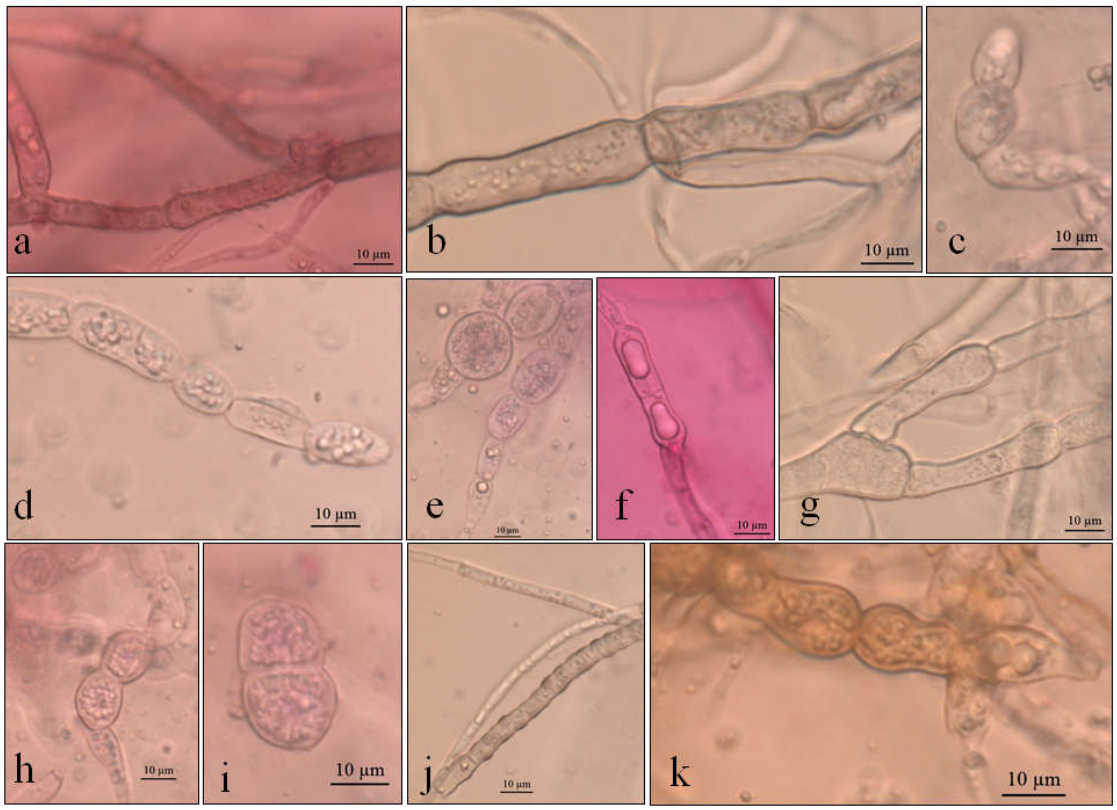

Fig. 2 Optical microscopy images of *Pyrenophora* cf. *chaetomioides* (a). Hyphae pigmented (lost in KOH); (b). Chlamydospores rectangular to globose at 5 °C in PDA; (c). Chlamydospores terminal at 5 °C in SAB; (d). Chlamydospores catenulate and larger at 10 °C in SAB; (e). Chlamydospores at 10 °C in GE; (f-g). Chlamydospores catenulade at the terminal hyphae at 20 °C in GE; (h-i). Globose chlamydospores at 23.5 °C in SAB; (j). Pigment in hyphae in PDA; (k). Hyphae turnin into chlamydospores at 23.5 °C in SAB.

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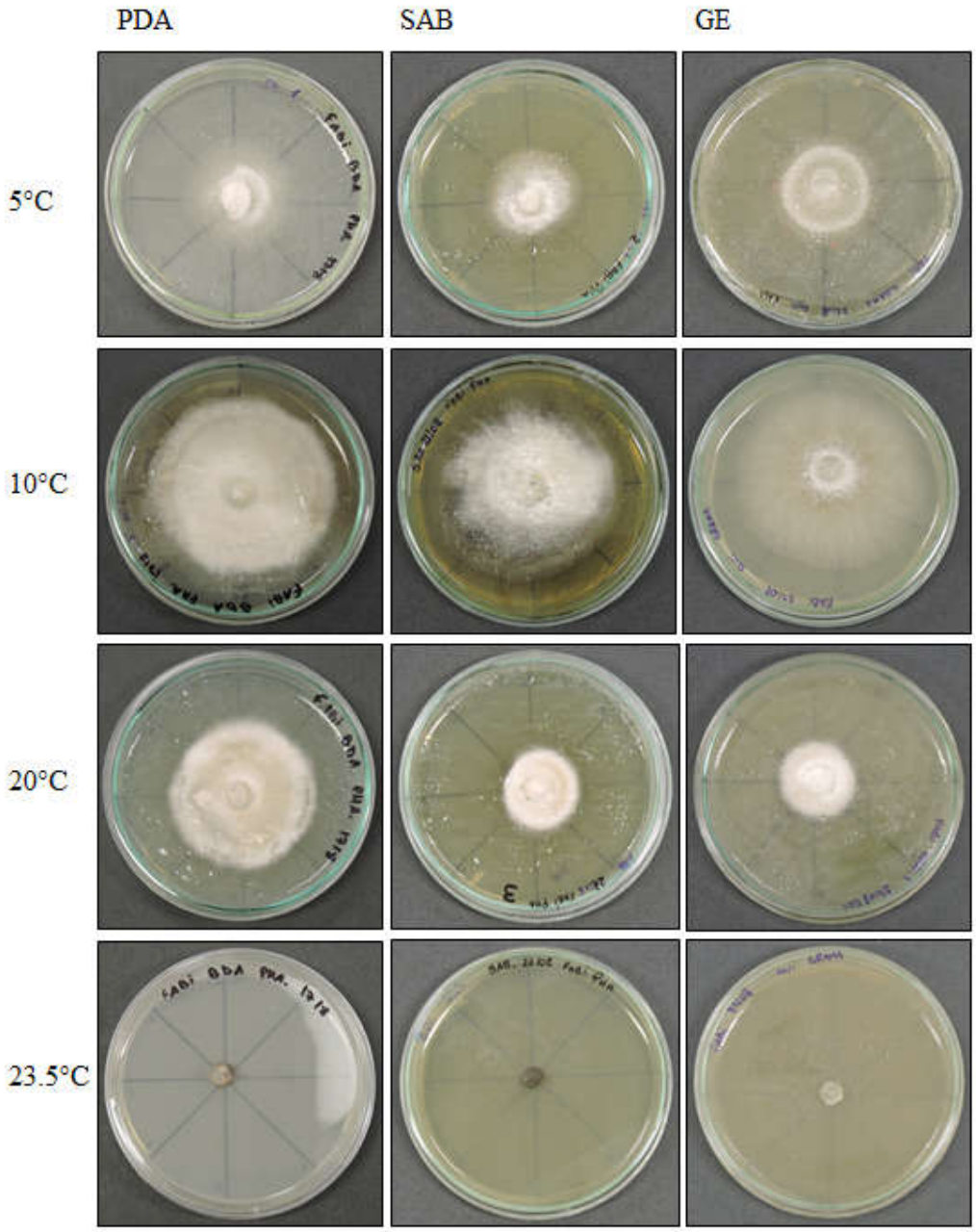

Fig. 3 *Phaeosphaeria elongate* colony morphology on three media (Potato Dextrose Agar - PDA, Sabouraud Agar - SAB, Grass Extract Dextrose Agar - GE) and growth at diferente temperatures (5º, 10º, 20º and 23.5º).

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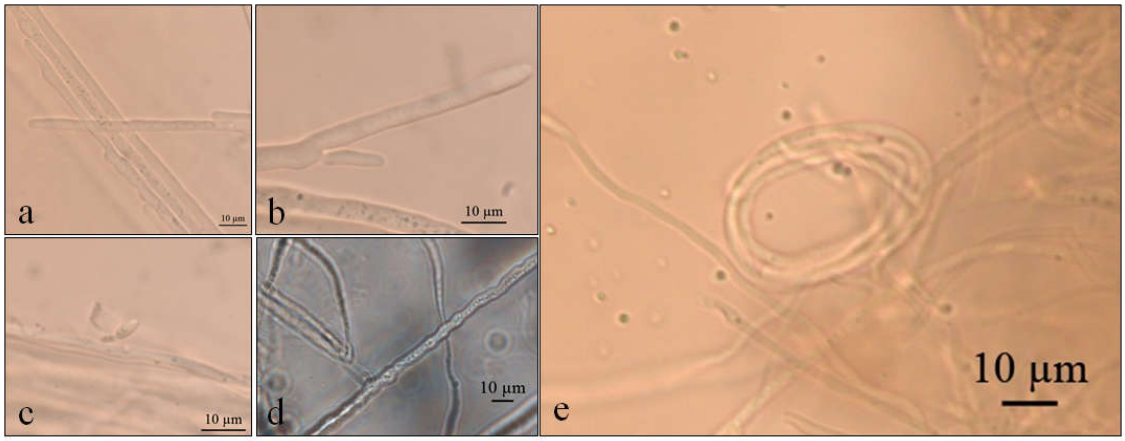

Fig. 4 Optical micrographs of *Phaeosphaeria elongata* (a-c). Conidial development in culture medium GE; (d). Sinuose hyphae at 5 °C in PDA; (e). Nematode capture hook.

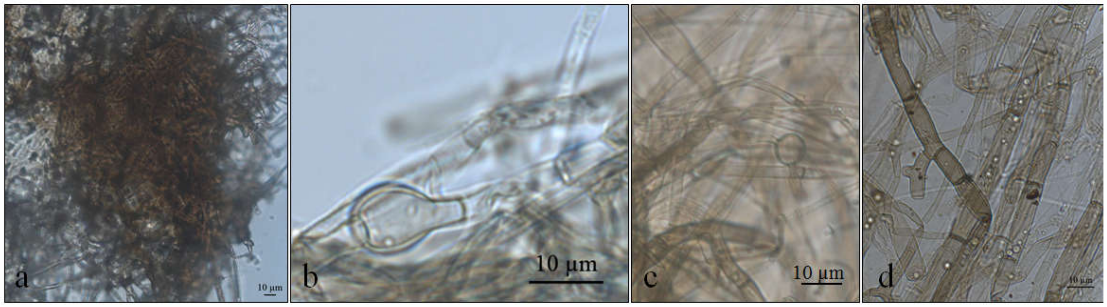

Fig. 5 Optical micrographs of *Neoascochyta paspali* (a). tufts in the oldest mycelium in PDA; (b-c). Chlamydospores; (d). Pigmented hyphae.

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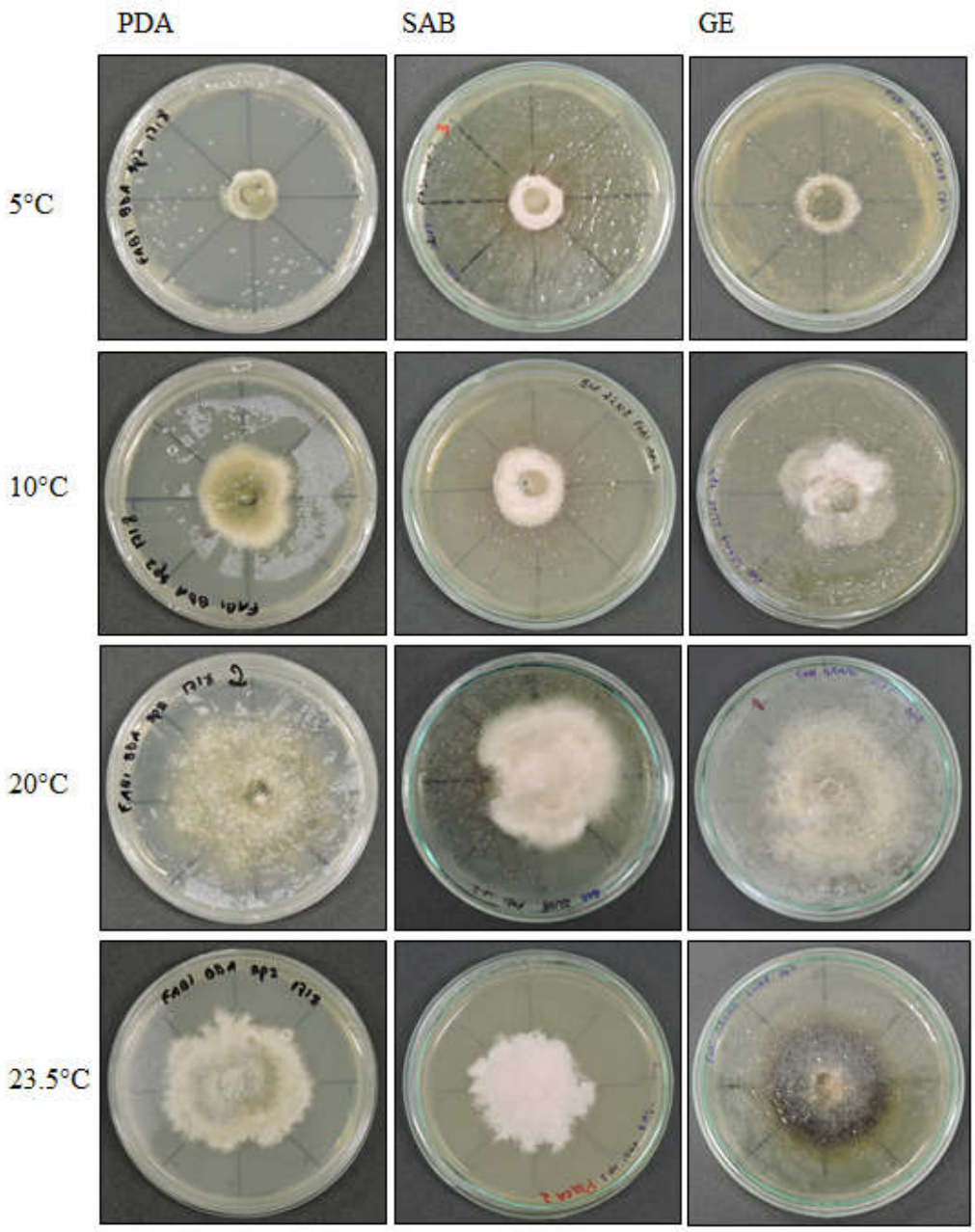

Fig. 6 *Alternaria* sp colony morphology on three media (Potato Dextrose Agar - PDA, Sabouraud Agar - SAB, Grass Extract Dextrose Agar - GE) and growth at diferente temperatures (5º, 10º, 20º and 23.5º).

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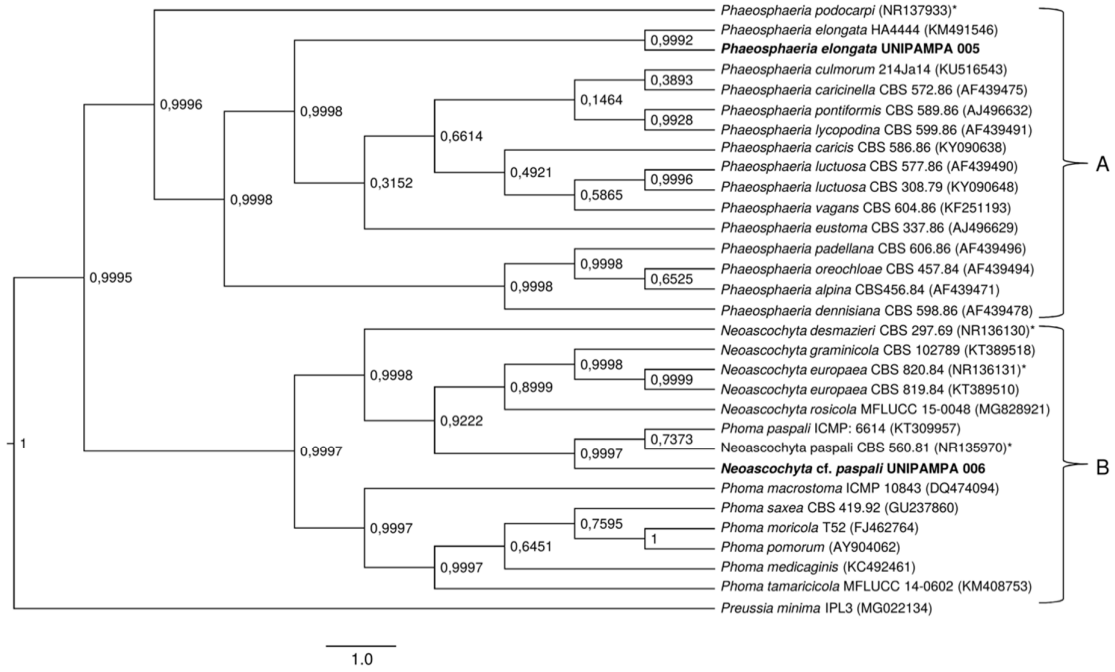

Fig. 7 Phylogenetic tree showing the relationship among *Deschampsia antarctica* associated fungi and other fungal species. The tree was constructed based on the rDNA sequence (ITS1-5.8S-ITS2) fragment by using the Bayesian Evolutionary Analysis Sampling Trees. The robustness of each node is represented by the posterior probability value obtained after 10.000.000 Monte Carlo Markov chains (MCMC). Sequences of type species (*). The tree was root using *Preussia minima* as outgroup due to be outside the clade of interest.

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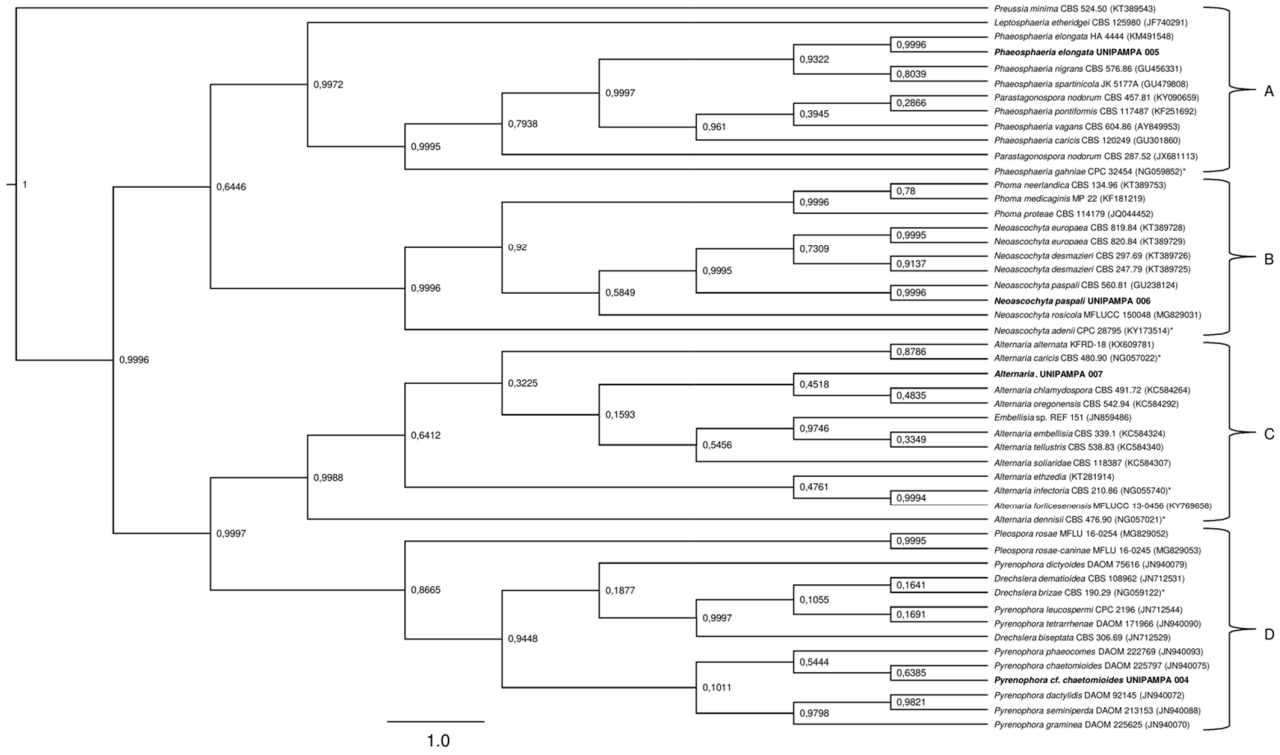

Fig. 8 Phylogenetic tree showing the relationship among *Deschampsia antarctica* associated fungi and other fungal species. The tree was constructed based on the nLSU region fragment by using the Bayesian Evolutionary Analysis Sampling Trees. The robustness of each node is represented by the posterior probability value obtained after 10.000.000 Monte Carlo Markov chains (MCMC). Sequences of type species (*). The tree was root using *Preussia minima* as outgroup due to be outside the clade of interest.

Table 1 Radial mycelial growth for fungi isolated from *Deschampsia antarctica* leaves in PDA medium to *Alternaria* sp. and *Phaeosphaeria* sp, and in GE medium for *Pyrenophora* sp.

Treatments	*Phaeosphaeria elongata*	*Pyrenophora* cf. *chaetomioides*
5°C	1.111 c	0.537 c
10°C	2.295 a	0.989 b
20°C	1.642 b	2.361 a
23.5°C	-	0.704 bc

*Different letters in the columns differ from each other values of significance levels in the Tukey test (α = 0,05).

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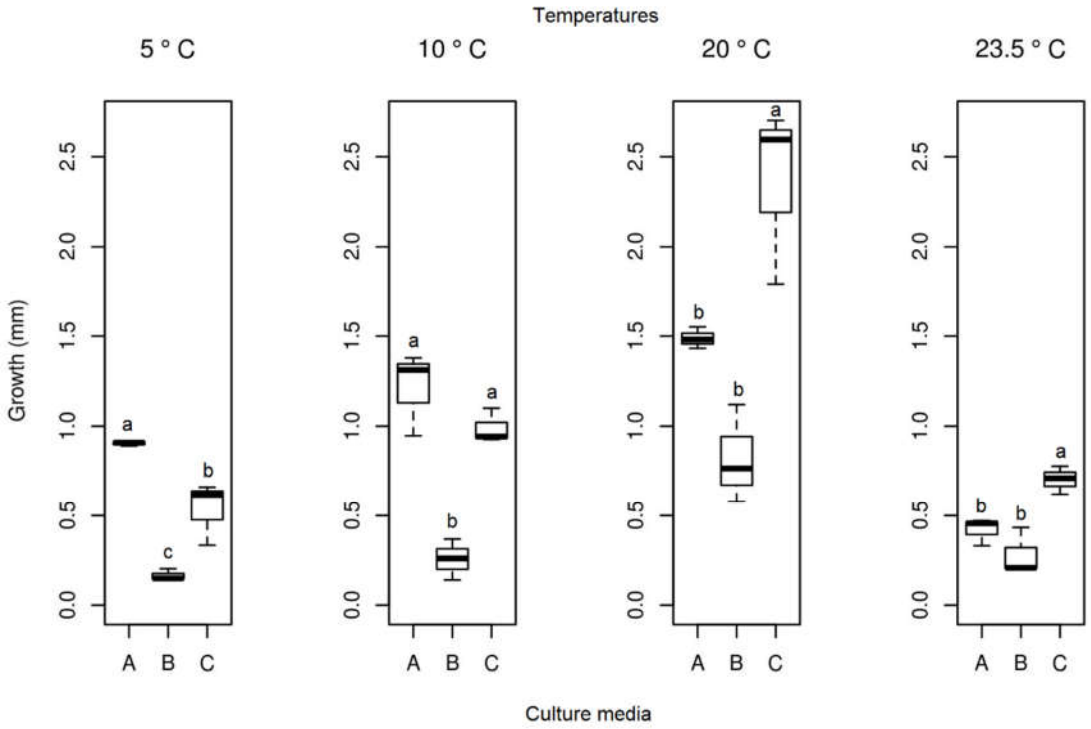

Fig. 9 Boxplot of mycelial growth of the isolate *Pyrenophora* cf. *chaetomioides* for the four temperatures studied. Boxplots with the same letter (lowercase), at the same temperature, do not differ statistically from each other by the Tukey test at 5% confidence. A = PDA, B = SAB and C = GE

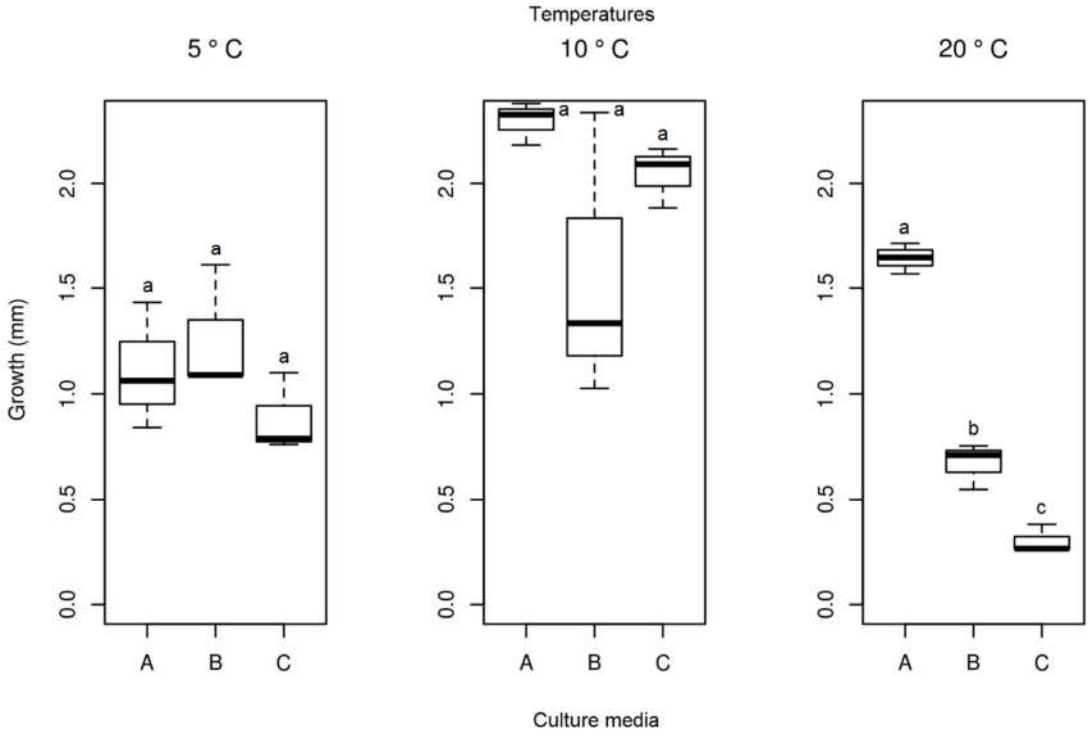

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Fig. 10 Boxplot of mycelial growth of the isolate *Phaeosphaeria elongata* for the four temperatures studied. Boxplots with the same letter (lowercase), at the same temperature, do not differ statistically from each other by the Tukey test at 5% confidence. A = PDA, B = SAB and C = GE.

4. Discussion

The major group of fungal endophytes in plants is represented by species of Ascomycota, which was confirmed in this study. The genera found are also widely distributed [44].

Previous studies have revealed a diversity of endophytic fungal communities associated with plants living in tropical, temperate, and boreal ecosystems, and their frequency seems to decrease in cold regions [45]. Saikkonenet al. [46] demonstrated a low incidence of endophytes from *Deschampsia flexuosa* (L.) Trin. and *Deschampsia cespitosa* (L.) P. Beauv. in the cold regions of Finland. Rosa et al. [14] isolated 18 fungi as endophytes from 273 leaf fragments of the Antarctic hairgrass resulting in 18 species. Our results also point to a small diversity of fungi associated with the *D. antarctica* leaf (four). However, our sampling effort was limited to two single islands in the Maritime Antarctic. This may reflect low isolated diversity since endophytic fungi can be restricted by geography but not by host [47]. Another possibility is that species diversity may vary with environmental factors at sample sites, but further investigation is required to confirm this.

One of the fungal genera reported in *D. antarctica* leaves is *Phaeosphaeria*, which is known as a pathogen that causes leaf spots on grasses and some other monocots. Dennis [48] was the first to report species of this genus from areas near Antarctica (South Georgia – sub-Antarctic Island). Some species are very specialized while others have a large host spectrum [49]. *Phaeosphaeria* is distributed over all South, Central, and North America as well as Africa and Asia [50,51]. The most related species to the *Phaeosphaeria* isolate identified in this study are *Phaeosphaeria elongata*, associated with terrestrial or near freshwater grasses [40]. Putzke and Pereira [4] described *Phaeosphaeria deschampsii* to Antarctica as a new species, showing that this genus is also formed by several unknown species in the area and associated with Antarctic hair-grass.

The genus *Neoascochyta* is one of the largest fungal genera, with more than 3,000 infrageneric taxa described. Species belonging to the genus *Neoascochyta* are often encountered as plant pathogens (mostly causing leaf stem spots) and as endophytes that use different hosts (including corn, citrus, and sorghum) [52]. The species most related to the UNIPAMPA 006 isolate is *Neoascochyta paspali* (=Phoma paspali). This species has not previously been reported in Antarctica and is considered an indigenous pathogen of grasses in Australia, New Zealand, and Europe [38]. Zhang and Yao [53} detected 31 known fungal species, most of which were originally reported in other habitats as endophytes in the leaves and stems of Arctic plants. *Phoma herbarum*, for example, was a widespread saprophyte and pathogen of plants, and has been found in diverse environments including Antarctica [54]. These results indicate the presence of specific psychrophilic and psychrotrophic fungi in various habitats in cold ecosystems. Furthermore, the wide distribution of these fungi suggests that they may be capable of long-distance dispersal.

Pyrenophora ssp. are another plant pathogen described as graminicolous, causing leaf spots in agronomically-important plants [55]. *Pyrenophora antarctica* was detected on Kerguelen Island (sub-Antarctic) on *Festuca antarctica* grass [56]. The UNIPAMPA 004 isolate

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is related to *Pyrenophora chaetomioides*, a specialized pathogen infecting various species of oats (*Avena* spp.) and some grasses [57]. Onofri et al. [2] reported no species of this genus to Antarctica as this was the first reference to the area.

The genus *Pyrenophora* is responsible for helminthosporiose leaf blight in wheat and barley, which causes a disease with great economic importance. These fungi can survive as mycelium in seed endosperms including during water stress, thus colonizing the radicular system since it is activated during germination [58]. As described by Farias et al. [54] and Benslimane et al. [60], this genus presents a significant range in conidia dimensions, including being formed directly from chlamydospores. The UNIPAMPA 004 isolated presented only rectangular to globose chlamydospores, terminal or intercalary, which makes identification impossible since no conidia was observed.

According to Ruisi et al. [17], geographic isolation, combined with environmental stress, make Antarctica an ideal location to research new species of endemic fungi. Endophytic fungi in relatively extreme environments as well as phylogenetically distinct plant strains are promising sources for recovering new species, which will be important in understanding fungal diversity [45].

The use of macro- and microscopic characters of anamorphic cultures does not offer enough information for taxonomic identification [61,62]. The UNIPAMPA 007 isolate exhibits morphological characteristics very close to those in anamorphic stage. The morphology that agrees with the teleomorph are the hyaline spores of the genus *Pleospora* [63,64]. This isolate presented characteristics close to those described by Grum-Grzhimaylo et al. [65], such as the development of thin conidia and terminal or intercalary chlamydospores.

Phaeosphaeria are parasites found in many grass cultures. The species are usually very specialized and can cause deadly diseases. Some species have a wide range of hosts, mostly among Poaceae and other monocots (Cyperaceae, Juncaceae, etc.), as well as *Lycopodium* and *Equisetum* [66]. The anamorph often belongs to species of the genus *Stagonospora*. These fungi normally grow in leaves or floral parts of Poaceae [67]. Our isolate *Phaeosphaeria elongata* is an anamorph of *Stagonospora* characterized by its solitary and hyaline cylindric conidia, and plane margins in PDA. The aerial mycelia are scarce, with a cream color at the beginning that turns pallid to olivaceous grey and then whitish with a dark reverse [68,69], such as the UNIPAMPA 005 isolate.

The anamorphic genus *Phoma* includes many important pathogenic fungi [70]. Aveskamp et al. [41] isolated *Phoma paspali* (*Neoascochyta paspali*) from the *Paspalum notatum grass*. Approximately 50% of *Phoma* species, redescribed by Boerema [71], were recognized as relevant phytopathogens. The morphologic characteristics of this fungus in PDA include regular margins with hyaline and white mycelia and colonies presenting hyaline to white radial spherical tufts that were densely clustered at the top, and later changed color to grey [72]. These results agree with our study. Unicellular dark brown to olivaceous terminal chlamydospores in aerial erect hyphae were described in Boerema et al. [71], which also correlates with the isolated UNIPAMPA 006 identified in this study. Phylogenetic and morphological analyses demonstrated that our isolate was *Neoascochyta paspali*, with a posterior probability of 0.99.

The genus *Phaeosphaeria* is known to present pathogenic and endophytic plant species. In addition, this genus presents a generalized distribution in grain crop areas [61, 68]. According to Jankowiaket al. [74], species belonging to the genus *Phaeosphaeria* were isolated from root fragments and cotyledons of *Abies alba* and incubated at temperatures of 22-25 °C. Cervelattiet al. [75] reported that the optimum temperature for *Phaeosphaeria* maydis ranges from 12 to 22 °C. The UNIPAMPA 005 isolate presented characteristics

like those observed in the previous study, growing at temperatures of 5, 10, and 20 °C, with the highest growth in 10°C of all the media used (BDA, SAB and GE).

Studies using plant extracts as culture medium have been carried out with the objective of verifying the development of morphological structures. The genus *Pyrenophora* is mainly characterized as plant pathogens, particularly of Poaceae. Borba et al. [76] demonstrated that the temperature for a better mycelial development for this genus in culture medium supplemented with grass extract is around 25 ± 1 °C. Our study showed that the best culture medium for the UNIPAMPA 004 isolate was GE, and the highest mycelial growth was in temperatures of 20 °C. Linhares et al. [77] demonstrated that 22 °C was the best incubation temperature for pathogens of the genus *Pyrenophora*. Khouri et al. [78] evaluated the effect of grass extracts of Ascomycota on fungi growth and concluded that *Cynodon dactylon* (Poaceae) and *Digitaria decumbens* (Poaceae) grass promoted better fungal growth. GE was supplemented with D. antarctica leaves, demonstrating greater mycelial growth at temperatures of 20 and 23.5 °C compared to other media. In addition, preference for plant species may be related to the nutritional requirement of the fungus [76]. According to Reis [79] species of this genus can be inoculated in PDA culture medium or supplemented with plant extracts, as these can provide carbon and sugar for their development [80].

The results of growth tests at different temperatures suggest that the fungi associated with *Deschampsia antarctica* in the Half Moon and King George Islands can grow at temperatures of 10 and 20 °C. Tosi et al. [81] demonstrated that most of the fungi isolated from mosses in Victoria Land could grow at temperatures ≤ 5 °C but exhibited optimum growth between 10-24 °C. Most endophytic fungi isolated from Antarctic mosses are also psychrotrophic and psychrophilic [29]. In addition, fungi may exhibit morphological adaptations, an example being the predominance of sterile fungi to grow at low temperature. These physiological and morphological mechanisms were cited for fungi present in Antarctica and other environments [17].

Based on the observations of *Newsham* et al. [82], future warming in Antarctica will lead to increases in fungal populations, and this will have negative consequences on biological productivity. These data agree with our study, considering that the *Pyrenophora* cf. *chaetomioides* isolate showed higher mycelial growth at 20 °C, and *Phaeosphaeria elongata* at 10 °C.

5. Conclusions

The Antarctic continent has unique environmental conditions that allows the isolation and identification of endemic and new species of fungi. Applying molecular and morphological approaches to the fungi isolated from *Deschampsia antarctica* we identified endophyte/pathogens fungi *Phaeosphaeria elongata*, *Pyrenophora* cf. *chaetomioides* and *Neoascochyta paspali* relating those species to cold environment and classifying them as psychrophilic organisms. The study of such group of species is very interesting since they could elucidate issues related to environmental changes and those associated with communities of antarctic plants.

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Author contribution statement

F.L. carried out the fungi isolation; growth experiments; DNA extraction; bioinformatical and statistical analysis, she wrote the manuscript with help from other authors. G.A.K.A contributes to the fungi isolation and growth experiment design. F.C.V conceived and co-directed the Project; contributes to the phylogenetic and molecular identification analysis. M.P.A. conceived the project; carried out the design of the experiments and contributes to the growth experiment, wrote, and revise the manuscript.

Conflict of Interest

The authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced it proposes.

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