1 **Effect of osmoprotectants on the survival of bacterial endophytes in lyophilized beet roots**

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27 Abstract

28 The increase of human population and associated increasing demand for agricultural products lead to
 29 soil over-exploitation. Biofertilizers based on lyophilized plant material containing living plant
 30 growth-promoting microorganisms (PGPM) could be an alternative to conventional fertilizers that fits
 31 into sustainable agricultural technologies ideas. We aimed to: (i) assess the diversity of endophytic
 32 bacteria in beet roots and (ii) determine the influence of osmoprotectants addition during
 33 lyophilization on bacterial density, viability, salt tolerance. Microbiome diversity was assessed based
 34 on 16S rRNA amplicons sequencing, bacterial density and salt tolerance was evaluated in cultures,
 35 while bacterial viability was calculated by using fluorescence microscopy and flow cytometry. Here
 36 we show that plant genotype shapes its endophytic microbiome diversity and determines
 37 physicochemical rhizosphere soil properties. Sea beet endophytic microbiome, consisting of genera
 38 characteristic for extreme environments, is more diverse and salt resistant than its crop relative.
 39 Supplementing osmoprotectants during root tissue lyophilization exerts a positive effect on bacterial
 40 community salt stress tolerance, viability and density. Trehalose improves these parameters more
 41 effectively than ectoine, moreover its use is economically advantageous.

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55 Introduction

56 Conventional agriculture practices negatively affect environment, e.g. by decreasing microbial
57 diversity, soil quality, water supply and plant productivity (Gveroska et al., 2014; Baez-Rogelio et al.,
58 2016). Wide adoption of sustainable agricultural technologies, e.g. biofertilizers, may significantly
59 decrease the use of chemical fertilizers, reducing negative consequences of agriculture on the
60 environment (Pretty, 2008; Malusá et al., 2011; Baez-Rogelio et al., 2016).

61 Biofertilizers are based on living plant growth-promoting microorganisms (arbuscular
62 mycorrhizal fungi - AMF, plant growth-promoting rhizobacteria - PGPR, nitrogen fixing bacteria -
63 NFB) and are key players in sustainable agriculture (Malusá et al., 2011). They can promote plant
64 growth in several different ways (e.g. increasing availability of nutrients, synthesizing phytohormones
65 or siderophores, fixing nitrogen), especially under unfavorable environmental conditions (e.g. drought,
66 salinity) (Szymańska et al., 2016; Hryniewicz et al., 2019). Most of commercially available
67 biofertilizers are based on two or more microbial beneficial strains, which is called 'consortium'
68 (Malusá et al., 2011). Compared to single strains, consortia display increased spectrum of beneficial
69 effect of inoculum on plants. However, criteria of strain selection are the crucial factor influencing the
70 inoculum's success and should be considered not only based on plant genotype compatibility but also
71 environmental factors.

72 The methods of biofertilizers production, storage and application are diverse. Inoculation
73 techniques are based on microorganisms application in liquid (sprays and drenches) or solid form
74 (lyophilizates delivered to soil/growth substrate; Alori and Babalola, 2018). The most important
75 problem in the preparation and storage technology of biofertilizers is maintaining high viability of
76 microorganisms (Alori and Babalola, 2018). Lyophilization is well known and widely used technique
77 extending viability (Prakash et al., 2013). To alleviate negative effect of low temperature on
78 microorganisms in this technology several different stabilizers can be used e.g. nonreducing
79 disaccharides, glycerol, skim milk (Park et al., 2002). Trehalose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-
80 glucopyranoside) is a disaccharide present in almost all prokaryotic and eukaryotic organisms and
81 exhibits high efficiency in protection of cells against low temperature, drying and osmotic stress (Park
82 et al., 2002; Lee et al., 2018). Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is
83 synthesized mostly by halotolerant and halophytic microorganisms and responsible for regulation of
84 osmotic pressure in microbial cells, increasing their tolerance to osmotic stress (salinity) (Oren, 2008;
85 Han et al., 2018 Roberts, 2005; Czech et al., 2018). Application of trehalose and ectoine in the process
86 of lyophilization of endophytic microbiomes was tested in our work for the first time.

87 Biofertilizer's efficiency analysed under laboratory conditions may not correspond to results
88 obtained under field conditions (Baez-Rogelio et al., 2016; Parnell et al., 2016). This effect may be
89 due to adverse effect of environmental conditions or autochthonic microorganisms on gene expression

90 in microbial cells (Baez-Rogelio et al., 2016) or low competitiveness of microorganisms used as
91 biofertilizers, i.e. they may be outgrown by autochthonic ones. This is why “plant microbiome” was
92 proposed as new generation of inoculants (Compant et al., 2019). Inoculation of crops with
93 microbiome and organic matter present in lyophilized plant roots seems to be a better solution to
94 enrich microbial biodiversity of soil and crops with new endophytes.

95 Endophytes are bacteria and fungi that colonize the internal plants tissues without causing
96 pathogenic symptoms (Hardoim et al., 2013) and can directly (nitrogen fixation, phosphate
97 solubilization, siderophore and phytohormone synthesis) and/or indirectly (biocontrol agents) promote
98 plant growth and development (Patle et al., 2018). Moreover, endophytes associated with halophytes
99 possess high tolerance to salt stress (Abbas et al., 2018; Szymańska et al., 2018). Application of
100 halotolerant microbes in sustainable agriculture e.g. in the increasing salinity tolerance of non-
101 halophytic crops, is well known and was extensively studied (Yadav and Saxen, 2018; Etesami and
102 Beattie, 2018; Szymańska et al., 2016, 2019).

103 Cultivated beets are one of crops whose direct ancestor (sea beet, *Beta vulgaris* ssp. *maritima*)
104 still grows in the wild. This feature enables comparing traits in plants that are very close genetically
105 (ca. 0.5% difference, Dohm et al. 2014), but whose ecology differs considerably. Moreover, as sea
106 beet is a halophyte (Rozema et al. 2015), it seems to be a good candidate for a source of
107 microorganisms that can be useful for crop beets improvement.

108 The goal of our study was twofold: i) to characterize root microbiomes of cultivated and wild
109 beet and ii) to check if addition of osmoprotectants during lyophilization changed root bacterial
110 community structure as well as microbiome salinity tolerance and viability. Specifically, we
111 formulated five hypotheses: i) rhizospheric soils would differ in physicochemical parameters and
112 bacterial community structure, ii) root endophytic communities would be different in the crop and its
113 wild ancestor, iii) alpha diversity in the crop roots would be lower, iv) there would be more halophilic
114 bacteria in sea beet roots, v) addition of osmoprotectants would change bacterial viability and
115 microbiome salinity tolerance.

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122 **Materials and methods**

123 ***Experimental design***

124 Sea beet (*Beta vulgaris* L. subsp. *maritima* L.) seeds were obtained from National Germplasm
125 Resources Laboratory, Beltsville, MD, USA, while in the case of sugar beet (*B. vulgaris* subsp.
126 *vulgaris* cv. 'Huzar') commercial seeds were bought from WHBC Poznań, Poland. Pot experiment was
127 conducted from mid-March through mid-May 2017 in a greenhouse (Nicolaus Copernicus University
128 in Toruń, Poland). Temperature was maintained at 22-24°C under natural lighting conditions. Healthy
129 and uniform-sized seeds were placed in 5 l pots filled with 2,5 kg of garden soil (one seed per pot; 5
130 pots were prepared for each genotype, in total 10 pots were used). All plants for both investigated
131 genotypes were arranged randomly on the green house benches. The plants were watered with tap
132 water every two days, amount depended on the plants demand. After three months plants and
133 rhizospheric soil samples were collected and analyzed as shown in Fig. 1.

134 ***Soil analysis***

135 Soil parameters (TOC, TN, CaCO₃, P_{cit}, pH, EC, Na, K, Ca, Mg, Cl, SO₄²⁻) were analyzed as
136 described earlier in Furtado et al., 2019.

137 ***Plant and soil samples preparation***

138 Plants were carefully uprooted, and 10 g of soil adhering to roots (rhizospheric soil) was collected,
139 frozen at -80°C and lyophilized before DNA isolation for metagenomic analysis. Roots were washed
140 with tap water to remove soil and were separated from shoots and leaves. Then, they were surface
141 sterilized with 70% ethanol and 15% hydrogen peroxide mixture (1:1 v:v) for 5 min and subsequently
142 rinsed six times with 0.9% NaCl. Efficiency of the sterilization process was evaluated by plating the
143 last rinse on Luria-Bertani (Difco™ LB Agar, Miller) and potato dextrose extract (Lab A Neogen
144 Company) media. Only properly sterilized plant material was used for subsequent analyzes.
145 Approximately 100 g of fresh root material was homogenized in 100 ml of 0,9% NaCl by using
146 surface sterilized (rinsed with 70% ethanol and UV-irradiated) blender. Homogenates were used to
147 evaluate bacterial density and to prepare lyophilizates.

148 ***Roots lyophilization***

149 Homogenated sugar and sea beet roots were used to prepare three variants of lyophilizates including
150 (1) control (without osmolytes addition) (2) trehalose and (3) ectoine supplemented. Three biological
151 replicates were prepared for each tested plant species (9 samples per plant species, in total 18 samples
152 were used for downstream analyzes). Either 1 ml of 0.9% NaCl (control) or xx g of trehalose (Tre) or

yy g of ectoine (Ect) were mixed with 50 g of homogenized roots. The mixtures were lyophilized in (nazwa liofilizatora) until completely dry (approximately 24 h).

155 *Estimation of bacterial density*

156 Serial dilutions were prepared directly from the homogenized fresh roots and lyophilizates
157 resuspended in 0.9% NaCl (1:9 m:v). The dilutions (10^{-3} to 10^{-8}) were plated in triplicates on LB
158 plates supplemented with nystatin (Sigma, 100 µg/ml) to prevent fungal growth, and the plates were
159 incubated for 5 days at 26°C. Colony counts (expressed as CFU per 1 g of fresh or dry weight for
160 homogenates and lyophilizates, respectively) were based on plates with 30-300 colonies. At least six
161 bacterial isolates were purified per experimental variant.

162 *Isolates identification by 16S rRNA gene sequencing*

163 Genomic DNA was isolated from purified strains using GeneMatrix Bacterial and Yeast Genomic
164 DNA Purification Kit (EurX) according to the manufacturer's protocol with modified homogenization
165 step (FastPrep®-24 bead-beater, one cycle of 20 seconds at 4.0 m/s). The DNA was analyzed
166 spectrophotometrically (NanoDrop 2000). 16S rRNA gene fragment was amplified using 27F and
167 1492R primers (Daffonchio et al., 1998), following the procedure described in Szymańska et al.
168 (2016). The products were purified with GeneMatrix PCR/DNA Clean-Up DNA Purification Kit
169 (EurX) according to the manufacturer's protocol. Sanger sequencing was performed with BrightDye
170 Cycle Sequencing kit (Nimagen), using 40 ng of template DNA, 1.5 pmol of primer and 1 µl of kit and
171 1.5 µl of BD buffer in 10 µl volume. The reactions were EtOH/NaAc precipitated and read out at IBB
172 PAS, Warsaw, Poland.

173 *Salt tolerance assessment*

174 Salt tolerance of root bacterial communities was measured as OD₆₀₀ after 5 days incubation at 26°C
175 using 96-wells microtiter plate reader (Biolog Micro Station). 140 µl of LB medium supplemented
176 NaCl to obtain final concentrations of 0, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900 mM
177 were used per well. Inoculates were prepared by suspending 2 g of mortar-ground lyophilized roots in
178 18 ml of 0.9% NaCl and diluting the mixture ten times. The inoculates were filtered through 40 µm
179 cell strainer (Biologix) to remove plant debris. Six test and two control wells were inoculated with 10
180 µl of filtered inoculate or 0.9% NaCl, respectively.

181 *Bacterial viability assessment: fluorescence microscopy and flow cytometry*

182 Ten-miligram samples of ground lyophilized roots were mixed with 10 ml of PBS (pH=7.4)
183 and incubated for 2 days at 26°C with mixing. The mixtures were filtered through a 40 µm cell strainer

(Biologix) and 2 ml were centrifuged for 3 min at 1000 x g at RT to pellet the residual plant debris. Cells in the supernatant were stained with Cell Viability kit (BectonDickinson) as per the manufacturer's protocol, than bacterial viability was analyzed using fluorescence microscopy (after 6 and 12 months of storage) and flow cytometer (after 12 months storage). Preparations were photographed in red and green channel under 40 x magnification upon fluorescence excitation with 433 nm light on Axiostar plus fluorescence microscope (Zeiss) equipped with Delta Optical camera. Percentage of live cells was based on counts from at least 30 view fields per sample. Flow cytometric analysis was performed on samples stained as described above with FACS Aria III (BectonDickinson) using 488 nm laser for excitation. Fluorescence was collected at 530±30 nm (for thiazole orange - TO) and 616±26 nm (for propidium iodide - PI) bands and seventy-micrometer nozzle was used. Parameters were optimized basing on pure environmental strains and their mixtures analyses and autoclaved lyophilizates samples served as negative controls.

196 *Statistical analysis and bioinformatics*

Bioinformatics analyses of Illumina reads was performed as described earlier (Thiem et al. 2018). Briefly, the reads were denoised, merged and chimeras were removed in dada2 (Callahan et al., 2016), then amplicon variant sequences were exported together with abundance information and processed in Mothur v.1.39 (Schloss et al., 2009): aligned against SILVA v.132 database, screened for those covering the 6428-22400 positions of the alignment, filtered to remove gap-only and terminal gap-containing positions, pre-clustered to remove residual noise and clustered into 0.03 dissimilarity OTUs. Representative OTU sequences were classified using naïve Bayesian classifier and SILVA database. Sanger reads were manually inspected in Chromas to remove obvious errors, the corrected sequences were merged with CAP3 (Huang and Madan, 1999), and classified using naïve Bayesian classifier (Wang et al., 2007).

Significance of differences between means was assessed with ANOVA test with Tukey's post-hoc analysis implemented in Statistica 10.0 (StatSoft). Normality of data was tested with Shapiro-Wilk's test and homogeneity of variance was assessed with Levene's test. When the assumptions were violated non-parametric Kruskal-Wallis test was used.

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215 Results

216 *Rhizospheric soil physicochemical parameters are different for sugar and sea beet*

217 Majority of tested parameters was higher in sugar beet soil, but only in cases of CaCO₃ and
218 Na⁺ the difference was statistically significant. On the other hand, OC, P, Ca²⁺, Mg²⁺ and N_t were
219 higher in sea beet soil and for the latter the difference was significant (Table 1).

220

221 *Bacterial diversity in sugar beet roots is lower than in its wild ancestor*

222 Bacterial diversity, evenness and species richness were the highest in rhizospheric soil,
223 regardless of the plant genotype. Lyophilized sea beet roots harbored more diverse community than
224 sugar beet (Fig 2). The number of OTUs was ca. three times higher in the wild beet than in the crop
225 (Fig. 2 AB), while the diversity was around 1.5 times higher (Fig. 2C), and evenness was ca. 1.3 times
226 greater (Fig. 2D).

227 *Both endophytic and rhizospheric soil bacterial community is dominated by Proteobacteria*

228 In total, 72 bacterial strains were identified, 35 coming from sugar beet and 37 from sea beet.
229 Proteobacteria were the most frequent phylum in fresh roots of both sugar and sea beet, followed by
230 Actinobacteria in the crop and Firmicutes in the wild plant. *Pseudomonas* and *Sphingomonas* were
231 characteristic for fresh roots of sugar beet, while *Bosea* and *Sphingopyxis* were found exclusively in
232 sea beet roots before lyophilization (Table 2).

233 There was no significant differences in taxonomic composition of rhizospheric soil bacterial
234 communities of sugar- and sea beet at the level of phylum (Fig 3A). At the level of genus, three taxa
235 were differentially represented, all of them belonging to Alphaproteobacteria: two Rhizobiales-
236 belonging genera, *Pedomicrobium* and an unknown genus of JG34.KF.361 family as well as
237 *Woodsholea* (Caulobacteraceae) were more abundant in the crop (fig. 3C). Differences in lyophilized
238 roots communities were more pronounced, although still there were no taxa significantly differentially
239 represented between osmolyte treatments. At the level of phyla Proteobacteria-derived reads were
240 more abundant in libraries from sugar beet lyophilized roots, while Actinobacteria, Bacteroidetes,
241 Acidobacteria, Verrucomicrobia and rare phyla were more abundant in its wild ancestor (Fig. 3B).
242 Among genera significant differences were observed for *Stenotrophomonas* and *Bacillus* that were
243 more abundant in the crop and for proteobacterial genera *Novosphingobium*, *Devosia*
244 (Alphaproteobacteria), *Hydrogenophaga*, *Polaromonas* (Betaproteobacteria), *Rhizobacter* and
245 *Tahibacter* (Gammaproteobacteria) as well as for rare and unclassified genera being more abundant in
246 sea beet (Fig. 3D)

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248 ***Culturable bacterial cell density in lyophilized roots depends on host genotype but not on osmolyte***

249 Density of culturable root endophytic bacteria was higher in sugar beet lyophilizates than in
250 sea beet (ANOVA, $F=...$, $p<0.05$, Fig. 4), regardless of the osmolytes addition. We observed no
251 influence of osmolytes on sea beet endophytes density, while trehalose increased slightly, but
252 significantly (ANOVA, $F=...$, $p<0.05$) the density in sugar beet samples (Fig. 4).

254 ***Sea beet endophytes are more salt tolerant than sugar beet ones***

255 Increasing salinity negatively affected growth of culturable fraction of microbiome regardless
256 of origin (sea- vs. sugar beet), however stronger effect was observed for *B. huzar*. In control treatment
257 the growth was inhibited (final cell density below the critical level of 0.2 OD₆₀₀) at 200 mM and 300
258 mM NaCl concentration for sugar and sea beet, respectively. Addition of osmolytes enhanced the
259 growth in general and increased the inhibitory concentration to 400 and 700 mM, respectively
260 (Supplementary Table 1). Influence of both osmolytes was similar with trehalose performing slightly
261 better at high NaCl concentrations, and it was greater for sea beet, than for sugar beet (Fig. 5).

263 ***Osmolytes enhance viability of Proteobacteria in lyophilized root samples***

264 No influence of osmolytes on diversity (Shannon's H'), observed, as well as estimated total
265 (Chao1) OTU richness and Shannon's evenness was observed in 16S rRNA gene libraries (Fig 2).
266 Lyophilization without addition of osmolytes eliminated Actinobacteria and promoted Firmicutes,
267 while addition of osmolytes (particularly trehalose) caused increase of Proteobacteria. (Table 2). To
268 the contrary, community structure assessed via 16S rRNA gene fragment sequencing was similar,
269 regardless of the osmolyte treatment (Fig. 2B,D).

271 ***Bacterial viability in lyophilized roots were associated with plant genotype and osmolyte***

272 Bacterial viability in lyophilized roots of sugar beet was consistently higher than in roots of its
273 wild relative, regardless of osmolyte treatment, storage time and measurement methodology. Both
274 osmolytes used increased the viability compared to control, regardless of genotype, but the effect of
275 trehalose was more pronounced (Fig. 6).

276 Discussion

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278 *Bacterial diversity in beet rhizosphere*

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280 Exudates play pivotal role in shaping the rhizosphere ecosystem (Bashir et al., 2016).
 281 Differences in rhizospheric soil physicochemical properties observed in our study, may be due to
 282 greater nutritional demands of the plant (TN, Na) or varying exudates composition (OC), as it was
 283 found that rhizodeposition is the primary organic carbon source in rhizosphere (Bashir et al., 2016).
 284 Alternatively, they might be caused by changes in microbial activity resulting from exudation or
 285 interaction between microorganisms (Nannipieri et al., 2008; Shi et al., 2011).

286 Greater microbiome diversity in rhizosphere compared to endosphere was commonly
 287 observed, and resulted from natural plant selection mechanisms (Kandel et al., 2017; Liu et al., 2017;
 288 Cheng et al., 2019). Accordingly, in our study, the higher bacterial diversity, evenness and species
 289 richness were noted for rhizosphere soil of both investigated genotypes, than for roots. At the same
 290 time, in spite of slightly different TN, OC and Na levels, microbiome composition and diversity were
 291 similar in rhizospheric soils of both studied beets. This observation could be explained by the use of
 292 the same garden soil and short culture period, not allowing the differences to fully manifest. Culture-
 293 independent analysis revealed that dominating bacterial phyla were the same as those observed in
 294 rhizosphere of many plant species e.g. barley, alfalfa, wheat (Velázquez-Sepúlveda et al., 2012;
 295 Bulgarelli et al., 2015; Kumar et al., 2019). Only a few differences were noted at the genus level,
 296 mainly concerning Alphaproteobacteria. *Pedomicrobium* as well as JG34.KF.361_ge, more frequent in
 297 sugar beet, represent *Rhizobiales*, an order known for organisms that establish beneficial interactions
 298 with plants and comprises numerous bacteria with nitrogen-fixing capability (Erlacher et al., 2015).
 299 The observed lower TN level in the sugar beet rhizosphere may indicate crop's higher demand for
 300 nitrogen. Tsurumaru et al. indicated that *Mesorhizobium* and *Bradyrhizobium*, also belonging to
 301 *Rhizobiales*, play an important ecological role in the taproot of sugar beet (2015). Moreover, Abdel-
 302 Motagally and Attia showed that higher levels of nitrogen (N) and potassium (K) significantly affect
 303 the growth parameters of sugar beet (2009). Both elements were generally recognized as crucial for
 304 obtaining higher yields of this crop, favorably affecting organic metabolites biosynthesis and
 305 improving nutritional status (Abdel-Motagally and Attia, 2009).

306

307 *Bacterial diversity in beet roots*

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309 The higher diversity both in rhizo- and endosphere of the wild plant compared to its crop
310 counterpart was observed (Zachow et al. 2014; Ofek-Lalzar et al., 2016). It was hypothesized that
311 beneficial endophytes associated with wild plants were absent or fewer in domesticated crops (Ofek-
312 Lalzar et al., 2016). Sugar beet as a cultivated plant grows under more controlled conditions regulated
313 by farmers, while sea beet grows mainly in highly saline and nutrients poor coastal soil (Tan et al.,
314 2017). Growth under adverse environmental conditions requires support of microorganisms with a
315 wide range of beneficial metabolic properties tailored for specific plant needs (Szymańska et al.,
316 2018). The loss of high tolerance to salt stress during the process of sea beet domestication was
317 demonstrated (Rozema et al., 2014) and might be associated with the loss of microbes that increased
318 tolerance of this plant to salinity. Concordantly, despite the lack of differences in rhizospheric soil
319 microbial composition, lower diversity of endophytes in sugar beet compared to its wild ancestor was
320 noted in our study. This difference might be explained by varying root system architecture, with
321 fibrous root system of sea beet providing more opportunities for bacteria to enter the endosphere
322 (Kandel et al., 2017), which affects stochastic community assembly. On the other hand microbe
323 selection can be driven by the genetic makeup of two studied subspecies. We observed that sea beet
324 caused decrease in the soil Na level, suggesting accumulation of Na ions in wild plant tissues.
325 Accordingly, there was an increase in community salinity resistance in this plant, which pointed at
326 higher level of halotolerant and halophytic microorganisms.

327 In general, endophytic microbiome diversity and composition is related to soil properties as
328 well as plant ecology and physiology (Miliute et al., 2015). Members of only three phyla
329 (Proteobacteria, Actinobacteria and Firmicutes) were cultured in our experiment, this may be related to
330 their high ability to grow on commercially available media (Miliute et al., 2015; Szymańska et al.
331 2016a, Szymańska et al. 2016b; Brígido et al., 2019). Miliute et al. (2015) emphasized that
332 Proteobacteria distinctly predominate among culturable plant endophytes, then the presence of
333 Firmicutes and Actinobacteria is common, and Bacteroidetes occur slightly less frequently.

334 16S rRNA libraries generated in our study were dominated by the four phyla (Proteobacteria,
335 Actinobacteria, Firmicutes, Bacteroidetes) commonly found in endosphere of glycophytes including
336 maize (*Zea mays* L.), *Dactylis glomerata* L., *Festuca rubra* L. and *Lolium perenne* L. as well as in
337 halophytes such as *Salicornia europaea* or para grass (*Urochloa mutica*) (Mukhtar et al., 2016; Zhao
338 et al., 2016; Wemheuer et al., 2017; Correa-Galeote et al., 2018; Szymańska et al., 2018). Sea beet was
339 characterized by significantly higher frequency of Actinobacteria, Bacteroidetes, Acidobacteria,
340 Verrucomicrobia and rare phyla compared to sugar beet, where Proteobacteria were observed more
341 often. Zachow et al. (2014) noted more Actinobacteria, Bacteroidetes and Verrucomicrobia in

rhizosphere of wild beet cultivated in coastal soil than in sugar beet. This fact, together with our results, may point at these bacterial taxa being preferred by sea beet regardless of soil.

Our 16S rRNA gene sequencing results also revealed significantly higher abundance of certain genera in sea beet endosphere, including: *Novosphingobium*, *Devosia* (Alphaproteobacteria), *Hydrogenophaga*, *Polaromonas* (Betaproteobacteria), *Rhizobacter* and *Tahibacter* (Gammaproteobacteria) as well as certain rare and unclassified bacteria. These microorganisms comprise extremophiles, e.g. *Polaromonas* (Margesin et al., 2012; Gawor et al., 2018) or *Hydrogenophaga* (Khan and Goel, 2008) and organisms modulating plant stress response, such as *Novosphingobium* (Etesami and Beattie, 2018; Vives-Peris et al., 2018). In our study, only *Stenotrophomonas* and *Bacillus* species were more frequent in roots of sugar beet than of sea beet. *Stenotrophomonas* and *Pseudomonas* sp. were identified in rhizospheric soil of sugar and sea beet while first of them and *Staphylococcus* sp. were mainly observed for crop rhizosphere. Sea beet microbiome was found to be more diverse than that of sugar beet (Zachow et al., 2014), which explains greater number of rare taxa. It was found that sugar beet rhizosphere was more frequently colonized by strains with antagonistic activity against plant pathogens and/or stress protection activity, while abiotic stress-releasing ones were more often found in sea beet's rhizosphere (Zachow et al., 2014). These facts together with our results suggest that pre-adaptation to stress observed in sea beet transcriptome (Skorupa et al., 2019) may also take place at the level of microbiome serving as a helper.

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Osmoprotectants enhance bacterial viability and diversity in lyophilized beet roots

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Significantly higher cell density of culturable bacteria observed in sugar beet lyophilized roots can be attributed to high content of sucrose (Haankuku et al., 2015). This sugar acts as a natural osmoprotectant, allowing a better viability of microorganisms during lyophilization (Bircher et al., 2018). Another explanation of obtained results can be associated with higher ability of sugar beet endophytes to grow on solid medium.

Sea beet endophytic microbiome was found to be more resistant to salinity. Microorganisms present in a more saline sea beet tissue most likely developed mechanisms of adaptation to high salt level, which provided them ability to grow in higher NaCl concentrations compared to the sugar beet microbiome. This fact may be related to higher sodium accumulation in this plant tissues (Skorupa et al. 2019), which caused drop in soil sodium concentration observed in our study.

Salinity-induced changes in community structure and adverse effects on microbial density, activity, biomass were reported by many scientists (Yan et al., 2015, Zhang et al., 2019). The decrease in number of culturable microorganisms related to increasing NaCl concentration was noted even in the case of endophytes associated with halophytes (*Aster tripolium*, *Salicornia europaea*) (Szymańska et al., 2013; Szymańska et al., 2016). Obtained results were in line with the above trend, but apart from negative effect of salinity on sugar and sea beet bacterial density, a beneficial impact of trehalose and ectoine on salt stress mitigation was demonstrated. Although ectoine is a major osmolyte in aerobic chemoheterotrophic bacteria and is considered as a marker for halophytic bacteria (Roberts, 2005), a slightly better effect of trehalose, was confirmed by the results of microscopic analyzes, flow cytometry and culture tests. Better performance of trehalose can be connected with its higher concentration used, which better counteracts the external osmotic pressure. Protective effect of trehalose is explained by “water replacement hypothesis” that states that the compound lowers the phase transition temperature of membrane phospholipids, by replacement of water molecules occurring around the lipid head groups (Berninger et al., 2017), thus protecting membrane structure (Nounjan and Theerakulpisut, 2012). The suggest that the use of trehalose is a better and more economic solution providing high viability of bacterial cells after lyophilization. In the case of sugar beet the above mentioned positive sucrose impact was enhanced by trehalose addition. Similar effect was observed by Pereira et al. (2012) for rhizobial strains, where trehalose worked better than sucrose/peptone mixture. In general, 16S rRNA gene sequencing results considering diversity of endophytes associated with sea and sugar beet root did not show any effect of applied osmoprotectants neither on alpha nor beta diversity of bacteria. This observation can be explained by the presence of ‘relic DNA’, i.e. DNA coming from non-viable cells (Carini et al., 2017) in lyophilized samples.

Bacillus sp. was the only species identified among the strains representing the Firmicutes phylum isolated from the lyophilized osmolytes treated roots of both investigated genotypes, in the control variant the presence of *Psychrobacillus* sp. and *Paenibacillus* sp. inside sea and sugar beet root was additionally found, respectively. The presence of the above-mentioned strains in the roots of the tested plants after lyophilization was associated with the commonly known their ability to endospore-forming and higher tolerance to environmental changes (Nicholson et al., 2000; Pham et al., 2015; Sáez-Nieto et al., 2017). Actinobacteria proved to be a very sensitive to lyophilization. Proteobacteria remarkably well tolerated the lyophilization, additional osmolytes promoted the incidence of culturable bacteria belonging to this phylum.

Conclusions

Our research revealed that plant genotype played a pivotal role in the shaping of its endophytic microbiome diversity and physicochemical rhizosphere soil properties, mainly on salinity, but not soil bacterial community structure. Bacterial diversity was lower in sugar beet roots than in its wild ancestor tissues. At the same time sea beet endophytic microbiome was more salt resistant and consisted of genera characteristic for extreme environments.

Supplementing osmoprotectants during root tissue lyophilization had a positive effect on bacterial salt stress tolerance, viability and density. Trehalose proved to improve these parameters more effectively than ectoine, moreover its use was economically advantageous.

Authors contribution

SS – performed experiments, analyzed data, drafted the manuscript, MS – performed experiments, helped in drafting the manuscript, KH – conceptualized the study, participated in writing the manuscript, JT – participated in writing the manuscript, AT – participated in writing the manuscript, MG – supervised the project, conceptualized the study, analyzed data, participated in writing the manuscript.

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Tables

Table 1. Physico-chemical rhizospheric soil parameters (mean and standard deviation) obtained after three months of cultivation of sugar- and sea beet.

Parameter\plant species	<i>B. huzar</i>	<i>B. maritima</i>
OC (%)	4.97 (1,646)	5.66 (1,210)
N _t (%)	0.28 (0.032)	0.34 (0.025) [↑]
CaCO ₃ (%)	1.88 (0.178) [↑]	1.6 (0.107)
P _{cytr.} [mg/kg]	1183,08 (116,312)	1253,29 (48.876)
pH	7.1 (0.077)	7.0 (0.074)
EC 1:5 [μS·cm ⁻¹]	176,93 (57,826)	142,28 (23,973)
Na ⁺ [mg·dm ⁻³]	16,80 (7,652) [↑]	7,35 (1,885)
K ⁺ [mg·dm ⁻³]	2,95 (0,644)	2,55 (1,111)
Ca ²⁺ [mg·dm ⁻³]	9,63 (2,291)	12,93 (2,916)
Mg ²⁺ [mg·dm ⁻³]	1,28 (0,306)	1,52 (0,223)
Cl ⁻ [mg·dm ⁻³]	46,02 (15,494)	38,94 (2,890)
SO ₄ ²⁻ [mg·dm ⁻³]	39,35 (5,211)	38,58 (7,998)
HCO ₃ ⁻ [mg·dm ⁻³]	94,55 (14,306)	88,45 (29,063)

[↑] significantly higher level based on Newman-Keuls test of rhizosphere soil parameter observed between the plant species.

Table 2. Identification of cultivable endophytic bacteria associated with roots of sugar- and sea beet before and after lyophilization without addition of any osmolyte (C) or supplemented either with ectoine (E) or trehalose (T).

Genotype		
treatment	<i>B. huzar</i>	<i>B. maritima</i>
before lyophilization	1 <i>Kocuria</i> sp. HU3-3 (A)	<i>Micrococcus</i> sp. BM2-4 (A)
	2 <i>Kytococcus</i> sp. HU3-20A (A)	<i>Bacillus</i> sp. BM2-13 (F)
	3 <i>Microbacterium</i> sp. HU1-2 (A)	<i>Bacillus</i> sp. BM2-7 (F)
	4 <i>Microbacterium</i> sp. HU1-14 (A)	<i>Paenibacillus</i> sp. BM1-5 (F)
	5 <i>Brevibacillus</i> sp. HU3-20B (F)	<i>Bosea</i> sp. BM1-16 (P)
	6 <i>Neorhizobium</i> sp. HU1-12 (P)	<i>Bosea</i> sp. BM1-9 (P)
	7 <i>Pseudomonas</i> sp. HU1-4 (P)	<i>Neorhizobium</i> sp. BM1-2 (P)
	8 <i>Pseudomonas</i> sp. HU3-10 (P)	<i>Neorhizobium</i> sp. BM2-15 (P)
	9 <i>Shinella</i> sp. HU1-3 (P)	<i>Shinella</i> sp. BM1-4 (P)
	10 <i>Sphingomonas</i> sp. HU3-5 (P)	<i>Shinella</i> sp. BM2-10 (P)
	11	<i>Sphingopyxis</i> sp. BM2-8 (P)
lyoph ilizzati	C 1 <i>Gordonia</i> sp. BH1CTR8 (A)	<i>Bacillus</i> sp. BM1CTR1 (F)
	2 <i>Bacillus</i> sp. BH1CTR2 (F)	<i>Bacillus</i> sp. BM1CTR10 (F)
	3 <i>Bacillus</i> sp. BH1CTR5 (F)	<i>Bacillus</i> sp. BM1CTR9 (F)

	4	<i>Bacillus</i> sp. BH3CTR4 (F)	<i>Bacillus</i> sp. BM3CTR10 (F)
	5	<i>Paenibacillus</i> sp. BH3CTR10 (F)	<i>Bacillus</i> sp. BM3CTR4 (F)
			<i>Psychrobacillus</i> sp. BM3CTR11 (F)
	6	<i>Acinetobacter</i> sp. BH2CTR5 (P)	
	7	<i>Pantoea</i> sp. BH4CTR1 (P)	
	8	<i>Pseudoxanthomonas</i> sp. BH3CTR6 (P)	
	9	<i>Pseudoxanthomonas</i> sp. BH3CTR9 (P)	
	10	<i>Shinella</i> sp. BH1CTR1 (P)	
E	1	<i>Bacillus</i> sp. BH1EKT5 (F)	<i>Bacillus</i> sp. BM1EKT11 (F)
	2	<i>Bacillus</i> sp. BH1EKT9 (F)	<i>Bacillus</i> sp. BM1EKT2 (F)
	3	<i>Bacillus</i> sp. BH4EKT3 (F)	<i>Bacillus</i> sp. BM2EKT5 (F)
	4	<i>Bacillus</i> sp. BH4EKT5 (F)	<i>Bacillus</i> sp. BM4EKT1 (F)
	5	<i>Pseudoxanthomonas</i> sp. BH1EKT3 (P)	<i>Bacillus</i> sp. BM4EKT10 (F)
	6	<i>Pseudoxanthomonas</i> sp. BH5EKT5 (P)	<i>Shinella</i> sp. BM1EKT6 (P)
	7	<i>Sphingobium</i> sp. BH1EKT10 (P)	<i>Stenotrophomonas</i> sp. BM4EKT2 (P)
	8	<i>Sphingobium</i> sp. BH2EKT1 (P)	<i>Stenotrophomonas</i> sp. BM4EKT3 (P)
	9		<i>Stenotrophomonas</i> sp. BM4EKT5 (P)
	10		<i>Stenotrophomonas</i> sp. BM4EKT8 (P)
T	1	<i>Bacillus</i> sp. BH3TRE4 (F)	<i>Nocardiopsis</i> sp. BM4TRE1
	2	<i>Pantoea</i> sp. BH4TRE2 (P)	<i>Bacillus</i> sp. BM1TRE9 (F)
	3	<i>Pantoea</i> sp. BH4TRE3 (P)	<i>Bacillus</i> sp. BM3TRE11 (F)
	4	<i>Pseudomonas</i> sp. BH4TRE5 (P)	<i>Bacillus</i> sp. BM3TRE8 (F)
	5	<i>Pseudomonas</i> sp. BH4TRE1 (P)	<i>Bacillus</i> sp. BM4TRE10 (F)
	6	<i>Shinella</i> sp. BH2TRE2 (P)	<i>Bacillus</i> sp. BM4TRE4 (F)
	7	<i>Shinella</i> sp. BH2TRE3 (P)	<i>Pseudomonas</i> sp. BM1TRE2 (P)
	8		<i>Pseudomonas</i> sp. BM3TRE2 (P)
	9		<i>Pseudoxanthomonas</i> sp. BM1TRE1 (P)
	10		<i>Shinella</i> sp. BM3TRE3 (P)

Supplementary Table 1. Osmolytes effect on growth of LB cultures inoculated with lyophilized sugar and sea beet roots untreated with any osmolyte (C), treated with ectoine (E) and trehalose (T). Means of minimum three replicates and standard deviations are given. Statistical significance was assessed with ANOVA and Tukey HSD, significant differences between salinity levels are denoted with different letters.

NaCl (mM)	<i>B. huzar</i>			<i>B. maritima</i>		
	C	E	T	C	E	T
0	0,395 (0,0269) i	0,445 (0,0502) i	0,472 (0,0412) i	0,338 (0,028) fg	0,528 (0,082) f	0,477 (0,0875) f
50	0,351 (0,0359) h	0,432 (0,0443) h	0,430 (0,0406) h	0,352 (0,051) fg	0,485 (0,125) f	0,476 (0,0537) f

100	0,310 (0,0450) g	0,380 (0,0482) g	0,386 (0,0322) g	0,359 (0,053) g	0,403 (0,058) e	0,462 (0,0472) f
150	0,272 (0,0472) f	0,316 (0,0399) f	0,378 (0,0332) f	0,357 (0,029) g	0,383 (0,049) e	0,438 (0,0616) f
200	0,194 (0,0469) e	0,235 (0,0355) e	0,267 (0,0493) e	0,312 (0,034) f	0,360 (0,051) de	0,425 (0,0752) f
300	0,158 (0,0418) d	0,203 (0,0277) d	0,202 (0,0359) d	0,190 (0,033) e	0,306 (0,056) cd	0,322 (0,0615) e
400	0,078 (0,0195) c	0,115 (0,0267) c	0,154 (0,0415) c	0,164 (0,040) de	0,277 (0,055) bc	0,266 (0,0570) d
500	0,053 (0,0106) bc	0,099 (0,0250) c	0,132 (0,0328) bc	0,145 (0,031) cd	0,254 (0,032) bc	0,241 (0,0542) Cd
600	0,041 (0,0132) b	0,059 (0,0135) b	0,109 (0,0226) b	0,125 (0,026) bc	0,226 (0,064) b	0,201 (0,0333) bc
700	0,004 (0,0015) a	0,007 (0,0037) a	0,007 (0,0039) a	0,102 (0,028) ab	0,157 (0,051) a	0,157 (0,0339) ab
800	0,004 (0,0024) a	0,006 (0,0025) a	0,007 (0,0024) a	0,078 (0,013) a	0,120 (0,040) a	0,141 (0,0297) a
900	0,003 (0,0021) a	0,004 (0,0029) a	0,007 (0,0029) a	0,070 (0,009) a	0,102 (0,017) a	0,140 (0,0346) a

Figures

Figure 1. Experimental design.

Figure 2. Species richness, evenness and diversity of bacterial communities in rhizospheric soils of

sugar beet (Bh) and sea beet (Bm) and lyophilized roots of these plants untreated (C), and treated with ectoine (E) or trehalose (T). Means (n=8-32) are presented, whiskers show standard error of the mean (SEM), and significant differences (ANOVA, $p < 0.05$) are denoted with different letters. Observed number of OTUs (A), estimated total number of OTUs (Chao1 index, B), Shannon's diversity index (H' , C), Shannon's evenness (D).

Figure 3. Taxonomic composition of bacteria communities in rhizospheric soils of sugar beet (Bh) and sea beet (Bm) (A, C) and lyophilized roots of these plants (B, D) untreated (Bh_C and Bm_C) and treated with ectoine (Bh_E, Bm_E) or trehalose (Bh_T, Bm_T) at the phylum (A, B) and genus (C, D) levels. Means (n=8-32) are presented, and significant differences between genotypes are marked with asterisks. No significant differences due to osmolytes were found.

Figure 4. Density of endophytic bacteria (expressed as log₁₀ CFU per g of dry weight) isolated from lyophilized sugar- and sea beet roots. Means (n = 3) ± standard deviation are presented. Significant differences between variants (ANOVA, p<0.05, with Tukey's HSD; C – control untreated with any osmolyte, ectoine (E) or trehalose (T) treated) were marked with different letters.

484 **Figure 5.** Osmolytes effect on growth of LB cultures inoculated with lyophilized sugar and sea beet
485 roots untreated with any osmolyte (C), treated with ectoine (E) and trehalose (T). Means (n = 4-6) ±
486 standard deviation are presented. Significant differences between treatments (ANOVA, p<0.05, with
487 Tukey's HSD) are marked with asterisks.
488

489 **Figure 6.** Bacterial viability in lyophilized beet roots. Viability measured with BD Cell Viability kit

490 under fluorescence microscope (AB) and using flow cytometer (C). Means are presented and
491 statistically significant differences are marked with differing letters.
492

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