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Bacterial seed endophytes of the holoparasitic endemic *Cistanche armena* (Orobanchaceae) from a semi-desert area in Armenia

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Abstract

We explored the seed-associated bacterial endophytic microbiome in seeds of the endemic holoparasitic species *Cistanche armena* from a saline and arid habitat in Armenia. A combination of culture-dependent and molecular techniques was employed for identifying the seed endomicrobiome (culturable and unculturable). From surface sterilized seeds, 10 phyla, 256 endophytic bacterial genera were identified. Of the culturable strains, we also investigated the plant growth-promoting (PGP) traits. Most of the isolates were spore forming, halotolerant, and alkaliphile *Bacillus* spp., indicating that the endophytic bacteria of *C. armena* seeds own traits related to the natural habitat of their host plant. Our results confirm that *Bacillus* species are common and dominated endophytes from plants growing on saline and arid soils. *Pantoea* spp. and *Stenotrophomonas* are more favourable PGP endophytes in seeds of *C. armena*. The PGP traits of these bacteria, such as production of auxins, ACC-deaminase and organic acids have the potential to improve the tolerance of their host plants against the abiotic stresses present in their natural habitat. To the best of our knowledge, that is the first report concerning bacterial seed endophytes of the *C. armena*.

Introduction

With approximately 4,750 species, parasitic plants constitute 1.6% of the angiosperms (Nickrent, 2020). Parasitism, especially holoparasitism, represents the most extreme interaction between plants, with strong associations between host and parasite biogeography, ecology, and probably with diversification (Schneider and Moore, 2017). Orobanchaceae is the largest parasitic plant family with 102 genera and over 2100 species (Nickrent, 2020). One of the most peculiar in this family is the genus *Cistanche* Hoffmanns. & Link, which includes approximately 25 species, and is found mainly in arid, semiarid and halophytic habitats across Eurasia and North Africa. These magnificent, achlorophyllous species, with fleshy stems, long underground stolons and intensely colored inflorescences grow as obligate parasite (holoparasite) on the roots of host-plant species mainly belonging to the Chenopodiaceae, Zygophyllaceae, Tamaricaceae, and Plumbaginaceae (Piwowarczyk et al., 2019). Species belonging to this genus have been widely used in traditional Chinese medicine for centuries (Li et al., 2016; Piwowarczyk et al., 2020a).

A particularity of parasitic plants is their production of huge numbers of seeds, which are also among the smallest of all seed plants. With a length of less than 1 mm they are often called 'dust seeds' (Yoneyama et al., 2008; Eriksson and Kainulainen, 2011; Piwowarczyk, 2013). The seeds possess a unique simple structure, contain only a reduced embryo, as a spherical body without a plumule, and radicle or cotyledons. The reticulated testa of these seeds with polygonal and sometimes deeply submerged walls might enhance the contact of the seed surface with water or facilitate the seed dispersal by wind. The endothelium (inner testa layer) containing mucilage and labyrinthine walls, allows rapid absorption of water, which is crucial for imbibition and subsequent germination (Piwowarczyk et al., 2020b). The cutinized endothelium has a protective role in the underground part of the plant life cycle (Dinesh et al., 2015; Piwowarczyk et al., 2019). Lipids are the main storage material in the seeds of Orobanchaceae (Ruraż et al., 2020). For germination, *Cistanche* seeds need to be very nearby their preferred host. Germination depends on hormones-strigolactones exuded from the host root (Yoneyama et al., 2008). Seeds of *Cistanche*, like related *Orobanche* s.l. species, seem to be resistant to harsh environmental conditions and stay viable in the soil for several decades (Joel et al., 2007). Among the wide range of plant protection mechanisms, the endophytic microbes have a specific role for improving the plant tolerance against different biotic and abiotic stresses (Shrivastava and Kumar, 2015).

Recently, the interest in plant endophytes from ecosystems with harsh environmental conditions, especially saline soils has increased (Hrynkiewicz et al., 2019; Manasa et al., 2020). Such endophytes can have the potential to mitigate the impacts of adverse conditions such as soil salinization, high concentrations of metals and climate change (Hallmann et al., 1997; Truyens et al., 2016; Manjunatha et al., 2017; Hemida and Reyad, 2019). Most of the seed associated bacteria are considered to have an environmental origin and to be important for the adaptation of their host to harsh environmental conditions (Frank et al., 2017). Therefore, tissues of halotolerant plants also contain halophilic bacterial communities (Etesami and Beattie, 2018) and the composition of seed-associated bacterial communities should be closely related to the soil bacterial communities. Besides of the obligate endophytes, plant tissues can be colonized by soil bacteria as well. This is explained by possible migration of bacteria from the soil to the seeds (Frank et al., 2017). According to Barret et al. (2016), the endophytes reach the seeds by: internal transmission through the vascular system and floral transmission (external transmission) through the stigma, fruits, or flowers. Indeed, during the early stages of seed development, the endophytes reach the seeds via the xylem and nonvascular plant tissues. Bacteria can also use the floral pathway to reach the seeds. Though, the floral route has a selective function, and only endophytes with biocontrol ability and nonhost pathogens can reach the seeds.

So far, ample of endophytes have been isolated from different seeds of many wild and agricultural/sylvicultural herbaceous and woody plant species (e.g., Ulrich et al., 2008; Truyens et al., 2013, 2014, 2016; Asaf et al., 2017; Glassner et al., 2018; Sánchez-López et al., 2018; Compant et al., 2019), including some holoparasitic species (tissue and seeds) such as *Phelipanche aegyptiaca*, *P. ramosa*, and *Orobanche hederae* (Iasur Kruh et al., 2017; Fitzpatrick and Schneider, 2020; Huet et al., 2020; Durlik et al., 2021). The microbiome of *P. aegyptiaca* in different developmental stages was investigated by Iasur Kruh et al. (2017). Surface-sterilized tissues of roots, haustoria and shoots harbored bacteria belonging to the Proteobacteria (*Rhizobium*, *Pseudomonas*, *Comamonadaceae* sp., *Sphingomonas* and *Burkholderia*, *Actinobacter* sp., *Bacillus* sp.). In addition, *Novosphingobium* and *Methylophilus* were reported as specific endophytes for this plant species (Iasur Kruh et al., 2017). A study of the endophytic microbiome of *O. hederae* reported that *Orobanche* leaves (scales) contain Acidobacteria, Proteobacteria, Verrucomicrobia and bacteria belonging to the *Enterobacteriaceae*, *Pseudomonadaceae*, and *Rhizobiaceae* (Fitzpatrick and Schneider, 2020). The first report about seed endophytes of the holoparasitic *P. ramosa* reported a dominance of four bacterial phyla, i.e., Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes (Huet et al., 2020). In another study on surface sterilized seeds of *P. ramosa*, culturable *Brevibacterium frigoritolerans* and *Bacillus simplex* were isolated (Durlik et al., 2021) (Table 1). Different bacterial phyla also have been isolated from plants growing in arid and semiarid regions, like *Larrea tridentata*, from the desert plant *Salsola* (Soussi et al., 2016) and the saline wetland species *Salicornia* (Szymańska et al., 2018). Furthermore, some authors argue that the bacterial phyla Proteobacteria, Bacteriodetes, Firmicutes, Planctomycetes, Actinobacteria, Fibrobacteres are common for halotolerant plants from arid and wetland soils (Soussi et al., 2016; Asaf et al., 2017; Szymańska et al., 2018).

Although many investigations highlight the importance of endophytes in plant health, the knowledge concerning communities of bacterial seed endophytes, especially about the microbiome of seeds of holoparasitic plant species is still limited (Iasur Kruh et al., 2017; Fitzpatrick and Schneider, 2020; Huet et al., 2020; Durlik et al., 2021). Therefore, the major objective of our study was to explore the bacterial endophytes (culturable and unculturable) from seeds of the holoparasitic endemic plant *Cistanche armena* (K. Koch) M.V. Agab. (Orobanchaceae) from a saline and semi-desert habitat of Armenia. The other aim was to

investigate the potential plant growth-promoting (PGP) traits of the culturable seed endophytes that might have a role in plant responses and tolerance to abiotic stresses.

The present study combined culture-dependent and molecular approaches. Moreover, the effectivity of the sterilization method is a crucial step to isolate just the seed endophytes. For this purpose, the micromorphology of the seeds was studied to help us to select the appropriate method of surface sterilization, due to the unique structure of the reticulated testa and the endothelium of the seed coat. Molecular techniques were used to identify the culturable bacteria and to describe the diversity of the microbial communities in seeds of the examined plant species. PGP traits such as the ability to produce Indole-3-acetic acid (IAA), ACC-deaminase, siderophores and organic acids of the culturable endophytic bacterial strains were also investigated.

To the best of our knowledge this is the first report about bacterial seed endophytes of the holoparasitic endemic plant species *C. armena*.

Materials and methods

Species natural habitat and plant material

Mature seeds of *Cistanche armena* (Orobanchaceae) were used. *C. armena* (K. Koch) M.V. Agab. is an endemic, critically endangered species. It is known only from the Ararat and Armavir provinces in central Armenia, in the Arax River valley and at the foot of Mount Ararat, NW of the village Lusarat, near the Khor Virap monastery (39°53″01′ N, 44°34″49′ E) at about 820–840 m above sea level (Piwowarczyk et al., 2017, 2019). This locality is one of the hottest and extremely arid regions of Armenia. The mean daily air temperature ranges from a maximum of 42˚C in July to a minimum of -33˚C in January. The average annual rainfall is 300 mm, while the annual evaporation reaches up to 1,000 mm. The area is characterized by strong salinity (total salt content of the soil 1–3%) with considerable carbonization (Panosyan et al., 2018). It is a semi-desert, with sandy, saline soils and a halophytic vegetation. *C. armena* parasitizes *Alhagi maurorum* (Fabaceae) and *Salsola dendroides* (Chenopodiaceae) (Fig. 1A, B).

The mature seeds were collected in June 2017. Seeds from at least 10 plant individuals of the total population from the region were collected. Mature and dry seeds were collected from dry fruits and used for further experiments. The seeds were collected and identified by Renata Piwowarczyk, and herbarium materials were deposited in the Herbarium of the Jan Kochanowski University in Kielce (KTC), Poland. The seeds were dried under natural conditions. Field studies, including the collection of plant and seed material complied with relevant local, institutional, national, and international guidelines, permissions, and legislation.

Microscopic observation and morphometric analysis of seeds

General seed morphology was studied using an Axio Zoom.V16 Stereo Zoom system (Carl Zeiss, Germany) in bright-field illumination (objective lenses PlanApo Z 1.5×, FWD = 30 mm) and processed in ImageJ software using Fiji macros. The terminology of seed surfaces was taken from Barthlott (1981), and Piwowarczyk et al. (2020b). At least 30 seeds were examined, and quantitative and qualitative morphological characteristics were determined several times for each seed (Fig. 2).

Method for seed surface sterilization and cultivation conditions of culturable seed endophytic bacteria

189 The aim of seed surface sterilization was to obtain only the endophytic bacterial communities
190 of the seeds. For this purpose, 50 mg seeds were transferred into 1.5-mL Eppendorf tubes,
191 submersed in 70% ethanol for 60 s, then 1 mL of 0.85% sterile NaCl solution was added,
192 followed by shaking on a vortex (8,000 rpm) at 21˚C for 2.5 h. Subsequently, the washed
193 seeds were kept at 4˚C for 15 min. Before rinsing with sterile double distilled water, the seeds
194 were centrifuged for 30 s at 12,000 rpm (13,400 × *g*). The washing process was repeated five
195 times with a decreasing time of shaking from 2 h to 30 min (2 h, 1.5 h, 60 min, 45 min, and 30
196 min). Each time samples were centrifuged for 30 s, rinsed with sterile double distilled water,
197 and kept at 4˚C for 15 min. The rinsing procedure was repeated three times. For proving the
198 effectiveness of the sterilization procedure, the last rinsing water was plated on previously
199 prepared Petri dishes with LB medium. The surface sterilized seeds were mechanically
200 homogenized using a sterile pellet pestle (Kimble®) in 0.5ml 10mM $MgSO_4$. Part of the
201 homogenous seed suspension was used for DNA extraction, another part for isolation of
202 culturable bacteria.

203 ***Total DNA extraction from seeds, library preparation, and Illumina sequencing***

204 For identification of the total (cultivable and uncultivable) bacterial community the
205 homogenized suspension of the surface sterilized seeds was used. The DNA isolation was
206 performed using the Mobio Power Plant protocol. The isolation of total bacterial DNA was
207 conducted in 4 replicates.
208 All DNA samples were subjected to bacterial 16S rRNA gene amplicon PCR. In the first
209 round of 16S rRNA gene PCR, an amplicon of 291 bp was generated, using primers 515F-
210 GTGYCAGCMGCCGCGGTAA and 806R- GGACTACNVGGGTWTCTAAT (Walters et
211 al., 2016), with an Illumina adapter overhang nucleotide sequence, resulting in the following
212 sequences, 515F-adaptor: 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-
213 3' and 806R-adaptor: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3'.
214 For the first round of PCR the Q5 High-Fidelity DNA Polymerase system (M0491, NEB), a
215 reaction volume of 25 µl per sample was prepared containing 1 µl of extracted DNA (final
216 DNA-concentration per reaction 1-10 ng), 1x Q5 Reaction Buffer with 2 mM $MgCl_2$, 200 µM
217 dNTP mix, 1x Q5 High GC Enhancer (for the seed and bacterial samples), 0.25 µM forward
218 or reverse primer, and 0.02 U μl^{-1} Q5 High-Fidelity DNA polymerase, and for the seed
219 endophytic extracts, additionally 0,5 µL mitoPNA blocker (2 µM final concentration added
220 from a 50 µM stock), 0,5 µL (seeds) plastidPNA blocker (2 µM final concentration from 50
221 µM stock) (Kusstatscher et al., 2021) were using. The PCR program started with an initial
222 denaturation for 3 min at 98 °C, followed by a 10 sec denaturation at 98˚C, a 30 sec annealing
223 at 56˚C for V3V4 (58˚C for ITS) and a 30 sec extension at 72 °C, all three steps were repeated
224 for a total of 30 cycles. The reaction was ended by a final 7 min extension at 72 °C. The
225 amplified DNA was purified using the AMPure XP beads (Beckman Coulter) and the
226 MagMax magnetic particle processor (ThermoFisher, Leuven, Belgium). Subsequently, 5 µl
227 of the cleaned PCR product was used for the second PCR attaching the Nextera indices
228 (Nextera XT Index Kit v2 Set A(FC-131-2001), and D (FC-131-2004), Illumina, Belgium).
229 For these PCR reactions, 5 µl of the purified PCR product was used in a 25 µl reaction
230 volume and prepared following the 16S Metagenomic Sequencing Library Preparation Guide.
231 PCR conditions were the same as described above, but the number of cycles reduced to 20,
232 and 55˚C annealing temperature. PCR products were cleaned with the Agencourt AMPure XP
233 kit, and then quantified using the Qubit dsDNA HS assay kit (Invitrogen) and the Qubit 2.0
234 Fluorometer (Invitrogen). Once the molarity of the sample was determined, the samples were
235 diluted down to 4 nM using 10 mM Tris pH 8.5 prior to sequencing on the Illumina MiSeq.
236 Samples were sequenced using the MiSeq Reagent Kit v3 (600 cycle) (MS-102-3003) and
237 15% PhiX Control v3 (FC-110-3001). For quality control, a DNA-extraction blank and PCR

238 blank were included throughout the process, and also the ZymoBIOMICS Microbial Mock
239 Community Standard (D6300) to test efficiency of DNA extraction (Zymo Research).
240
241 ***Bioinformatic processing of reads***

242 Sequences were demultiplexed using the Illumina Miseq software, and subsequently quality
243 trimmed and primers removed using DADA2 1.10.1 (Callahan et al., 2016) in R version 3.5.1.
244 Parameters for length trimming were set to keep the first 290 bases of the forward read and
245 200 bases of the reverse read, maxN=0, MaxEE=(2,5) and PhiX removal. Error rates were
246 inferred, and the filtered reads were dereplicated and denoised using the DADA2 default
247 parameters. After merging paired reads and removal of chimeras via the
248 removeBimeraDenovo function, an amplicon sequence variant (ASV) table was built and
249 taxonomy assigned using the SILVA v138 training set (Quast et al., 2013; Yilmaz et al.,
250 2014). The resulting ASVs and taxonomy tables were combined with the metadata file into a
251 phyloseq object (Phyloseq, version 1.26.1) (McMurdie and Holmes, 2013). Contaminants
252 were removed from the dataset using the package Decontam (version 1.2.1) applying the
253 prevalence method with a 0.5 threshold value (Davis et al., 2018). A phylogenetic tree was
254 constructed using a DECIPHER/Phangorn pipeline as described before (Murali et al., 2018).

255 ***Data visualization and statistical analyses***

256 The ASV table was further processed removing organelles (chloroplast, mitochondria), and
257 prevalence filtered using a 2% inclusion threshold (unsupervised filtering) as described by
258 Callahan et al. (2016). Alpha-diversity metrics such as Chao1, Simpson's and Shannon's
259 diversity indexes were calculated on unfiltered data using scripts from the MicrobiomeSeq
260 package. Hypothesis testing was done using analysis of variance (ANOVA) and the Tukey
261 Honest Significant Differences method (Tukey HSD). When assumptions of normality and
262 homoscedasticity were not met, a Kruskal-Wallis Rank Sum test and a Wilcoxon Rank Sum
263 test was performed. The results were summarized in boxplots. Relative abundances were
264 calculated and visualized in bar charts using Phyloseq. All performed statistical tests were
265 corrected for multiple testing and alpha < 0.05 was considered as statistically significant. All
266 graphs were generated in R version 4.0.4.

267 ***Isolation of culturable endophytes***

268 The first part of the suspension obtained after crushing the seeds (see above) was used for
269 DNA extraction, the second part for isolation of culturable bacteria. Serial dilutions were
270 made 10^6 cfu ml^{-1} and then 100 µl was plated onto 1/869 rich medium with composition:
271 0.035 g L^{-1} $CaCl_2$ x $2H_2O$, Glucose D 0.1 g L^{-1}, NaCl 0.5 g L^{-1}, Trypton 1 g L^{-1}, Yeast Extract
272 0.5 g L^{-1}, Agar 15 g L^{-1} (Eevers et al., 2015) and incubated at 30˚C for 7 days. For further
273 experiments, single, morphological diverse colonies were picked and purified. Subsequently,
274 they were grown in 96-well master blocks and triplicated: one block was used for DNA-
275 extraction, the second one was used for PGP tests and the third was stored at -45˚C in 15%
276 glycerol (75 g glycerol, 4.25 g NaCl, 425 ml dH_2O).

277 ***Genomic DNA extraction and taxonomic identification of the culturable endophytic***
278 ***bacterial strains***

279 The DNA isolation was performed using standard procedure for DNA isolation from bacterial
280 pellets with MagMAX. DNA was quantified with a Qubit® 2.0 Fluorometer
281 (ThermoScientific, US) and checked for purity on a Nanodrop spectrophotometer
282 (ThermoScientific, US) with an A260/A280 ratio of 1.7–2.0. The near full-length sequences

of the 16S rRNA gene were amplified with the primers 27f (5-AGAGTTTGATCMTGGCTCAG-3) and 1492r (5-GGTTACCTTGTTACGACTT-3). The products were checked on agarose gel and then shipped to Macrogen for 16S rRNA Sanger sequencing. Sequencing results were quality filtered using Geneious v4.8, were analyzed over the ribosomal database SILVA (https://www.arb-silva.de/aligner/) and NCBI GenBank databases using the program Standard Nucleotide BLAST and database RDP (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Plant growth promoting (PGP) characteristics

In order to evaluate the ability of the isolated strains to induce plant growth promotion, *in vitro* PGP tests were performed. All tests were performed at least two times.

The IAA production ability was tested using the Salkowski test. Bacteria were grown in a 1/10 869 medium containing tryptophan (Patten and Glick, 2002). 25 µl of bacterial suspension with 0.7 ml IAA medium were incubated for 4 days at 30˚C and shaken at 150 rpm in the dark. Thereafter, the suspension was centrifuged for 10 min at 4000 rpm. 1 ml Salkowski reagent was added to 0.5 ml supernatant. After 20 min reaction time colored pink means positive for IAA production.

To check for organic acid production the method of Cunningham & Kuiack was used. The bacteria were cultivated in a Sucrose Tryptone (ST) medium with composition: sucrose 20 g L^{-1}, tryptone 5 g L^{-1}, 10 ml trace element solution SET ($Na_2MoO_4.2H_2O$ 20 mg L^{-1}, H_3BO_3 200 mg L^{-1}, $CuSO_4.5H_2O$ 20 mg L^{-1}, $FeCl_3$ 100 mg L^{-1}, $MnCl_2.4H_2O$ 20 mg L^{-1}, $ZnCl_2$ 280 mg L^{-1}). The bacterial suspension was incubated for 5 days at 30˚C and 200 rpm, after which the pH-sensitive color indicator 100 µL Alizarine Red S 0,1% was added (Cunningham and Kuiack, 1992). The organic acid production was checked after 15 min reaction time: yellow = positive, pink = negative.

ACC-deaminase activity was tested in SMN medium with 5 mM ACC as N-source with HCl and autoclaved (Belimov et al., 2005). SMN medium composition: 970mL: 0,4g L^{-1} KH_2PO_4, 2 g L^{-1} K_2HPO_4 (pH 6,6), 10 mL $MgSO_4$ solution, 10 mL $CaCl_2$ solution and 10mL micronutrient stock were added after filter sterilization. 50 mL C-mix stock with 2 g L^{-1} glucose, 2 g L^{-1} sucrose, 2 g L^{-1} Na-acetate, 2 g L^{-1} Na-citrate, 2 g L^{-1} Malic acid and 2 g L^{-1} Mannitol and 10 mL ACC-stock were added. 250 µL of the bacterial suspension added to 1.2 mL SMN medium with 5 mM ACC as N-source were incubated for 3 days at 30˚C and centrifuged at 4000 rpm for 15 min. The pellet was resuspended in 100 µL 0,1M Tris-HCl buffer (pH 8,5) and 3 µL toluene was added for cell lysis, and vortexed for 5 min. In next step 10 µL 0,5 M ACC and 100 µL 0,1M Tris-HCl buffer (pH 8,5), vortexed and incubated for 30 min at 30˚C and 150 rpm. 690 µL 0,56N HCl and 150 µL 0,2% 2,4-dinitrophenylhydrazine in 2N HCl and 1 mL 2N NaOH were added. The obtained results were evaluated: brown = positive, yellow = negative.

Production of siderophores was studied by using the 284 medium with 0.25 µl optimal iron concentration with CAS solution (Schwyn and Neilands, 1987). Tris 6.06 g L^{-1}, NaCl 4.68 g L^{-1}, KCl 1.49 g L^{-1}, NH_4Cl 1.07 g L^{-1}, Na_2SO_4 0.43g L^{-1}, $MgCl_2.6H_2O$ 0.2 g L^{-1}, $CaCl_2.2H_20$ 0.03 g L^{-1}, $Na_2HPO_4.2H_20$ 0.04 g L^{-1}, S17 trace elements 1 ml, 0.25 mM Fe(III)Citrate solution, Sodium lactate (sol. 50%) 0.7 ml, D-(+)-glucose 0.52 g L^{-1}, D-gluconic acid sodium salt 0.66 g L^{-1}, D-(+) fructose 0.54 g L^{-1}, Sodium succinate.$6H_2O$ 0.81 g L^{-1}. The 284 medium with 0 µl and 3 µl were used as control. 800 µL 284 medium (0 µM, 0,25 µM and 3 µM Fe) with 20 µL of the bacterial suspension were incubated for 5 days at 30˚C and 200 rpm. 100 µL Chroom-Azurol S Solution (CAS-Solution) were added. After 4 h reaction time, orange = positive, blue = negative.

Results

Seed micromorphology

C. armena seeds are dark brown, 541–1003 µm long, 347–631 µm wide with a 1.1–2.3 length-to-width ratio and 164333–445987 μm^2 area. The shape was oblongoid to ovoid, rarely subrectangular. The seed ornamentation was constantly alveolate. The testa of the seeds had smooth, thin outer periclinal walls adjacent to the inner periclinal wall with perforated (pitted) sculpture. The seed coat surface was formed by polygonal and isodiametric cells with different sizes, 41–159 µm long and 33–96 µm wide with a 1.0–3.1 length-to-width ratio. The number of cells along the seed longitudinal axis was 7–13; in the lateral view; it varied from 34 to 79. The anticlinal walls were of slight depth with a width of 7.7–14.6 µm (Fig. 2).

Seed endophytic bacterial community composition

The number of paired raw Illumina reads after filtering low quality reads, adapters, barcodes and primers, there were about 2300 effective read for the 4 replicates of *C. armena* seeds. The Shannon-Wiener biodiversity index, Chao1 and Simpson indexes for the seed endophytes of *C. armena* were 2.82, 27, 13.9 respectively (Supplementary Figure S1) with P-value 0.05. A total of 75 different Operational Taxonomic Unit (OTU)s on genus level was found from 10 phyla. The relative abundance of the dominant bacteria comprising the seed endophytic community at different taxonomic levels is presented in Supplementary Figure S2.

From the surface sterilized seeds, 10 phyla and 256 bacterial genera were identified. The taxonomy of the sequences was described primarily at the phylum level. For the *C. armena* seeds, we determined Proteobacteria, Firmicutes and Actinobacteriota, whereas the Bacteroidetes, Acidobacteria, Verrucomicrobia, Mixococcota, Planctomycetes, Patescibacteria and Chloroflexi were less abundant (Supplementary Figure S2). Firmicutes were the predominating phylum in the seeds of the examined plant population, followed by Proteobacteria and Actinobacteriota. The phylum Actinobacteriota was classified only in 3 biological replicates. Only Bacilli, Gammaproteobacteria and Actinobacteria dominated at the class level (Table 2). Indeed, Bacilli were the most abundant class (Supplementary Figure S2). The majority of endophytic bacterial community of seeds of *C. armena* belonged to the order Bacillales that at genus level was represented by *Psychrobacillus*, *Bacillus* and *Domibacillus*. The most abundant family of Firmicutes identified in examined seeds was Planococcaceae with *Paenisporosarcina* as a predominant genus.

The Gammaproteobacteria were identified as another abundant class, that at the order level was represented by Xanthomonadales, Pseudomonadales and Enterobacterales. At genus level *Pseudomonas*, *Stenotrophomonas* and *Serratia* dominated (Table 2). Finally, *Microbacterium* and *Curtobacterium* were the dominating genera of the phylum Actinobacteriota. Unclassified groups were found also at different taxonomic levels. The results are presented based on the most representative and dominating OTUs (identified at genus level with a relative abundance higher than 1%).

Diversity of cultivable endophytes from surface-sterile seeds and in vitro characterization of PGP bacteria

43 bacterial strains were picked up from the 1/869 medium. Using 16S rRNA gene Sanger sequencing we found that 35 bacteria (81.4%) of the total isolates were Firmicutes and only 18.6% were Proteobacteria with *Stenotrophomonas maltophilia* and different strains of *Pantoea*. The majority of Firmicutes isolates belonged to the genera *Bacillus* and *Paenibacillus*.

36 strains scored positive for IAA production and only 3 strains of *Bacillus* spp. tested positive for siderophore production. Relatively similar outcomes were obtained for production of ACC-deaminase and organic acids: 26 and 27 strains respectively showed positive (Fig. 3). In the *in vitro* tests *Pantoea* spp. and *Stenotrophomonas maltophilia* demonstrated higher growth promoting capacities compared to *Bacillus* spp. and other isolates (Fig. 3).

Discussion

The seed surfaces of holoparasitic *C. armena* possess an alveolate ornamentation with perforated (pitted) sculpture formed by polygonal and isodiametric cells with different sizes. The quite coarse structure of the seed coat (Fig. 2) can complicate the surface sterilization of the seeds. The preliminary results obtained by applying the generally used sterilization protocols (Watts et al., 1993; Metwaly et al., 2018) showed to be inadequate. We assumed that the sterilizing agents could not always sufficiently reach the deepest zones of the coarse seed surface. Due to this, not all bacteria residing on the surface of the seeds could be eliminated. Finally, the combination of 70% ethanol and 0.85% NaCl sterile solution together with intense shaking showed to be adequate to remove all bacteria from the surfaces of *C. armena* seeds. This allowed us to isolate only the bacteria present inside the seeds. It is known that the majority of plant associated bacteria are unculturable, and it is often assumed that only 0.001-1% can be grown in laboratory conditions (Eevers et al., 2015). Consequently, in order to obtain more information about the composition of the total endophytic bacterial communities of the seeds (culturable and unculturable) of *C. armena*, molecular techniques were used. The Illumina MiSeq data showed that the seeds of *C. armena* were mainly inhabited by Gram-positive, spore forming *Bacilli* (36.8%) (Supplementary Figure S2). In case of a holoparasitic plant, like *C. armena*, this is very plausible because these seeds, similarly to *Orobanche* s.l., have to stay viable in the soil for several decades (Joel et al., 2007). Plant colonization by spore forming *Bacillus* spp. that possess potential to mitigate environmental stress can help plants to survive in harsh environmental conditions. *C. armena* adapted to the arid and saline environment of specific areas in Armenia (Piwowarczyk et al., 2017, 2019). We demonstrated that *C. armena* was colonized by halotolerant, alkalophilic, spore forming, motile *Bacillus spp*. strains (Petrosyan et. al., 2022). Some isolated strains were also thermophilic. They are able to produce one or more hydrolytic enzymes, especially cellulase and protease. Some strains also produced amylase and pectinase too. Production of auxins (IAA) and gibberellins (GA) and phosphate solubilization was also characteristic for the *Bacillus* spp. isolated from the seeds of *C. armena*.

Our results demonstrated that at genus level *Paenibacillus*, *Bacillus*, *Psychrobacillus*, *Domibacillus and Paenisporosarcina* were well represented in the seeds of the investigated population of *C. armena* (Table 2). The dominating *Paenisporosarcina* have been described as *gen. nov*. and not sufficiently investigated (Parte, 2018). However, some members of the family Planococcaceae were isolated from a semi-arid tropical soil from India (Raj et al., 2013). Thus, their presence in the examined seeds is not surprising because of the natural habitats of *C. armena* (Fig. 1B).

Forty-three isolated strains were well adapted to the growing conditions of their host plant and showed potential PGP traits (production of organic acids, ACC-deaminase, IAA and siderophores). Most of the isolated strains (83.7%) were positive for IAA production (Fig. 3). Endophytic bacteria can increase plant growth through their ability to produce plant growth hormones, particularly auxins. Auxin producing PGP endophytes improve plant growth even under stress by effectively mitigating the effects of all the growth inhibiting conditions (Grobelak et al., 2018). Respectively 26 and 27 of the isolates produced ACC-deaminase and organic acids, and only 3 *Bacillus* spp. could produce siderophores (Fig. 3). All these traits have potential to improve plant growth also under stress conditions (Grobelak et al., 2018;

Shameer and Prasad, 2018). Hassan and Bano (2016) explored the IAA production of *Stenotrophomonas maltophilia* strains isolated from a halophytic herb *Cenchrus ciliaris* and mentioned that bacterial IAA production played a positive role in the salt tolerance of their host plant.

Compared to *Bacillus* spp. and *Paenibacilus* spp. strains that demonstrated relatively low levels of production of PGP compounds, *Pantoea* spp. and *Stenotrophomonas maltophilia* demonstrated a high production of IAA (100%), ACC-deaminase (100%) and organic acids (96.3%) (Fig. 3), which is in agreement with earlier reports (Singh and Jha, 2017; Lumactud and Fulthorpe, 2018). The production of various organic acids by seed endophytic *Paenibacillus* sp., *Pantoea* sp., and *Bacillus* sp. inhibits the growth of pathogens and can significantly enhance plant growth and resistance against plant pathogens (Herrera et al., 2016; Shahzad et al., 2017). The high levels of IAA production among *P. agglomerans* and *S. maltophilia* strains correspond with findings of other authors (Ambawade and Pathade 2015; Luziatelli et al., 2020).

Conclusion

We explored the endophytic bacterial community of the seeds of the endemic holoparasite *C. armena*. The sterilization procedure for the seed surface was optimized. Ten phyla and 256 bacterial genera were identified. However, also some unclassified and unexplored taxonomic groups were found in the seeds.

Our results confirm that spore forming *Bacillus* spp. are common and dominated endophytes from seeds of plants growing in harsh environmental conditions, especially from arid saline soils. *Pantoea* spp. and *Stenotrophomonas* seem the most favourable PGP endophytes in seeds of *C. armena*. The PGP traits of these bacteria, such as production of IAA, ACC-deaminase and organic acids seem correlated with the natural habitat of their hosts and have the potential to improve plant tolerance against abiotic stresses. To elucidate the effective benefits of these endophytic bacteria for their host plants, particularly for the seeds, seed germination and development of the seedling, more research is required.

Data availability. The sequence data available in the NCBI Genbank (https://www.ncbi.nlm.nih.gov/) Sequence Read Archive with accession number PRJNA819412.

Supplementary material.

Supplementary Figure S1. The values of reads of *Cistanche armena* seed-endophytes after filtering **(a)** Chao1 index was 27, **(b)** Shannon-Wiener biodiversity index was 2.82, **(c)** Simpson index 13.9 for total 75 different OTUs. P-value: 0.05.

Supplementary Figure S2. The relative abundances of the dominated bacteria comprising the seed associated endophytic community of *Cistanche armena* species at different taxonomic levels at **a)** Phylum, **b)** Class, **c)** Order, **d)** Genera, **e)** Families.

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preparation, K.P., R.P and K.R.; writing the review and editing, R.P., W.K., J.V.; visualization, K.P., R.P, S.T. and K.R. All authors read and approved the final manuscript.

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Conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures and tables captions:

Figure 1. General habit of the studied species and its habitats: (A) parasitic plant *Cistanche armena*, (B) semi-deserts with halophytic vegetation - the natural habitat of *C. armena*. Photos by R. Piwowarczyk.

Figure 2. ZOOM microscopy micrographs of seeds of *Cistanche armena*.

Figure 3. PGP activity of tested bacteria and relative PGP traits between isolated bacterial species (%). The left figure presents the PGP activity for all tested isolates. The figure on right shows the relative IAA (blue), ACCD (violet), siderophore (green) and organic acids (red) production ability among the isolated bacterial genera.

Table 1. Endophytic bacterial taxa isolated from different tissues of holoparasitic plant species

Table 2. Cumulative list of dominating endophytic bacteria in the seeds of *Cistanche armena* and their taxonomic information

772 **Table 1.** Endophytic bacterial taxa isolated from different tissues of holoparasitic plant species

Holoparasitic plant	Endophytic bacteria
Phelipanche aegyptiaca, host plant: tomato (*Lycopersicum esculentum*) Iasur Kruh et al., 2017	Pre-haustorium stage *α*,*β*,*γ*,*δ* Proteobacteria, Actinobacteria, Flavobacteria, Sphingobacteria Spider stage *α*,*β*,*γ*,*δ* Proteobacteria, Flavobacteria, Sphingobacteria, Firmicutes Shoots *α*,*β*,*γ* Proteobacteria, Actinobacteria, Sphingobacteria, Clostridia, Flavobacteria, Firmicutes
Orobanche hederae, host plant: ivy (*Hedera* sp.) Fitzpatrick and Schneider, 2020	Roots Armatimonadetes, Bacteroidetes, Proteobacteria, Actinobacteria, Acidobacteria, Verrucomicrobia Leaves Bacteroidetes, Actinobacteria, Proteobacteria
Phelipanche ramosa, host plants: oilseed rape (*Brassica napus*), hemp (*Cannabis sativa*), tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), sunflower (*Helianthus annuus*), melon (*Cucumis melo*) Huet et al., 2020; Durlik et al., 2021	Seeds Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes *Brevibacterium frigoritolerans*, *Bacillus simplex*

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779 **Table 2.** Cumulative list of dominating endophytic bacteria in the seeds of *Cistanche armena* and their
780 taxonomic information

Phyla	**Classes**	**Orders**	**Families**	**Genera**
Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	*Paenibacillus*
		Bacillales	Bacillaceae	*Psychrobacillus* *Bacillus* *Domibacillus*
			Planococcaceae	*Paenisporosarcina*
Proteobacteria	γProteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
		Enterobacterales	Yersiniaceae	*Serratia*
Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	*Microbacterium* *Curtobacterium*

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