



Study of physiological and enzymatic properties and characterization of pathogenic activity of a fungus isolated from moss *Sanionia uncinata* (Hedw.) Loeske in Antarctica

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Abstract

In Antarctica, fungi occupy different niches and interact with different living things; but its importance in these niches and interactions is still poorly understood. An example of an interaction reported from Antarctica involves fungi and the Antarctic mosses, in which the fungi formed rings on the carpets of mosses. However, due to the complexity of these fungi, information about these is limited, and they have not been completely characterized yet. The Antarctic region is vulnerable to climatic change, and abiotic factors can influence the growth of fungi. This may impact the pathogenic interactions between the mosses and the fungi. The aim of this study was to identify, characterize, and evaluate the pathogenic potential of a fungus isolated from moss samples *Sanionia uncinata* (Hedw.) Loeske. The material for this study was collected from King George Island during the Brazilian Antarctic Expedition XXXI. Through taxonomic, molecular, and phylogenetic methods, the isolate was identified as belonging to the genus *Trichoderma*. The isolate inhibited the growth of the moss *Physcomitrium acutifolium* Broth. in vitro and caused complete discolouration of its gametophytes. The physiological characterization of the isolate revealed that it was psychrotolerant with optimal growth at 20 °C, producing amylase and protease at temperatures of both 10 and 30 °C and cellulase at 10 °C only. These results suggest that an increase in temperature may enhance the occurrence of ring-forming fungi in mosses in Antarctica.

Keywords Pathogenicity · *Sordariomycetes* Link · Fungi · Ring-forming · Antarctica

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Introduction

The distribution of fungi in Antarctica is related to the distribution of “hosts” such as birds, invertebrates and vegetation consisting mainly of lichens and bryophytes (Tosi et al. 2002). The presence of fungal pathogens of mosses has been

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reported since the mid-nineteenth century (Racovitza 1959), and it is posited to play important roles in nutrition cycle, population dynamics by causing small-scale disturbances that change community composition in bryophyte-dominated ecosystems, and explore different nutritional micro-niches within the gametophyte. There are reports of fungi forming concentric rings on carpets of mosses in regions of Antarctica (Wilson 1951; Hawksworth 1973; Longton 1973; Fenton 1983; Tojo et al. 2012). Studies show that bryophytes are a rich source of fungal material (Azmi and Seppelt 1998; Tosi et al. 2002). However, mosses do not produce “pathogen targets” such as storage structures rich in nutrients, or specialized transport tissues rich in photosynthetic products such as those found in vascular plants. In spite of this, fungal pathogenesis in mosses have been reported more frequently (Davey and Currah 2006).

Abiotic factors such as temperature and availability of water and nutrients influence fungal growth on natural environment. Nutrition can have a big impact on the pathogenic interaction between mosses and fungi (Dix and Webster 1995; Davey and Currah 2006). The vast majority of nonlichenized fungi described in Antarctica until now are cosmopolitan. Many of these fungi may be transient propagules introduced by air currents or by human and animal activities. However, some species seem to be well established in individual ecological niches (Bridge and Spooner 2012). One of the characteristics that may play a role in the establishment of a species in a particular niche is its ability to produce secondary metabolites. The genus *Trichoderma* Pers. is important in food, pharmaceutical, and biotechnology industry, due to its properties like pathogenicity and its application as a biocontrol agent (Benítez et al. 2004). They play an important role in ecology by helping in recycling organic matter of all types. They could be used as biological indicators because they are the common contaminants of humans, food, and materials (Duong 1996; McRae and Seppelt 1999; Prusky et al. 2004).

Species belonging to *Trichoderma* are cosmopolitan and have been frequently isolated from samples of Antarctic mosses (Azmi and Seppelt 1998; McRae and Seppelt 1999; Tosi et al. 2002; Zhang et al. 2013; Fenice 2016). They can produce a large variety of chitinolytic enzymes for the repression of catabolism. However, these enzymes are very specific with respect to their function. Some of these enzymes can be detected in all species, while others are produced by certain species in response to specific environmental conditions (Esposito and Azevedo 2004). Specific fungal enzymes may contribute to the ability to grow and survive in a hostile environment characterized by low temperatures and limited availability of organic matter (Bradner et al. 1999).

The aim of this study was to characterize the fungi isolated from moss *Sanionia uncinata* (Hedw.) Loeske collected from King George Island. The study identified the

pathogenic potential of fungi and their ability to produce secondary metabolites.

Material and methods

The samples were collected from King George Island (62°09'S 58°28'W), in the South Shetlands Islands, Antarctica, during the austral summer of 2012–2013. Moss samples were stored in sterile plastic bags and transported to Brazil by members of the Antarctic Expedition of the Brazilian Antarctic Program. The ring-forming fungi were isolated with the aid of tweezers, pulling small fragments from the rings that were present on the moss *S. uncinata*. The fungus has been deposited at the Bruno Edgar Irgang Herbarium of the Universidade Federal do Pampa—Campus São Gabriel/RS, Brazil (strain number FIMA 665-5). The strain was inoculated in Petri plates containing Sabouraud agar medium culture (dextrose agar 4%—Acumedia) with the addition of 1% Tartaric Acid (Labsynth), adjusted to pH 4.0 and maintained in greenhouse photoperiod (model Q315F25—Quimis) at 15 °C, in the absence of light. The mycelial growth was monitored every 24 h after isolation.

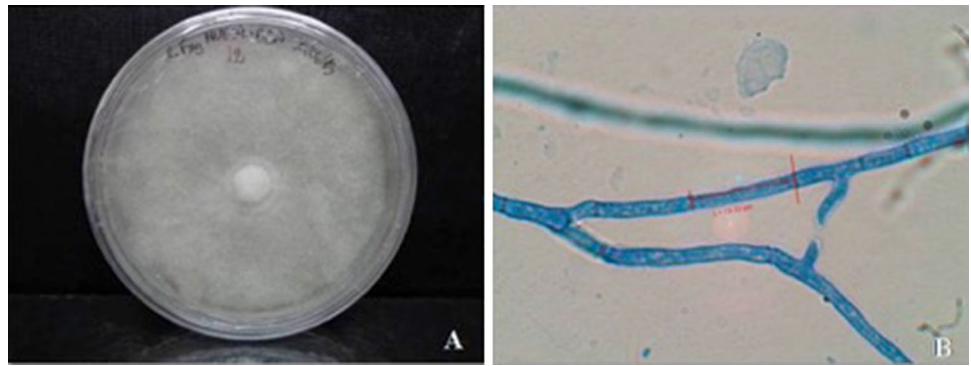
Optimization of culture media required for radial mycelial growth

Different culture media like PDA (potato dextrose—Labsynth, pure bacteriological agar - Vetec), MEA (malt extract power—Himedia, agar) and Sabouraud agar (4% dextrose agar—Acumedia) were tested. Active mycelium (9 mm plugs) was inoculated at the center of Petri plates and was kept in greenhouse photoperiod at 2 °C in the absence of light for 24 h. Twenty replicates were used for each treatment. The diameter of the colony was measured with a digital caliper (Mitutoyo) on the back of each plate every 24 h in 4 directions, until the mycelial growth of a colony reached the edge of the board (Fig. 1). The mean diameter of the replicates was calculated for each of the treatments. The results were subjected to analysis of variance to compare the means and to Tukey test ($p < 0.05$) using the Statistix 8.0 software (de Albuquerque et al. 2011).

Optimization of temperature required for radial mycelial growth

Active mycelium (9-mm plugs) was inoculated at the center of Petri plates containing Sabouraud agar and was kept in greenhouse photoperiod at temperatures of – 6 °C, 1 °C, 5 °C, 10 °C, 20 °C, 26 °C, and 30 °C in the absence of light for 24 h. Twenty replicates were used for each treatment. The diameter of the colony was measured with a digital caliper (Mitutoyo) on the back of each plate every 24 h in four

Fig. 1 Morphological representation (macroscopical and microscopical) of the isolated fungus from *Sanionia uncinata* (Hedw.) Loeske sampled in Antarctica. Culture of the isolate FIMA 665-5 (a). Septate hyphae—19.3 μm (b)



directions, until the mycelial growth of a colony reached the edge of the board. The mean diameter of the replicates was calculated for each of the treatments. The results were subjected to analysis of variance to compare the means and to Tukey test ($p < 0.05$) using the Statistix 8.0 software (de Albuquerque et al. 2011).

DNA extraction, PCR amplification and purification

DNA extraction from the sample was performed with Norgen (Biotec Corp.) plant/fungi DNA isolation kit according to the manufacturer's instructions. Amplification of the internal transcribed spacers (ITS) region was performed using the primers S3126T (5'-ATA TGC TTA AGT TCA GCG GGT-3'), S2234C (5'-GTT TCC GTA GGT GAA CCT GC-3'), FFITS4R (5'-TCC TCC GCT TAT TGA TAT GC-3'), FFITS5F (5'-GGA AGT AAA AGT CGT AAC AAG G-3'). The amplification was performed using the following procedure: 95 °C for 2 min followed by 30 cycles at 95 °C for 1 min, 50 °C for 30 s and 75 °C for 2 min, and a final extension at 72 °C for 5 min. The amplicon was purified using PROMEGA Wizard® purification kit (SV Gel and PCR Clean-Up) according to the manufacturer's instructions. Purified PCR products were sequenced using ABI—PRISM 3500 Genetic Analyzer (Applied Biosystems). The ITS markers were chosen since those are the official DNA bar-coding markers for species-level identification of fungi (Raja et al. 2017).

Phylogenetic analysis and construction of Phylogenetic tree

The sequences were aligned and edited using the BioEdit Sequence Alignment Editor program. The sequences were analyzed using MEGA 5.1 program and using the GenBank database to in order to classify the organism. The closest matches followed a 70% query coverage and $1e^{-10}$ for e-value. The ingroups and the outgroups were selected before the first alignment and by the phylogenetic signal obtained from Maximum-Likelihood analysis. To determine

the similarities and differences, the sequence of the sample was used along with the closest sequence and for those fungi reported to Antarctica obtained from GenBank database, which acted as the reference sequence. An initial ML tree was constructed (data not shown), and the monophyletic groups were adjusted according to the best model of nucleotide substitution, through the jModelTest application (Posada 2008). An appropriate model (GTR + Γ) was selected to perform the Bayesian analysis in BEAST software (Drummond et al. 2012), using a strict molecular clock, constant size as tree prior, random starting tree, and a MCMC chain run of 100,000,000 states, sampling every 1000 and discarding the first 25% as burn-in. This analysis was chosen as this was a natural way to accommodate the uncertainty in phylogenies when performing comparative analyses (Losos and Miles 1994). The trees obtained were used to build the 50% majority rule consensus tree. The inference of ITS region related to our target will be evaluated according to the methodology used by Victoria et al. (2012) and the images of the phylogenetic trees were built using Figtree software (Rambaut 2014).

Enzyme assays

To verify the production of enzymes amylase, pectinase, cellulase and protease, the isolates (9 mm plugs) were inoculated on Petri plates containing suitable culture media and incubated at 10 °C and 30 °C until 12 days. Four replicates were used for each treatment. The amylase production was determined in culture medium containing agar (1%), starch (1%) and 1 M phosphate citrate buffer at pH 5.0. The identification of amylase activity was performed by adding 3 ml of iodine tincture. The formation of a yellowish halo around the colony indicates the degradation of starch. The yellow area is termed hydrolysis halo (Soares et al. 2010). To check the protease production culture medium containing agar (1.8%), colorless gelatine (1%), skimmed milk powder (1%) and 0.1 M citrate–phosphate buffer was used at pH 5.0. The formation of a translucent halo during mycelial growth on the dish indicates the production of protease. The

cellulase activity was detected in culture medium containing agar (1.8%), carboxymethylcellulose (1%) and 0.1 M sodium acetate buffer at pH 5.0. The identification of cellulase activity was performed by adding the dye Congo red (0.1 g/100 ml—Vetec). The pectinase production was determined in culture medium containing agar (1.8%), citrus pectin (1.0%—Delaware) and 0.1 M sodium acetate buffer at pH 5.0. The identification of pectinase production was verified with the formation of a halo, after addition of 5 N HCl solution (Teixeira 1994).

Preparation of fungal extract

The fungal isolate was cultured in Sabouraud Agar at a temperature of 10 °C, until sufficient mycelial mass was obtained. The aqueous extract was prepared according to Stein and Klingauf (1990), with adaptations. The material was macerated and immersed in 1000 ml of distilled water and kept at room temperature. After 24 h, it was stirred and filtered. For each 1000 ml of distilled water, 250 g of the isolate was used. The extraction was performed using sterilized filter paper, until a less viscous solution was obtained. Subsequently, the extract was subjected to filtration for decontamination using syringes containing supports (Millipore) with a diameter of 13 mm and membranes (Millipore) with a pore size of 0.22 µm. All procedures were performed in a hood with laminar flow.

In vitro test for chemical pathogenicity

The fungal aqueous extract was added in different concentrations (25 and 50%, equivalent to 25 and 50 ml) after diluting it with Murashigie and Skoog (MS) medium (Murashige and Skoog 1962). The other nutrients in the culture medium were not modified. For this experiment, 4 replicates with 4 gametophytes per Petri dish were used for each treatment and an untreated control was maintained for comparison. Four healthy gametophytes of the moss *Physcomitrium acutifolium* Broth. were inoculated in culture media, kept in greenhouse photoperiod at 25 °C with a photoperiod of 16 h light/8 h dark and monitored for 25 days.

Confrontation test in vitro

The tests were performed using disk diffusion method adapted from Bauer et al. (1966). In this method, disks (9 mm) with mycelium were inoculated in the center of Petri dishes containing MS medium along with 6 healthy gametophytes of the moss *P. acutifolium* (Online Resource 1). The plates were kept in greenhouse photoperiod with a photoperiod of 16 h light/8 h dark at 25 °C. Ten replicates were used for this experiment.

Table 1 Comparison of the radial mycelial growth of the isolated FIMA 665-5 in different culture media

Culture media	Mean
Sabouroud	23.055AB
PDA	27.188A
MEA	21.585B

The letters represent statistical averages ($p < 0.05$)

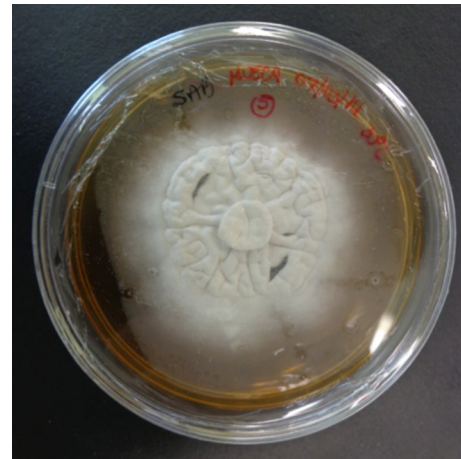


Fig. 2 Formation of cracks and deep grooves in the isolated colony FIMA 665-5 in sabouraud medium at 30 °C

Results

Optimization of culture media required for radial mycelial growth

Tests using different culture media demonstrated that the PDA culture medium had more favorable nutritional conditions for mycelial growth of the isolate as compared to other culture media (Table 1). The isolate required 4 days to achieve complete growth (reach the edge of the plate) in PDA and sabouraud culture media, whereas it took 6 days in MEA medium.

Optimization of temperature required for radial mycelial growth

Tests for the optimization of temperature demonstrated that there was no radial mycelial growth at -6 °C. At 5 °C, the isolate took 27 days to reach the edge of the plate but the mycelium was more vigorous. The rate of mycelial growth was highest at a temperature of 20 °C, reaching the edge of the plate in 5 days though the mycelium was found to be flimsier. (Online Resource 2) (Fig. 2).

Phylogenetic analysis and construction of Phylogenetic tree

The ITS DNA phylogeny demonstrated that the isolated fungus probably belonged to the genus *Trichoderma* Pers., clustering with other species from this genus (Fig. 3). Our sequence was re-aligned with 14 other sequences of *Trichoderma* selected through BLAST analysis. Phylogenetic analysis of the isolate FIMA_665_5 showed a close relationship with *Trichoderma viride*. This was supported by a higher posterior value (0.99) when compared with ITS homologues obtained from GenBank. Detailed Bayesian Inference consensus tree of ITS region of the sequences of FIMA 665_5 with the nearest taxa obtained from GenBank (Fig. 3) showed that our isolate formed a clade composed of three *Trichoderma viride* accesses, which included the type species *T. viride* (AJ133431), which had grouped together

with it. The other group of this clade was formed by a *T. viride* isolate from Antarctica (AF218788) and a strain of *Hypocrea rufa* (Pers.) Fr., the teleomorph of these species (Fig. 4). Most of the groups from the Genbank database used in the phylogenetic analysis were samples from Polar regions and were associated with bryophytes.

Enzyme assays

The production of amylase was observed at temperatures of 10 °C and 30 °C after 4 and 8 days of inoculation, respectively. The maximum amount of the enzyme was produced at a temperature of 30 °C (Fig. 5a, b). The production of protease was observed only at 30 °C after 3 days inoculation of the isolate (Fig. 5c). The isolate did not produce pectinase at 10 °C and did not present the growth of the colony at 30 °C.

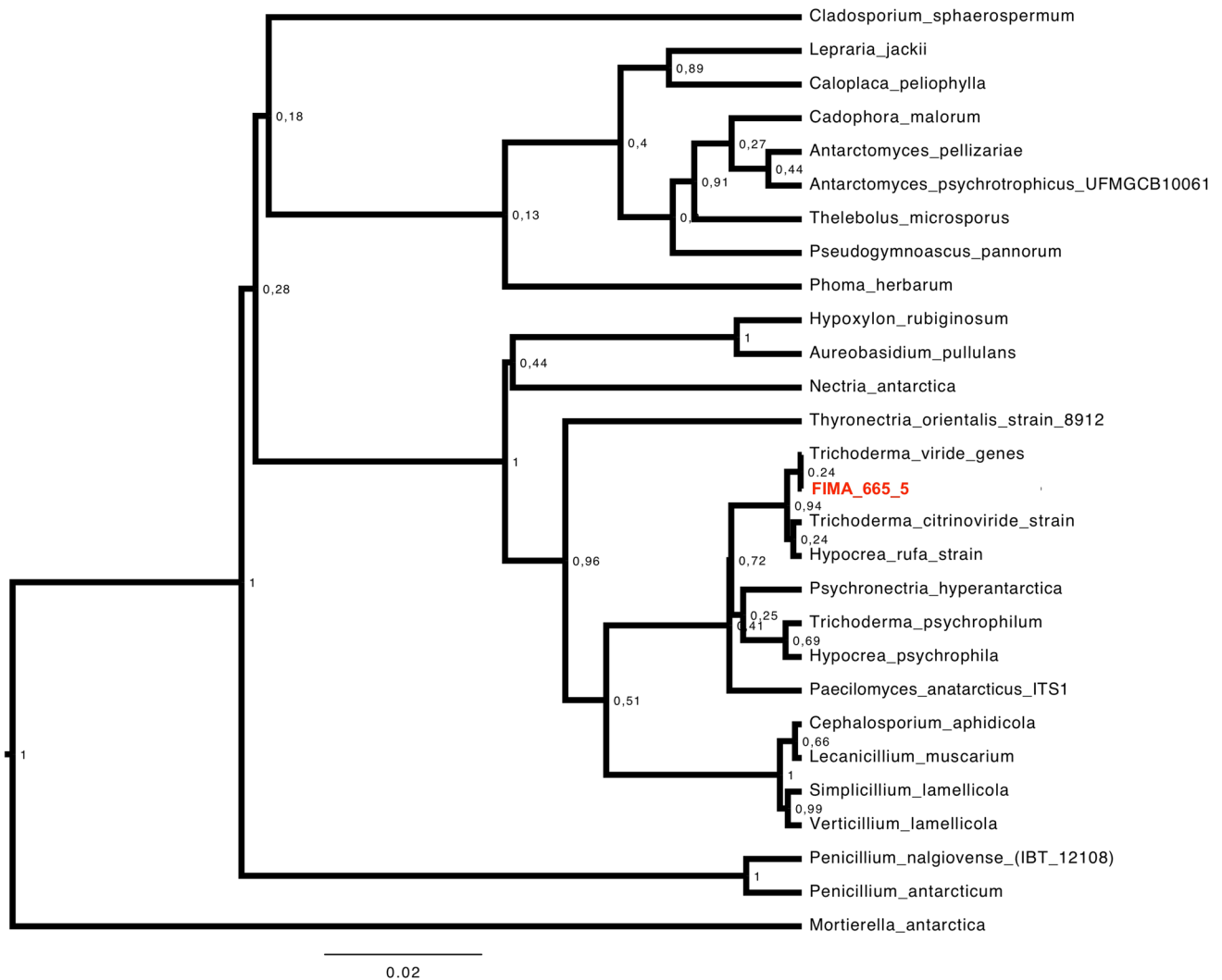


Fig. 3 Phylogenetic tree based on analyses showing the relationships between different species fungi with isolated FIMA 665-5. Being the external group formed by *Mortierella antarctica* Linnem

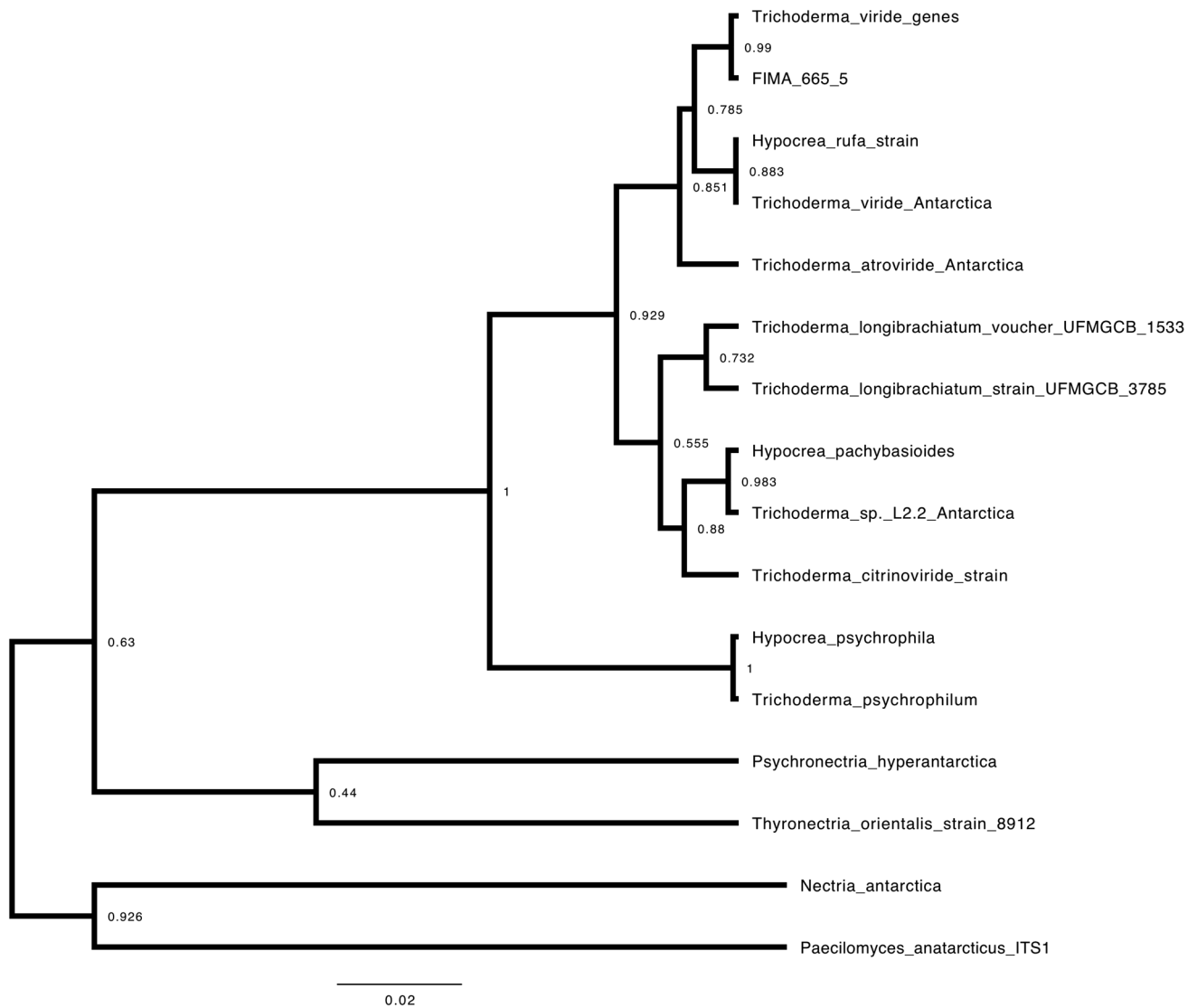


Fig. 4 Phylogenetic tree based on analyses showing the relationships between different species of *Trichoderma* with a fungus isolated in this study. Being the external group formed by *Paecilomyces antarcticus* Bridge, M.S. CLarck & D.A. Pearce

Production of cellulase was verified only at a temperature of 10 °C after 12 days of inoculation of the isolate (Fig. 5d).

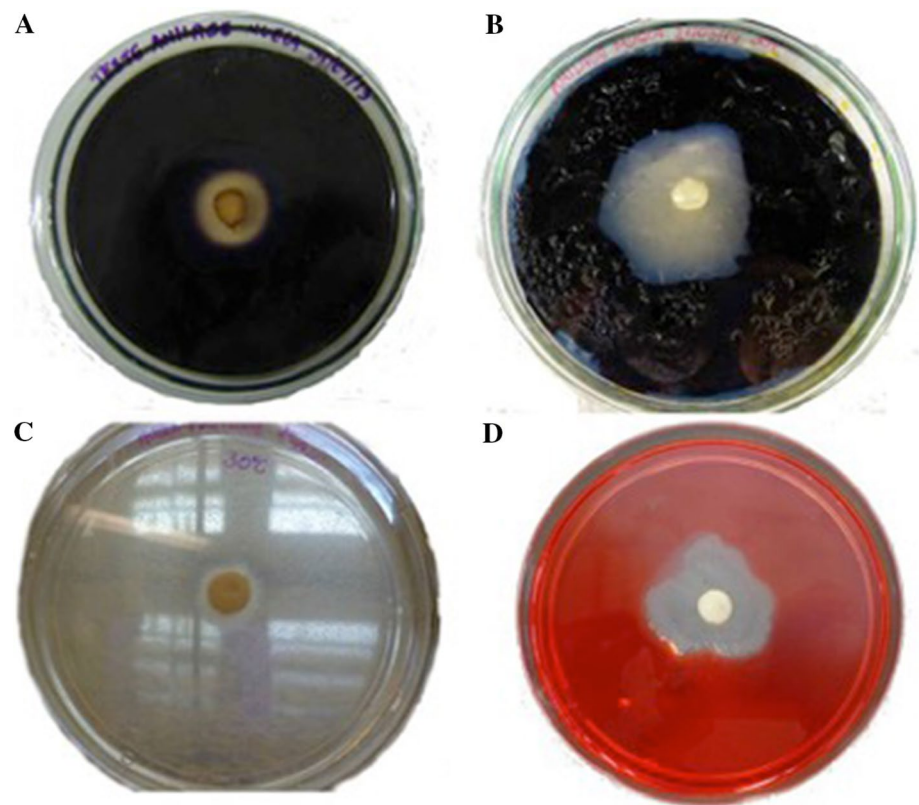
In vitro test for chemical pathogenicity

The pathogenicity test in vitro demonstrated that the aqueous extract of isolate had an inhibitory effect on the growth of moss *P. acutifolium* while the control showed the formation of rhizoids and new gametophytes in vitro. Culture media containing 25 and 50% of the fungal aqueous extract showed no development. Out of 16 gametophytes that were inoculated, 5 gametophytes exhibited complete yellowing after 7 days in culture medium in the presence of 50% aqueous extract (Online Resource 3).

Confrontation test in vitro

After 10 days of incubation, the gametophytes showed no development and some of them started to show yellowing of leaves at the base. On the 12th day of incubation, over half of the gametophytes showed complete discolouration, and healthy gametophytes showed no development (Online Resource 4). After 15 days of incubation, all gametophytes showed total discolouration (Online Resource S4c). These results showed that the fungal isolate had an inhibitory effect on the growth of mosses and was also its pathogen.

Fig. 5 Production of amylase at 10 °C and 30 °C (**a, b**); production of protease at 30 °C (**c**); production of cellulase at 10 °C (**d**)



Discussion

Phylogenetic analysis showed that isolate in this study belongs to the genus *Trichoderma*, one of the most common genera found in the regions of Antarctica (Fletcher et al. 1985; Broady et al. 1987; Mercantini et al. 1989; McRae and Seppelt 1999; Corte et al. 2000; Tosi et al. 2002; Connell et al. 2006; Godinho et al. 2013). The identification of species belonging to the genus *Trichoderma* is not an easy task because the genus has several species common in all environment and altitudes. Several isolates of this genus have already been obtained from soil, water and plant samples in Antarctica. Moreover, this genus has been isolated more often from plant specimen collected from nature. Hence, we rule out the possibility of an opportunistic contamination of our sample. At least three more species of fungi have been isolated from mosses: *Pythium polare* Tojo, van West & Hoshino, *Thyronectria antarctica* (Speg.) Seeler, and *Psychronectria hyperantarctica* (D. Hawksw.) J. Pawłowska, Istel, Wrzosek & D. Hawksw (Putzke and Pereira 2012; Tojo et al. 2012; Pawłowska et al. 2017). Putzke and Pereira (2012) report *T. antarctica* as the most common ring-forming fungi on the antarctic mosses, but those authors do not show either a diagnosis about their sample on Elephant Island, or any other data that confirm their identification, basing its taxonomic determination only on the macroscopic characteristics of mycelial rings on moss mats, such as color,

type of growth, and the way in which the plants are necrotizing. Other Nectriaceae are also known as ring-forming fungi on Antarctic mosses, as *Psychronectria hyperantarctica* (D. Hawksw.) J. Pawłowska, Istel, Wrzosek & D. Hawksw., *Thyronectria antarctica* var. *hyperantarctica*, *Coleroa turfosorum*, *Bryosphaeria megaspora*, and *Epibryon chorisodontii* Spooner (Hawksworth 1973; Longton 1973; Fenton 1983; Pawłowska et al. 2017), all of which present characteristics of very similar mycelial rings, without more diagnostic characters, only molecular analyses allow the correct determination of these species. Yamazaki et al. (2011) report *Trichoderma polysporum* (Link) Rifai as pathogenic fungi to *S. uncinata* from Svalbard region, but without mentioning if this was a ring-forming fungi in the plants analyzed by them. Based in the molecular phylogeny, the fungi isolated in the present study are not close to the species found by Yamazaki et al. (2011), suggesting that it is a species other than *T. polysporum*, being more related to the isolates of *T. viride* already sampled in other studies for Antarctica (Bradner et al. 1999; Saili et al. 2014). Thus, our isolate represents the fourth among the ring-forming species to be isolated from mosses and the first within the genus *Trichoderma*. Different species have distinct nutritional requirements (Griffin 1994) and have generally exhibited different patterns of substrate utilization (Leung et al. 2011). Among all the culture media used, the highest mycelial growth was seen in PDA medium. The knowledge of nutritional requirement of the

fungi from the Antarctic might indicate the conditions for its development in different environments. According to Davey et al. (2009), the rapid growth and abundant production of mycelia are two important factors for the fungi to spread and survive in environmental conditions. The isolate showed rapid growth when subjected to milder temperature ranges, possibly a strategy for colonization in the Antarctic environment during the austral summer when thawing occurs and temperatures are milder than winter. Earlier studies in polar regions have demonstrated that most of the fungal isolates have a maximum growth rate at temperature between 15 and 25 °C (Kerry 1990; Zucconi et al. 1996; Robinson 2001; Tosi et al. 2002; Tojo et al. 2012). Microorganisms with a minimum temperature of growth between – 5 °C and 5 °C and a maximum temperature between 30 °C and 35 °C are called as psychrotrophs (Gava et al. 2009). Hence, the species isolated in this study will fall under this category. The fungi psychrotrophs, which grows at lower temperatures, dominate the fungal floras in certain polar habitats (Kerry 1990). Apart from the availability of a suitable food source, examples of other factors that influence the growth of fungi are moisture, temperature, pH, and oxygen (Alexopoulos et al. 1996). The isolate in this study had a rapid growth at temperatures ranging between 20 and 26 °C, presenting a more fragile mycelium. At a temperature between 1 and 10 °C, the isolate presented a slow growth but its mycelium was denser. The growth rate and ambient temperature is an important determinant for the success and colonization of a species, and low temperature is a limiting factor for the development of fungi (Kerry 1990).

Fungal species have the ability to occupy different environments. This great diversity of environments gave these organisms the ability to synthesize a number of enzymes with different characteristics. Several physiological mechanisms adopted by fungi for cold tolerance have been proposed, and one of these strategies would be the production of cold-active enzymes (Robinson 2001). The production of amylase at 10 °C may be one of the mechanisms used by this isolate of the *Trichoderma* sp. to survive in the Antarctic environment. Robinson (2001) had pointed out that the enzymes active at low temperatures were produced by species of mycorrhizal fungi decomposing in the Arctic and Antarctic environment. Singh and Singh (2012) did chemical analysis in strains of filamentous fungi and yeast found in the cryoconite holes from Midre Lovénbreen glacier. This analysis showed that the secretion of enzymes by cold-adapted fungi carried out the process of degradation of organic macromolecules, thus contributing to the nutrient cycling in subglacial environments.

In the disk diffusion and confrontation tests, *Trichoderma* sp. produced secondary metabolites that inhibited the growth of moss and even led to the complete discoloration of the gametophyte, suggesting that this strain had to compete

with moss and hence exhibited toxic or inhibitory effects (Khaldi et al. 2010), perhaps also could be the cause of brown and yellow stains on the moss *S. uncinata*, in the Antarctic environment. But, Tojo et al. (2012) have identified *Pythium polare* causing brown discoloration in the same moss species. Putzke and Pereira (2012) also identified *T. hyperantarctica* causing the same discoloration in *S. uncinata*. These data suggest that different species of fungi may be associated with the brown discoloration in this moss species. *Trichoderma* is a genus known worldwide for its production of extracellular enzymes and secondary metabolites of commercial value (Benítez et al. 2004; Fenice 2016). Species of this genus have been reported in association with the Antarctic mosses and understanding the ecological relationships that occur between these organisms is important. *Trichoderma* sp. was able to cause complete discoloration of gametophytes in vitro, and this biological characteristic of the isolate may be occurring in a way similar to the natural Antarctic environment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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