PeerJ Preprints NOT PEER-REVIEWED

Root fungal endophytes improve the growth of antarctic plants through an enhanced nitrogen acquisition

Romulo Oses Pedraza Corresp., [1, 2] , **Cristian Torres-Díaz** [3] , **Paris Lavin** [4] , **Patricio Retamales-Molina** [5] , **Cristian Atala** [6] , **Ian Acuña-Rodríguez** [7] , **Marco A. Molina-Montenegro** [2, 7, 8]

[1] Centro Regional de Investigación y Desarrollo Sustentable de Atacama (CRIDESAT), Universidad de Atacama, Copiapo, Region of Atacama, Chile
[2] Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Facultad de Ciencias del Mar, Universidad Católica del Norte, Coquimbo, Chile
[3] Laboratorio de Genómica y Biodiversidad (LGB), Departamento de Ciencias Naturales, Universidad del Bío-Bío, Chillán, Región de Ñuble, Chile
[4] Laboratorio de Complejidad Microbiana y Ecología Funcional, Instituto Antofagasta, Universidad de Antofagasta, Antofagasta, Región de Antofagasta, Chile
[5] Laboratorio de Bacteriología Molecular, Facultad de Medicina, Universidad Diego Portales, Santiago, Región Metropolitana, Chile
[6] Laboratorio de Anatomía y Ecología Funcional de Plantas (AEF), Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Región de Valparaíso, Chile
[7] Centro de Ecología Molecular y Funcional, Instituto de Ciencias Biológicas, Universidad de Talca, Talca, Región del Maule, Chile
[8] Research Program "Adaptation of Agriculture to Climate Change" PIEI A2C2, Universidad de Talca, Talca, Región del Maule, Chile

Corresponding Author: Romulo Oses Pedraza
Email address: romulo.oses@uda.cl

Mutualistic symbiosis with fungal endophytes has been suggested as a possible mechanism for extreme environment colonization by Antarctic vascular plants. Fungal endophytes improve plant stress tolerance and performance by increasing plant hormone production and the uptake of water and nutrients. However, there are still gaps regarding the mechanisms by which these process ocurr. This work explores the role of root fungal endophytes in the production of exolytic enzymes involved in endophyte-mediated mineralization and nutrient uptake, as well as their impact on the performance of Antarctic plants. Hence, we evaluated the ability of fungal endophytes isolated from the two native Antarctic vascular plants, *Colobanthus quitensis* and *Deschampsia antarctica*, to enzymatically degrade different nutrient sources, mediate nitrogen mineralization and enhance growth of the host plant. Single-spore derived isolates were identified using molecular and morphological approaches. *Penicillium chrysosgenum* and *Penicillium brevicompactum* were identified as the dominant root endophytes in *C. quitensis* and *D. antarctica*, respectively. Root endophytes exhibited hydrolytic and oxidative enzymatic activities involved in carbohydrate or protein breakdown and phosphorus solubilization. In addition, the rates and percentages of nitrogen mineralization, as well as the final total biomass were significantly higher in *C. quitensis* and *D. antarctica* individuals with root endophytes relative to those without endophytes. Our findings suggest that root endophytes exert a pivotal ecological role based not only on their capability to breakdown different nutrient sources but also accelerating nitrogen mineralization, improving nutrient

acquisition and promoting plant growth in limited nutrient soils in Antarctic terrestrial ecosystems

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED

1 **ROOT FUNGAL ENDOPHYTES IMPROVE THE GROWTH OF ANTARCTIC PLANTS**
2 **THROUGH AN ENHANCED NITROGEN ACQUISITION**
3
4 **Running head: N mineralization and hydrolytic-oxidative enzymes from root Antarctic**
5 **endophytes**
6
7 Rómulo Oses[1,2*], Cristian Torres-Díaz[3], Paris Lavín[4], Patricio Retamales-Molina[5], Cristian Atala[6],
8 Ian S. Acuña-Rodríguez[7] and Marco A. Molina-Montenegro[2,7,8]
9
10 *1. Centro Regional de Investigación y Desarrollo Sustentable de Atacama (CRIDESAT) –*
11 *Universidad de Atacama, Av. Copayapu N° 485, Copiapó, Chile*
12 *2. Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Facultad de Ciencias del Mar,*
13 *Universidad Católica del Norte, Larrondo N°1281, Coquimbo, Chile*
14 *3. Laboratorio de Genómica y Biodiversidad (LGB), Departamento de Ciencias Naturales,*
15 *Universidad del Bío-Bío, Chillan, Chile*
16 *4. Laboratorio de Complejidad Microbiana y Ecología Funcional, Instituto Antofagasta,*
17 *Universidad de Antofagasta, Chile*
18 *5. Laboratorio de Bacteriología Molecular, Facultad de Medicina, Universidad Diego Portales*
19 *(UDP), Santiago, Chile*
20 *6. Laboratorio de Anatomía y Ecología Funcional de Plantas (AEF), Instituto de Biología,*
21 *Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Campus Curauma, Chile.*
22 *7. Centro de Ecología Molecular y Funcional, Instituto de Ciencias Biológicas, Universidad de*
23 *Talca, Campus Talca, Avda. Lircay s/n, Talca, Chile*

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

24 *8. Research Program "Adaptation of Agriculture to Climate Change" PIEI A2C2, Universidad de*
25 *Talca, Talca, Chile*
26
27 *Corresponding author: Rómulo Oses; E-mail: romulo.oses@uda.cl, *Av. Copayapu N° 485,*
28 *Copiapó, Chile*
29

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

30 **ABSTRACT**

31 Mutualistic symbiosis with fungal endophytes has been suggested as a possible mechanism for
32 extreme environment colonization by Antarctic vascular plants. Fungal endophytes improve plant
33 stress tolerance and performance by increasing plant hormone production and the uptake of water
34 and nutrients. However, there are still some gaps regarding the mechanisms by which these
35 processes occur. This work explores the role of root fungal endophytes in the production of
36 exolytic enzymes involved in endophyte-mediated nitrogen mineralization and nutrient uptake, as
37 well as their impact on the performance of Antarctic plants. Hence, we evaluated the ability of
38 fungal endophytes isolated from the two native Antarctic vascular plants, *Colobanthus quitensis*
39 and *Deschampsia antarctica*, to enzymatically degrade different nutrient sources, mediate nitrogen
40 mineralization and enhance growth of the host plant. Single-spore derived isolates were identified
41 using molecular and morphological approaches. *Penicillium chrysogenum* and *Penicillium*
42 *brevicompactum* were identified as the dominant root endophytes in *C. quitensis* and *D. antarctica,*
43 respectively. Root endophytes exhibited hydrolytic and oxidative enzymatic activities involved in
44 carbohydrate or protein breakdown and phosphorus solubilization. In addition, the rates and
45 percentages of nitrogen mineralization, as well as the final total biomass, were significantly higher
46 in *C. quitensis* and *D. antarctica* individuals with root endophytes relative to those without
47 endophytes. Our findings suggest that root endophytes exert a pivotal ecological role based not
48 only on their capability to breakdown different nutrient sources but also accelerating nitrogen
49 mineralization, improving nutrient acquisition and promoting plant growth in limited nutrient soils
50 in Antarctic terrestrial ecosystems.

51

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

52 **Keywords**: fungal endophytes, *Penicillium,* enzymes, mineralization, nitrogen uptake, Antarctic
53 vascular plants.
54

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

55 **INTRODUCTION**

56

57 Microbial endophytes are defined as symbiotic microorganisms capable of migrate into the plant
58 endosphere, colonizing healthy plant tissues inter-and/or intracellularly and persisting for the
59 whole or part of the life cycle without cause disease symptoms in the host plant (Porras-Alfaro et
60 al 2011; Hardoim et al 2015). In mutualistic symbiosis, microbial endophytes can enhance nutrient
61 acquisition and transfer, promote growth, increase reproductive success, confer biotic defense or
62 abiotic stress tolerance to their host plant (Bacon and White 2000; Hardoim et al 2015; Bacon and
63 White 2016). For example, it has been proposed that the interaction between both, plant and
64 mycorrhiza (van der Heijden et al. 2008) and plant and microbial endophytes (Rodríguez et al.
65 2009) is a key factor for the performance and survival of plants in terrestrial ecosystems (Mandyam
66 and Jumpponen 2005). Nevertheless, in cold-stressed habitats, where mycorrhizas are usually
67 absent, fungal endophytes frequently act as the main root mutualistic symbionts (Mandyam and
68 Jumpponen 2005; Upson et al. 2009).

69 Root-associated dark septae endophytes (DSE) share numerous similarities with
70 mycorrhizal; nevertheless, most endophytes do not have an obligate biotrophic life stage and live
71 at least part of their life cycle away from the plant (Mandyam and Jumpponen 2005; Newsham
72 2011). The mechanisms for plant colonization and the nature of the symbiosis are poorly
73 understood when compared with mycorrhizal fungi (Saikkonen 2007). In this context, the ability
74 of fungal endophytes to transfer nutrients to the host is a relatively new discovery and the
75 mechanisms of this transfer are unknown (Behie et al. 2012, 2014). Previous reports have
76 demonstrated that fungal endophytes are able to produces extracellular enzymes and use different
77 sources of carbon, nitrogen and phosphorus suggesting that fungal endophytes can access to and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

78 process organic nutrient pools (Caldwell et al. 2000, Mandyam et al. 2010). Since almost all plants
79 in natural ecosystems appear to be symbiotic with fungal endophytes (Rodriguez et al. 2009), it is
80 important to investigate the potential role of root fungal symbionts in nutrient uptake and transfer
81 (Behie et al. 2014) as well as their impact on the adaptation of symbiotic plants to stressful
82 environments characterized by nutrient shortage.

83 The Antarctic continent represent one of the most stressful environments on Earth for plant
84 life (Pointing et al 2015) entirely covered by permanent ice and snow, with only 2-3% of its surface
85 area available for plant colonization and development (Convey et al. 2008). It is subject to severe
86 environmental conditions, including cold temperatures and shortage of water and organic nutrients
87 (carbon, organic matter, nitrogen and phosphorus) in its acidic soils (Convey et al. 2011, Convey
88 et al. 2014). In these extreme conditions, only two native vascular plants, *Colobanthus quitensis*
89 (Antarctic pearlwort) and *Deschampsia antarctica* (Antarctic hairgrass) occur naturally (Lewis-
90 Smith 2003).

91 In Antarctic terrestrial ecosystems, nitrogen (N) supply regulates primary productivity,
92 then the ability to acquire N at an early stage of availability is relevant to the success of
93 photosynthetic organisms (Hill et al. 2011). The accumulation of faeces from penguins in rookeries
94 (ornithogenic soils) represents an abundant source of organic nitrogen (Lindeboom 1984), which
95 is mineralized for the benefit of Antarctic plants by soil microbial activities (Roberts et al. 2009).
96 Nevertheless, in areas where there are low inputs of animal faeces, plants are largely dependent on
97 organic nitrogen, which enters the soil as protein, in the form of short peptides (Hill et al. 2011).
98 Thus, the growth of Antarctic plants may be limited both by slow rates at which proteins are
99 decomposed to amino acids, as well as by low nitrogen mineralization rates to ammonium (NH_4^+)
100 and nitrates (NO_3^-), the main forms in which antarctic plants are able to acquire and use this

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

101 nutrient (Rabert et al. 2017). But despite these limitations, antarctic plants are able to take up
102 organic nitrogen directly either as peptides or amino-acids, conferring a key advantage for the
103 acquisition of limiting nutrients in this stressful ecosystem (Hill et al. 2011).

104 Upson and coworkers (2009) reported the activity of DSE root-endophytic fungi in the
105 nitrogen uptake involving organic nitrogen sources (mainly peptides), thus pointing out a possible
106 second route of nitrogen assimilation. In this context, the role of these endophytes in litter protein
107 breakdown and amino-acid mineralization could appear to be determinant for nitrogen acquisition.
108 However, besides the study of Upson et al. (2009) and Hill et al. (2011) with *Deschampsia*
109 *antarctica*, this complementary route for nitrogen acquisition has been seldom evaluated, for
110 which the involved mechanism remains unknown for other species like *Colobanthus quitensis*.

111 To date, there have been few attempts to characterize the exolytic enzyme production
112 mediated by fungal endophytes and its impact on the nutrient uptake process (but see, Mandyam
113 and Jumpponen 2005; Mandyam et al. 2010). In fact, as far away we know, this mechanism has
114 not been evaluated among antarctic vascular plants. Accordingly, the present study aims: ***i)*** to
115 identify dominant root fungal endophytes associated with both native antarctic vascular plants, ***ii)***
116 to evaluate the ability of antarctic fungal endophytes to produce enzymatic extracellular machinery
117 able to degrade the main nutrient sources, ***iii)*** to test the ability of plant-endophyte partnership to
118 mineralize nitrogen, and ***iv)*** to assess the effect of fungal endophytes on growth of *C. quitensis* and
119 *D. antarctica*.

120

121

122

123

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

124 **METHODS**

125

126 ***Isolation and identification of fungal endophytes***

127 We count with the authorization given by Chilean Antarctic Institute (INACH) to collect all these
128 plants (authorization number: 1060/2014). Plants of *D. antarctica* and *C. quitensis* were collected
129 from sites around of the Antarctic Polish Station "Henryk Arctowski" on King George Island,
130 South Shetland Islands (62º09´S; 58º27´W during growing season (January 2013-2014). The study
131 site corresponds to a fringe parallel to coast line (Western shore of Admirantly Bay, King George
132 Island) with smooth slopes, influence of salt spray, abundant sandy substrate. This site experiences
133 prevailing northwest winds which generate a high evaporative demand for plant and substrates
134 with low water availability (Kozeretska et al. 2010; Molina-Montenegro et al. 2013). In these
135 coastal areas, the ocean is the primary source of mineral salts and contributes to the salinity of
136 freshwater bodies and soils (Kozeretska et al. 2010). Vegetation in this area is typical of the
137 Maritime Antarctic ice-free soils and dominated mainly by flowering plants such as *D. antarctica*
138 and *C. quitensis*, with these species occupying fairly large area adjacent to the cost line.

139 For isolation of root-inhabiting fungi, individual plants from *D. antarctica* and *C. quitensis*,
140 were sampled and surface sterilized with bleach (0.5% active ingredient sodium hypochlorite) for
141 1 min, washed with sterile water and treated with 70% alcohol for 2 min followed by several
142 washes with sterile distilled water. The root fragments were cultured on agar water (1.5%) and
143 incubated up to two months at 25 ºC in darkness. Roots were routinely observed under a dissecting
144 microscope, and the emerging fungi were transferred onto cornmeal agar and potato dextrose agar
145 (CMA; PDA; Becton Dickinson & Co, Maryland). Single-spore derived isolates were obtained,
146 purified and maintained by routine sub-culturing. After incubation and growing on solid media

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

147 plates, individual cultures were stored at 4ºC. Additionally, mycelia pieces were kept in sterile
148 glycerol at -80ºC for long-term preservation. Colonies growing out from individual root fragments
149 were identified according to taxonomical keys (Kieffer and Morelet 2000). Afterwards, the identity
150 of single-spore derived fungal isolates was confirmed using molecular tools as described below.

151 The frequency of root fungal endophytes (FFE) was calculated according to the following
152 formula: [(Nrc / Nra) x 100], where Nrc and Nra, are the number of roots from which fungi were
153 independently isolated and the total number root fragments analyzed, respectively (Rosa et al.
154 2010). The relative isolation frequency (RIF) corresponds to a measure of abundance of each of
155 the fungal endophytes calculated by the following formula: percentage abundance of single fungal
156 endophyte = (occurrence of single fungal endophyte x 100 / occurrence of total fungal endophytes)
157 (Hoff et al. 2004). The values of relative isolation frequency (RIF) were considered to determine
158 the most abundant fungal morphotypes in *D. antartica* and *C. quitensis*.

159 The most abundant single-spore derived isolates from each Antarctic plant were used in
160 the subsequent experiments including screening of enzymatic activities and nitrogen
161 mineralization assays. The inoculum of fungal isolate was derived from single spore fungal isolates
162 cultured on potato dextrose agar (PDA) medium diluted ten times and supplemented with 50–100
163 mg/ml of ampicillin, tetracycline, and streptomycin. Fungal cultures were incubated at 22 ºC with
164 a 12 h light regime. After 5–14 days of growth, conidia were harvested from plates by adding 10
165 ml of sterile water and gently scraping off spores with a sterile glass slide. The spore suspension
166 was adjusted to 100 ml of 0.05% Tween-80, sterilized solution, filtered through four layers of
167 sterile cotton cheesecloth gauze and spore concentration adjusted to 10^7–10^8 spores/ml.

168

169

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

170 ***Extracellular enzymes activities***

171 Extracellular hydrolytic and oxidative enzymatic activities were assayed in solid media using five
172 Petri dishes plates per assay (Krishnam et al. 2016). A basal medium composed of mineral salts
173 was used and supplemented with the appropriate target substrate as described below. Each plate
174 containing the tested culture media was inoculated with a 5-mm fungal plug cored from PDA
175 slants. Petri dishes of each isolates were incubated in darkness in different growth chambers, over
176 a period of four weeks at three different temperatures 4°C, 15° C and 25°C (Krishnam et al. 2016).
177 For detection of amylase, cellulase and hemicellulase activities (polysaccharides hydrolases), 1%
178 of starch [α-1,4 glucan], cellulose-azure (Green and Highley 1997) and xylan were used as carbon
179 sources. The strength of activity was classified based on the diameter of the hydrolyzed zone or
180 the development or disappearance of color in the solid media. All halo tests were performed
181 incorporating negative controls, consisting of plugs with active mycelia subjected to a process of
182 inactivation by pulses of UV light (5.6 J/cm^2 per pulse, each pulse of 15 minutes, three pulses)
183 according to protocols described by Krishnamurthy and coworkers (2007). Hydrolysis of fatty acid
184 ester (1% Tween 40) was determined by an opaque halo of calcium palmitate crystals. Protein
185 hydrolysis was determined by formation of a clear halo in basal medium supplemented with gelatin
186 (4 g/l) as the sole nitrogen source. A positive test was indicated by liquefaction in the tube after
187 chilling for a period of 30 min as previously described (Mandyam et al. 2010). Urease activity was
188 tested on urea agar plates with 2% urea and phenol red and confirmed by the formation of a pink-
189 colored clear zone surrounding plugs (MacFaddin 2000). Pikovskaya's agar medium was used to
190 assay phosphate solubilization (Nautiyal 1999). Phenoloxidase activity was assayed using
191 lignolytic indicator dyes, Poly R-478, Remazol Brilliant Blue and O-dianisidine (0.01%) (Oses et
192 al. 2006).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

193 ***Plant growth and axenic production of individuals***

194 Soil samples from field were submitted to sterilization process (121°C/1.5 atmospheres for 2.5
195 hour) in order to obtain sterilized soil for the following experiments. After this procedure, soil sub-
196 samples were cultivated on PDA plates using a serial dilution method with the purpose to check
197 microbial removal from soil before transplanting endophyte-free plants to pots. Current condition
198 for total nitrogen content was based on the average of 15 soil samples taken from the study site
199 (7.8 ± 0.8 mg/kg). It is well known that thermal soil sterilization may affect nutrient availability
200 and soil samples were therefore tested for differences in total nitrogen in sterilized and non-
201 sterilized soil samples (n = 5); no difference was found between both soils samples (t = 0.93; p =
202 0.77).

203 To assess the effect of fungal endophytes on the nitrogen mineralization process in *C.*
204 *quitensis* and *D. antarctica*, a manipulative experiment was conducted involving endophyte-free
205 plants (hereafter, E- plants or control) and endophyte-free plants re-inoculated with single spore
206 fungal endophyte, growing on sterilized Antarctic soil (hereafter, E+ plant or treatment).
207 Endophyte-free plants of each species were obtained from samples collected at the study site.
208 Samples were transported and planted in 300 ml plastic pots filled with Antarctic soil from the
209 study site and maintained in a climatic chamber at 4º C with a photon flux density (PFD) of 190
210 µmol m^{-2} s^{-1} and 16/8 h light/dark photoperiod mimicking Maritime Antarctica climatic conditions
211 for four months.

212 To remove fungal endophyte infections from Antarctic plants, the commercially available
213 fungicide Benlate (containing benomyl [methyl [1-butylamino carbonyl]-1H-benzimidazol-2-yl]
214 carbamate, DuPont, Wilmington, DE, USA) was used as treatment. Benomyl was chosen because
215 no phytotoxic effects have been detected on perennial ryegrass (Spiering et al. 2006). Leaves and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

216 roots were completely submerged in tap water containing 2 g/l of Benlate and maintained for 1 h
217 at room temperature. Thereafter 10-12 tillers (6.0 ± 0.3 cm height) were transplanted to pots (50
218 ml) filled with sterilized soil taken from the study site. In order to reinforce the removal treatment
219 of fungal endophytes, tillers were sprinkled with Benlate solution (2 g/l) once a week. After 4–5
220 weeks of growth, newly emerged tillers were examined microscopically in order to evaluate the
221 success of endophyte elimination after Benlate treatment. Detection of fungal infection was tested
222 using two approaches including detection by light microscopy techniques, and culture-based
223 methods (Bacon and White 2000).

224 Firstly, to assess the success of endophyte elimination after Benomyl treatment, the
225 infection status was checked microscopically on a subset of at least 10% of new emerged plants (n
226 = 50). Clearing and staining methods were conducted to quantify percentage of endophytic
227 colonization or percentage of infested root length (Bacon and White 2000; Spiering et al. 2006).
228 Endophyte occurrence in tissues was quantitatively determined by counting aniline blue-stained
229 endophyte hyphae in leaf and roots cross-sections. This method is a reliable and direct measure of
230 the amount of viable endophyte mycelium (Spiering et al. 2006). Secondly, after Benlate treatment,
231 endophyte-free plants were submitted to re-isolation protocols using culturing-based methods
232 described previously. The samples of plant tissue that showed no outgrowth of fungi into the
233 surrounding solid media were considered clean or endophyte-free plants suitable for use in the
234 subsequent experiments. This procedure was carried out to ensure that nitrogen mineralization
235 experiments included endophyte-free plants only. Thus, an endophyte-free individual of *D.*
236 *antarctica* corresponds to a tussock composed by 10 tillers, of 6.0 ± 0.3 cm height, 550.5 ± 8.5 mg
237 of weight and for *C. quitensis* corresponds to 6 rosettes, of 3.0 ± 0.1 cm height, 825.0 ± 5.5 mg of
238 weight (fig. 1B).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

239 Endophyte-free plants obtained after Benomyl treatment were re-inoculated with an
240 inoculum of fungal endophyte derived from single spore isolates to re-generate endophyte-plant
241 symbiotic association but with a single isolate. The most dominant fungal endophytes isolated
242 from each host plant were used as inoculum. Re-inoculated plants with single spore derived
243 endophytic isolates (10 ml of 10^8 spores per pot) and non-inoculated plants (10 ml of 10^8 spores
244 suspension per pot previously sterilized) of *D. antarctica* and *C. quitensis* were cultivated in
245 growth chambers in the same conditions described earlier. After this period, with the aim to assess
246 the re-colonization of aseptically grown tillers with a single dominant fungal endophyte, the
247 infection status was checked microscopically and re-isolation protocols using culturing-based
248 methods were conducted as described previously.
249

250 ***Assisted plant growth and nitrogen mineralization***

251 Individuals of both Antarctic plant species, endophyte-free plants (E-) and endophyte-free plant
252 but re-inoculated (E+) with a single dominant endophyte were transferred to pots (300 ml), filled
253 with sterilized soil taken from the study site and put in a growth chamber to obtain enough
254 vegetative material. The most regular and homogeneous individuals of both Antarctic plant
255 species, endophyte-free plants (E-) and endophyte-free plant but re-inoculated (E+) with a single
256 dominant endophyte (n = 7) were selected for the nitrogen mineralization experiments. At the onset
257 and at the end of the nitrogen mineralization experiment (t = 60 days), in order to determine the
258 status of endophytic colonization of roots, three random individuals of *C. quitensis* and *D.*
259 *antarctica* were microscopically checked for endophytic infection (percentage of infested root
260 length).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

261 Considering the total nitrogen content in Antarctic soils at the start of the growing season,
262 we applied the nitrogen equivalent to 8.0 mg/kg (Roberts et al. 2009) before the start of the
263 experiment (t = 0 days). According to a previous report, urea pellets were added to the soil as a
264 source of nitrogen (Jumpponen et al. 1998). Urea was chosen because is an intermediate compound
265 in the degradation pathway of uric acid. Uric acid is rapidly degraded by aerobic and anaerobic
266 microorganisms, through allantoin and urea and then to ammonium in summer when soils thaw
267 (Lindeboom 1984). Further urea dissolves slowly over a 4-5-months period then nitrogen pulses
268 were avoided during the experiment (Jumpponen et al. 1998).
269 Total nitrogen determination was performed using a Kjeldahl digestion method (Allen 1989).
270 Approximately 0.05 g of catalyst (lithium sulphate : copper sulphate in 10:1 ratio) and 1mL of
271 digest reagent (33 g of salicylic acid, 1 L of concentrated sulphuric acid) was added to 0.2 g of soil
272 sample in a digestion tube, and then heated at 370 ºC in a digestion block for 6 hours or until the
273 solution goes clear. The digested soil sample was cooled, and then cautiously diluted with about
274 10ml of water followed by filtration through Whatman N° 44 filter paper. The filtrate was further
275 diluted to 50 mL in a volumetric flask, and the concentrations of the individual elements were
276 determined through flame atomic absorption spectrometry. Organic nitrogen (nitrate and
277 ammonium) was extracted from 5 g of air dried soil sample with 50 mL 2M potassium chloride
278 solution for 30 min and filtered through Whatman paper Nº 42. The samples were later analyzed
279 colorimetrically for nitrate and ammonium using a continuous flow injection analyzer (FIAflow2,
280 Burkard Scientific, Uxbridge, UK) (Knepel 2003).
281 Each E+ and E- plant received 50 ml of tap water every four days. Pots positions within the
282 growth chambers were changed every five days to avoid block effect. The experimental treatments
283 were carried out over 60 days and percentage of nitrogen mineralization was estimated as the

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

284 percentage of N-urea (source of nitrogen) transformed to N-NH_4^+, measured in 50 g soil sub-
285 samples. Final total biomass was also measured in each individual plant at the end of the
286 experimental period, including fallen leaves. All tissues were oven-dried at 70 ºC for 72 h, and
287 weighted with an electronic precision balance (Boeco BBl-54, Germany).

288

289 **DATA ANALYSIS**

290 Nitrogen mineralization percentage was compared between treatments with repeated measures
291 ANOVA, with the endophyte infection status being the independent variable for each species. The
292 final percentages of nitrogen mineralization as well as the final total biomass were compared
293 between inoculated and non-inoculated individuals with a t-test for independent samples, as
294 implemented in the R language software (R-Core Team, 2015). All analyses were performed
295 independently for each species after testing for normality and homogeneity of variances using the
296 Shapiro-Wilks and Bartlett tests, respectively (Zar 1999).

297

298 **RESULTS**

299

300 ***Isolation and identification of fungal endophytes***

301 Different fungal endophytes were isolated from roots of symptomless individuals of *D. antarctica*
302 and *C. quitensis* host plants (Table 1). Seven different fungal morphotypes were independently
303 isolated with different relative isolation frequencies (RIFs) from both Antarctic plants;
304 corresponding to fungi from the genus *Penicillium* sp.- I (78%), *Alternaria* sp (13.2%), and
305 *Phaeosphaeria* sp (8.8%) were isolated from *D. antarctica*, and *Penicillium* sp - II (65%),
306 *Geomyces sp* (19%) and *Microdochium* sp (16%) were isolated from *C. quitensis.* Single-spore

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

307 derived fungal isolates *Penicillium* sp - I and *Penicillium* sp – II were independently recovered
308 from 133 individuals of *D. antarctica* (73.8%) and from 96 individuals of *C. quitensis* (64%),
309 respectively (Table 1). On the base of the values of relative isolation frequencies (RIFs), the most
310 frequent fungal endophytes from each species was isolated from a single spore, purified and used
311 in later experiments. According to Molina-Montenegro et al. (2016), the two most-abundant fungal
312 morphotypes corresponded to two isolates of Antarctic fungal endophyte, hereafter, AFE (AFE001
313 and AFE002), which represented more than 65% and 78% of the total fungal morphotypes
314 recorded in *C. quitensis* (AFE001) and *D. antarctica* (AFE002), respectively. Isolates, AFE001
315 (Genebank accession number: KJ881371) and AFE002 (GeneBank accession number KJ881370)
316 were identified as *P. chrysogenum* and *P. brevicompactum*, respectively.
317

318 ***Extracellular enzymes activities***

319 The most dominant single-spore derived fungal endophyte *P. chrysogenum* and *P.*
320 *brevicompactum*, isolated from *C. quitensis* and *D. antarctica*, respectively, were able to exhibit
321 hydrolytic and oxidative enzymatic activities involved in nutrient conversion and assimilation such
322 as carbon, nitrogen and phosphorus (Table 2). The maximum intensities of enzymatic reactions
323 under controlled conditions were obtained within the first two weeks at 15ºC and at four weeks at
324 4ºC while in 25°C the intensities decreased in all enzymes in both fungal endophytes. *P.*
325 *chrysogenum* and *P. brevicompactum* isolates displayed positive reaction for amylase, cellulase,
326 laccase, gelatinase and urease. *P. chrysogenum* was able to use most of the carbon, nitrogen and
327 phosphate substrates (Table 2). Proteins and urea were both hydrolyzed by *P. chrysogenum* and *P.*
328 *brevicompactum* isolates. The fatty acid ester, Tween 40, was hydrolyzed by both dominant
329 isolates. The intensities of some enzymatic hydrolysis and oxidations were different between the

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

330 isolates and also under different temperatures of incubation. The intensity of cellulase at 4°C was
331 higher in *P. chrysogenum* than in *P. brevicompactum*. However, the intensity of hemicellulase
332 displayed by *P. chrysogenum* was higher at 15°C rather than 4°C. The intensity esterease was
333 higher *P. chrysogenum* than *P. brevicompactum* in both temperatures. *P. chrysogenum* and *P.*
334 *brevicompactum* showed no lignolytic activities when Poly R-478 and RBB were used as substrate
335 but reacted positively with O-dianisidine.
336
337 ***Assisted plant growth and nitrogen mineralization***
338 At the initial stage of the experiment (t = 0), as expected, there was no evidence of endophytic root
339 infection in non-inoculated *C. quitensis* and *D. antarctica* individuals. However, after inoculation
340 with dominant root endophytes, root systems of all inoculated individuals were colonized by the
341 mycelium superficial or intercellularly. An encroachment of fungal endophytes into the root tissue
342 was detectable. Morever, an infection pattern characterized by black microsclerotia and pigmented
343 hypha distributed irregularly along the roots was observed. The fungal infection progressed during
344 the experimental period. By the end of nitrogen mineralization experiment (t = 60 days), the
345 percentage of infested root length in *C. quitensis* inoculated with *P. chrysogenum* reached 88.5 ±
346 1.6 % and 91.2 ± 0.9 % in *D. antarctica* inoculated with *P. brevicompactum*.
347 The total biomass accumulation at the end of the experiment was significantly higher in *C.*
348 *quitensis* (27%) and *D. antarctica* (22%) individuals infected with root endophytes (E+) compared
349 to endophyte-free (E-) plans (*t*-test = 9.11; $p < 0.0001$ and *t*-test = 6.13; $p < 0.0001$, respectively
350 (Fig. 1). The final percentage and rate of mineralization in *C. quitensis* infected with *P.*
351 *chrysogenum* (E+) was significantly higher (*t*-test = 10.19; $p < 0.0001$) than in individuals without
352 endophytes (E-) (Fig. 2). The repeated measures ANOVA revealed that the percentage of

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

353 mineralization increased significantly with time ($F_{4,\,112}$ = 503.03; $p < 0.001$) in both treatments.
354 Furthermore, the percentage of nitrogen mineralization increase in the presence of endophytes was
355 more pronounced than in their absence, as shown by the significant interaction between treatment
356 (E+ and E-) and time ($F_{4,\,112}$ = 9.04; $p < 0.01$; Fig. 2). Similarly, *D. antarctica* individuals
357 inoculated with *P. brevicompactum* showed a significantly higher final percentage of
358 mineralization (*t*-test = 2.84; $p = 0.016$) compared to those without the inoculums (Fig. 2). In the
359 same way, final percentage of mineralization significantly increased with presence of endophyte
360 ($F_{4,\,112}$ = 7.01; $p = 0.031$) and time ($F_{4,\,112}$ = 206.97; $p < 0.001$, respectively; Fig. 2A).
361

362 **DISCUSSION**

363 In the present work, the dominant fungal endophytes *Penicillium chrysogenum* and *Penicillium*
364 *brevicompactum* were found to inhabit roots of antarctic plants improving its growth through of
365 accelerated mineralization and exolytic enzymes production. A previous report documented the
366 occurrence of dark septate endophytes (most of them members of Heliotales) associated to
367 Antarctic plants (Upson et al. 2009) which are different to those fungal endophytes reported in the
368 present study. The *Penicillium* group is a large and polyphyletic group, which comprises the most
369 catabolically and anabolically diverse microorganisms described to date (Houbraken et al 2011).
370 Being part of this cosmopolitan genus several species of *Penicillium* species has been previously
371 reported in alpine and tundra soils (Gunde-Cimerman et al. 2003). Accordingly, Zucconi and
372 coworkers (1996) pointed out that the majority of Antarctic fungi are ecotypes of cosmopolitan
373 species that show mesophilic-psychrotolerant behavior as an adaptation to the cold Antarctic
374 climate. Particularly in Antarctica, various *Penicillium* species has been documented to be part of
375 the endophytic community of the native plants, appearing in rhizoids of the non-vascular leafy

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

376 liverwort *Cephaloziella varians* (Newsham 2010) and the moss *Bryum argenteum* (Bradner et al.
377 2000).
378 Several studies indicate that fitness benefits conferred by mutualistic fungi contribute to -
379 or are- responsible for plant adaptation to abiotic stress (Stone et al 2000; Bacon and White 2016).
380 Mutualistic fungi may confer tolerance to drought, metals, disease, heat and herbivory, and/or
381 promote growth and nutrient acquisition. According to previous reports, there is a consensus that
382 plants without fungal endophytes apparently are unable to tolerate habitat-imposed abiotic and
383 biotic stresses (Malinowski et al. 2005; Rodriguez et al. 2008; Gond et al. 2015).
384 In Maritime Antarctic soils the release of nitrogen slowly as decomposition is limited by
385 low temperatures that impose several restrictions for plant life. Then the ability to acquire N at an
386 early stage of availability during a short growth season is key to the success of vascular plants.
387 Previous works have addressed two nitrogen assimilation pathways available for plants in
388 Antarctic environments: those of the plant itself (Hill et al. 2011) and those derived from the
389 mutualistic symbiosis between plant and fungal endophytes (Upson et al. 2009). However, the
390 mechanisms involving nitrogen uptake and transfer to the host mediated by fungal endophytes
391 remain unknown. Regarding the later, our work shows that, in addition of being root endophytes,
392 *P. chrysogenum* and *P. brevicompactum,* are able to exudate a wide range of hydrolytic and
393 oxidative enzymes such as amylase, cellulase, lipase, protease, urease and xylanase under suitable
394 conditions. In our study, we found no evidence for lignolytic enzymes using complex polymeric
395 dyes (Remazol Brilliant Blue and Poly R-478), however, a weak lignolytic activity was found only
396 when O-dianisidine was used as a substrate. These differential lignolytic activities suggest that the
397 exolytic enzymes of these fungi are capable of degrading just some types of simple lignin

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

398 structures, but not more complex lignin molecules that require high oxidation potentials for
399 degradation (Oses et al 2006).

400 The root endophytes *P. chrysogenum* and *P. brevicompactum* could behave in a facultative
401 biotrophic way (Fesel and Zuccaro 2016) and be involved in the metabolizing of complex
402 substances accumulated in organic matter pools as in Antarctic rhizospheric soil (Yergeau and
403 Kawalchuk 2008, Krishnam et al. 2016). These facultative biotrophic behaviors have been
404 proposed as a competitive advantage over other saprotrophs (Fesel and Zuccaro 2016). But
405 moreover, this endophytic lifestyle (i.e. facultative-biotrophic) has been recognized as an ancestral
406 feature and an important part of the model of plant-fungal endophyte interactions known as
407 "mutualism-parasitism continuum paradigm" (Mandyam and Jumpponen 2015; Fesel and Zuccaro
408 2016).

409 Considering the short growing season in Antarctica and its narrow window of opportunities
410 for plants, the ability to quickly produce and activate complex enzymatic machinery may represent
411 a key ecological advantage to symbiotic plants in accelerating of recycling and assimilation of
412 limiting nutrients. In this context, it has been proposed that a balanced symbiosis mechanism (or
413 "fine-tuning") regulating these enzymatic activities under environmental, physiological and
414 genetic control within the host plant tissues could provide huge ecological advantages resulting in
415 fitness benefits for both partners (Kogel et al. 2006). The higher nitrogen mineralization rates from
416 urea to NH_4^+ and increase in total plant biomass found in inoculated (E+) individuals of both *C.*
417 *quitensis* and *D. antarctica*, when symbiotic are consistent with the presence of extracellular
418 hydrolytic enzymatic activities (proteinase and urease) observed in fungal cultures, strongly
419 suggesting not only an efficient uptake and nutrient transfer mechanisms, at least for the scarce
420 nitrogen sources, but also, that fungal endophytes are not detrimental to the host plant (Jumpponen

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

421 et al. 1998; Upson et al. 2009; Behie and Bidochka 2014). Indeed, the positive symbiotic status
422 (E+) was consistent with the presence of extracellular hydrolytic enzymatic activities (proteinase
423 and urease) observed in fungal cultures. Therefore, our results suggest that being symbiotic with a
424 target Antarctic fungal endophyte partner could be a critical advantage in terms of plant survival
425 and development, particularly, when competing for limiting nitrogen resources with soil microbes
426 and other non-vascular plants (Upson et al. 2009; Hill et al 2011). Our results support the
427 hypothesized mechanisms to explain microbe-mediated enhanced plant growth that include
428 increase the absorption of nutrients by plants from the rhizosphere due to activities of microbes on
429 roots (Bacon and White 2016)
430 Our work is consistent with previous report, in which have identified dark septate
431 endophytes have been identified out as relevant agents during inorganic nitrogen uptake,
432 increasing the plant biomass in *D. antarctica* (Upson et al. 2009). Also, our findings are similar to
433 those found by Jumpponen et al. (1998), who found that inoculation with fungal endophytes
434 significantly, increased the biomass of the host plant species using urea as a main nitrogen source.
435 This finding suggests that mutualistic association between plant-root fungal endophytes, could
436 facilitate and increment the rate of acquisition of organic nitrogen in both Antarctic vascular plants.
437 In a recent work, Molina-Montenegro and workers (2016) found that cross-inoculation of antarctic
438 root endophyte described in this work also improve the ecophysiological performance and yield in
439 lettuce (*Latuca sativa*) under drought condition showing potential for biotechnological
440 approaches.
441
442
443

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

444 **CONCLUSIONS**

445

446 Our study demonstrates how root fungal endophytes improve the growth of Antarctic vascular
447 plants *D. antarctica* and *C. quitensis* through an enhanced nitrogen acquisition. Firstly, it was
448 demonstrated that fungal endophytes *Penicillium chrysogenum* and *Penicillium brevicompactum*
449 dominant root endophytes isolated from *C. quitensis* and *D. antarctica,* respectively; exhibited
450 enzymatic activities involved in carbohydrate or protein breakdown and phosphorus solubilization.
451 Secondly, the rates and percentages of nitrogen mineralization, as well as the final total biomass,
452 were significantly higher in *C. quitensis* and *D. antarctica* individuals with root endophytes
453 relative to those without endophytes. This work contributes to understand how the higher
454 mineralization rate of organic nitrogen performed by plant-endophyte association and the exolytic
455 enzymes produced by fungal endophytes (proteases and ureases) may drive an efficient nitrogen
456 conversion, access and recycling, increasing plant growth in Antarctic environments. Our results
457 showed that endophyte-assisted nitrogen mineralization is an advantageous mechanism to compete
458 for organic nitrogen resources with soil microbes and other plants, preferentially for NH_4^+, as
459 suggested in previous works (Hill et al. 2011; Behie and Bidochka 2014; Bacon and White 2016).
460 Therefore, this symbiotic mechanism of nitrogen uptake could explain, at least in part, the survival
461 and establishment of vascular plants in Antarctic environments (Smith-Lewis, 2003; Upson et al.
462 2009, Hill et al. 2011). We are far from a clear understanding of mechanisms underlying nutrient
463 uptake by plants in the poor soils of Antarctic environment, this study has identified a promising
464 aspect in endophyte-assisted nitrogen mineralization as part of nitrogen uptake and transfer
465 mechanisms for plants and the relevance of mutualistic symbiosis in extreme environments.
466 Finally, we suggest that the future researches should be addressed to explore the molecular and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

467 functional dissection the plant-fungal endophytes relationship, identifying mechanisms underlying
468 to this symbiosis to help explain the colonization, performance and spread of plants inhabiting
469 stressful environments as those found in Antarctica (Fesel and Zuccaro 2016).

470

471 **ACKNOWLEDGEMENTS**

472

473 We thank Chilean Antarctic Institute (INACH), the Chilean Navy (AP-41 "Aquiles" and AP-46
474 "Almirante Oscar Viel") and the Arctowski Polish Antarctic Station for their logistical support.
475 We want to thank C. Fardella and M.A. Montoya for his valuable help in the lab and growth
476 chamber experiments. This work is dedicated to Professor Hugo I. Moyano† (October 2014),
477 Zoology Department, University of Concepción for his contribution to Chilean Antarctic Research
478 and to the memory of Dr. Jaime Rodríguez G. †, Director of Center of Biotechnology, University
479 of Concepción (February 2017).

480

481 **REFERENCE**

482

483 **Allen SE. 1989**. Chemical Analysis of Ecological Materials. 2nd Edn., Backwell Scientific
484 Publications.
485 **Bacon CW, White JF. 2000.** Microbial Endophytes. Marcel Dekker, New York
486 **Behie SW, Zelisko PM, Bidochka MJ. 2012**. Endophytic insect-parasitic fungi translocate
487 nitrogen directly from insects to plants. *Science* 336, 1576–1577. DOI
488 10.1126/science.1222289

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

489 **Behie SW, Bidochka MJ. 2014**. Nutrient transfer in plant–fungal symbioses. *Trends in Plant*
490 *Science* 19: 734–740. 10.1016/j.tplants.2014.06.007
491 **Bölter M. 2011**. Soil development and soil biology on King George Island, Maritime Antarctic.
492 *Polish Polar Research* 32: 105-318 116. DOI 10.2478/v10183-011-0002-z
493 **Bradner JR, Sidhu RK, Yee B, Skotnicki ML, Selkirk PM, Nevalainen KM. 2000**. A new
494 microfungal isolate, *Embellisia* sp., associated with the Antarctic moss *Bryum argenteum*.
495 *Polar Biology* 23: 730–732. DOI 10.1007/s003000000161
496 **Caldwell BA, Jumpponen A, Trappe JM. 2000.** Utilization of major detrital substrates by dark-
497 septate root endophytes. *Mycologia* 92: 230-232. DOI 10.2307/3761555
498 **Cannone N. 2011.** The secret of success. *Nature Climate Change* 1: 28–29. DOI
499 10.1038/nclimate1077
500 **Convey P, Gibson JA, Hillenbrand CD, Hodgson DA, Pugh PJ, Smellie JL, Stevens MI. 2008.**
501 Antarctic terrestrial life –challenging the history of the frozen continent?. *Biological Review*
502 *Cambridge Philosophy Society* 83: 103-117. DOI 10.1111/j.1469-185X.2008.00034.x
503 **Convey P, Chown SL, Clarke A, Barnes DK, Bokhorst S, Cummings V, Ducklow HW, Frati**
504 **FT, Green A, Gordon Sh, Griffiths HJ, Howard-Williams C, Huiskes AdHL,**
505 **Laybourn-Parry JW, Lyons B, McMinn A, Morley SA, Lloyd SP, Quesada A,**
506 **Robinson SA, Schiaparelli S, Wall DH. 2014.** The spatial structure of Antarctic
507 biodiversity. *Ecology Monograph* 84: 203-244. DOI 10.1890/12-2216.1
508 **Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J,**
509 **Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A.**
510 **2011.** Geneious v5.4. <http://www.geneious.com>

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

511 **Fesel PH, Zuccaro A. 2016**. Dissecting endophytic lifestyle along the parasitism / mutualism
512 continuum in *Arabidopsis*. *Current Opinion Microbiology* 32: 103-112 DOI
513 10.1016/j.mib.2016.05.008
514 **Ghaffari MR, Ghabooli M, Khatabi B, Hajirezaei MR, Schweizer P, Salekdeh GH. 2016.**
515 Metabolic and transcriptional response of central metabolism affected by root endophytic
516 fungus *Piriformospora indica* under salinity in barley. *Plant Molecular Biology* 90: 699-
517 717. DOI10.1007/s11103-016-0461-z
518 **Gond SK, Torres MS, Bergen MS, Helsel Z,White JF. 2015**. Induction of salt tolerance and up-
519 regulation of aquaporin genes in tropical corn by rhizobacterium *Pantoea agglomerans*.
520 *Letter Applied Microbiology* 60: 392–399. DOI 10.1111/lam.12385
521 **Green III F, Highley TL. 1997.** Mechanisms of brown-rot fungi decay: paradigm or paradox.
522 *International Biodeterioration Biodegradation* 39: 113-124. DOI 10.1016/S0964-
523 8305(96)00063-7
524 **Gunde-Cimerman N, Sonjak S, Zalar P, Frisvad JC, Diderichsen B, Plemenitas A. 2003.**
525 Extremophilic fungi in arctic ice: a relationship between adaptation to low temperature and
526 water activity. *Physico-Chemical Earth* 28: 1273–1278. DOI 10.1016/j.pce.2003.08.056
527 **Hill PW, Farrar J, Roberts P, Farrell M, Grant H, Newsham KK, Hopkins DW, Bardgett**
528 **RD, Jones DL. 2011.** Vascular plant success in a warming Antarctic may be due to efficient
529 nitrogen acquisition. *Nature Climate Change* 1: 50-53. DOI 10.1038/nclimate1060
530 **Hoff JA, Klopfenstein NB, McDonald GI, Tonn JR, Kim MS, Zambino PJ, Hessburg PF,**
531 **Rogers JD, Peever TL, Carris LM. 2004**. Fungal endophytes in woody roots of Douglas-
532 fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*). *Forest Pathology* 34:
533 255–271. DOI 10.1111/j.1439-0329.2004.00367.x

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

534 **Hogg ID, Cary SC, Convey P, Newsham KK, O'Donnell AG, Adams BJ, Wall DH. 2006.** Biotic interactions in Antarctic terrestrial ecosystems: are they a factor?. *Soil Biology and Biochemistry* 38: 3035-3040. **DOI 10.1016/j.soilbio.2006.04.026**

537 **Houbraken J, Samson RA. 2011** Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families. *Studies in Mycology* 70: 1-51. doi:10.3114/sim.2011.70.01

540 **Jumpponen A, Mattson KG, Trappe JM. 1998**. Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza* 7: 261-265. DOI 10.1007/s005720050190

543 **Kiffer E, Morelet M. 2000**. The Deuteromycetes, Mitosporic Fungi, Classification and Generic keys. Science Publishers, Inc. USA

545 **Knepel K. 2003.** Determination of Nitrate in 2 M KCl Soil Extracts by Flow Injection Analysis. QuikChem Method 12-107-04-1-B. Lachat Instruments, Loveland, CO.

547 **Kogel KH, Franken P, Hückelhoven R. 2006.** Endophyte or Parasite—What Decides? *Current Opinion Plant Biology* 9: 358-363. DOI 10.1016/j.pbi.2006.05.001

549 **Kozeretska IA, Parnikoza IYu, Mustafa O, Tyschenko OV, Korsun SG, Convey P (2010)** Development of Antarctic herb tundra vegetation near Arctowski station, King George Island. *Polar Science* 3: 254–261. http://dx.doi.org/10.1016/j.polar.2009.10.001

552 **Krishnamurthy K, Demirci A, Irudayaraj JM. 2007.** Inactivation of *Staphylococcus aureus* in milk using flow-through pulsed UV-light treatment system. *Journal of Food Science* 72: 233-239. DOI 10.1111/j.1750-3841.2007.00438.x

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

555 **Krishnan A, Convey P, Gonzalez-Rocha G, Alias SA (2016)** Production of extracellular
556 hydrolase enzymes by fungi from King George Island. *Polar Biology* 39: 65-76. DOI
557 10.1007/s00300-014-1606-7

558 **Leitão AL, Enguita FJ. 2016.** Gibberellins in *Penicillium* strains: Challenges for endophyte-plant
559 host interactions under salinity stress. *Microbiological Research* 183: 8-18. DOI
560 10.1016/j.micres.2015.11.004

561 **Lewis-Smith RI. 2003**. The enigma of *Colobanthus quitensis* and *Deschampsia antarctica*. In:
562 Huiskes AHL, Gieskes

563 **WWC, Rozema J, Schorno RML, van der Vies SM, Wolff WJ**, (eds). Antarctic biology in a
564 global context. Backhuys Publishers Leiden, The Netherlands, pp 234–239

565 **Lindeboom HJ. 1984**. The nitrogen pathway in a penguin rookery. *Ecology* 65: 269-77. DOI
566 10.2307/1939479

567 **MacFaddin JF. 2000** Biochemical tests for the identification of medical bacteria. Lippincott
568 Williams & Wilkins, Philadelphia

569 **Malinowski MP, Belesky DP, Lewis GC. 2005.** Abiotic stresses in endophytic grasses. In:
570 Roberts C, West CP, Spiers DE (eds) *Neotyphodium* in cool-season grasses Blackwell
571 publishing. Ames, Ohio, pp. 187–199

572 **Mandyam K, Jumpponen, A. 2005.** Seeking the elusive function of the root-colonising dark
573 septate endophytic fungi. *Studies in Mycology* 53: 173–189. doi: 10.3114/sim.53.1.173

574 **Mandyam K, Jumpponen A. 2015.** Mutualism–parasitism paradigm synthesized from results of
575 root-endophyte models. *Frontiers in Microbiology* 5: 1-13. doi: 10.3389/fmicb.2014.00776

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

576 **Mandyam K, Loughin T, Jumpponen A. 2010.** Isolation and morphological and metabolic
577 characterization of common endophytes in annually burned tallgrass prairie. *Mycologia* 102:
578 813-821. doi:10.3852/09-212.
579 **Molina-Montenegro MA, Ricote-Martínez N, Muñoz-Ramírez C, Gómez-González S,**
580 **Torres-Díaz C, Salgado-Luarte C, Gianoli E .2013.** Positive interactions between the
581 lichen *Usnea antarctica* (Parmeliaceae) and the native flora in maritime antarctica. *Journal*
582 *of Vegetation Science* 24: 463-472. DOI 10.1111/j.1654-1103.2012.01480.x
583 **Molina-Montenegro, M.A., Oses, R., Torres-Díaz, C., Atala, C., Zurita-Silva, A., & Ruiz-**
584 **Lara, S. 2016**. Root-endophytes improve the ecophysiological performance and production
585 of an agricultural species under drought condition. *AoB PLANTS*, 8plw062
586 **Nautiyal CS. 1999.** An efficient microbiological growth medium for screening phosphate
587 solubilizing microorganisms. *FEMS Microbiology Letters* 170: 265-270.
588 doi:10.1111/j.1574-6968.1999.tb13383.x
589 **Newsham KK. 2010.** The biology and ecology of the liverwort *Cephaloziella varians* in
590 Antarctica. *Antarctic Science* 22: 131-143. DOI 10.1017/S0954102009990630
591 **Ortiz R, Navarrete H, Navarrete J, Parraga M, Carrasco I, Vega EDL, Ortiz M, Herrera P,**
592 **Blanchette RA. 2014.** Deterioration, decay and identification of fungi isolated from wooden
593 structures at the Humberstone and Santa Laura saltpeter works: A world heritage site in Chile
594 *International Biodeterioration Biodegradation* 86: 309-316.
595 http://dx.doi.org/10.1016/j.ibiod.2013.10.002
596 **Oses R, Valenzuela S, Freer J, Baeza J, Rodríguez J. 2006.** Evaluation of fungal endophytes
597 for lignocellulolytic enzyme production and wood biodegradation. *International*
598 *Biodeterioration Biodegradation* 57: 129-135. DOI 10.1016/j.ibiod.2006.01.002

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

599 **Porras-Alfaro, A and Bayman, P. 2011.** Hidden fungi, emergent properties: endophytes and
600 microbiomes. *Annual Review. Phytopathology*. 49, 291–315. DOI 10.1146/annurev-phyto-
601 080508-081831
602 **R Core Team 2015** R: A language and environment for statistical computing. R Foundation for
603 Statistical Computing, Vienna, Austria. URL http://www.R-project.org/
604 **Rabert C, Reyes-Díaz, M, Corcuera LJ., Bravo, LA., Alberdi, M. 2017.** Contrasting nitrogen
605 use efficiency of Antarctic vascular plants may explain their population expansion in
606 Antarctica. *Polar Biology*, 1-12.
607 **Roberts P, Newsham KK, Bargett RD, Farrar JF, Jones DL. 2009** Vegetation cover regulates
608 the quantity, quality and temporal dynamics of dissolved organic carbon and nitrogen in
609 Antarctic soils. *Polar Biology* 32: 999-1008. DOI 10.1007/s00300-009-0599-0
610 **Rodriguez RK, Henson J, van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y-O,**
611 **Redman RS. 2008.** Stress tolerance in plants via habitat-adapted symbiosis. The *ISME*
612 *Journal Multidisciplinary Journal of Microbial Ecology* 2(4):404–416
613 **Rodríguez RJ, White Jr JF, Arnold AE, Redman RS (2009)** Fungal endophytes: diversity and
614 functional roles. *New Phytology* 182: 314-330. DOI 10.1111/j.1469-8137.2009.02773.x
615 **Rosa LH, Vaz AB, Caligiorne RB, Campolina S, Rosa CA 2009** Endophytic fungi associated
616 with the Antarctic grass *Deschampsia antarctica* Desv. (Poaceae). *Polar Biology* 32: 161-
617 167. doi:10.1007/s00300-008-0515-z
618 **Rosa LH, Almeida Vieira MD, Santiago IF, Rosa CA .2010.** Endophytic fungi community
619 associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth)
620 Bartl.(Caryophyllaceae) in Antarctica. *FEMS Microbiology Ecology* 73: 178-189. doi:
621 10.1111/j.1574-6941.2010.00872.x

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

622 **Saikkonen K (2007)** Forest structure and fungal endophytes. *Fungal Biology Review***.** 21, 67–74
623 https://doi.org/10.1016/j.fbr.2007.05.001
624 **Schoch CL, KA Seifert, S Huhndorf, V Robert, JL Spouge, CA Levesque, W Chen, Fungal
625 Barcoding Consortium. 2012.** Nuclear ribosomal internal transcribed spacer (ITS) region
626 as a universal DNA barcode marker for Fungi. *Proceedings of National Academic and*
627 *Science USA* 109: 6241-6246. doi: 10.1073/pnas.1117018109
628 **Schulz B, Boyle C .2005.** The endophytic continuum. Mycol Res 109: 661-686. DOI
629 10.1017/S095375620500273X
630 **Spiering MJ, Greer DH, Schmid J 2006.** Effects of the Fungal Endophyte, *Neotyphodium lolii*,
631 on Net Photosynthesis and Growth Rates of Perennial Ryegrass (*Lolium perenne*) are
632 Independent of In Planta Endophyte Concentration. *Annals of Botany* - London 98: 379-387.
633 doi:10.1093/aob/mcl108
634 **Stenström E, Ndobe NE, Jonsson M, Stenlid J, Menkis A 2014.** Root-associated fungi of
635 healthy-looking *Pinus sylvestris* and *Picea abies* seedlings in Swedish forest nurseries.
636 *Scandinavian Journal Forest Research* 29: 12-21. DOI
637 dx.doi.org/10.1080/02827581.2013.844850
638 **Stone JK, Bacon CW, White JF. 2000.** An overview of endophytic microbes: endophytism
639 defined. In: Bacon CW, White JF, editors. Microbial endophytes. New York: Marcel Dekker,
640 Inc; 2000. pp. 3–30.
641 **Schoch CL, KA Seifert, S Huhndorf, V Robert, JL Spouge, CA Levesque, W Chen, Fungal
642 Barcoding Consortium. 2012).** Nuclear ribosomal internal transcribed spacer (ITS) region
643 as a universal DNA barcode marker for Fungi. *Proceeding National Academy Science* DOI
644 10.1073/pnas.1117018109

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

645 **Upson R, Newsham KK, Read DJ. 2008.** Root–fungal associations of *Colobanthus quitensis* and
646 *Deschampsia antarctica* in the maritime and sub-Antarctic. *Arctic Antarctic Alpine Research*
647 40: 592–599. DOI 10.1657/1523-0430(07-057)[UPSON]2.0.CO;2
648 **Upson R, Read DJ, Newsham KK. 2009.** Nitrogen form influences the response of *Deschampsia*
649 *antarctica* to dark septate root endophytes. *Mycorrhiza* 20: 1-11. DOI 10.1007/s00572-009-
650 0260-3
651 **van der Heijden MG, Bardgett RD, van Straalen NM 2008** The unseen majority: soil microbes
652 as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11:
653 296-310. DOI 10.1111/j.1461-0248.2007.01139.x
654 **Vilgalys R, Hester M 1990** Rapid genetic identification and mapping of enzymatically amplified
655 ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172: 4239-4246
656 **Walton DWH 1985** Cellulose decomposition and its relationship to nutrient cycling at South
657 Georgia. In: Siegfried WR, Condy PR, Laws RM (eds). Antarctic Nutrient Cycling and Food
658 Webs. Proceedings of the 4th SCAR Symposium on Antarctic Biology, Springer-Verlag,
659 Berlin, pp 192–199
660 **White TJ, Bruns T, Lee S, Taylor J. 1990.** Amplification and direct sequencing of fungal
661 ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ
662 (eds.) PCR protocols: A guide to methods and applications. Academic Press, New York, pp
663 315–322
664 **Yergeau E, Kowalchuk GA 2008** Responses of Antarctic soil microbial communities and
665 associated functions to temperature and freeze-thaw cycle frequency. *Environmental*
666 *Microbiology* 10: 2223–2235. DOI: 10.1111/j.1462-2920.2008.01644.x
667 **Zar JH. 1996.** Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

668 **Zucconi L, Pagano S, Fenice M, Selbmann L, Tosi S, Onofri S. 1996** Growth temperature
669 preferences of fungal strains from Victoria Land, Antarctica. *Polar Biology* 16: 53–61.
670 doi:10.1007/BF01876829

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

Figure 1(on next page)

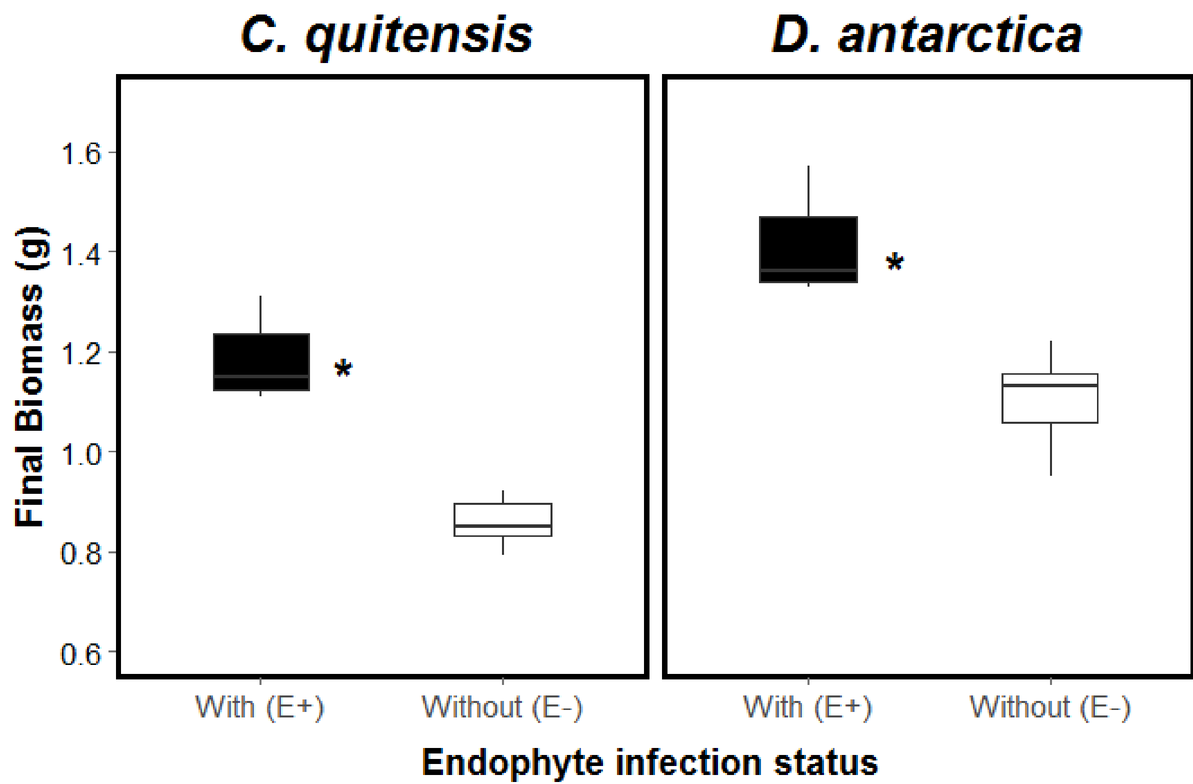
Total biomass accumulation in individuals of *Colobanthus quitensis* and *Deschampsia antarctica* with (E+) and without (E-) root fungal endophytes after mineralization assay

Penicillium chrysosgenum and *Penicillium brevicompactum* were used as root fungal endophytes for *C. quitensis* and *D. antarctica*, respectively. The mean and quartile distribution of individual plants (n = 7) are indicated. Asterisks denote significant differences between treatments ($p < 0.05$) as determined by a *t*-test

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED

Figure 1.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

Figure 2(on next page)

Kinetic of nitrogen mineralization in soils in contact with axenic individuals of *Colobanthus quitensis* and *Deschampsia antarctica* inoculated (E+) or not inoculated (E-) with root fungal endophytes

Individual plants (n = 7) emerging from benomyl-treated cultures were cultured in 300 ml pots containing autoclaved antarctic soil supplemented with urea as unique nitrogen source. Mineralization was estimated as the percentage of N-urea transformed to N-NH_4^+. Asterisks denote significant mean differences between treatments

PeerJ Preprints NOT PEER-REVIEWED

Figure 2.

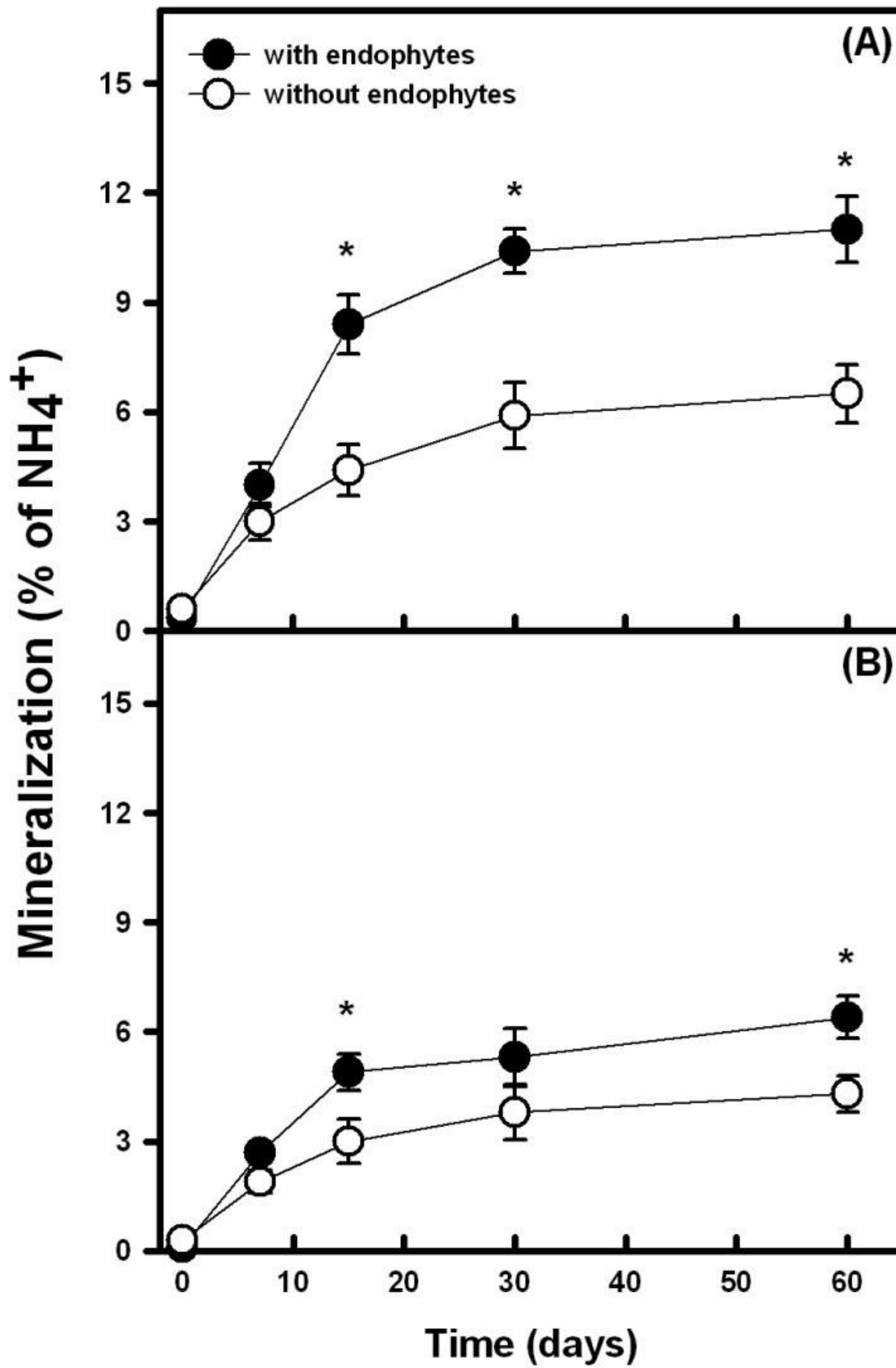


Table 1 (on next page)

Relative isolation frequencies (RIF) from root fragments of the Antarctic vascular plants *Colobanthus quitensis* and *Deschampsia antarctica* growing in coastal area in King George Island, South Shetland Islands

(a) The relative isolation frequencies (RIF) for each fungus are calculated as percentage between the number of roots colonized with single isolate divided by number of colonized root

PeerJ Preprints NOT PEER-REVIEWED

1 **Table 1.** Relative isolation frequencies (RIF) from root fragments of the Antarctic vascular plants *Colobanthus quitensis* and *Deschampsia antarctica*
2 growing in coastal area in King George Island, South Shetland Islands.

3
4
5
6
7
8

Host plant	N° of plants analyzed	N° of plants with isolated endophyte [n° (%)]	N° of roots analyzed	N° of colonized roots [n° (%)]	N° of root with single isolate [n° (%)]	Relative isolation frequency RIF (%)(a)	Isolated fungal endophyte
D. antarctica	180	133 (73.8)	1160	963 (83)	752 (64.8)	78.0	Penicillium sp.- I
		15 (8.3)			92 (7.9)	9.6	Alternaria sp I
		12 (6.6)			74 (6.4)	7.7	Alternaria sp II
		8 (4.4)			45 (3.9)	4.7	Phaeosphaeria sp
C. quitensis	150	96 (64)	1250	839 (67)	562 (45)	65.0	Penicillium sp - II
		31 (20.6)			164 (13)	21.9	Geomyces sp
		18 (12)			113 (9.0)	13.1	Microdochium sp
Total	330		2410	1802			

27

28 (a) The relative isolation frequencies (RIF) for each isolated fungus are calculated as a percentage between the number of roots colonized with single isolate divided
29 by the number of colonized root.

Table 2 (on next page)

Hydrolytic and oxidative enzymes present in the fungal endophyte *Penicillium chrysogenum* and *Penicillium brevicompactum* isolated from vascular plants Colobanthus quitensis and Deschampsia antarctica growing in the King George Island, South She

a) *Cellullose azure assay*: (-) no color release under or around mycellium; (+) blue color < 2 cm diam; (++) blue color about 2 cm diam; (+++) blue color > 2 cm diam

b) *Amylase / hemicellulose assay*: (-) absence of clearing halo around mycellium, negative for amylase; (+) clearing 1-3 cm diam; (++) clearing 3-6 cm diam; (+++) opaque halo 6 cm diam.

c) *Esterase assay*: (-) absence of opaque halo around mycellium, negative for esterase; (+) opaque halo 1-3 cm diam; (++) opaque halo 3-6 cm diam; (+++) opaque halo 6 cm diam.

d) *Phenoloxidase assay*: (-) remaining blue color (Remazol Brilliant Blue) or red color (Poly R-478) or absence of pink color (O-dianisidine) under or around mycellium, means negative for phenoloxidase; (+) discoloration of blue or red color or formation of pink color under mycellium at the center, visible only on the underside of the plate; (++) discoloration of blue or red color or formation of a pink color under most of mycellium but not extending to margin seen from under side of the plate; (+++) discoloration of blue or red color or formation of pink color extending beyond margin of fungal colony and visible from the topside of the plate. Wood decay fungi *Trametes versicolor* was used as positive control.

e) *Gelatine assay*: (-) absence of liquefaction at 4° C, negative for gelatinase; (+) liquefaction < 25% medium; (++) liquefaction 26-50% medium; (+++) liquefaction 51-75% medium; (++++) liquefaction 76-100% medium.

f) *Phosphate-solubilising assay*: (-) strong discoloration of magenta color under or around mycellium; (+) magenta color < 2 cm diam; (++) magenta color about 2 cm diam; (+++) magenta color > 2 cm diam.

g) *Urease-assay*: (-) pink color remains under or around mycellium plug; (+) discoloration of pink color < 2 cm diam; (++) discoloration about 2 cm diam; (+++) discoloration about > 2 cm diam.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

PeerJ Preprints NOT PEER-REVIEWED

1 **Table 2** Hydrolytic and oxidative enzymes present in the fungal endophyte *Penicillium chrysogenum* and *Penicillium brevicompactum* isolated from

2 the Antarctic vascular plants *Colobanthus quitensis* and *Deschampsia antarctica* growing in the King George Island, South Shetland Islands.

Hydrolytic and oxidative enzymes produced by fungal endophytes isolated from Antarctic vascular plants							
Assay	**Substrate**	*P. chrysogenum*			*P. brevicompactum*		
		4º C	**15 ºC**	**25° C**	**4º C**	**15 ºC**	**25° C**
Cellulase [a]	Cellulose-azure	++	+++	+	+	+++	-
Amylase [b]	Starch	++	+++	+	++	+++	-
Hemicellulase [b]	Xylan	+	++	+	+	+	-
Esterease [c]	Fatty acid ester (Tween 40)	+++	++++	+	++	+++	+
Phenoloxidase [d]	Remazol Brilliant Blue	-	-	-	-	-	-
	Poly R-478	-	-	-	-	-	-
	O-dianisidine	+	+	-	+	+	-
Proteinase [e]	Gelatin	++	+++	+	++	+++	+
Phosphate-solubilizing[f]	Tricalcium phosphate	++	++	+	+	+	+
Urease [g]	Urea	++	+++	+	++	+++	+

3

4

5

6

7

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26774v1 | CC BY 4.0 Open Access | rec: 27 Mar 2018, publ: 27 Mar 2018

[a] *Cellulose Azure assay*: (-) no color release under or around mycelium; (+) blue color, <2 cm diam; (++) blue color about 2 cm diam; (+++) blue color □ 2 cm diam.
[b] *Amylase / hemicellulase assay*: (-) absence of clearing halo around mycelium, negative for amylase; (+) clearing 1–3 cm diam; (++) clearing 3–6 cm diam; (+++) clearing .6 cm diam.
[c] *Esterase assay*: (-) absence of opaque halo around mycelium, negative for esterase; (+) opaque halo 1–3 cm diam; (++) opaque halo 3–6 cm diam; (+++) opaque halo 6 cm diam.
[d] *Phenoloxidase assay*: (-) remaining blue color (Remazol Brilliant Blue) or red color (Poly R-478) or absence of pink color (O-dianisidine) under or around mycelium, means negative for phenoloxidase; (+) discoloration of blue or red color or formation of pink color under mycelium at the center, visible only on the underside of the plate; (++) discoloration of blue or red color or formation of pink color under most of mycelium but not extending to margin, seen from under side of the plate; (+++) discoloration of blue or red color or formation of pink color extending beyond margin of fungal colony and visible from the topside of the plate. Wood decay fungi *Trametes versicolor* was used as positive control.
[e] *Gelatinase assay*: (-) absence of liquefaction at 4º C, negative for gelatinase; (+) liquefaction, <25% medium; (++) liquefaction 26–50% medium; (+++) liquefaction 51–75% medium; (++++) liquefaction 76–100% medium.
[f] *Phosphate-solubilizing assay*: (-) strong discoloration of magenta color under or around mycelium; (+) magenta color, <2 cm diam; (++) magenta color about 2 cm diam; (+++) magenta color >2 cm diam
[g] *Urease-assay*: (-) pink color remains under or around mycelium plug; (+) discoloration of pink color, <2 cm diam; (++) discoloration about 2 cm diam; (+++) discoloration about >2 cm diam

29