

The “Trojan horse” strategy: Seed fungal endophyte symbiosis helps to explain the invasion success of the grass, *Poa annua*, in Maritime Antarctica

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Abstract

Aim: *Poa annua* L. (annual bluegrass) is presently the sole invasive vascular plant species to have successfully established in Maritime Antarctica, where it poses a significant conservation threat to native plant species. However, the reasons for its success in the region have yet to be established. Here, we determined whether the invasiveness of *P. annua*, and its competitiveness with the native Antarctic hairgrass *Deschampsia antarctica*, is influenced by symbioses formed with seed fungal endophytes, and whether plants derived from seeds from four global regions differ in their performance.

Locations: Four regions (Maritime Antarctica, sub-Antarctica, South America and Europe).

Methods: Endophyte frequency was measured in *P. annua* seeds collected from the four regions. The germination, survival, biomass accumulation, flowering and competitiveness with *D. antarctica* of *P. annua* plants grown from endophyte-uncolonised and uncolonised seeds was determined in the laboratory. The effects of endophytes on *P. annua* seed germination and survival and seedling osmoprotection were also assessed in the Maritime Antarctic natural environment using locally-sourced seeds.

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Results: Endophytes were at least twice as frequent in seeds from Maritime Antarctica than in those from other regions. A higher proportion of endophyte-colonized seeds germinated and survived than did uncolonised seeds, but only when they originated from Maritime Antarctica. Seed endophytes increased the competitiveness of *P. annua* with *D. antarctica*, but only for plants grown from Maritime Antarctic seeds. In the field, endophyte-colonized seeds from Maritime Antarctica germinated and survived more frequently than uncolonised seeds, and osmoprotection was higher in seedlings grown from colonized seed.

Main Conclusions: The findings indicate beneficial effects of seed endophytes on invasion-related traits of *P. annua*, such as survival, germination success and flowering. Together with vegetative and reproductive traits facilitating the colonization process, the seed-fungal endophyte symbiosis can be invoked as an important factor explaining the invasiveness of *P. annua* in Maritime Antarctica.

KEYWORDS

annual bluegrass (*Poa annua*), Antarctic hairgrass (*Deschampsia antarctica*), biological invasion, *Cladosporium*, King George Island, Maritime Antarctica, seed fungal endophytes, symbiosis

1 | INTRODUCTION

Biological invasions are one of the greatest threats to natural ecosystems worldwide (Pyšek et al., 2020). They can have profound negative consequences for ecosystem functioning and processes, biodiversity and the conservation of native species, and are hence of major global concern (Bertelsmeier et al., 2013; Mačić et al., 2018). With species introductions being enhanced by increasing anthropogenically assisted dispersal and climate change, establishing the impacts of invasive species on natural ecosystems is pivotal to identifying high-risk species and habitats in the future (Chown et al., 2012; Kumshick & Richardson, 2013). Historically, Antarctica has been thought to be at relatively low risk from biological invasions, owing to its geographical isolation and harsh climatic conditions (Hughes et al., 2015, 2020). However, developing human activity, particularly since the 1950s, has increased the unintentional introduction of non-native organisms to the continent (Chown et al., 2012; Hughes et al., 2020; Znoj et al., 2017). Moreover, since the mid-20th century, Maritime Antarctica (i.e., the Antarctic Peninsula and Scotia Arc) has been strongly affected by regional climatic change (Chown et al., 2022; Turner et al., 2014), reflected in rapid warming and an expansion of terrestrial ice-free areas with increased availability of liquid water (Frenot et al., 2005; Lee et al., 2017; Siegert et al., 2019). These milder environmental conditions favour the establishment and spread of invasive plant species (Atala et al., 2019; Malfasi et al., 2020), posing serious threats to Antarctic ecosystems (Galera et al., 2018; Molina-Montenegro et al., 2019). Moreover, the low diversity (poor biotic resistance) and remote and patchy biogeography (island susceptibility) of Maritime Antarctic ecosystems make them particularly susceptible to invasion by non-native species (Perterra et al., 2022), with biological invasions being

identified as one of the primary conservation risks for the broader Antarctic (Chown et al., 2012; Duffy et al., 2017).

Poa annua L. (annual bluegrass) is considered to be one of the most invasive plant species worldwide (Gardarin et al., 2009), and, at present, is the sole invasive vascular plant species present in Maritime Antarctica (Chwedorzewska et al., 2015; Molina-Montenegro et al., 2019). Since 1953, the species has been repeatedly introduced to at least eight different locations in the region between Signy Island (60° S) and Galíndez Island (65° S), typically in areas close to research stations or those visited frequently by tourists (Chwedorzewska et al., 2015; Malfasi et al., 2020). Clothing or cargo is considered to be the main vectors for the introduction of seeds and other propagules from other continents (Chown et al., 2012; Hughes et al., 2010; Huiskes et al., 2014). Although *P. annua* has been eradicated from most of the locations in which it has been discovered, on King George Island (South Shetland Islands, 62° S), where the founder population was initially left uncontrolled, the species has rapidly invaded local natural tundra, where it competes vigorously with the two native Antarctic vascular plant species, *Deschampsia antarctica* and *Colobanthus quitensis* (Molina-Montenegro et al., 2019; Olech & Chwedorzewska, 2011), negatively impacting their biomass, fecundity and survival (Ballesteros et al., 2022; Cavieres et al., 2018; Molina-Montenegro et al., 2012, 2019). However, despite the successful establishment of *P. annua* in Maritime Antarctica, and the threat posed by the species to the conservation of native vascular plants, the mechanisms underlying its environmental tolerance and competitiveness in the region have seldom been assessed (but see Ballesteros et al., 2022; Perterra et al., 2017).

Symbioses between plants and endophytic fungi are ubiquitous components of natural ecosystems, where they exert profound impacts on communities through their effects on plant fitness and

stress tolerance (Rodríguez et al., 2009). The effects of such symbioses on plant invasion success have been investigated in different ecosystems (Dickie et al., 2017; Molina-Montenegro et al., 2015; Traveset & Richardson, 2020; van der Putten et al., 2007), with some studies suggesting that plant-microbe symbioses are key strategies or even “novel weapons” contributing to invasiveness (Callaway & Ridenour, 2004; Elsheikh et al., 2021). Several studies indicate that beneficial symbioses with fungal endophytes that colonize seeds and are transmitted vertically between generations may allow vascular plants to survive harsh Antarctic environmental conditions (Acuña-Rodríguez et al., 2020; Barrera et al., 2020; Hereme et al., 2020). A recent study has shown that the release of allelochemicals – chiefly phenolic compounds – by seed endophytes of *P. annua* negatively affect the biomass and fecundity of *D. antarctica* and *C. quitensis*, possibly enhancing the competitiveness of *P. annua* in the natural environment (Ballesteros et al., 2022). Although the role of biotic interactions in mediating plant invasions is now well established, the role of endophytes in this regard remains poorly studied (but see Traveset & Richardson, 2020). Moreover, the putative role of seed fungal endophytes as a novel acquired strategy accounting for the success of *P. annua* in Maritime Antarctica has not previously been examined.

Here, we hypothesized that, compared with other global regions (Europe, South America and sub-Antarctica), the harsher environmental conditions of the Maritime Antarctic might select for *P. annua* individuals forming symbioses with seed fungal endophytes. We compared the frequency of endophytes in *P. annua* seeds from these regions and also explored the possibility that endophytes have differential effects on *P. annua* performance in Maritime Antarctica, either in monoculture or in competition with *D. antarctica*. Finally, we hypothesized that *P. annua* individuals originating from seeds colonized by endophytes would perform better in the Maritime Antarctic natural environment compared with those from seeds without endophytes, thereby contributing to the success of the plant species in the region.

2 | METHODS

2.1 | Frequency of fungal endophytes in *P. annua* seeds and its relationship with regional macroclimate

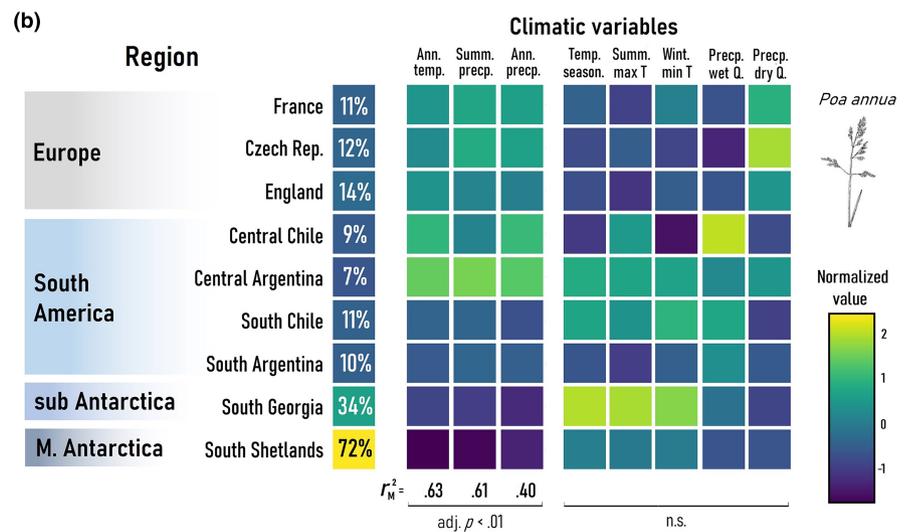
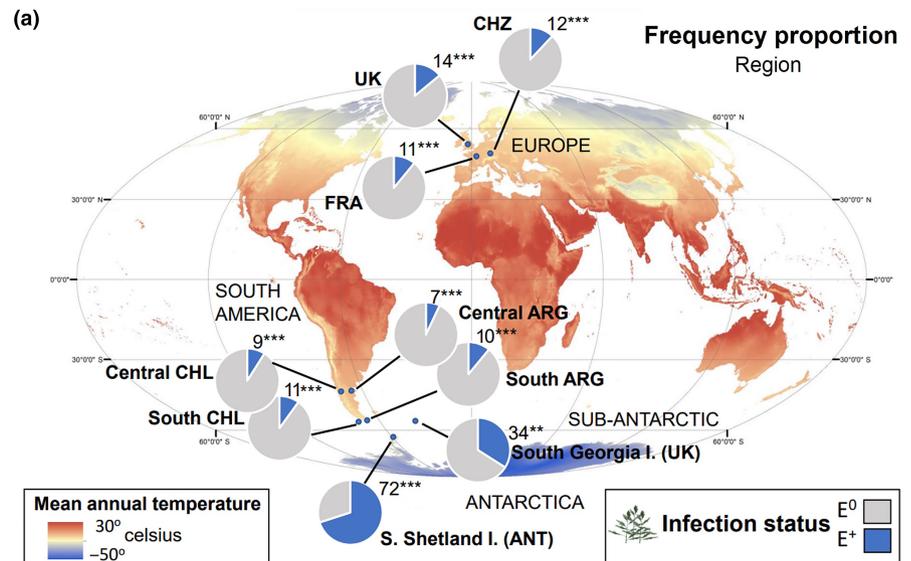
Mature seeds of *P. annua* were collected in 2019 from nine populations clustered in four global regions, viz., (1) Europe: England (51°35' N; 00°46' E), Czech Republic (50°04' N; 14°36' E) and France (48°58' N; 02°42' E); (2) South America: Chile (33°02' S; 71°36' W and 53°07' S; 70°53' W) and Argentina (34°33' S; 58°24' W and 54°48' S; 68°17' W); (3) sub-Antarctica: South Georgia (54°17' S; 36°30' W) and (4) Maritime Antarctica: King George Island (62°09' S; 58°28' W) (Figure 1). Europe is within the native range of *P. annua*, whereas the species is naturalized in South America, and is currently spreading in sub-Antarctica and Maritime Antarctica (Molina-Montenegro et al., 2012; Pertierra et al., 2017).

One hundred adult *P. annua* plants, typically separated by at least 2 m, were selected in eight of the nine populations. Only 49 plants could be located in the Maritime Antarctic population. All of the selected plants had mature spikes and no apparent damage to leaves or reproductive structures. At least three seeds were collected from a minimum of five different spikelets on each plant in its reproductive stage, with seed status being visually assessed just before collection to ensure that only mature and undamaged seeds were collected. Then, three seeds from each individual plant were randomly selected to assess for the presence of endophytic fungi. First, two seeds were cut in half transversely with a razor blade and were stained with aniline blue prior to observation at 400× magnification (Motic BA310) to record stained fungal hyphae. Second, the remaining seed from each plant was pressed into potato dextrose agar (PDA, Difco) in 90 mm diameter Petri dishes prior to being incubated at 18°C for 2 weeks. Maternal plants were considered to be endophyte-free when there was no visual presence of any endophytic fungal hyphae in seeds and no outgrowth of hyphae into the PDA medium, and were considered to be colonized by endophytes when stained hyphae were observed in seeds and when hyphae grew from seeds onto the medium. The remaining seeds from each of 10 plants in each population that were endophyte-free, and 10 that were endophyte-colonized, were used in the experiments described in Sections 2.2 and 2.3. These seeds are hereafter referred to as E⁰ and E⁺, respectively.

2.2 | Effects of fungal endophytes on *P. annua* performance under controlled conditions

To assess the effects of seed endophytes on different ecophysiological traits (germination, survival, reproductive structures and biomass), we conducted an experiment using a total of 720 *P. annua* seeds, consisting of 180 seeds from each region. The seeds were sown in pots (50 mL capacity) containing an autoclaved mixture of sterile soil collected from King George Island, sand and peat (2:1:1). For each region, 180 individual pots, 90 containing E⁺ seeds and 90 containing E⁰ seeds, were divided equally between three automated growth chambers (Bioref 38 L, PITEC) and were maintained for 90 days under simulated Maritime Antarctic summer conditions (temperature 4°C, photosynthetic photon flux density 350 μmol photons m⁻² s⁻¹, 20/4 h light/dark photoperiod and 75% relative humidity). Each pot was watered every 4 days with 7 mL of water. To avoid a potential chamber (block) effect, all plants were randomized within each chamber during irrigation and were rotated between chambers weekly. The effect of presence of seed endophytes on germination rate was measured by counting the number of new seedlings emerging every 2 days, while plant survival was recorded weekly. Plant reproductive structures were measured by recording the number of spikelets on each individual every week. Total fresh biomass was measured after washing and weighing plant tissues on a digital electronic balance (Boeco BBL-52) to 0.01 g precision. Total dry biomass was recorded after oven-drying tissues at 70°C for 70 h.

FIGURE 1 (a) Incidence of fungal endophytes (%) in seeds of *Poa annua* individuals from different sites: England (UK), Czech Republic (CHZ), France (FRA), Central Chile (Central CHL), Central Argentine (Central ARG), Southern Chile (South CHL), Southern Argentina (South ARG), South Georgia (SubANT), and the South Shetland Islands, Maritime Antarctica (ANT). In the pie charts, grey denotes the absence of fungal endophytes from *P. annua* seeds (E^0), while blue denotes the presence of the endophytes (E^+). The map of mean annual temperature was obtained from WorldClim 2.0. (b) fungal endophyte frequencies in *P. annua* seeds from nine populations in four regions. The heatmap shows the climatic variables and Mantel correlation coefficients (r_M^2) between each variable and the frequency of endophytes. Ann. prec., annual precipitation; Ann. temp., mean annual temperature; Precp. dry Q., precipitation of driest quarter; Precp. wet Q., precipitation of wettest quarter; Summ. max T, maximum temperature of hottest month; Summ. prec., precipitation of the hottest quarter; Wint. min T, minimum temperature of coldest month. Asterisks denote significant differences (** $p < .001$; *** $p < .0001$).



2.3 | Effects of fungal endophytes on the competitive ability of *P. annua*

The influence of seed endophytes on the ability of *P. annua* to compete with *D. antarctica* was tested by calculating the relative competition intensity index (RCI; Grace, 1995), using final biomass as the response variable (Molina-Montenegro et al., 2015). For each region, 60 pots (50 mL capacity) containing the same mixture of sterile soil, sand and peat as described above were prepared. Fifteen of the 60 pots were each planted with two *P. annua* individuals grown from E^+ seeds, and another 15 pots were each planted with two *D. antarctica* individuals. The remaining 30 pots were each planted with *P. annua* and *D. antarctica* individuals in pairs, with the *P. annua* individuals in 15 of these pots having been grown from E^+ seeds and those in the other 15 having been grown from E^0 seeds. The 60 pots from each region were divided equally between the three automated growth chambers under the same conditions as described above. The pots were again randomized and rotated between chambers as previously described. After 90 days, plants were harvested as described above

and the relative competitive impact of *P. annua* on *D. antarctica* was determined as $RCI_{Da} = (\lg_{Da:Da} - \lg_{Da:Pa}) / \lg_{Da:Da}$, and the resistance of *D. antarctica* to invasion by *P. annua* as $RCI_{Pa} = (\lg_{Pa:Pa} - \lg_{Da:Pa}) / \lg_{Pa:Pa}$, where Pa and Da represent the biomasses of *P. annua* and *D. antarctica*, respectively (Molina-Montenegro et al., 2015).

2.4 | Effects of seed endophytes on *P. annua* performance in the field

Growth chamber experiments were conducted to assess the effects of seed endophytes on *P. annua* performance (biomass production and survival) and traits related to osmoprotection (leaf proline accumulation and late embryogenesis abundant [LEA] gene expression) in the Maritime Antarctic natural environment. Leaf proline concentration and LEA1 gene expression, which are influenced by the presence of endophytes, have been suggested to be mechanisms used by plants to survive harsh abiotic conditions (Acuña-Rodríguez et al., 2022; Azizi et al., 2021). Seeds were collected

from inflorescences of *P. annua* plants in the vicinity of Arctowski Station on King George Island (62°09' S; 58°28' W) in January 2019 and were air-dried and maintained in clean paper bags until January 2020. The presence of absence of endophytes in maternal plants was determined using the methods described in Section 2.1, and seed status was assessed visually shortly before the assays to ensure that undamaged seeds were used, with empty, broken, or damaged seeds being rejected. The E⁺ seeds were divided into two groups. One group was maintained as seeds with fungal endophytes, while the other group, hereafter referred to as E⁻, was submersed in the fungicide Benlate® (2 g L⁻¹; DuPont) for 30 min at room temperature to remove endophytes. Benlate has a broad spectrum of action and is harmless to the seeds of Antarctic plants (Ballesteros et al., 2022; Barrera et al., 2020; Hereme et al., 2020). Hence, three different groups of seeds (E⁺, E⁰ and E⁻) were obtained at the start of the experiment. A subset of seeds from each group was also tested to assess viability by transversely cutting the seed in half with a razor blade after 24 h of immersion in tetrazolium chloride. This test is a colorimetric method that differentiates live (red) from dead (colourless) seeds, based on the activity of respiratory enzymes in the seeds (Franca-Neto & Krzyzanowski, 2019).

Field experiments were conducted in two ways. First, 30 seeds of *P. annua*, 10 each from the E⁺, E⁰ and E⁻ groups, were sown into each of five plastic trays (50 × 30 × 10 cm) filled with soil from King George Island that had been sterilized by autoclaving. Seeds were sown at 1–2 cm depth, which is the approximate depth at which *P. annua* seeds are found at the study site (pers. obs.). The trays were then sunk into the ground in pits separated by ≥ 3 m in the vicinity of Arctowski Station. The experiment ran for 2 months, with the number of seedlings that emerged being recorded every 2 days. Second, to examine the effect of seed endophytes on *P. annua* survival and osmoprotection in the natural environment, 75 seeds from each group (E⁺, E⁰ and E⁻) were germinated and 50 individuals of approximately equal (5–6 cm) height were planted into five seed trays of the same size, and containing the same sterile soil, as those described above. The seedlings in each tray were separated by at least 5 cm. As above, the five trays were placed into pits separated by ≥ 3 m close to Arctowski Station. The experiment ran for 3 months, during which seedling survival was recorded every 5 days. Proline concentrations in *P. annua* leaves were measured using a modification of the protocol of Bates et al. (1973). After 90 days, approximately 25 mg of foliar tissue collected from 15 plants from each of the three groups was frozen in liquid nitrogen and pulverized in 3% sulfosalicylic acid. The extract was then centrifuged at 16,000 g at room temperature for 20 min. An aliquot of the supernatant was added to 2 mL of ninhydrin reagent and was incubated in a water bath at 90°C for 1 h to allow colour development. The solutions were cooled, and 2 mL of toluene were added to separate the chromophore. Absorbance was measured at 525 nm and proline concentrations were calculated and expressed as μmol g⁻¹ FW (Molina-Montenegro et al., 2016).

The relative expression of the LEA1 gene was measured in five *P. annua* plants from each of the three treatment groups by

extracting total RNA from leaves after 3 weeks of growth using a modified perchlorate method (5 M sodium perchlorate, 300 mM Tris-HCl pH 8, 1% v/v SDS, 2% v/v PEG 20,000, 8.5% p/v PVPP, 3% v/v 2-mercaptoethanol; Davies & Robinson, 1996). DNA was removed from the samples by DNase treatment using Turbo DNase (Ambion). Single-stranded cDNAs were then synthesized from 2 μg of total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Determination of relative transcript abundance of the LEA1 gene was carried out by quantitative real-time PCR analysis. For this gene, specific forward and reverse LEA1 primers (Fw: 5' TGAGCGTGATGAGG AAGTCG 3' - Rv: 5' CACCAACCCATACTCCCACC 3') were designed using the NCBI PrimerBlast tool, to the reference sequence GCZY01014682.1, obtained from a *P. annua* transcriptome assembly available at NCBI (Chen et al., 2016). Each PCR reaction contained 2 μL of diluted cDNA (50 ng), 10 μL of Maxima SYBR Green PCR Master Mix (Thermo Fisher Scientific), 6.4 μL of nuclease free-water and 0.8 μL of each forward and reverse primer (each at 10 μM). All reactions were carried out in triplicate (three technical replicates per biological replicate) using the Mx3000P qPCR system (Agilent) under the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s, with a final cycle at 95°C for 15 s, 54°C for 15 s and 95°C for 30 s. The relative expression levels of the LEA1 gene in *P. annua* leaves from three biological replicates per experimental group were calculated using the comparative 2^{-ΔΔCT} method (Livak & Schmittgen, 2001) with elongation factor 1α as the normalizer gene, following LaForest et al. (2021).

2.5 | Data analyses

Chi-squared (χ^2) tests of independence were used to determine if the proportion of seeds infected with fungal endophytes was independent of the region of origin. Mantel tests were used to assess correlations between the matrix of distances produced by the differences between populations in endophyte infection frequencies, and the respective distance matrix of each of eight bioclimatic variables from WorldClim data (1970–2000; Fick & Hijmans, 2017), four of which were associated with temperature (Bio1: mean annual temperature, Bio4: temperature seasonality, Bio5: maximum temperature of hottest month, Bio6: minimum temperature of coldest month) and four with precipitation (Bio12: annual precipitation, Bio13: precipitation of wettest quarter, Bio14: precipitation of driest quarter, Bio18: precipitation of the warmest quarter). Bioclimatic variables were obtained for each location by extracting the variable value from the respective 10-min resolution Bioclim layer with the *terra* R-package (Hijmans, 2022). Raw data for each variable were then transformed to a “between-locations” distance matrix, and the correlation between the distance matrix derived from the data on fungal infection proportion and each environmental distance matrix was analysed with the “mantel” function from the *vegan* R-package (Oksanen et al., 2022).

Linear mixed models (LMMs) were used to test the effects of seed endophytes on *P. annua* germination, survival, final biomass and the production of reproductive structures within each region using the “lme” function in the R-package *nlme* (Pinheiro et al., 2021), including experimental block (i.e., growth chamber) as a random factor in the model error structure (Pinheiro & Bates, 2000). LMMs were also used to evaluate the effects of seed endophytes on RCI, using independent models to test competition in allospecific and conspecific pairs of individuals. Finally, LMMs were used to test for the effects of endophytes on *P. annua* performance (germination, survival, leaf proline concentration and LEA1 gene expression) in plants derived from E⁺ and E⁻ seeds compared with those from E⁰ seeds. To establish specific pair-wise differences between the three experimental groups, a comparison of their marginal mean values was performed through the *emmeans* R-package (Lenth, 2021). All data were corrected for multiple comparisons using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). Analyses were performed in the R environment for statistical computing v.4.2.0 (R Core Team, 2021).

3 | RESULTS

3.1 | Frequency of fungal endophytes in *P. annua* seeds and relationships with macroclimatic patterns

Fungal endophytes in *P. annua* seeds from Maritime Antarctica were significantly more frequent than in seeds sampled from Europe, South America and sub-Antarctica (Figure 1a, Figure S1; all $\chi^2 = 221.8$, $df = 8$, $p \leq .0001$). In seeds from Europe and South America, mean endophyte frequencies ranged from 7% to 14%, with a frequency of 34% being recorded in seeds from South Georgia (Figure 1a). In each case, the seeds from these three regions were less frequently colonized by endophytes than would be expected by chance (Figure S1). In contrast, the frequency of endophytes in *P. annua* seeds from Maritime Antarctica was 70% (Figure 1a), with these seeds being more frequently colonized than expected by chance ($\chi^2 = 19.36$, $df = 1$, $p \leq .0001$). The higher incidence of seed endophytes in Maritime Antarctica was associated with macroclimatic gradients, with Mantel tests indicating significant negative correlations between endophyte incidence and mean annual temperature (bio1) and summer (bio18) and annual precipitation (bio12), and with the strongest negative correlations being recorded in the Maritime Antarctic (Figure 1b). Five other macroclimatic predictors, encompassing seasonality, maximum and minimum temperatures and seasonal precipitation, were not significantly associated with the frequency of seed endophytes in any region (Figure 1b).

3.2 | Effects of fungal endophytes on *P. annua* performance under controlled conditions

Linear mixed models indicated that endophytes had highly significant ($p < .0001$) effects on the germination of Maritime Antarctic

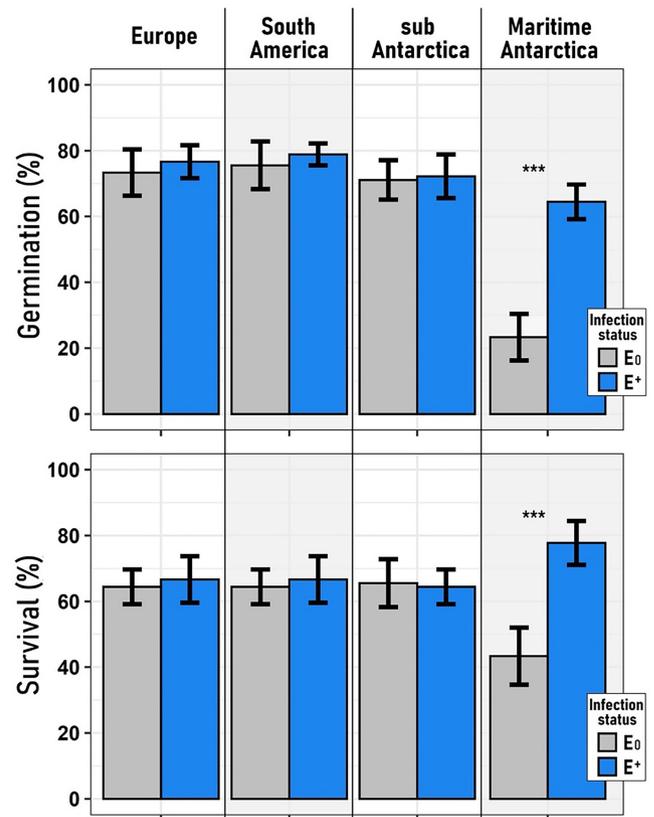


FIGURE 2 Germination and survival of *Poa annua* plants from Europe, South America, sub Antarctica and Maritime Antarctica grown from uncolonised seeds (E⁰) or seeds colonized by fungal endophytes (E⁺). Values are means ($n = 30$) \pm SD. Asterisks denote significant differences (***) $p < .0001$ between E⁰ and E⁺ means in LMM analyses. See Table S1 for random effects in each model.

P. annua seeds under controlled conditions (Table S1), with endophytes approximately tripling germination from 23% for E⁰ seeds to 64% for E⁺ seeds (Figure 2). In contrast, LMMs showed that endophytes had no effects on the germination of seeds from Europe, South America and sub-Antarctica (Table S1), with the germination of seeds from these regions ranging from 71% to 79% regardless of endophyte infection status (Figure 2). Survival was also strongly affected by the presence of endophytes, but, as for germination, only for Maritime Antarctic seedlings, with LMMs similarly indicating a highly significant ($p < .0001$) effect of endophytes on the survival of plants from this region (Table S1). For Maritime Antarctic seedlings, endophytes almost doubled survival from 43% for those grown from E⁰ seeds to 78% for those grown from E⁺ seeds (Figure 2). The survival of seedlings from other regions, which ranged from 64% to 66%, was unaffected by the presence of endophytes (Table S1; Figure 2).

Seedling biomass followed a different pattern, with endophytes only exerting significant ($p = .0048$) effects on the mass of seedlings from sub-Antarctic South Georgia, and not on that of seedlings from the other regions (Table S2). Biomass increased from 2.4 g to 2.6 g for E⁰ and E⁺ seedlings from South Georgia, respectively (Figure 3). Reproductive investment (i.e., number of flowers) increased for plants

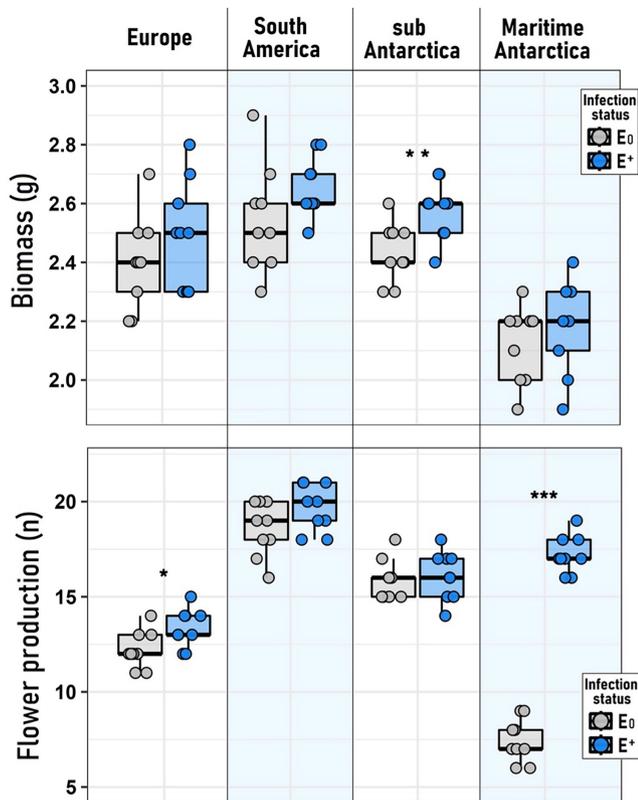


FIGURE 3 Biomass and flower production of *Poa annua* plants from Europe, South America, sub Antarctic and Maritime Antarctica grown from uncolonised seeds (E^0) or seeds colonized by fungal endophytes (E^+). Boxplots show the interquartile data distribution ($n=10$) of each group, and the dots correspond to individual values. Asterisks denote significant differences (* $p < .05$; ** $p < .001$; *** $p < .0001$) between E^0 and E^+ means in LMM analyses. See Table S2 for random effects in each model.

derived from endophyte-infected seeds from Maritime Antarctica and Europe ($p=.0001$ and $p=.0456$, respectively; Table S2). The mean numbers of flowers on plants grown from E^0 and E^+ seeds from Maritime Antarctica were 7.4 and 17.0, and were 12.0 and 13.0 on plants from European E^0 and E^+ seeds, respectively (Figure 3).

3.3 | Effects of fungal endophytes on the competitive ability of *P. annua*

Poa annua from each of the four regions outcompeted the native Antarctic grass *D. antarctica*, as shown by the typically positive and negative RCI values for the two species, respectively (Figure 4). For *P. annua* seeds from Europe, South America and sub-Antarctica, LMMs indicated that the infection status of the seeds from which the *P. annua* seedlings were derived had no effects on the outcomes of interactions with *D. antarctica* (Table S3; Figure 4a–c). In contrast, LMMs showed that endophytes had highly significant ($p < .0001$) effects on the outcomes of competitive interactions between the two species when *P. annua* had been grown from E^+ seeds from Maritime Antarctica (Table S3). For this region, mean RCI for

D. antarctica grown in competition with *P. annua* seedlings from E^0 seeds was -0.17 , which declined to -0.45 when the former species was grown with *P. annua* derived from E^+ seeds (Figure 4d). In contrast, for *P. annua* from the Maritime Antarctic, mean RCI was -0.04 for seedlings derived from E^0 seeds, whereas endophytes exerted a strong positive effect on the competitiveness of these plants, with a mean RCI of 0.2 being recorded for seedlings derived from E^+ seeds (Figure 4d).

3.4 | Effects of seed endophytes on *P. annua* performance in the field

The two field experiments both indicated positive effects of endophytes on *P. annua* performance in natural Maritime Antarctic tundra. Seed germination increased significantly ($p < .0001$) from 20% to 22% in seedlings grown from E^0 and E^- seeds to 62% in seedlings from E^+ seeds (Table S4; Figure 5a). Similarly, endophytes significantly ($p < .0001$) increased the survival of *P. annua* seedlings in the field, with 18% and 26% survival of seedlings from E^0 and E^- seeds, respectively, and 48% survival of those from E^+ seeds (Table S4; Figure 5b). Endophytes also increased seedling osmoprotection, with mean proline concentrations of 5.2 and $5.8 \mu\text{mol g}^{-1}$ FW in the leaves of seedlings grown from E^0 and E^- seeds, a mean proline concentration of $7.7 \mu\text{mol g}^{-1}$ FW in seedlings from E^+ seeds (Figure 5c), and highly significant ($p < .0001$) differences between each of the three means (Table S4). Similarly, the relative expression level of the LEA1 gene was increased by seed endophytes, with mean expression levels of 2.4 and 2.7 in the leaves of seedlings grown from E^0 and E^- seeds, a mean expression level of 3.8 in those of seedlings from E^+ seeds (Figure 5d), and highly significant ($p < .0001$) differences between all three means (Table S4).

4 | DISCUSSION

Our data indicate that the formation of the symbiosis with seed fungal endophytes may be a successful strategy used by *P. annua* to cope with the harsh environmental conditions encountered in Maritime Antarctica. The frequency of the symbiosis in Maritime Antarctic seeds was more than fourfold that in European and South American seeds, and was more than double that in seeds from sub-Antarctic South Georgia, suggesting that plants in Maritime Antarctic environments may rely on the symbiosis for their successful establishment (Ballesteros et al., 2022; Znój et al., 2021). Our observations further support the view that beneficial plant-microbial symbioses increase in prevalence in more stressful environments (*c.f.* Saona et al., 2010; Newsham, 2011). Laboratory and field experiments both pointed to significant positive effects of seed endophytes on invasion-related traits of *P. annua*, such as survival, germination rate and fecundity, relative to plants grown from seeds lacking endophytes. Importantly as a factor explaining the invasiveness of the plant species in Maritime Antarctica and its impacts on the conservation of native

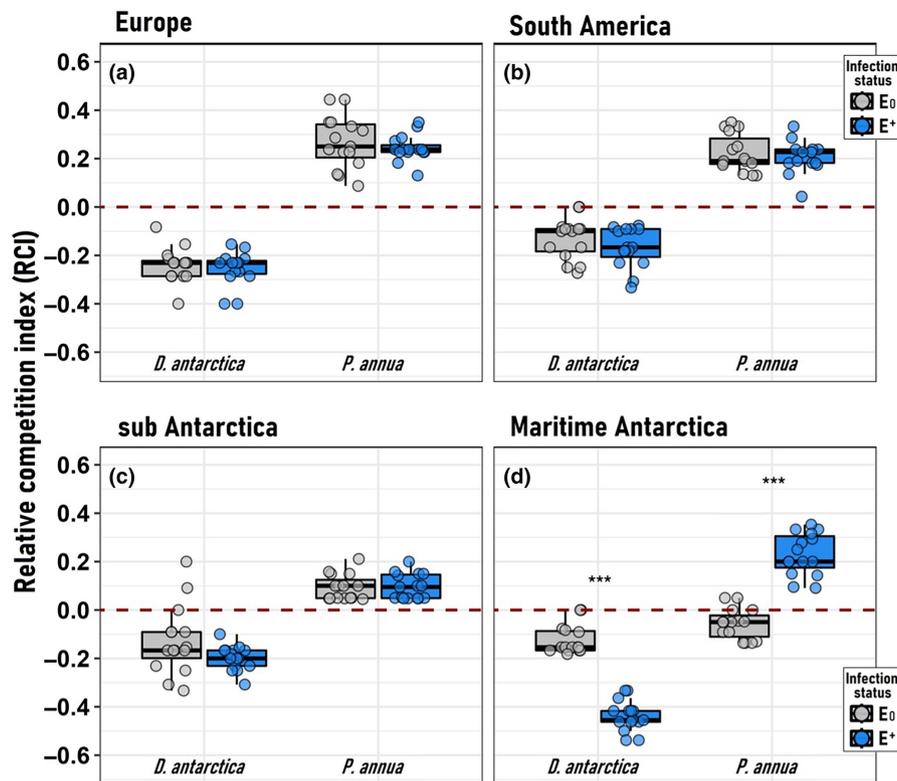


FIGURE 4 Relative competition index (RCI) of *Poa annua* from (a) Europe, (b) South America, (c) sub Antarctica and (d) Maritime Antarctica grown from uncolonised seeds (E^0) or seeds colonized by fungal endophytes (E^+) in conspecific pairs or allospecific pairs with *Deschampsia antarctica*. RCI values of zero, denoted by the horizontal red lines, indicate the absence of a competitive effect, whereas positive values indicate a competitive gain (i.e., increased biomass when grown in allospecific pairs, compared with conspecific pairs), and negative values indicate competitive loss (i.e., reduced biomass when grown in allospecific pairs, compared with conspecific pairs). Boxplots represent the interquartile data distribution ($n=10$) of each group and the dots correspond to individual values. Asterisks denote significant differences ($***p < .0001$) between E^0 and E^+ means in LMM analyses. See Table S3 for random effects in each model.

Antarctic vascular plant species (Chown et al., 2012; Chwedorzewska et al., 2015; Molina-Montenegro et al., 2019), we also found that endophytes in *P. annua* seeds increased the competitiveness of the species when grown with *Deschampsia antarctica*, with this effect only being evident for *P. annua* plants derived from Antarctic seeds. The seed-endophyte symbiosis may also help to explain why *P. annua* is able to grow in locations beyond the species' predicted range envelope, with a previous study finding substantial unexplained niche novelty for its southernmost populations (Perterra et al., 2017).

Root fungal endophytes have been shown to improve the performance and environmental stress tolerance of the native Antarctic plant species *Colobanthis quitensis* through physiological and molecular osmoprotection mechanisms (Hereme et al., 2020). Similarly, our observations indicate that seed endophytes may confer benefits on *P. annua* in Maritime Antarctica, possibly through increased foliar proline concentrations and LEA1 gene expression, which are common responses of plants to a range of environmental stresses (Vujanovic et al., 2022). Proline is a stress-related amino acid involved in broad-based antioxidant responses, in which it suppresses reactive oxygen species (ROS) or inhibits ROS-mediated cellular apoptosis (Vujanovic et al., 2022). LEA proteins act as molecular chaperones and have roles in the maintenance of protein and

membrane structures, the sequestration of ions and the binding of water. Together, these roles are pivotal to osmotic adjustment and responses to stresses such as aridity (Dastogeer, 2018). It is plausible that proline and LEA proteins, which are transcribed from plant LEA genes (Jin et al., 2019), increase in the leaves of *P. annua* when plants are exposed to abiotic stress factors, and that these responses are amplified in the presence of endophytic microbes (Dastogeer et al., 2017; Pedrosa et al., 2015). This suggests that the osmoprotective responses observed here are a consequence of endophytic fungi triggering plant primary regulatory genes involved in stress responses, such as phytohormone signalling, lipid metabolism and the production of osmolytes (Ganie et al., 2022). However, these responses may also depend on host-endophyte interactions, as, in some cases, a reduction in proline concentration has been observed when endophyte-colonized plants are exposed to stressful conditions (Dastogeer, 2018).

Alternatively, it is possible that the increased concentrations of proline in the leaves of *P. annua* growing from endophyte-colonized seeds might arise directly from fungal metabolism (Khan & Lee, 2013). Proline is synthesized by a wide range of fungi (Jastrzębowska & Gabriel, 2015), including members of the genus *Cladosporium*, which, whilst apparently absent from soils around plants on King George

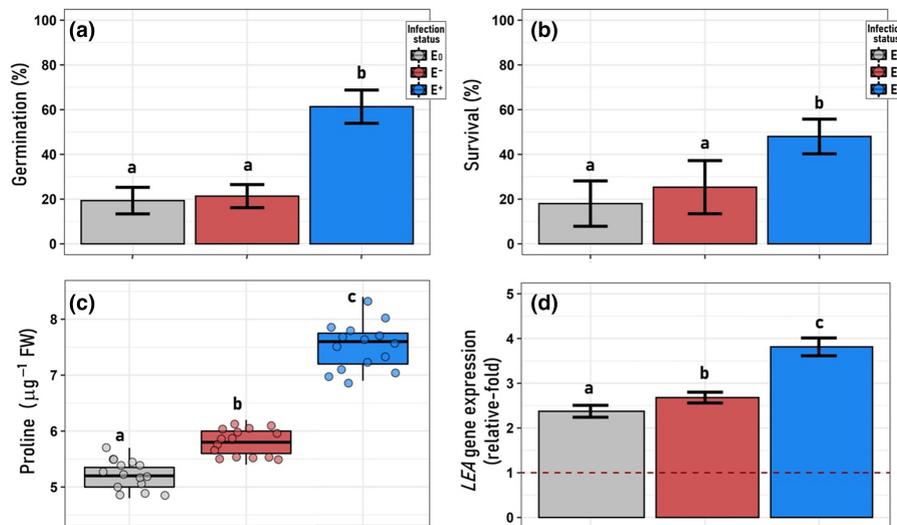


FIGURE 5 (a) Germination, (b) survival, (c) foliar proline concentration and (d) LEA1 gene expression of Antarctic *Poa annua* seedlings grown under field conditions. Seedlings were grown from *P. annua* seeds from Maritime Antarctica that were either uncolonised or colonized by fungal endophytes (E⁰ and E⁺, respectively) or were treated with Benlate to remove endophytes (E⁻). Values in (c) and (d) are means of 15 replicates and those in other panes are means of 50 replicates. Bars show SD. The red line in (d) indicates the respective expression of the normalizer gene (elongation factor 1 α) in each treatment group. Bars marked with distinct letters indicate significant differences ($p < .05$) following pairwise marginal mean comparisons.

Island, are present at very high frequency (~95%) in the tissues of *P. annua* plants, and particularly seeds, growing in these soils (Ballesteros et al., 2022). *Cladosporium* species, which are common fungal endophytes in many plant tissues and seeds (Depetris et al., 2020; Murphy et al., 2018), are adapted to cold environments and are known to synthesize proline (Ma et al., 2018), perhaps accounting for the increased concentrations of the amino acid in *P. annua* leaves observed here. Members of the genus confer protection against different biotic and abiotic stresses, producing and releasing beneficial secondary metabolites, biostimulants and allelochemicals, and have positive effects on crop, growth rate, seed germination and survival (Murphy et al., 2018; Pan et al., 2020; Răut et al., 2021). Hence, the improved performance and competitive ability of *P. annua* plants from Maritime Antarctica originating from seeds harbouring *Cladosporium*, and possibly other fungi, could arise not only from the increased frequency of the endophytes in seeds, but also from metabolites produced by endophytes conferring enhanced environmental tolerance and competitive abilities on the host plant (Ballesteros et al., 2022).

It has previously been reported that allelochemicals released from roots enable alien plant species to successfully invade new habitats (Lorenzo et al., 2013, and references therein). These allelochemicals can be thought of as “novel weapons” deployed by invasive species (sensu Callaway & Ridenour, 2004). They impact native plant species by reducing stomatal conductance and photosynthesis, decreasing nutrient uptake and increasing ROS concentrations, which trigger a cell death cascade in susceptible plants (Weir et al., 2004). Although endophytes are known to synthesize allelochemical compounds in plant tissues (Aschehoug et al., 2014; Cipollini et al., 2012), further studies into the secondary metabolites synthesized in the tissues of *P. annua* are required to establish whether the competitiveness

of Maritime Antarctic *P. annua* with native vascular plant species (Ballesteros et al., 2022; Cavieres et al., 2018; Molina-Montenegro et al., 2012, 2019; Olech & Chwedrozweska, 2011) arises from the deployment of these weapons by seed fungal endophytes. It should also be established whether the lower competitiveness of *P. annua* from sub-Antarctica, South America and Europe is caused by the reduced frequency of endophytes in seeds from these regions, or by differences in the allelochemicals synthesized by their endophytic fungi. Indeed, phylogenetic distances among plant species or assemblages of species correlate with genetic distances between communities of soil symbionts (Bouffaud et al., 2014), suggesting that plant species inhabiting separate geographical regions have evolved in concert with different symbionts with distinct functional roles. Further research is thus required to establish how these roles vary with plant biogeography.

Poa annua is able to survive in unstable and stressful habitats, which has allowed the species to persist for approximately 40 years on disturbed soil surrounding Arctowski Station on King George Island (Chwedrozweska et al., 2015). In the last decade, its local distribution has rapidly expanded into natural Antarctic terrestrial ecosystems on the island, including ice-free bare ground, where it grows together, and successfully competes, with native plant species, with significant implications for their conservation (Galera et al., 2015; Molina-Montenegro et al., 2019; Olech & Chwedrozweska, 2011). This successful range expansion has previously been attributed to the high phenotypic and genotypic variability and plastic life cycle of *P. annua* (Chwedrozweska et al., 2015; Galera et al., 2015; Rudak et al., 2019). However, recent studies show that bacterial and fungal symbionts may facilitate the establishment and development of *P. annua* in Maritime Antarctica (Ballesteros et al., 2022;

Znoj et al., 2021). It seems likely that seed fungal endophytes such as *Cladosporium* and *Epichloë*, which are transmitted vertically between generations (Ballesteros et al., 2022; Bulgheresi, 2010; Nissinen et al., 2019; Rosenberg et al., 2010), confer more immediate benefits on their hosts than horizontally-transmitted endophytes. The latter, such as dark septate endophytes (Newsham, 2011), colonize root tissues from surrounding soil and neighbouring plants, which, under the low temperatures and aridity encountered in Maritime Antarctic soils, is most probably a lengthy process. In contrast, vertically-transmitted seed endophytes benefit the host plant soon after germination and during its establishment (Jeong et al., 2021), presenting it with a selective advantage during the short Maritime Antarctic growing season.

The observations reported here, showing increased competitiveness, fecundity and fitness of *P. annua* plants grown from seeds colonized by fungal endophytes, support the view that the widely reported, but previously unexplained, invasiveness of the grass species in Maritime Antarctica (Chwedorzewska et al., 2015; Molina-Montenegro et al., 2019; Olech & Chwedorzewska, 2011) may arise from symbioses formed with seed endophytic fungi, and indicate that seeds act as long-distance vectors for fungal endophytes. The invasion of natural Antarctic ecosystems by *P. annua* from human-influenced microsites, as recorded over the last decade (Olech & Chwedorzewska, 2011), also supports the view that the higher environmental tolerance and competitive ability of plants forming symbioses with seed endophytes may allow an enlargement of niche sizes in Antarctica (Rodríguez-Cabal et al., 2012). These observations suggest that the high capacity of the Poaceae to colonize, persist and to transform environments (the “Viking syndrome”, sensu Linder et al., 2018) might be associated in some species, such as *P. annua*, with the presence of seed endophytes. Thus, interactions with endophytes, as well as other features of *P. annua*, such as its plastic biomass allocation (Rudak et al., 2019) and high capacity for forming soil seed banks (Galera et al., 2021), deserve further attention in the quest to understand and potentially manage the escalating problem created by the presence of the species in Maritime Antarctica. We propose that *P. annua*, one of the most successful and widespread invasive plant species worldwide (Gardarin et al., 2009), employs a “Trojan horse” strategy to invade Maritime Antarctica by forming symbioses with hidden endophytes, enabling host plants to cope with extreme environmental conditions and to compete with native species at one of the final frontiers for plant life on Earth.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All materials, raw data, and protocols used in the article are available upon request and without restriction, and all data will be made publicly available in a public repository (Figshare) upon publication. Figshare: <https://doi.org/10.6084/m9.figshare.22182904>.

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BIOSKETCH

The research team possess complementary expertise ranging from molecular biology, microbiology, ecophysiology to macroecology with a great emphasis in the cold ecosystems. The main research goals are put on microorganisms, insects and plants, as well as in the interactions among them or with the surrounding environment.

Author contributions: MAM-M, ISA-R, GIB and KKN conceived the research, and along with EMB and PC conducted fieldwork. MAM-M, GIB and KKN drafted the first version of the article, which was further developed by all other authors. All authors approved the submitted version of the article.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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