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PRELIMINARY ESTIMATES OF NUMBER AND DIVERSITY OF THE CULTURABLE ENDOPHYTIC BACTERIA FROM *DESCHAMPSIA ANTARCTICA* AND *COLOBANTHUS QUITENSIS*

IE. PREKRASNA[1], A. DZHULAI[1], I. PARNIKOZA[1,2,3]

[1] State Institution National Antarctic Scientific Center, Ministry of Education and Science of Ukraine
Ukraine, 01601, Kyiv, Shevchenko Ave., 16

[2] Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine
Ukraine, 03143, Kyiv, Akad. Zabolotnogo St., 150

[3] National University of «Kyiv-Mohyla Academy»
Ukraine, 04070, Kyiv, Skovorody St., 2
e-mail: preckrasna@gmail.com

Endophytes are able to promote the plant's growth and are essential for their hosts to overcome biotic and abiotic stress. Plant-promoting capacities of these microorganisms can be crucial for Antarctic plants. ***The aim*** *of the study was to estimate the number and diversity of culturable endophytic microorganisms from Deschampsia antarctica and Colobanthus quitensis growing in different localities of the West Antarctic Peninsula.* ***Methods.*** *Serial dilutions of the surface-sterilized plant biomass were inoculated on the CASO (Merk, USA) and R2A (Merk, USA) media and cultured at room temperature for a week. Number of colonies and their morphotypes were estimated.* ***Results.*** *The number of colony forming units in the aboveground part and roots of D. antarctica was* $4\times10^6 \pm 2\times10^6$ *and* $7\times10^6 \pm 2\times10^6$ *per g of biomass, respectively. The colony forming units CFU number in the aboveground part of C. quitensis was* $3\times10^6 \pm 1\times10^6$ *per g of biomass. The highest number of CFU was in the roots of D. antarctica from Galindez Island on both media (*$n\times10^7$*). The highest CFU number in C. quitensis was in plants from Deception Island on nutrient-poor (*7×10^6*) and -rich (*1×10^7*) media. The lowest value was found for C. quitensis from Cape Pérez (*7×10^3 *on R2A and* 1×10^4 *on CASO). There was no significant difference in the number of CFU grown on nutrient-poor and nutrient-rich medium, but the morphology of the CFU varied on the two media. 112 pure cultures of endophytes were isolated. The vast majority (78 %) of the isolates were gram-negative rods. The number of cultured endophytes of Antarctic vascular plans varied across the samples, which can be affected both by the features of the individual plants and the ecology of sites where they grow. Bacterial communities did not significantly vary in number depending on the medium but did somewhat differ in morphology. A collection of 112 endophyte isolates was developed, which is important to study their genetic and physiological traits and mechanisms of plant-bacteria interaction.* ***Conclusions.*** *Isolation of the endophytic microorganisms is important to study their genetic and physiological traits and mechanisms of plant-bacteria interaction.*

Keywords: *Antarctic hairgrass, Antarctic pearlwort, maritime Antarctica, symbionts of plants.*

Introduction. *Deschampsia antarctica* É. Desv. (1854) and *Colobanthus quitensis* (Kunth) Bartl. (1831), are the only two vascular plant species in the maritime Antarctic (Alberdi et al., 2002; Parnikoza et al., 2011). The Antarctic hairgrass and Antarctic pearlwort grow by some clusters along the coast of West Antarctic Peninsula and adjacent islands (Komárková et al., 1985; Komárková et al., 1990) from c. 61°S on the North to 69°S on the South in Lazarev Bay (Convey et al., 2011). These plants complete their life cycles under constant low temperature (Alberdi et al., 2002; Day et al., 1999) and are frequently exposed to low temperatures and freeze-thaw cycles even during the Austral summer (Day et al., 1999). The other abiotic factors that influence the plants' growth are the constant effects of ultraviolet radiation and visible light during the summer season, and drought (Alberdi et al., 2002; Parnikoza et al., 2011). The stepwise distribution of both species clearly shows their ability to withstand the hardships of the Antarctic environment.

Microorganisms can develop beneficial associations with plants and promote plant growth (Santoyo et al., 2016). Endophytic bacteria, which inhabit the internal part of the plants, is one of the known plant-growth promoting groups of the bacteria (Brader et al., 2014; Hardoim et al., 2015). Endophytic bacteria can carry out a number of functions such as the synthesis of antifreeze compounds, modulating the phytohormone level, and stimulating antioxidant activity (Devi et al., 2017). These effects can be essential for their plant hosts to overcome abiotic stresses found in the Antarctic. For these reasons we made an assumption of the crucial role of endophytes in adaptation of Antarctic vascular plants to the environment.

Less than 5 % of the microbial communities found in the environments are cultured (Rappé & Giovannoni, 2003), while others are unculturable or require special conditions. Nevertheless, the cultured part of the communities, e.g. endophytic one, can shed some light on the diversity, physiology and plant-microbe interaction. Endophytic bacteria were studied from various types of hosts that include agricultural and wild plants (Nair & Padmavathy, 2014; Zinniel et al., 2002). Roots, stems, leaves, seeds, fruits, tubers, ovules and nodules were shown to host endophytic bacteria (Senthilkumar et. al., 2011). Growth promotion effect of endophytic bacteria was shown on canola, tomato, wheat, rice, potato, and lots of other plant species (Mei & Flinn, 2010; Sturz & Nowak, 2000). In many cases the growth-promoting effect was provided by bacteria isolated from the same plants.

Endophytic bacteria from Antarctic plant tissues were studied earlier (Podolich et al., 2021). However, the study involves only two locations on the Antarctic Peninsula (King George and Galindez Islands) and endophytes of *D. antarctica* solely. Advanced understanding of endophytic bacteria of Antarctic vascular plants is timely and necessary, and for this reason the aim of the study is to assess the number and diversity of culturable endophytic microorganisms of *D. antarctica* and *C. quitensis*. The study has the following tasks: i) to quantify the culturable bacteria colonizing endosphere of *D. antarctica* and *C. quitensis* from sites along the West Antarctic Peninsula; ii) estimate the effectiveness of bacteria isolation on different culture media; iii) isolate the pure cultures of endophytic bacteria in order to estimate the mechanisms of the plant-bacteria interaction in future.

Materials and methods

Sampling of the material. Samples of *D. antarctica* and *C. quitensis* were collected during the 25th Ukrainian Antarctic expedition (January–March 2020) along West Antarctic Peninsula (Fig. 1). Alive plants were aseptically collected in plastic containers and delivered to the Ukrainian Antarctic Akademik Vernadsky station for further processing. All laboratory work was done at the Antarctic station. Samples of *D. antarctica* included the root systems and the aboveground parts(stems and leaves). Samples of *C. quitensis* included the aboveground part only.

The list and description of samples is presented in Table 1, and location of the sampling points is presented on Fig. 1.

Table 1. List and description of the samples

# of sample	Sampling location	Plant	Coordinates
1	Lahille Island	*D. antarctica*	-65.553580°, -64.394883°
2	Ronge Island	*D. antarctica*	-64.683430°, -62.644170°
3	Galindez Island, Argentine Islands	*D. antarctica*	-65.244807°, -64.255709°
5	Santos Peak, Graham Passage	*D. antarctica*	-64.405750°, -61.547410°
6	Lagotellerie Island	*D. antarctica*	-67.88486°, -67.38765°
7	Waugh Mt.	*D. antarctica*	-65.511814°, -64.083470°
8	Lahille Island	*C. quitensis*	-65.553580°, -64.394883°

# of sample	Sampling location	Plant	Coordinates
9	Deception Island	*C. quitensis*	-62.982010°, -60.520370°
10	Berthelot Island	*C. quitensis*	-65.329090°, -64.161650°
11	Darboux Island	*C. quitensis*	-65.395220°, -64.214920°
12	Cape Perez	*C. quitensis*	-65.407730° , -64.097250°
13	Lagotellerie Island	*C. quitensis*	-67.88486° , -67.38765°

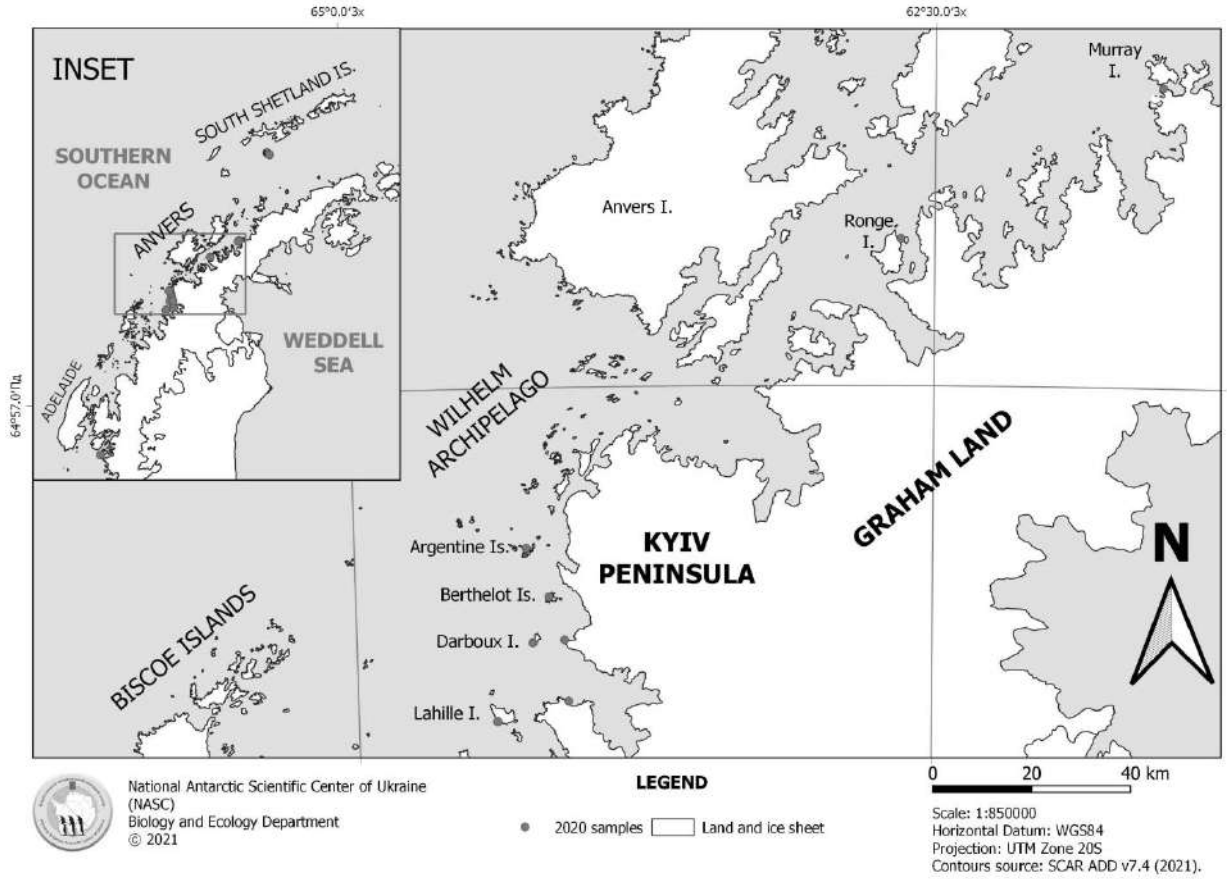

Fig. 1. Location of the sampling points of Antarctic vascular plants along the West Antarctic Peninsula.

Surface sterilization of the plants. Surface sterilization of the plants was done to the work with endophytic bacteria only. Sterilization of the plant material was carried out according to (Barra et al., 2016) with modifications. The duration of several steps of the sterilization was prolonged, because the described method was not effective enough with the Antarctic plant material. Roots and aboveground parts of *D. antarctica* were sterilized separately. The following steps were performed:

1. Plant material was washed in the tap water to get rid of crude soil particles.
2. Plant material was vortexed in sterile distilled water for 3 min. This step was repeated twice, and fresh distilled water was used for each time.

3. Vortexing of the plant material in 70 % ethanol for 2 min.
4. Treatment of the plant material in 5.6 % $NaClO_4$for 10 min.
5. Vortexing of the plant material in 70 % ethanol for 2 min.
6. Vortexing of the plant material in sterile distilled water for 3 min. This step was repeated three times, and fresh distilled water was used for each time.

Water (0.1 mL) from the last wash step was inoculated on the nutrient agar medium CASO (Merck, USA) to control the sterility of the material.

Number of CFU in the plant biomass. Surface-sterilized plant biomass was crushed in a sterile mortar with a pestle, and 0.1 g of crushed biomass was diluted and resuspended in 0.9 mL sterile NaCl (0.9 %). The suspension (10^{-1} mg/mL dilution) was used to prepare tenfold dilutions (10^{-2}, 10^{-3} and 10^{-4}) of plant biomass. 0.1 mL of each dilution was inoculated on solid nutrient-rich media CASO (Merk, USA) and nutrient-poor media R2A (Merk, USA) supplemented with 1 % ethanol extract of *D. antarctica* and 1 % methanol. The R2A media has lower concentration of peptone (0.05 %) comparatively to the CASO media (1.7 %) and facilitates growth of slow-growing species. Bacteria were cultured at room temperature. Number of CFU and the variety of colonies' morphotypes were estimated after seven days of cultivation.

Isolation and description of pure microbial isolates. Distinct bacterial colonies were inoculated repeatedly on the corresponding nutrient media (the same as media of isolation) to receive pure isolates of endophytic bacteria from Antarctic vascular plants. Bacteria were stained with Gram Stain Kit (Difco, USA) and viewed at 1000× (Konus Academy microscope, Italy). To preserve pure bacteria isolates they were transferred to 2 mL tubes with nutrient media and stored at +4 °C.

Results

Number of CFU in the plant biomass. Microorganisms inhabiting the interior part of the vascular plants can have fastidious and largely unknown culturability conditions. To include microorganisms with different trophic preferences, they were cultured in nutrient-rich and nutrient-poor conditions. There was no significant difference (t-test, p = 0.41) between the number of CFU grown on the CASO and R2A media. Similarly, it did not affect the number of colony morphotypes (t-test, p = 0.37). The number of colonies' morphotypes varied from 1 to 12 with mode value of 3. The majority of the colonies grown on the CASO medium were beige (33 %) and yellow (27 %). Most of the colonies grown on the R2A medium were white (41 %) and yellow (23 %). Colorless, orange, pink, red, peach color colonies were less abundant on the both types of media.

Quantity of endophytic microorganisms inhabiting roots, stems and leaves of *D. antarctica* and above-ground part of *C. quitensis* was estimated (Fig. 2a). Roots of *D. antarctica* plants sampled on Galindez Islands harbored the highest number of CFU cultured on both nutrient-poor and nutrient-rich media ($3 \times 10^7 \pm 2 \times 10^6$ and $3 \times 10^7 \pm 2 \times 10^6$ CFU/g of biomass, respectively). In the leaves the highest number of CFU was in the plants collected in Lahille Island on both CASO and R2A media. There was significant difference between number of CFU (t-test, p-value = 0.013) and no significant difference in number of colony morphotypes (t-test, p-value = 0.4) in *D. antarctica's* leaves and roots.

C. quitensis collected on Deception Island had the highest number of CFU cultured on both CASO and R2A medium, followed by plants from Lagotellerie and Berthelot Islands (Fig. 2b). CFU number of plants sampled in Cape Pérez and Darboux Island were the lowest on both media.

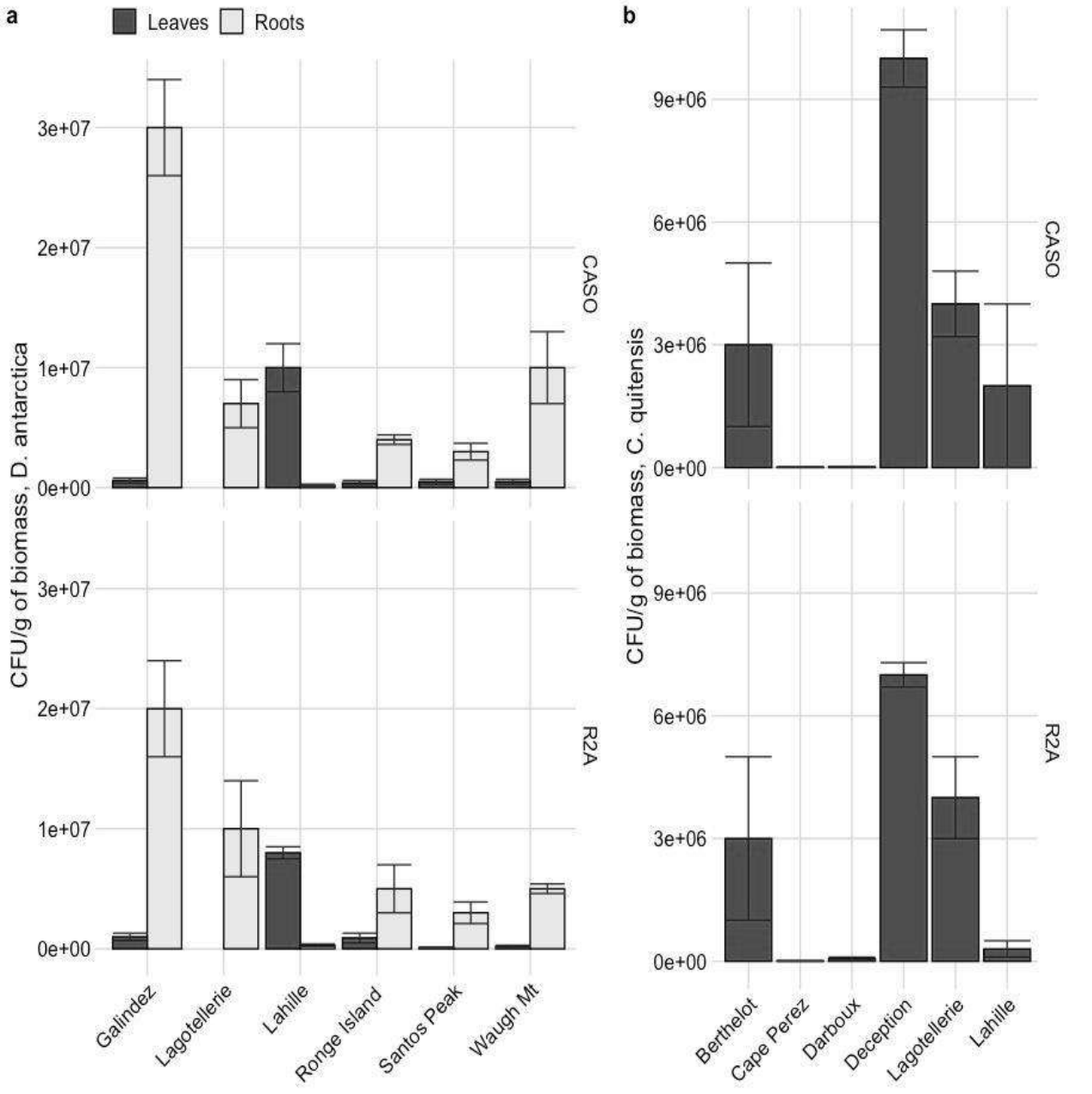

Fig. 2. CFU number per g of wet biomass a) *D. antarctica*; b) *C. quitensis* sampled from different locations of West Antarctic Peninsula.

Isolates from endosphere of Antarctic vascular plants. Overall 112 isolates were obtained from the endosphere of *D. antarctica* and *C. quitensis*. The number of isolates from plants sampled in different localities is presented on Fig. 3. The highest number of isolates was obtained from *D. antarctica* sampled on Ronge (26) and Lahille (14) Islands and from *C. quitensis* from Berthelot Island (17).

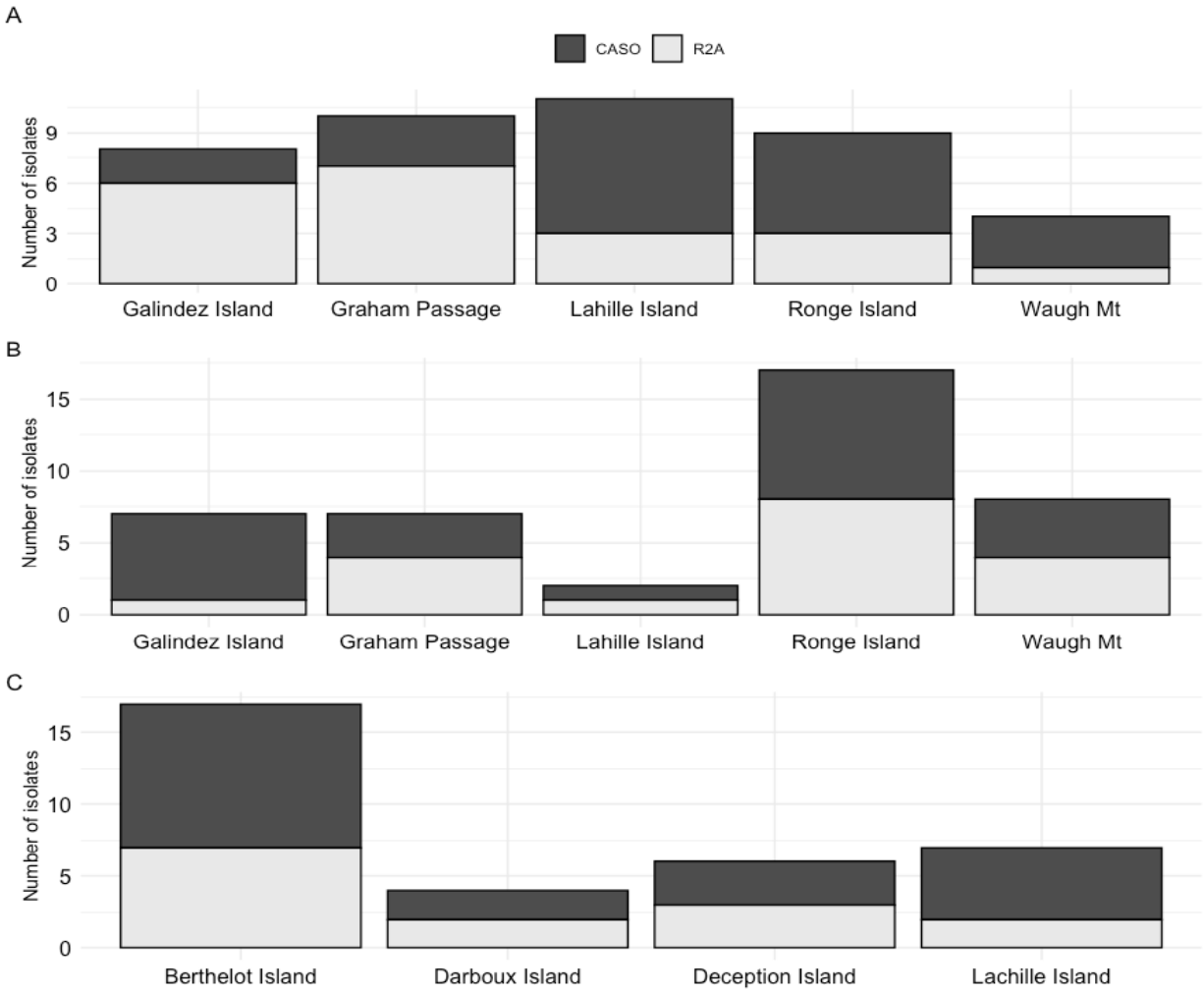

Fig. 3. Number of microbial cultures isolated from: A) *D. antarctica*'s roots; B) *D. antarctica*'s leaves; C) *C. quitensis.*

The majority of isolates were gram-negative rods (Fig. 4) followed by gram-positive rods and gram-positive cocci. One yeast culture was isolated from *D. antarctica*'s leaves.

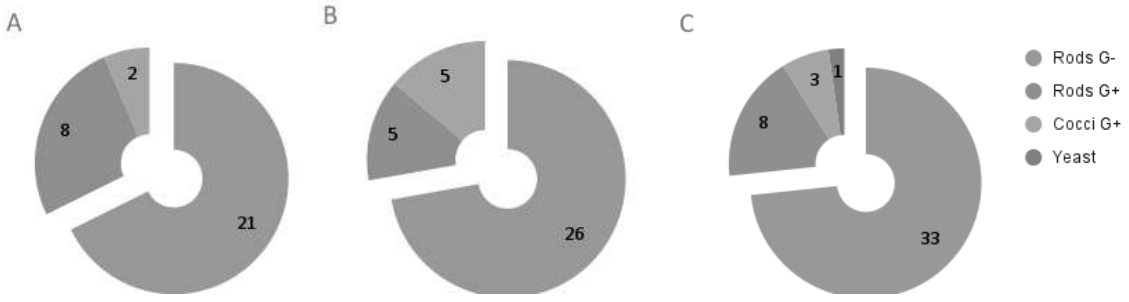

Fig. 4. Cell morphology of microorganisms isolated from: A) *C. quitensis*; B) *D. antarctica* aboveground part; C) *D. antarctica* roots. Number of microorganisms with respective morphology is indicated on the chart segments. Total amount of microorganisms isolated on nutrient-rich and nutrient-poor media is presented.

Discussion

Number and morphological diversity of microorganisms in the internal parts of Antarctic vascular plants was studied. Cultured bacteria usually represent a tiny proportion of the whole bacterial community (Rappé & Giovannoni, 2003). Other bacteria are stubborn to cultivation and require specific and usually unknown culturing conditions. Nevertheless the minor cultured part of the bacterial community enables studying the physiological, genetic traits of endophytic bacteria and bacteria's effect on the plants' performance. To harvest as much of the endophytic bacteria as possible, nutrient-rich and nutrient-poor media were used. Microbial communities of *D. antarctica*'s roots and aboveground parts (leaves and stems) were studied separately. Only the microbial community of the pearwort's aboveground parts was included in the study. Number of CFU indicates the high variance of the cultured part of Antarctic endophyte microbial communities. The highest CFU number was revealed in the roots of *D. antarctica* from Galindez Island on both nutrient-poor and -rich media. The highest CFU number in *C. quitensis* biomass was in plants growing on Deception Island, and the lowest — in plants from Cape Perez and Darboux Island. Bacterial populations inhabiting the endosphere of Antarctic vascular plants can be shaped by diverse environmental factors. Composition and diversity of the endophytic microbial community are driven by the ecology of the plant and soil (Edwards et al., 2015). Apart from the host plant species and soil conditions, microbial composition is affected by plant genotype, organ type (Hameed et al., 2015), developmental stage (de Almeida Lopes et al., 2016), geographical location, and host plant nutrient status (Hameed et al., 2015).

Numbers of CFU in the endosphere of Antarctic vascular plants are comparable with the previous reports. To compare the CFU values with literature data we provide the log-transformed results. In the endosphere of roots and leaves of Antarctic hairgrass, the CFU number was on average 6.6 and 6.8 log10 CFU/g of biomass respectively. The CFU number in the aboveground part of Antarctic pearlwort was 5.7 log10 CFU/g of biomass. Number of endophytes in the tissues of alfalfa, sweet corn, sugar beets, squash, cotton and potatoes varied from 2.0 to 6.0 log10 CFU/g of biomass (Kobayashi & Palumbo, 2000), and in the tissues of prairie plants it was from 3.5 to 7.7 (Zinniel et al., 2002). The CFU number in the biomass of beans was 2.6–3.4 log10 CFU/g of biomass (Costa et al., 2012). All above mentioned CFU numbers together with our data have comparatively low values. Yet, the colonization rates by non-pathogenic microorganisms are usually lower, while concentration of pathogenic bacteria in the infected plants can reach up to 10 log10 CFU/g of biomass(Grimault & Prior, 1994).

Number and morphological diversity of bacteria grown in nutrient-rich and nutrient-poor media exhibited no significant difference. However, the morphologies of the colonies grown on two kinds of media were slightly different. Cultured bacteria from the endosphere of Antarctic plants are likely able to exploit both media, and communities isolated on both media are likely to be quite similar. On the other hand, nutrient-poor conditions can promote growth of the slow-growing bacteria that are outcompeted by fast-growing bacteria in the nutrient-rich environment. Both these assumptions will be checked by the identification of microbes derived on both kinds of media.

In most samples of *D. antarctica*, e.g. plants collected on Galindez and Ronge Islands, Graham Passage and Mt. Waugh, the CFU number was higher in the roots compared to the aboveground part. In plants collected on Lahille Island, the CFU number was higher in leaves. Composition of endophytic communities in each part of the plant is affected by epiphytic bacteria (Mano et al., 2006; Mano & Morisaki, 2008). Bacterial communities are considered to be more numerous in roots compared to the stems and leaves (Lamb et al., 1996; Rosenblueth & Martínez-Romero, 2006). The root system tightly interacts with the highly populated rhizosphere, which supplies bacteria to the endosphere of the plants (Mano et al., 2006; Mano & Morisaki, 2008). Endosphere of the leaves is mainly colonized by bacteria arriving from the leaf surfaces. Phyllosphere and leaf endosphere of the Antarctic hairgrass is a less favourable habitat in the Antarctic environments compared to the more isolated rhizosphere. The aboveground parts of the Antarctic plants experience more intense temperature fluctuations, and undergo virtually continuous impact of the UV radiation during the Austral summer. That is why the phyllosphere of Antarctic plants can be assumed to have less impact on the composition of the endophytic bacteria, and endophytic communities inhabiting leaves are less numerous in most of the samples studied. Differential abundance of the bacteria in different compartments of the Antarctic hairgrass agrees with other studies. Podolich et al., 2021 have shown different composition of root and stem endophytic communities in *D. antarctica* sampled from several locations on King George and Galindez Island by group-specific PCR. T-test

comparisons of data from our study reveal a significant difference in CFU number in roots and leaves. This observation should be studied the means of 16S rRNA amplicon sequencing or qPCR.

Pure microbial isolates (112 in total) were isolated from the endosphere of Antarctic hairgrass and pearlwort. Most of the isolated bacteria were gram-negative rods, while the proportion of gram-positive rods and gram-positive cocci was notably lower. Our data resonates with previous studies on the Antarctic vascular plants' microbiome composition. Yet, the planned phylogenetic analysis and identification of the isolates will provide a much more clear result. Predominance of *Proteobacteria*, followed by *Firmicutes* and *Actinobacteria* in bacterial communities associated with Antarctic vascular plants was previously shown with the means of 16S rRNA gene sequencing (Zhang et al., 2019). Among the 12 bacterial strains isolated from *D. antarctica*'s biomass, the majority belonged to the *Pseudomonas* sp., followed by *Bacillus* sp. and *Micrococcus* sp. (Podolich et al., 2021).

Bacterial isolates from the endosphere of the Antarctic plants will be used to study their effect on plant growth and promotion and mechanisms of the plant-bacteria interaction. Similar research was done for other plants and their endophytic bacteria, and the majority of bacterial isolates promoted the plant growth. For example, *Herbaspirillum* spp., *Methylobacterium* spp., and *Brevundimonas* spp. isolated from Zijuan tea cultivars promoted these plants' species growth (Yan et al., 2018). Stains isolated from buds of *Mimosa pudica* were identified as *Enterobacter* sp. and *Serratia* sp. These strains were able to mobilize phosphates, produce auxins, cellulase, chitinase and inhibit growth of pathogenic micromycetes (Sánchez-Cruz et al., 2019). Isolates from *D. antarctica* and *C. quitensis* might facilitate their survival in the harsh Antarctic conditions.

Conclusions

The study addressed the quantity and morphological diversity of the culturable bacteria residing in the interior parts of the Antarctic vascular plants. It involved plants of *C. quitesis* and *D. antarctica* growing in different spots along the West Antarctic Peninsula from Deception Island as the northernmost point and Lagotellerie Island as the southernmost point. The number of cultured endophytes of Antarctic plans varied across the samples in a range $n\times10^3$ - $n\times10^7$ per gram of biomass, which can be affected by environmental factors. The CFU numbers of endophytes grown on CASO and R2A media did not differ, however the cultivation on different media resulted in development of different types of colonies. Bacterial communities isolated on two kinds of media can have quite similar compositions. However, the nutrient-poor media can facilitate the slow-growing bacteria, which can be tested by the further identification of isolates. Overall, 111 bacterial and 1 yeast cultures were isolated from the endosphere of the plants. Most of the bacteria were gram-negative rods. Isolation of the endophytic microorganisms is important to study their genetic and physiological traits and mechanisms of plant-bacteria interaction.

Author contributions. Ie. P. — conceptualization, lab work and data analysis, writing; A. D. — sampling; I. P. — conceptualization, sampling, editing.

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Conflict of interest. Authors declare no conflict of interest.

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ПОПЕРЕДНЯ ОЦІНКА КІЛЬКОСТІ ТА РІЗНОМАНІТТЯ КУЛЬТИВОВАНИХ ЕНДОФІТНИХ БАКТЕРІЙ З *DESCHAMPSIA ANTARCTICA* ТА *COLOBANTHUS QUITENSIS*

Є. Прекрасна[1], А. Джулай[1], І. Парнікоза[1,2,3]

[1] Державна установа Національний антарктичний науковий центр, Міністерство освіти і науки України
Україна, 01601, м. Київ, пр. Шевченка, 16

[2] Інститут молекулярної біології і генетики
Національна академія наук України
Україна, 03143, м. Київ
вул. Академіка Заболотного, 150

[3] Національний університет
«Києво-Могилянська Академія»
Україна, 04070, м. Київ, вул. Сковороди, 2
e-mail: preckrasna@gmail.com

Ендофіти здатні сприяти росту рослин та забезпечувати їхній захист від стресових факторів. Їх здатність сприяти росту рослин може бути критичною для антарктичних рослин. **Метою** роботи було визначити кількість та різноманіття культивованих ендофітних мікроорганізмів з *Deschampsia antarctica* та *Colobanthus quitensis* зрізних локалітетів уздовж західної частини Антарктичного півострова. **Методи.** Для цього серійні розведення поверхнево-стерилізованої біомаси рослин інокулювали на середовище CASO (Merk, USA) і R2A (Merk, USA) та культивували при кімнатній температурі протягом тижня. Після цього визначали кількість колоній та їх морфотипів. **Результати.** Кількість колоніє-утворюючих одиниць (КУО) в наземній і кореневій частинах *D. antarctica* була $4 \times 10^6 \pm 2 \times 10^6$ і $7 \times 10^6 \pm 2 \times 10^6$ на грам біомаси відповідно. Кількість КУО в наземній частині *C. quitensis* була $3 \times 10^6 \pm 1 \times 10^6$ на грам біомаси. Найвища кількість КУО була в коренях *D. antarctica* з острову Галіндез на обох видах середовищ ($n \times 10^7$). Найвища кількість КУО в біомасі *C. quitensis* була в рослинах з о. Десепшн на середовищі з низьким (7×10^6) та високим (1×10^7) вмістом поживних речовин. Найнижча кількість була в зразках *C. quitensis* з мису Перез (7×10^3 на R2A і 1×10^4 на CASO). Достовірної різниці у кількості КУО, що виросли на різних видах середовища, не було. Проте морфологія колоній дещо варіювала. Було виділено 112 ізолятів ендофітів. Переважна більшість (78%) ізолятів були грам-негативними паличками. Кількість культивованих ендофітів варіювала в різних зразках, що може бути зумовлено умовами навколишнього середовища, зокрема особливостями кожної рослини. Бактеріальні угруповання, виділені на двох видах середовища не різнились у кількості, проте мали різницю у морфології колоній. Була створена колекція з 112 ізолятів, що важливо для вивчення їх генетичних, фізіологічних особливостей та механізмів взаємодії з рослиною. **Висновки.** Ізоляція ендофітних мікроорганізмів необхідна для вивчення їх генетичних та фізіологічних особливостей та взаємодії рослини з бактеріями.

Ключові слова: щучник антарктичний, перлинниця антарктична, морська Антарктика, симбіонти рослин.