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DIVERSITY AND ECOLOGICAL TOLERANCE OF BACTERIA ISOLATED FROM THE RHIZOSPHERE OF HALOPHYTON PLANTS LIVING NEARBY KISKUNSÁG SODA PONDS, HUNGARY

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Many halophytes and halophilic microorganisms are capable to adapt to the extremities of saline habitats. This study reveals the taxonomic diversity and ecological tolerance of bacteria isolated from the rhizosphere of three different halophytes (*Bolboschoenus maritimus, Puccinellia limosa* and *Aster tripolium*) living in the vicinity of Kiskunság soda ponds. Following a sampling in September 2013, altogether 76 bacterial strains were isolated using two different media. The strains were identified on the basis of 16S rRNA gene sequencing following ARDRA grouping. Salt and pH tolerance of the strains were examined by measuring their growth in broths containing 0–15% NaCl (w/V) and characterized with pH 7–12 values. Among the strains genera of *Anaerobacillus, Bacillus* and *Exiguobacterium* (Firmicutes), *Agromyces, Isoptericola, Microbacterium, Micrococcus, Nocardiopsis, Nesterenkonia* and *Streptomyces* (Actinobacteria), *Halomonas* and *Idiomarina* (Proteobacteria) and *Anditalea* (Bacteroidetes) were identified. The *Bolboschoenus* and *Puccinellia* samples characterized with the highest pH and electric conductivity values were dominated by *Bacillus, Halomonas* and *Nesterenkonia,* respectively. The salt tolerance of the bacterial strains was strongly dependent on the sampling location and plant species. In contrast, growth of bacterial strains in broths with alkaline pH values was more balanced. The strains from the *Puccinellia* sample showed the widest salt and pH tolerance.

Keywords: soda soil, rhizosphere bacteria, cultivation, 16S rRNA gene, pH and NaCl tolerance

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Introduction

In Europe, in addition to soda lakes, the proportion of salt affected soils (SAS) in Hungary is the highest compared to the total area (approximately 13% of the country) [1]. The Danube–Tisza Interfluve can be characterized by Solonchak and Solonchak-Solonetz soil types which are developed on the alluvial sandy soils and classified as "Calcareous Sodic soils". In these soils sodium-carbonate, bicarbonate, sulphate and chloride are the dominant salts. Calcium-carbonate can also be found in large quantity in the whole soil profile because of the frequent occurrence of loessial and carbonatic deposits of River Danube. The electric conductivity of the saturated soil extracts for SAS varies widely (2–78 mS cm^{-1}), and the pH value is generally higher than 9.0 [1].

The vegetation shows characteristic zonation nearby the Kiskunság soda ponds. Typical plant associations develop due to the changes in topography and groundwater level which decisively influence the moisture and salinity of the upper layer of soda soils. In the salt marshes located in the shore zone of the extremely shallow soda ponds, mosaic associations are formed from homogeneous population of *Bolboschoenus maritimus* and mosaic plant association of *Bolboschoenus-Phragmitetum*. Under typical circumstances marsh vegetation is permanently water covered. "Szikfok" vegetation is characteristic in the deeper areas with repeated waterlogging away from the soda ponds. *Puccinellia limosa* is one of its typical endemic salt-tolerant plant species. The halophyton *Aster tripolium* living in soils with high salt concentration is a xero-indicator of habitats with long dry periods [2].

Studying the arbuscular mycorrhizal fungi colonization of dominant halophytes living in the Great Hungarian Plain, a characteristic seasonal dynamism was found. Mycorrhizal colonization was maximal from late spring to early summer and had a second peak later in autumn. Arbuscule formation and overall mycorrhizal colonization inversely correlated with the intensity of rainfall. It was also demonstrated that colonization depends on the plant species and their physiological status [3, 4]. In a previous study, the community level physiological profile and genetic diversity of rhizosphere microbial communities of different plant species growing nearby Kiskunság soda ponds were compared [5]. The results suggested that geographical location, soil physical and chemical properties and the vegetation type were all important factors influencing the metabolic activity and genetic diversity of rhizosphere microbial communities [5].

The aim of the present study was to reveal and compare the taxonomic diversity and ecological tolerance of bacteria cultivated from the rhizosphere of three typical salt-tolerant plant species living in the vicinity of Kiskunság soda ponds.

Materials and Methods

Study sites and samplings

In each sampling site located near Böddi-szék, Kelemen-szék and Zab-szék soda ponds in the Kiskunság National Park approximately 500–500 g of rhizosphere samples from the halophyton plant species (*Bolboschoenus maritimus* [B16, K19, Z23], *Puccinellia limosa* [B17] and *Aster tripolium* [K21, Z24]) were collected into sterile jars in September 2013. Samples were kept cool (6–8 °C) until laboratory processing within 24 hours. Prior to laboratory tests, roots and other visible plant residues were removed using a 2 mm mesh sieve.

The exact location of the sampling sites and the measured physical and chemical properties of the samples at the time of sampling are presented elsewhere [5].

Cultivation and ecological tolerance testing of bacterial strains

In order to gain insight into the abundance and species composition of rhizosphere bacterial communities, cultivation based examination was carried out using Horikoshi alkaline (DSMZ 940) and modified R2A (DSMZ 830; www.dsmz.de) media. During the laboratory work, the composition of R2A medium (DSMZ 830) was supplemented with 5.0 g Na_2CO_3 and the pH was adjusted to 9.0.

From the composite samples produced by homogenization of equal amounts of three replicate samples, tenfold serial dilutions were made and used for plating. Following a 7–14 day incubation period at 25 °C, the viable counts were determined, and colonies with different morphology were isolated. All isolates were purified and maintained on the isolation medium.

The pH range for growth was determined in a modified nutrient broth (DSMZ 1) supplemented with 50 g l^{-1} NaCl, and the pH was adjusted to 7.0–12.0 with KOH at intervals of 1.0 pH units. The salt requirement for growth was stud-

ied in nutrient broth adjusted to pH 9.0 and supplemented with 0, 5, 7, 10, 12 and 15% (w/v) NaCl. Optical density change of the medium was detected at 620 nm after 7 days of incubation.

For the boxplot analysis of NaCl and pH tolerance results, Past Paleontological Statistics V2.08 software was applied [6].

DNA extraction, ARDRA grouping and 16S rRNA gene-based identification

From each cultivated strain, a mechanical cell disruption method was applied to extract the DNA. In this procedure, a loopful of 24–48-hour slant culture was suspended in an Eppendorf tube containing 100 µl of DEPC treated water and approximately 0.1 g of glass beads. This mixture was shaken in a Mixer Mill MM301 (Retsch, Germany) at 30 sec^{-1} frequency for 2 min. The obtained cell lysates were centrifuged at 10 000 rcf for 1 min and denatured in an Applied Biosystems Gene Amp PCR System 2700 at 98 °C for 5 min. The samples were then centrifuged at 10 000 rcf for 3 min to pellet cell debris. Supernatants were transferred to new tubes, and were used as template in polymerase chain reaction.

The 16S rRNA gene was amplified using 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1401r (5′-GGG TGT GTA CAA GAC CC-3′) bacterial primer pair in a Gene Amp PCR System 2700 (Applied Biosystems). The PCR mixtures contained 1.5 mM $MgCl_2$, the enzyme buffer, 200 µM dNTPs, 0.3 µM of each primer and 1 U of *Taq* DNA polymerase. The following PCR temperature protocol was used: initial denaturation at 95 °C for 3 min, followed by 32 cycles of annealing at 52 °C for 30 s, extension at 72 ºC for 1 min and denaturation at 94 ºC 30 s, and a final extension at 72 ºC for 10 min.

The PCR products of bacterial strains were visualized in 1% agarose gel and grouped by ARDRA (Amplified Ribosomal DNA Restriction Analysis) method [7] using enzymes *Hin*6I and *Bsu*RI (Fermentas, Vilnius, Lithuania). Each reaction contained 2.5 µl Y+/Tango Buffer (Fermentas), 15.2 µl sterile distilled water, 3 U of enzyme and 7 µl of PCR product. Digestions were made at 37 °C, for 3 hours. Digestion products were separated in 2% agarose gels (Gibco), and visualized with UV excitation.

The identification of representative bacterial strains was based on partial 16S rRNA gene sequence analysis. The sequencing reaction using 27f primer was performed by the LGC Genomics (Berlin, Germany). Sequences were identified using the EzTaxon database [8]. The phylogenetic dendrograms were constructed by neighbor-joining method, using MEGA 5.0 software [9]. The accession numbers of bacterial strains are presented in the phylogenetic trees (Figs 1–3).

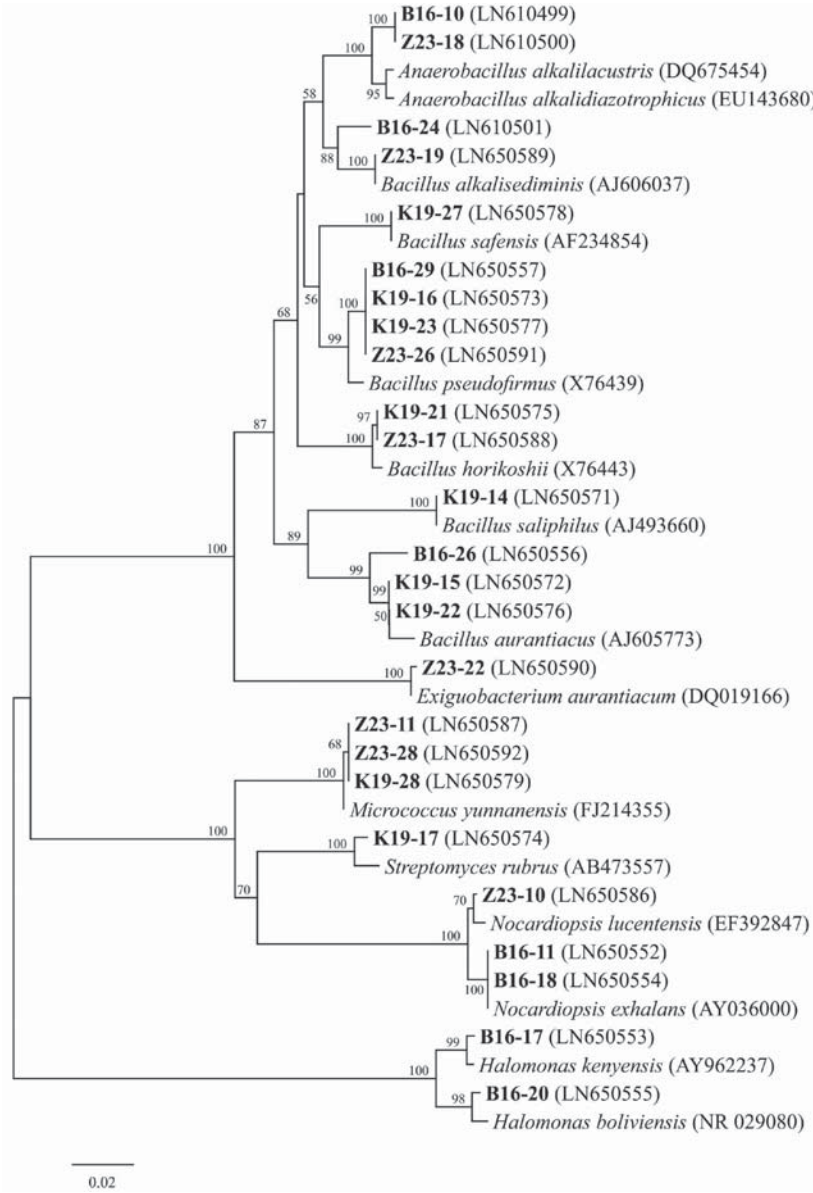

Figure 1. 16S rRNA gene sequence based neighbor-joining phylogenetic tree of the bacterial strains isolated from the rhizosphere of *Bolboschoenus maritimus.*

Notes: GenBank accession numbers are given in brackets.
The bacterial strains of this study appear in bold. Only bootstrap values above 50% are shown (1000 replications). Bar, 2 substitutions per 100 nucleotide positions

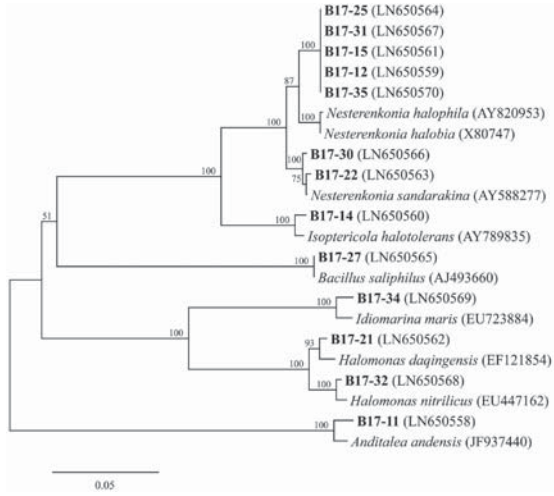

Figure 2. 16S rRNA gene sequence based neighbor-joining phylogenetic tree of the bacterial strains isolated from the rhizosphere of *Puccinellia limosa*.

Notes: GenBank accession numbers are given in brackets.
The bacterial strains of this study appear in bold. Only bootstrap values above 50% are shown (1000 replications). Bar, 5 substitutions per 100 nucleotide positions

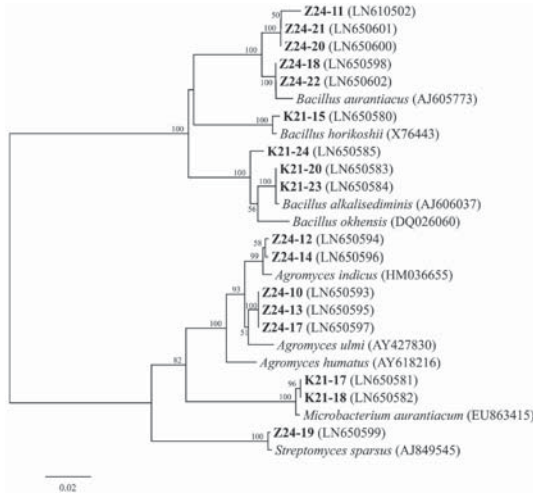

Figure 3. 16S rRNA gene sequence based neighbor-joining phylogenetic tree of the bacterial strains isolated from the rhizosphere of *Aster tripolium*.

Notes: GenBank accession numbers are given in brackets.
The bacterial strains of this study appear in bold. Only bootstrap values above 50% are shown (1000 replications). Bar, 2 substitutions per 100 nucleotide positions

Results and Discussion

Taxonomic diversity of bacterial strains

From the rhizosphere of the studied three typical halophytes altogether 76 bacterial strains were isolated, and altogether 55 ARDRA representatives (25, 13 and 17 among the *Bolboschoenus*, *Puccinellia* and *Aster* isolates) were sequenced (Figs 1–3). The strains were identified as members of genera *Anaerobacillus, Bacillus* and *Exiguobacterium* (Firmicutes), *Agromyces, Isoptericola, Microbacterium, Micrococcus, Nocardiopsis, Nesterenkonia* and *Streptomyces* (Actinobacteria), *Halomonas* and *Idiomarina* (Proteobacteria) and *Anditalea* (Bacteroidetes). From the samples representatives of 29 different bacterial species were revealed (Figs 1–3). However, some strains showed less than 97% sequence similarities to described species, therefore they may represent new species. Regarding the number of identified species (19 from the DSMZ 940 and 18 from the modified DSMZ 830), the two media showed a similar degree of selectivity. Bacterial strains belonging to some *Bacillus* (e.g. *B. alkalisediminis, B. pseudofirmus, B. horikoshii, B. saliphilus* and *B. aurantiacus*) and *Nesterenkonia* (e.g. *N. halophila* and *N. halobia*) species as well as *Micrococcus yunnanensis* were isolated from both media.

Most of the *Bolboschoenus* rhizosphere isolates were related to different *Bacillus* species (Fig. 1). Representatives of *Bacillus* species are frequently isolated from different soda lakes and soils worldwide [10–16]. Several alkaliphilic and/or halophilic *Bacillus* species (e.g. *B. alkalicola*, *B. alkalinitrilicus*, *B. bogoriensis, B. chagannorensis, B. daliensis, B. daqingensis, B. locisalis*) were described from alkaline and/or saline lakes and soils [17–23], including the Hungarian soda ponds, as well. From the rhizosphere of *Bolboschoenus* surrounding all three studied soda ponds, besides representatives of *B. horikoshii, B. pseudofirmus, B. saliphilus* and *B. safensis*, a number of strains belonging to *B. aurantiacus* [24] and *B. alkalisediminis* [25] described from the sediment of Kiskunság soda ponds were recovered (Fig. 1). Two other strains which may represent novel taxa (Fig. 1), showed the highest sequence similarities to the strictly fermentative, aerotolerant and alkaliphilic species of *Anaerobacillus alkalidiazotrophicus* [26] described from a Mongolian soda soil and to the strictly anaerobic and obligate alkaliphilic species of *Anaerobacillus alkalilacustris* [27] described from the sediment of Khadyn soda lake (Russia). From the *Bolboschoenus* rhizosphere only one strain showed high sequence similarities to the alkaliphilic species of *Exiguobacterium aurantiacum* [28, 29].

Among the isolates from the *Bolboschoenus* rhizosphere, the phylum Actinobacteria was represented by strains closely related to *Micrococcus yun-*

nanensis, Streptomyces rubrus and *Nocardiopsis* species (Fig. 1). The species of *M. yunnanensis* was recently described from surface-sterilized *Polyspora axillaris* roots [30]. Members of the genus *Streptomyces* are rarely isolated from soda lakes [12] but plant growth promoting activity of a *Streptomyces* isolate under saline soil conditions has been reported [31]. Representatives of the genus *Nocardiopsis* were isolated only from the rhizosphere of *Bolboschoenus maritimus* (Fig. 1), and were identified as *N. lucentensis* and *N. exhalans*. The latter species was detected also by cultivation from the shallow haloalkaline Lake Elmenteita, Kenya [12].

Members of different *Halomonas* species are also frequently isolated from soda lakes and soils [11–16]. In the rhizosphere of *Bolboschoenus maritimus,* strains identified as species of haloalkaliphilic *H. kenyensis* described from sediment samples of Kenyan soda lakes [32] and alkalitolerant and moderately halophilic *H. boliviensis* [33] described from a soil sample around the hypersaline lake Laguna Colorada (Bolivia) were found (Fig. 1).

The majority of the strains, isolated exclusively from the rhizosphere of *Puccinellia limosa* (Fig. 2), were related to moderately halophilic species of *Nesterenkonia halobia* [34], *N. sandarakina* [35] and *N. halophila* [36], described from ponds of a saltern located in Spain and from saline soil samples located in the eastern desert of Egypt and north-west China, respectively. In addition, strains affiliated with the recently described haloalkaliphilic *Bacillus saliphilus* [37], *Isoptericola halotolerans* [38], *Idiomarina maris* [39] as well as the moderately halophilic *Halomonas daqingensis* [40] and the haloalkaliphilic *Halomonas nitrilicus* [41] were identified (Fig. 2). Only one strain isolated also from the rhizosphere of *Puccinellia limosa* represented the phylum Bacteroidetes and was closely related to the alkaliphilic and halotolerant species of *Anditalea andensis* [42].

Members of different *Agromyces* species are among the bacteria usually detected by cultivation from various soil and rhizosphere samples. In a previous study, representatives of this genus were cultivated from the sediment of Zab-szék soda pond [13]. In this study, all strains identified as *Agromyces ulmi* [43], *A. humatus* [44] and *A. indicus* [45] originated from the rhizosphere of *Aster tripolum* in the vicinity of Zab-szék soda pond (Fig. 3). Within the phylum Actinobacteria, strains representing species of *Microbacterium aurantiacum* and *Streptomyces sparsus* were also detected. Furthermore, strains belonging to alkaliphilic or alkalitolerant and moderately halophilic or halotolerant species of *Bacillus alkalisediminis*, *B. aurantiacus*, *B. horikoshii* and *B. okhensis* [46] were identified from the rhizosphere of *Aster tripolum*, as well (Fig. 3).

Based on the results of this study, the highest taxonomic diversity with 15 different species was revealed from the rhizosphere of *Bolboschoenus mariti-*

mus. Although there was no substantial difference in the number of cultivated species among the sampling sites, their species composition was partly different.

Both the *Bolboschoenus* and *Puccinellia* rhizosphere samples originated from the shore of Böddi-szék soda pond and characterized with high pH and electric conductivity values [5] were dominated by representatives of *Bacillus* and *Halomonas* species. On the other hand, in the rhizosphere of *Aster tripolum* at Zab-szék soda pond where very low electric conductivity was detected [5], members of the genus *Agromyces* were typical. In contrast, members of the genus *Nesterenkonia* were cultivated only from the rhizosphere of *Puccinellia limosa* near the Böddi-szék soda pond where the highest electric conductivity was measured [5].

Ecological tolerance of bacterial strains

The NaCl and pH tolerance results of bacterial strains are presented in boxplot diagrams (Figs 4 and 5) according to the studied halophytes.

On the basis of the NaCl tolerance results, most strains were able to grow best in broths supplemented with 5, 7 and 10% NaCl (the highest 50th percentiles), and less growth was observed in broths without NaCl and supplemented with 12 and 15% NaCl, regardless of the sample types (Fig. 4). However, substantial differences can be seen among the growth rate of bacterial strains. NaCl tolerance results of bacterial strains isolated from the *Bolboschoenus* rhizosphere are similar to those of the soda lakes and can be characterized with very small H-spread values (the difference between the 75th and 25th percentiles) at 0% and 15% NaCl concentrations and relatively large number of outside values at all NaCl concentrations (Fig. 4A). However, the H-spread values are the highest in case of *Puccinellia* rhizosphere isolates (Fig. 4B). Changes in salt concentration are the highest in the habitat of *Puccinellia* in accordance with the periodic wetting drying cycles which is reflected in the salt tolerance of bacterial strains. The widest salt tolerance (from 0% to 15% NaCl concentrations) was also detected in case of strains isolated exclusively from the *Puccinellia* rhizosphere and identified as members of the genus *Nesterenkonia*. In contrast, the most uniform growth in broths with different NaCl concentrations and the lowest level of salt tolerance was detected in case of *Aster* rhizosphere isolates. In the boxplot diagram outside values are indicated almost exclusively at 0% NaCl concentration (Fig. 4C).

Compared to the NaCl tolerance results, the growth of bacterial strains was balanced in the examined pH range in all three sample types (Fig. 5). This may be related to the fact that the pH value of the examined habitats is constantly alkaline regardless of the changes in salinity. Therefore, presence of facultative and

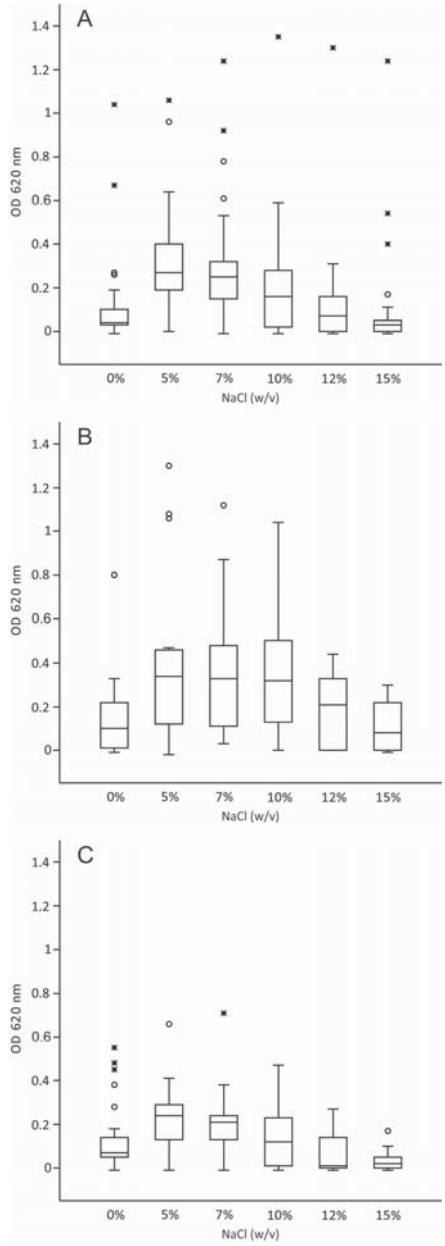

Figure 4. Boxplot diagrams of NaCl tolerance results of bacterial strains isolated from the rhizosphere of *Bolboschoenus maritimus* (A), *Puccinellia limosa* (B) and *Aster tripolium* (C). (Bacterial growth at different NaCl concentrations was detected by the changes of optical density (OD) of the medium)

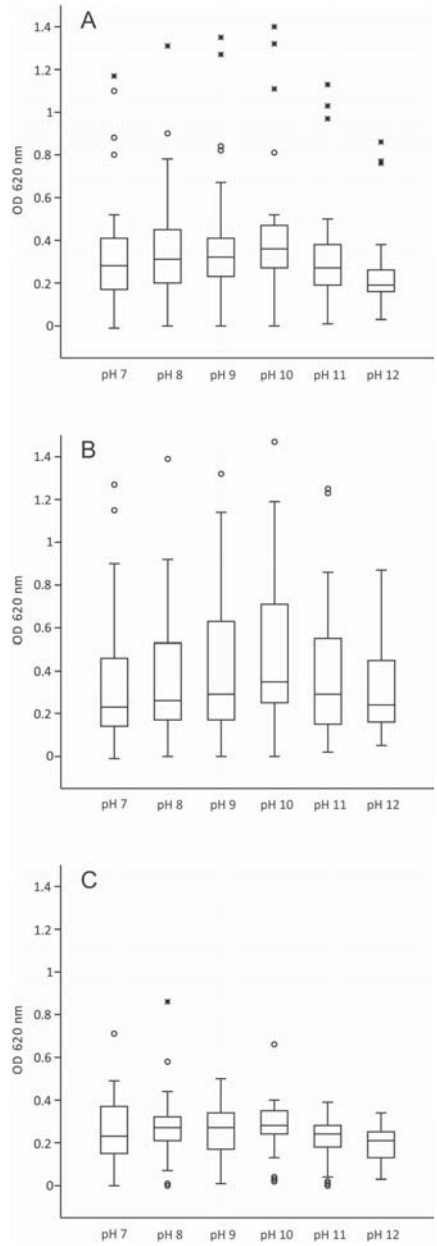

Figure 5. Boxplot diagrams of pH tolerance results of bacterial strains isolated from the rhizosphere of *Bolboschoenus maritimus* (A), *Puccinellia limosa* (B) and *Aster tripolium* (C). (Bacterial growth at different pH values was detected by the changes of optical density (OD) of the medium)

obligate alkaliphilic bacteria (e.g. *Bacillus* species) is typical. Nevertheless, similarly to the NaCl tolerance results, sample type characteristics (e.g. the highest number of the outlier points at the *Bolboschoenus* sample, the highest H-spread values at the *Puccinellia* sample) can be observed in the case of pH tolerance, as well.

Conclusions

Regardless of the media used for cultivation, most strains isolated from the rhizosphere of halophytes living nearby the Kiskunság soda ponds showed the highest 16S rDNA sequence similarity to haloalkaliphilic bacterial species described from soda and saline lakes or soils in the recent years. Both bacterial species composition and ecological tolerance of bacterial strains were partly different in accordance with the difference in the physical and chemical properties of the studied rhizosphere samples.

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Conflict of Interest

None.

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