

Research Paper

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Characterization and diversity of seed endophytic bacteria of the endemic holoparasitic plant *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, comprising 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* spp. are more favourable PGP endophytes in seeds of *C. armena*. The PGP traits of these bacteria, such as production of indole, a precursor of auxin, 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, this is the first report concerning bacterial seed endophytes of the *C. armena*.

Introduction

With approximately 4750 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, semi-arid and halophytic habitats across Eurasia and North Africa. These magnificent, achlorophyllous species, with fleshy stems, long underground stolons and intensely coloured 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 (Eriksson and Kainulainen, 2011). 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, and 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 *Orobanchae* 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 (Hryniewicz 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 the obligate endophytes, plant tissues can be colonized by soil bacteria as well. This is explained by the possible migration of bacteria from the soil to the seeds (Frank et al., 2017; Johnston-Monje et al., 2021). 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. However, the floral route has a selective function, and only endophytes with biocontrol ability and nonhost pathogens can reach the seeds. However, the seeds endophytic microbiome composition, diversity and bacterial proportions depend on plant species genotype, natural habitat of host plant, seeds dormancy and storage conditions, which was not investigated sufficiently (Jonkers et al., 2022).

So far, ample 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 *Orobanchae hederiae* (Iasur Kruh et al., 2017; Fitzpatrick and Schneider, 2020; Huet et al., 2020; Durlík 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 harboured bacteria belonging to the Proteobacteria (*Rhizobium*, *Pseudomonas*, Comamonadaceae, *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. hederiae* reported that *Orobanchae* 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 (Durlík et al., 2021; Table 1). Different bacterial phyla also have been isolated from plants growing in arid and semi-arid 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, Bacteroidetes, Firmicutes, Planctomycetes, Actinobacteria and 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; Durlík 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

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

Holoparasitic plant	Endophytic bacteria
<i>Phelipanche aegyptiaca</i> , host plant: tomato (<i>Lycopersicon esculentum</i>) Iasur Kruh et al., 2017	Pre-haustorium stage $\alpha, \beta, \gamma, \delta$ Proteobacteria, Actinobacteria, Flavobacteria, Sphingobacteria Spider stage $\alpha, \beta, \gamma, \delta$ Proteobacteria, Flavobacteria, Sphingobacteria, Firmicutes Shoots α, β, γ Proteobacteria, Actinobacteria, Sphingobacteria, Clostridia, Flavobacteria, Firmicutes
<i>Orobanchae hederiae</i> , host plant: ivy (<i>Hedera</i> sp.) Fitzpatrick and Schneider, 2020	Roots Armatimonadetes, Bacteroidetes, Proteobacteria, Actinobacteria, Acidobacteria, Verrucomicrobia Leaves Bacteroidetes, Actinobacteria, Proteobacteria
<i>Phelipanche ramosa</i> , host plants: oilseed rape (<i>Brassica napus</i>), hemp (<i>Cannabis sativa</i>), tomato (<i>Solanum lycopersicum</i>), tobacco (<i>Nicotiana tabacum</i>), sunflower (<i>Helianthus annuus</i>), melon (<i>Cucumis melo</i>) Huet et al., 2020; Durlík et al., 2021	Seeds Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes <i>Brevibacterium frigoritolerans</i> , <i>Bacillus simplex</i>

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 southern 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 1000 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).

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, and Yerevan State University (ERCB), Armenia. 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. 1).

Seed surface sterilization and cultivation conditions of culturable seed endophytic bacteria

The aim of seed surface sterilization was to obtain only the endophytic bacterial communities of the seeds. For this purpose, 50 mg of seeds were transferred into 1.5 ml Eppendorf tubes, submerged in 70% ethanol for 60 s, then 1 ml of a 0.85% sterile NaCl solution was added, followed by shaking on a vortex (8000 rpm) at 21°C for 2.5 h. Subsequently, the washed seeds were kept at 4°C for 15 min. Before rinsing with sterile double distilled water, the seeds were centrifuged for 30 s at 12,000 rpm (13,400 × g). The washing process was repeated five times with a decreasing time of shaking from 2 h to 30 min (2 h, 1.5 h, 60 min, 45 min and 30 min). Each time samples were centrifuged for 30 s, rinsed with sterile double distilled water, and kept at 4°C for 15 min. The rinsing procedure was repeated three times. For proving the effectiveness of the sterilization procedure, the last rinsing water was plated on previously prepared Petri dishes with LB medium. The surface-sterilized seeds were mechanically homogenized using a sterile pellet pestle (Kimble®) in 0.5 ml,

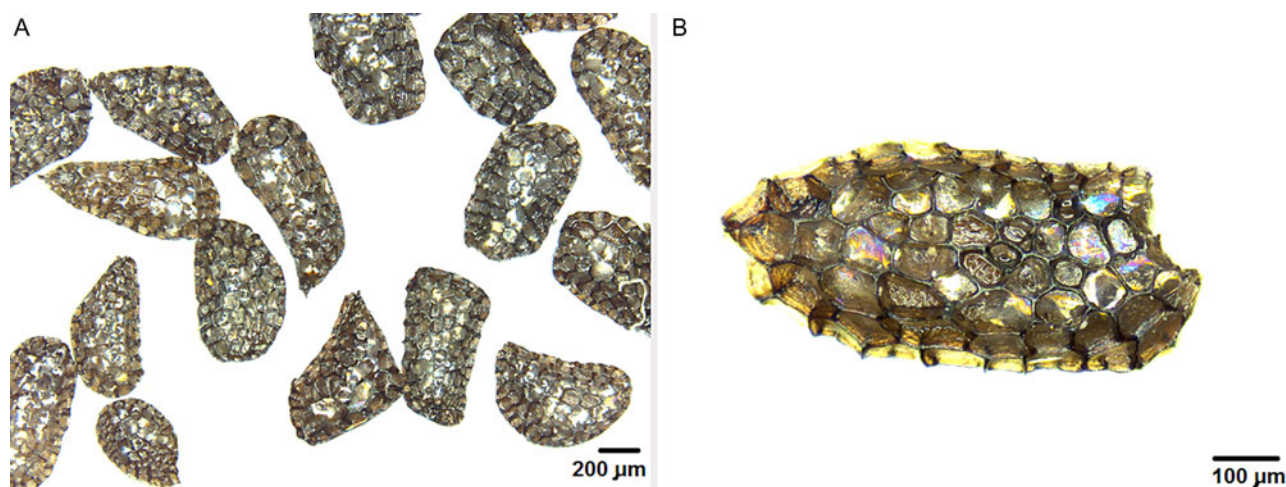


Fig. 1. ZOOM microscopy micrographs of seeds of *Cistanche armena*. Photo by Y. Krasnylenko.

10 mM MgSO₄. Part of the homogenous seed suspension was used for DNA extraction, another part for isolation of culturable bacteria.

Total DNA extraction from seeds, library preparation and Illumina sequencing

For identification of the total (cultivable and uncultivable) bacterial community, the homogenized suspension of the surface-sterilized seeds was used. The DNA isolation was performed using the Mobio Power Plant protocol. The isolation of total bacterial DNA was conducted in four replicates.

All DNA samples were subjected to bacterial 16S rRNA gene amplicon PCR. In the first round of 16S rRNA gene PCR, an amplicon of 291 bp was generated, using primers 515F-GTGYCAGCMGCCGCGGTAA and 806R-GGACTACNVGG GTWTCTAAT (Walters et al., 2016), with an Illumina adapter overhang nucleotide sequence, resulting in the following sequences, 515F-adaptor: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3' and 806R-adaptor: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3'. For the first round of PCR the Q5 High-Fidelity DNA Polymerase system (M0491, NEB), a reaction volume of 25 µl per sample was prepared containing 1 µl of extracted DNA (final DNA-concentration per reaction 1–10 ng), 1 × Q5 Reaction Buffer with 2 mM MgCl₂, 200 µM dNTP mix, 1 × Q5 High GC Enhancer (for the seed and bacterial samples), 0.25 µM forward or reverse primer, and 0.02 U µl⁻¹ Q5 High-Fidelity DNA polymerase, and for the seed endophytic extracts, additionally 0.5 µl mitoPNA blocker (2 µM final concentration added from a 50 µM stock), 0.5 µl (seeds) plastidPNA blocker (2 µM final concentration from 50 µM stock) (Kusstatscher et al., 2021) were used. The PCR program started with an initial denaturation for 3 min at 98°C, followed by a 10 s denaturation at 98°C, a 30 s annealing at 56°C for V3V4 (58°C for ITS) and a 30 s extension at 72°C; all three steps were repeated for a total of 30 cycles. The reaction was ended by a final 7 min extension at 72°C. The amplified DNA was purified using the AMPure XP beads (Beckman Coulter) and the MagMax magnetic particle processor (ThermoFisher, Leuven, Belgium). Subsequently, 5 µl of the cleaned PCR product was used for the second PCR attaching the Nextera indices (Nextera XT Index Kit v2 Set A (FC-131-2001), and D (FC-131-2004), Illumina, Belgium). For these PCR reactions, 5 µl of the purified PCR product was used in a 25 µl reaction volume and prepared following the 16S Metagenomic Sequencing Library Preparation Guide. PCR conditions were the same as described above, but the number of cycles reduced to 20, and 55°C annealing temperature. PCR products were cleaned with the Agencourt AMPure XP kit, and then quantified using the Qubit dsDNA HS assay kit (Invitrogen) and the Qubit 2.0 Fluorometer (Invitrogen). Once the molarity of the sample was determined, the samples were diluted down to 4 nM using 10 mM Tris pH 8.5 prior to sequencing on the Illumina MiSeq. Samples were sequenced using the MiSeq Reagent Kit v3 (600 cycle) (MS-102-3003) and 15% PhiX Control v3 (FC-110-3001). For quality control, a DNA-extraction blank and PCR blank were included throughout the process, and also the ZymoBIOMICS Microbial Mock Community Standard (D6300) to test efficiency of DNA extraction (Zymo Research).

Bioinformatic processing of reads

Sequences were demultiplexed using the Illumina Miseq software, and subsequently quality trimmed and primers removed using

DADA2 1.10.1 (Callahan et al., 2016) in R version 3.5.1. Parameters for length trimming were set to keep the first 290 bases of the forward read and 200 bases of the reverse read, maxN=0, MaxEE=(2.5) and PhiX removal. Error rates were inferred, and the filtered reads were dereplicated and denoised using the DADA2 default parameters. After merging paired reads and removal of chimeras via the removeBimeraDenovo function, an amplicon sequence variant (ASV) table was built and taxonomy assigned using the SILVA v138 training set (Quast et al., 2013; Yilmaz et al., 2014). The resulting ASVs and taxonomy tables were combined with the metadata file into a phyloseq object (Phyloseq, version 1.26.1) (McMurdie and Holmes, 2013). Contaminants were removed from the dataset using the package Decontam (version 1.2.1) applying the prevalence method with a 0.5 threshold value (Davis et al., 2018). A phylogenetic tree was constructed using a DECIPHER/Phangorn pipeline as described before (Murali et al., 2018).

Data visualization and statistical analyses

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

Isolation of culturable endophytes

The first part of the suspension obtained after crushing the seeds (see above) was used for DNA extraction, the second part for isolation of culturable bacteria. Serial dilutions were made 10⁶ cfu ml⁻¹ and then 100 µl was plated onto 1/869 rich medium with composition: 0.035 g l⁻¹ CaCl₂ × 2H₂O, Glucose D 0.1 g l⁻¹, NaCl 0.5 g l⁻¹, Trypton 1 g l⁻¹, Yeast Extract 0.5 g l⁻¹, Agar 15 g l⁻¹ (Eevers et al., 2015) and incubated at 30°C for 7 d. For further experiments, single, morphological diverse colonies were picked and purified. Subsequently, they were grown in 96-well master blocks and triplicated: one block was used for DNA-extraction, the second one was used for PGP tests and the third was stored at -45°C in 15% glycerol (75 g glycerol, 4.25 g NaCl, 425 ml dH₂O).

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

DNA isolation was performed using standard procedure for DNA isolation from bacterial pellets with MagMAX. DNA was quantified with a Qubit® 2.0 Fluorometer (ThermoScientific, US) and checked for purity on a Nanodrop spectrophotometer (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-AGAGTTTGTATCMTGGCTCAG-3) and

1492r (5-GGTTACCTTGTTCAGACTT-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 tryptophanase activity was tested using the Salkowski test (Patten and Glick, 2002). Bacteria were grown in a 1/10 869 medium containing tryptophan. 25 μ l of bacterial suspension with 0.7 ml of medium with tryptophan were incubated for 4 d 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 coloured 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 (Na₂MoO₄·2H₂O 20 mg l⁻¹, H₃BO₃ 200 mg l⁻¹, CuSO₄·5H₂O 20 mg l⁻¹, FeCl₃ 100 mg l⁻¹, MnCl₂·4H₂O 20 mg l⁻¹, ZnCl₂ 280 mg l⁻¹). The bacterial suspension was incubated for 5 d at 30°C and 200 rpm, after which the pH-sensitive colour indicator 100 μ l 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: 970 ml: 0.4 g l⁻¹ KH₂PO₄, 2 g l⁻¹ K₂HPO₄ (pH 6.6), 10 ml MgSO₄ solution, 10 ml CaCl₂ solution and 10 ml 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 μ l of the bacterial suspension added to 1.2 ml SMN medium with 5 mM ACC as N-source were incubated for 3 d at 30°C and centrifuged at 4000 rpm for 15 min. The pellet was resuspended in 100 μ l 0.1 M Tris-HCl buffer (pH 8.5) and 3 μ l toluene was added for cell lysis, and vortexed for 5 min. In the next step, 10 μ l 0.5M ACC and 100 μ l 0.1 M Tris-HCl buffer (pH 8.5), vortexed and incubated for 30 min at 30°C and 150 rpm. 690 μ l 0.56N HCl and 150 μ l 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 μ l 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, NH₄Cl 1.07 g l⁻¹, Na₂SO₄ 0.43 g l⁻¹, MgCl₂·6H₂O 0.2 g l⁻¹, CaCl₂·2H₂O 0.03 g l⁻¹, Na₂HPO₄·2H₂O 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·6H₂O 0.81 g l⁻¹. The 284 medium with 0 and 3 μ l were used as control. 800 μ l 284 medium (0 μ M, 0.25 μ M and

3 μ M Fe) with 20 μ l of the bacterial suspension were incubated for 5 d at 30°C and 200 rpm. 100 μ l Chroom-Azurool 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 μ m long, 347–631 μ m wide with a 1.1–2.3 length-to-width ratio and 164,333–445,987 μ m² area. The shape was oblongoid to ovoid, rarely sub-rectangular. 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 μ m long and 33–96 μ m 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 μ m (Fig. 1).

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, and 13.9, respectively (Supplementary Fig. S1) with *P*-value 0.05. A total of 75 different Operational Taxonomic Units (OTUs) 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 Fig. 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 Fig. 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 three biological replicates. Only Bacilli, Gammaproteobacteria and Actinobacteria dominated at the class level (Table 2). Indeed, Bacilli were the most abundant class (Supplementary Fig. 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

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

Phyla	Classes	Orders	Families	Genera
Firmicutes	Bacilli	Paenibacillales Bacillales	Paenibacillaceae Bacillaceae Planococcaceae	<i>Paenibacillus</i> <i>Psychrobacillus</i> <i>Bacillus</i> <i>Domibacillus</i> <i>Paenisporosarcina</i>
Proteobacteria	γ Proteobacteria	Xanthomonadales Pseudomonadales Enterobacteriales	Xanthomonadaceae Pseudomonadaceae Yersiniaceae	<i>Stenotrophomonas</i> <i>Pseudomonas</i> <i>Serratia</i>
Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	<i>Microbacterium</i> <i>Curtobacterium</i>

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

Forty-three 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* (Table 3).

A total of 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. 2). In the *in vitro* tests, *Pantoea* spp. and *Stenotrophomonas maltophilia* demonstrated higher growth-promoting capacities compared to *Bacillus* spp. and other isolates (Fig. 2).

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. 1) 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.

The aim of current work was the identification of the total endophytic bacterial community and the culturable fraction of the endophytes isolated from the seeds of *C. armena*. It is known that the majority of plant associated bacteria are unculturable, and it is often assumed that only 0.001–1% of the total bacterial community can be grown in laboratory conditions (Evers 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 Fig. 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 the 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

Table 3. Cumulative list of cultivable endophytic bacteria in the seeds of *Cistanche armena* and their taxonomic information

Phyla	Classes	Orders	Families	Genera
Firmicutes	Bacilli	Paenibacillales Bacillales	Paenibacillaceae Bacillaceae	<i>Paenibacillus</i> (27.90%) <i>Bacillus</i> (41.86%) Others (11.82%)
Proteobacteria	γ Proteobacteria	Xanthomonadales Enterobacteriales	Xanthomonadaceae Enterobacteriaceae	<i>Stenotrophomonas</i> (4.47%) <i>Pantoea</i> (13.95%)

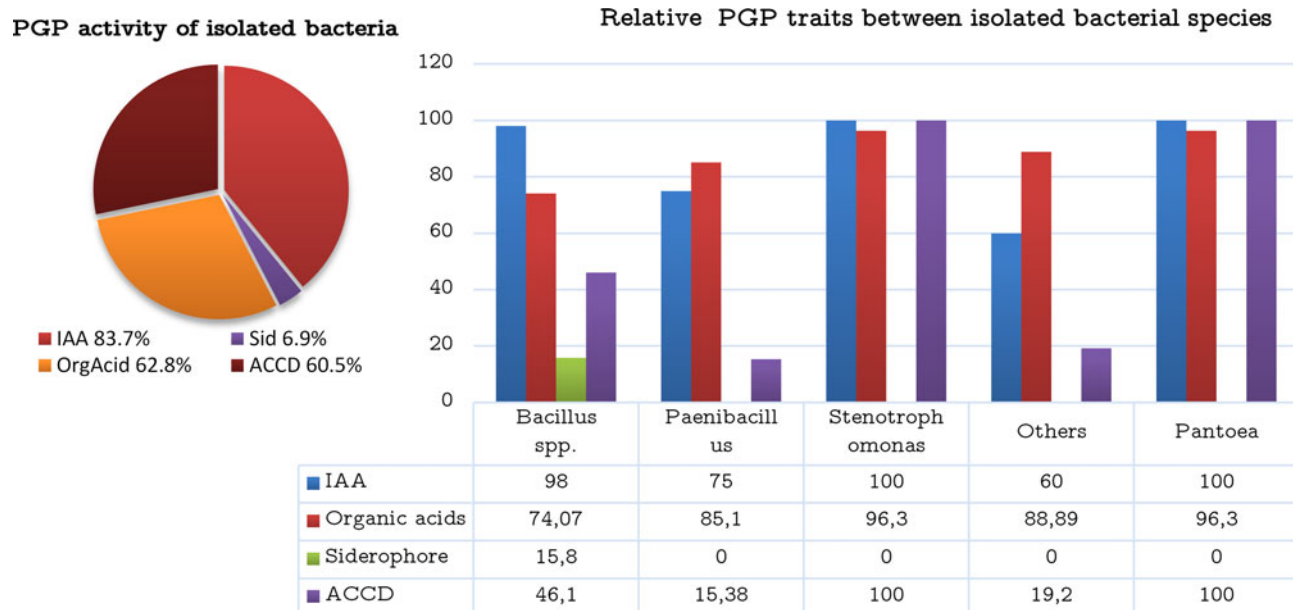


Fig. 2. PGP activity of tested bacteria and relative PGP traits between isolated bacterial species (%). The left figure presents the PGP activity for all tested isolates. The figure on right shows the relative IAA (blue), ACCD (violet), siderophore (green) and organic acids (red) production ability among the isolated bacterial genera.

et al., 2013). Thus, their presence in the examined seeds is not surprising because of the natural habitats of *C. armena*. From surface-sterilized seeds of *C. armena*, we could isolate 43 cultivable bacterial strains using culture-dependent microbiological methods.

Our results indicated that all cultivable strains isolated from the seeds of *C. armena* were belonging to the bacterial phyla Firmicutes and Proteobacteria (Tables 2 and 3). Thirty-five bacterial strains (81.4%) of the total isolates were Firmicutes from which different strains of *Bacillus* (41.86%), *Paenibacillus* (27.90%) and other genera of family Bacillaceae (11.82%) and only 18.6% were Proteobacteria with *Stenotrophomonas maltophilia* (4.47%) and different strains of *Pantoea* (13.95%). The majority of Firmicutes isolates belonged to the genera *Bacillus* and *Paenibacillus* (Table 3).

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, indole and siderophores). Most of the isolated strains (83.7%) were positive for indole production (Fig. 2). 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 (Grobela 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. 2). All these traits have potential to improve plant growth also under stress conditions (Grobela 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 *Paenibacillus* spp. strains that demonstrated relatively low levels of production of PGP compounds, *Pantoea* spp. and *Stenotrophomonas maltophilia* demonstrated a high production of indole (100%), ACC-deaminase

(100%) and organic acids (96.3%) (Fig. 2), 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* spp. seem the most favourable PGP endophytes in seeds of *C. armena*. The PGP traits of these bacteria, such as production of indole, 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.

Supplementary material. To view supplementary material for this article, please visit: <https://doi.org/10.1017/S0960258522000204>.

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

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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.

References

- Ambawade MS and Pathade GR** (2015) Production of indole acetic acid (IAA) by *Stenotrophomonas maltophilia* BE25 isolated from roots of banana (*Musa* spp). *International Journal of Science and Research* **4**, 2644–2650.
- Asaf S, Aaqil Khan M, Latif Khan A, Waqas M, Shahzad R, Kim A-Y, Kang S-M and Lee I-J** (2017) Bacterial endophytes from arid land plants regulate endogenous hormone content and promote growth in crop plants: an example of *Sphingomonas* sp. and *Serratia marcescens*. *Journal of Plant Interactions* **12**, 31–38. doi:10.1080/17429145.2016.1274060
- Barret M, Guimbaud J-F, Darrasse A and Jacques M-A** (2016) Plant microbiota affects seed transmission of phytopathogenic microorganisms. *Molecular Plant Pathology* **17**, 791–795. doi:10.1111/mpp.12382
- Barthlott W** (1981) Epidermal and seed surface characters of plants: systematic applicability and some evolutionary aspects. *Nordic Journal of Botany* **1**, 345–355. doi:10.1111/j.1756-1051.1981.tb00704.x
- Belimov AA, Hontzas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S and Glick BR** (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biology and Biochemistry* **37**, 241–250. doi:10.1016/j.soilbio.2004.07.033
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ and Holmes SP** (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**, 581–583. doi:10.1038/nmeth.3869
- Compant S, Samad A, Faist H and Sessitsch A** (2019) A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *Journal of Advanced Research* **19**, 29–37. doi:10.1016/j.jare.2019.03.004
- Cunningham JE and Kuiack C** (1992) Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. *Applied Environmental Microbiology* **58**, 1451–1458. <https://journals.asm.org/doi/10.1128/aem.58.5.1451-1458.1992>
- Davis NM, Proctor DM, Holmes SP, Relman DA and Callahan BJ** (2018) Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226. doi:10.1186/s40168-018-0605-2
- Dinesh R, Anandaraj M, Kumar A, Bini YK, Subila KP and Aravind R** (2015) Isolation, characterization, and evaluation of multi-trait plant growth promoting rhizobacteria for their growth promoting and disease suppressing effects on ginger. *Microbiological Research* **173**, 34–43. doi:10.1016/j.micres.2015.01.014
- Durlik K, Żarnowiec P, Piwowarczyk R and Kaca W** (2021) Culturable endophytic bacteria from *Phelipanche ramosa* (Orobanchaceae) seeds. *Seed Science Research* **31**, 69–75. doi:10.1017/S0960258520000343
- Eevers N, Gielen M, Sánchez-López A, Jaspers S, White JC, Vangronsveld J and Weyens N** (2015) Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. *Microbial Biotechnology* **8**, 707–715. doi:10.1111/1751-7915.12291
- Eriksson O and Kainulainen K** (2011) The evolutionary ecology of dust seeds. *Perspectives in Plant Ecology, Evolution and Systematics* **13**, 73–87. doi:10.1016/j.ppees.2011.02.002
- Etesami H and Beattie GA** (2018) Mining halophytes for plant growth-promoting halotolerant bacteria to enhance the salinity tolerance of non-halophytic crops. *Frontiers in Microbiology* **9**, 148. doi:10.3389/fmicb.2018.00148
- Fitzpatrick CR and Schneider AC** (2020) Unique bacterial assembly, composition, and interactions in a parasitic plant and its host. *Journal of Experimental Botany* **71**, 2198–2209. doi:10.1093/jxb/erz572
- Frank AC, Saldierna Guzmán JP and Shay JE** (2017) Transmission of bacterial endophytes. *Microorganisms* **5**, 70. doi:10.3390/microorganisms5040070
- Glassner H, Zchori-Fein E, Yaron S, Sessitsch A, Sauer U and Compant S** (2018) Bacterial niches inside seeds of *Cucumis melo* L. *Plant and Soil* **422**, 101–113. doi:10.1007/s11104-017-3175-3
- Grobela K, Kokot P, Świątek J, Jaskulak M and Rorat A** (2018) Bacterial ACC deaminase activity in promoting plant growth on areas contaminated with heavy metals. *Journal of Ecological Engineering* **19**, 150–157. doi:10.12911/22998993/89818
- Hallmann J, Quadt-Hallmann A, Mahaffee WF and Kloepper JW** (1997) Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* **43**, 895–914. doi:10.1139/m97-131
- Hassan TU and Bano A** (2016) Comparative effects of wild type *Stenotrophomonas maltophilia* and its indole acetic acid-deficient mutants on wheat. *Plant Biology (Stuttgart, Germany)* **18**, 835–841. doi:10.1111/plb.12477
- Hemida KA and Reyad AM** (2019) Improvement salt tolerance of safflower plants by endophytic bacteria. *Journal of Horticulture and Plant Research* **5**, 38–56. doi:10.18052/www.scipress.com/JHPR.5.38
- Herrera SD, Grossi C, Zawoznik M and Groppa MD** (2016) Wheat seeds harbour bacterial endophytes with potential as plant growth promoters and biocontrol agents of *Fusarium graminearum*. *Microbiological Research* **186–187**, 37–43. doi:10.1016/j.micres.2016.03.002
- Hryniewicz K, Patz S and Ruppel S** (2019) *Salicornia europaea* L. as an underutilized saline-tolerant plant inhabited by endophytic diazotrophs. *Journal of Advanced Research* **19**, 49–56. doi:10.1016/j.jare.2019.05.002
- Huet S, Pouvreau J-B, Delage E, Delgrange S, Marais C, Bahut M, Delavaul P, Simier P and Poulin L** (2020) Populations of the parasitic plant *Phelipanche ramosa* influence their seed microbiota. *Frontiers in Plant Science* **11**, 1075. doi:10.3389/fpls.2020.01075
- Iasur Kruh L, Lahav T, Abu-Nassar J, Achdari G, Salami R, Freilich S and Aly R** (2017) Host-parasite-bacteria triangle: the microbiome of the parasitic weed *Phelipanche aegyptiaca* and tomato-*Solanum lycopersicum* (Mill.) as a host. *Frontiers in Plant Science* **8**, 269. doi:10.3389/fpls.2017.00269
- Joel DM, Hershenthorn Y, Eizenberg H, Aly R, Ejeta G, Rich PJ, Ransom JK, Sauerborn J and Rubiales D** (2007) Biology and management of weedy root parasites, pp. 267–349 in Janick J. (Ed.) *Horticultural reviews*, vol. 33. New York, John Wiley & Sons. doi:10.1002/9780470168011.ch4
- Johnston-Monje D, Gutiérrez JP and Lopez-Lavalle LAB** (2021) Seed-transmitted bacteria and fungi dominate juvenile plant microbiomes. *Frontiers in Microbiology* **12**, 737616. doi:10.3389/fmicb.2021.737616
- Jonkers W, Gundel PE, Verma SK and White JF** (2022) Editorial: seed microbiome research. *Frontiers in Microbiology* **13**, 943329. doi:10.3389/fmicb.2022.943329
- Kusstatscher P, Adam E, Wicaksono WA, Bernhart M, Olimi E, Müller H and Berg G** (2021) Microbiome-assisted breeding to understand cultivar-dependent assembly in *Cucurbita pepo*. *Frontiers in Plant Science* **12**, 642027. doi:10.3389/fpls.2021.642027
- Li Z, Lin H, Gu L, Gao J and Tzeng C-M** (2016) Herba *Cistanche* (Rou Cong-Rong): one of the best pharmaceutical gifts of traditional Chinese medicine. *Frontiers in Pharmacology* **7**. doi:10.3389/fphar.2016.00041

- Lumactud R and Fulthorpe RR (2018) Endophytic bacterial community structure and function of herbaceous plants from petroleum hydrocarbon contaminated and non-contaminated sites. *Frontiers in Microbiology* **9**, 1926. doi:10.3389/fmicb.2018.01926
- Luziatelli F, Ficca AG, Bonini P, Muleo R, Gatti L, Meneghini M, Tronati M, Melini F and Ruzzi M (2020) A genetic and metabolomic perspective on the production of indole-3-acetic acid by *Pantoea agglomerans* and use of their metabolites as biostimulants in plant nurseries. *Frontiers in Microbiology* **11**, 1475. doi:10.3389/fmicb.2020.01475
- Manasa KM, Vasanthakumari MM, Nataraja KN and Uma Shaanker R (2020) Endophytic fungi of salt adapted *Ipomea pes-caprae* L. R. Br: their possible role in inducing salinity tolerance in paddy (*Oryza sativa* L.). *Current Science* **118**, 1448–1453. doi:10.18520/cs/v118/i9/1448-1453
- Manjunatha BS, Asha AD, Nivetha N, Bandeppa, Govindasamy V, Rathi MS and Sangeeta P (2017) Evaluation of endophytic bacteria for their influence on plant growth and seed germination under water stress conditions. *International Journal of Current Microbiology and Applied Sciences* **6**, 4061–4067. doi:10.20546/ijcmas.2017.611.475
- McMurdie PJ and Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* **8**, e61217. doi:10.1371/journal.pone.0061217
- Metwaly A, Salama GMY and Ali GA (2018) Using hydrogen peroxide for reducing bacterial contamination in date palm tissue culture. *International Journal of Advances in Agricultural Science and Technology* **5**, 25–33.
- Murali A, Bhargava A and Wright ES (2018) IDTAXA: a novel approach for accurate taxonomic classification of microbiome sequences. *Microbiome* **6**, 140. doi:10.1186/s40168-018-0521-5
- Nickrent DL (2020) Parasitic angiosperms: how often and how many? *Taxon* **69**, 5–27. doi:10.1002/tax.12195
- Panosyan H, Hakobyan A, Birkeland N-K and Trchounian A (2018) Bacilli community of saline-alkaline soils from the Ararat Plain (Armenia) assessed by molecular and culture-based methods. *Systematic and Applied Microbiology* **41**, 232–240. doi:10.1016/j.syapm.2017.12.002
- Parte AC (2018) LPSN — list of prokaryotic names with standing in Nomenclature (bacterio.net), 20 years on. *International Journal of Systematic and Evolutionary Microbiology* **68**, 1825–1829. doi:10.1099/ijsem.0.002786
- Patten CL and Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology* **68**, 3795–3801. doi:10.1128/AEM.68.8.3795-3801.2002
- Petrosyan K, Piwowarczyk R, Ruraż K, Thijs S, Vangronsveld J and Kaca W (2022) Seed associated microbial communities of holoparasitic *Cistanche* species from Armenia and Portugal, p. 125 in Proceedings from XVI International Conference on Plant Physiology and Plant Science, January 2022, Zurich, Switzerland.
- Piwowarczyk R (2013) Seed productivity in relation to other shoot features for endangered parasitic plant *Orobanche picridis* F.W. Schultz (Orobanchaceae). *Polish Journal of Ecology* **61**, 55–64.
- Piwowarczyk R, Kwolek D, Góralski G, Denysenko M, Joachimiak AJ and Aleksanyan A (2017) First report of the holoparasitic flowering plant *Cistanche armena* on Caspian Manna (*Alhagi maurorum*) in Armenia. *Plant Disease* **101**, 512–512. doi:10.1094/PDIS-10-16-1469-PDN
- Piwowarczyk R, Sánchez Pedraja Ó, Moreno Moral G, Fayvush G, Zakaryan N, Kartashyan N and Aleksanyan A (2019) Holoparasitic Orobanchaceae (*Cistanche*, *Diphelypaea*, *Orobanche*, *Phelipanche*) in Armenia: distribution, habitats, host range and taxonomic problems. *Phytotaxa* **386**, 001–106. doi:10.11646/phytotaxa.386.1.1
- Piwowarczyk R, Ochmian I, Lachowicz S, Kapusta I, Sotek Z and Błaszak M (2020a) Phytochemical parasite-host relations and interactions: a *Cistanche armena* case study. *Science of The Total Environment* **716**, 137071. doi:10.1016/j.scitotenv.2020.137071
- Piwowarczyk R, Ruraż K, Krasnylenko Y, Kasińska J and Sánchez-Pedraja Ó (2020b) Seed micromorphology of representatives of holoparasitic Orobanchaceae genera from the Caucasus region and its taxonomic significance. *Phytotaxa* **432**, 223–251. doi:10.11646/phytotaxa.432.3.1
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J and Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**, D590–D596. doi:10.1093/nar/gks1219
- Raj PS, Sasikala C, Ramaprasad EVV, Subhash Y, Busse H-J, Schumann P and Ramana C (2013) *Chryseomicrobium amylolyticum* sp. nov., isolated from a semi-arid tropical soil, and emended descriptions of the genus *Chryseomicrobium* and *Chryseomicrobium imtechense*. *International Journal of Systematic and Evolutionary Microbiology* **63**, 2612–2617. doi:10.1099/ijms.0.044552-0
- Ruraż K, Piwowarczyk R, Gajdoś P, Krasnylenko Y and Čertík M (2020) Fatty acid composition in seeds of holoparasitic Orobanchaceae from the Caucasus region: relation to species, climatic conditions and nutritional value. *Phytochemistry* **179**, 112510. doi:10.1016/j.phytochem.2020.112510
- Sánchez-López AS, Pintelon I, Stevens V, Imperato V, Timmermans J-P, González-Chávez C, Carrillo-González R, Van Hamme J, Vangronsveld J and Thijs S (2018) Seed endophyte microbiome of *Crotalaria pumila* unpeeled: identification of plant-beneficial *Methylobacteria*. *International Journal of Molecular Sciences* **19**, 291. doi:10.3390/ijms19010291
- Schneider AC and Moore AJ (2017) Parallel Pleistocene amphitropical disjunctions of a parasitic plant and its host. *American Journal of Botany* **104**, 1745–1755. doi:10.3732/ajb.1700181
- Schwyn B and Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**, 47–56. doi:10.1016/0003-2697(87)90612-9
- Shahzad R, Khan AL, Bilal S, Asaf S and Lee I-J (2017) Plant growth-promoting endophytic bacteria versus pathogenic infections: an example of *Bacillus amyloliquefaciens* RWL-1 and *Fusarium oxysporum* f. sp. *lycopersici* in tomato. *PeerJ* **5**, e3107. doi:10.7717/peerj.3107
- Shameer S and Prasad TNKV (2018) Plant growth promoting rhizobacteria for sustainable agricultural practices with special reference to biotic and abiotic stresses. *Plant Growth Regulation* **84**, 603–615. doi:10.1007/s10725-017-0365-1
- Shrivastava P and Kumar R (2015) Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences* **22**, 123–131. doi:10.1016/j.sjbs.2014.12.001
- Singh RP and Jha PN (2017) The PGPR *Stenotrophomonas maltophilia* SBP-9 augments resistance against biotic and abiotic stress in wheat plants. *Frontiers in Microbiology* **8**, 1945. doi:10.3389/fmicb.2017.01945
- Soussi A, Ferjani R, Marasco R, Guesmi A, Cherif H, Rolli E, Mapelli F, Ouzari HI, Daffonchio D and Cherif A (2016) Plant-associated microbiomes in arid lands: diversity, ecology and biotechnological potential. *Plant and Soil* **405**, 357–370. doi:10.1007/s11104-015-2650-y
- Szymańska S, Borruso L, Brusetti L, Hulisz P, Furtado B and Hryniewicz K (2018) Bacterial microbiome of root-associated endophytes of *Salicornia europaea* in correspondence to different levels of salinity. *Environmental Science and Pollution Research* **25**, 25420–25431. doi:10.1007/s11356-018-2530-0
- Truyens S, Weyens N, Cuypers A and Vangronsveld J (2013) Changes in the population of seed bacteria of transgenerationally Cd-exposed *Arabidopsis thaliana*. *Plant Biology* **15**, 971–981. doi:10.1111/j.1438-8677.2012.00711.x
- Truyens S, Jambon I, Croes S, Janssen J, Weyens N, Mench M, Carleer R, Cuypers A and Vangronsveld J (2014) The effect of long-term Cd and Ni exposure on seed endophytes of *Agrostis capillaris* and their potential application in phytoremediation of metal-contaminated soils. *International Journal of Phytoremediation* **16**, 643–659. doi:10.1080/15226514.2013.837027
- Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A and Vangronsveld J (2016) The effects of the growth substrate on cultivable and total endophytic assemblages of *Arabidopsis thaliana*. *Plant and Soil* **405**, 325–336. doi:10.1007/s11104-015-2761-5
- Ulrich K, Ulrich A and Ewald D (2008) Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiology Ecology* **63**, 169–180. doi:10.1111/j.1574-6941.2007.00419.x
- Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, Apprill A and Knight R (2016) Improved bacterial 16S rRNA gene (V4 and V4-5) and

- fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems* **1**, e00009–15. doi:10.1128/mSystems.00009-15
- Watts JE, de Villiers OT and Watts L** (1993) Sterilization of wheat seeds for tissue culture purposes. *South African Journal of Botany* **59**, 641–642. doi:10.1016/s0254-6299(16)30683-4
- Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W and Glöckner FO** (2014) The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Research* **42**, D643–D648. doi:10.1093/nar/gkt1209
- Yoneyama K, Xiaonan X, Sekimoto H, Takeuchi Y, Ogasawara S, Akiyama K, Hayashi H and Yoneyama K** (2008) Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. *New Phytologist* **179**, 484–494. doi:10.1111/j.1469-8137.2008.02462.x