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Bacterial endophytes from horseradish (*Armoracia rusticana* G. Gaertn., B. Mey. & Scherb.) with antimicrobial efficacy against pathogens

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Abstract: The current study aimed to determine the diversity of culturable endophytic bacteria associated with horseradish (*Armoracia rusticana* G.Gaertn., B.Mey.&Scherb.) grown in Chatkal Biosphere Reserve of Uzbekistan and their antimicrobial potentials. The bacteria were isolated from plant leaves and root tissues using culture-dependent techniques. The 16S rRNA sequences similarities of endophytic bacteria isolated from *A. rusticana* showed that isolates belong to species *Paenibacillus*, *Raoultella*, *Stenotrophomonas*, *Pseudomonas*, *Serratia*, *Microbacterium*, *Enterobacter*, *Achromobacter*, *Brevibacterium*, *Pantoea*, and *Erwinia*. The isolated endophytic bacteria *Stenotrophomonas maltophilia* KRT1, *Serratia ficaria* KRT5, and *Pantoea agglomerans* KLT4 possess antimicrobial activities against human pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans.* The endophytic bacteria *Paenibacillus typhae* KRN1, *Stenotrophomonas maltophilia* KRT1, *Pseudomonas kilonensis* KRT11, *Pseudomonas umsongensis* KRT21, *Brevibacterium frigoritolerans* KLT2 and *Pantoea agglomerans* KLT4 inhibited phytopathogenic fungi *Rhizoctonia solani*, *Fusarium culmorum*, and *F. solani*. These findings indicate that plant endophytic bacteria with antimicrobial activity could be a source for producing agriculturally and pharmaceutically important antimicrobial compounds.

Keywords: medicinal plant; Brassicaceae; plant microbiome; bacterial diversity

Horseradish (*Armoracia rusticana* G.Gaertn., B.Mey.&Scherb.) is a well-known member of the Brassicaceae family representing approximately 350 genera with 3 000 species (Nguyen et al. 2013). The perennial plant grows under multiple natural environmental conditions, including farmland, weedy areas, home gardens, and roadsides in various regions all over the world and have been considered as food and spice or as medicine (Agneta et al. 2013). The antimicrobial activity of horseradish secondary metabolites isothiocyanates was reported against pathogenic microbes such as *Candida albicans* and *Fusobacterium nucleatum* (Park et al. 2013). Similar findings were reported by Choi et al. (2017), where the isothiocyanates inhibited the growth of fungi *Epidermophyton floccosum*, *Microsporum cani*, *Trichophyton rubrum*, and *T. mentagrophytes*.

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Currently, many studies reported the phytochemical and pharmacological properties of plants with medicinal benefits. The medicinal plants endemic to the region may host novel and diverse endophytic microbes, and these have rarely been studied and characterised (Köberl et al. 2013). The plant microbiome contains various microbial communities that provide benefits to plants through supplying nutrients, modulating physiological processes, and inducing systemic resistance to abiotic and biotic stresses (Köberl et al. 2013, Egamberdieva et al. 2019). These physiological properties and activities of endophytic microbes living in plant tissues are strongly affected by secondary metabolites of plants (Chaparro et al. 2014). In previous studies, endophytic microbes with antimicrobial activity were isolated from *Hypericum perforatum* L. and *Ziziphora capitata* L. plants, which also showed antibacterial activity against human pathogenic microbes (Egamberdieva et al. 2017). However, limited information is available about bacterial endophytes from horseradish. Thus, the aim of our study was: (1) to isolate and identify cultivable endophytic bacteria associated with *A. rusticana* G.Gaertn., B.Mey.&Scherb. grown in the Chatkal Biosphere Reserve of Uzbekistan by using 16S rRNA gene analysis, and (2) to evaluate the antimicrobial activity of endophytic bacteria.

MATERIAL AND METHODS

Plant sample collection. *A. rusticana* G.Gaertn., B.Mey.&Scherb. was collected from the Chatkal Biosphere Reserve of Uzbekistan, which is an isolated and protected area in Western Tien Shan province in June 2017. Three individual plants at a distance of 10–12 m were collected as a whole and stored in zip-lock plastic bags using sterile gloves and transported to the laboratory of the Faculty of Biology, National University of Uzbekistan. The plant material was identified by the Institute of Botany, Uzbek Academy of Sciences, Uzbekistan.

Isolation of endophytic bacteria. The bacteria were isolated from leaves and root tissues of a horseradish using culture-dependent techniques, as described by Egamberdieva et al. (2017) with minor modifications. For the isolation of endophytic bacteria, the root and leaves of horseradish were separated with a sterile scalpel, washed with water, and sterilised with 99.9% ethanol for 2 min, subsequently treated with 10% NaClO and rinsed five times in distilled water. The sterile leaves and roots (10 g fresh weight) were macerated using a sterile mortar and pestle. The macerated tissue (1 g) was transferred into plastic tubes with 9 mL sterile phosphate-buffered saline (PBS) and then serially diluted (10^1–10^5). 100 µL aliquots from the appropriate dilutions were spread on tryptic soy agar (TSA) (BD, Difco Laboratories, Detroit, USA) supplemented with nystatin 50 µg/mL, and plates were incubated for four days at 28 °C. Visually homogenous colonies of different sizes, shapes, and colours were used for DNA isolation.

DNA isolation and polymerase chain reaction (PCR). For DNA extraction, the heat treatment method (Dashti et al. 2009) was used. Extracted DNA was used as a template for 16S rRNA gene analysis. The 16S rRNA genes were amplified with PCR using the following primers: 16SF 5'-GAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich, St. Louis, USA) and 16SR 5'-GAAAGGAGGTGATCCAGCC-3' (Sigma-Aldrich, St. Louis, USA). The PCR was performed using a thermocycler (PTC-200, BioRad Laboratories, Inc., Hercules, USA).

Restriction fragment length polymorphism (RFLP) analysis. To identify identical isolates and reduce the number of strains to be sequenced, we conducted a RFLP analysis of 16S rRNA genes as described by Jinneman et al. (1996).

Sequencing and phylogenetic analysis. Before being sequenced, the PCR products were purified with the USB® ExoSAP-IT® PCR Product Cleanup Kit (Affymetrix, USB® Products, Santa Clara, USA) according to the protocol of the manufacturer. Sequencing was performed using ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) by the protocol of the manufacturer. Received data were analysed and corrected using Chromas (v. 2.6.5) software (Technelysium Pty. Ltd., Brisbane, Australia). Corrected sequences were merged manually using the EMBOSS Explorer (http://emboss.bioinformatics.nl/).

The basic local alignment search tool (BLAST) was used to identify the sequences and compared with the GenBank® (NCBI) (http://www.ncbi.nlm.nih.gov/). The 16S rRNA sequences were deposited to GenBank® under the accession numbers MH165369–MH165378 for root endophytes and MH165379–MH165384 for leaf endophytes. All sequences were multiply aligned using ClustalX 2.1 software (Dublin, Ireland), and the FASTA format file was used to construct a phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987).

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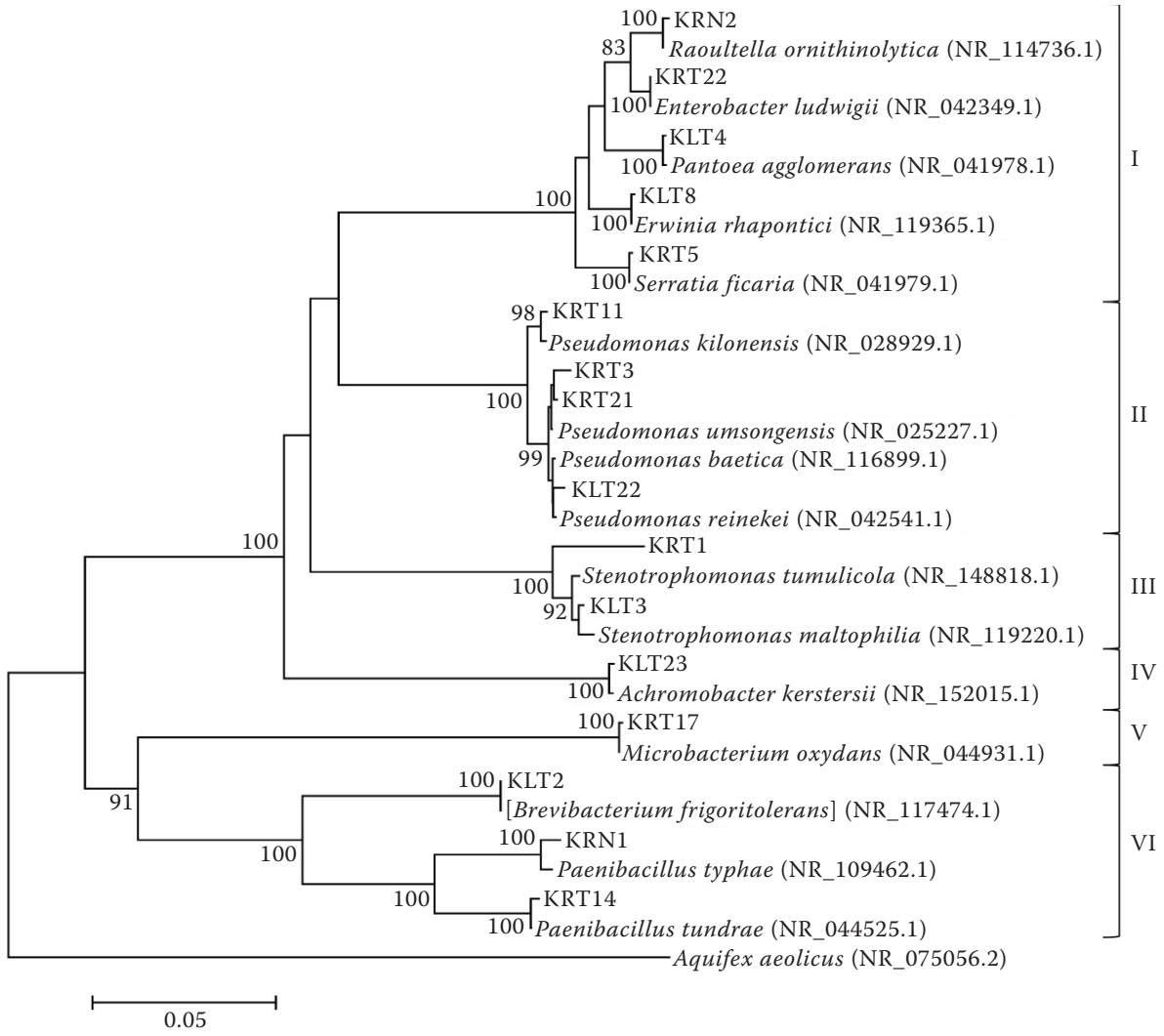

Figure 1. Phylogenetic tree of bacterial endophytes isolated from *Armoracia rusticana* G.Gaertn., B.Mey.& Scherb. and their closest relatives from GenBank. The isolated endophytes represent six orders (clusters): I – Enterobacteriales; II – Pseudomonadales; III – Xanthomonadales; IV – Burkholderiales; V – Micrococcales; VI – Bacillales

The optimal tree with the sum of branch length = 1.03011234 is demonstrated in Figure 1. The maximum composite likelihood method (Tamura et al. 2004) was used to analyse the evolutionary distances. Evolutionary analyses were conducted using MEGA6 (Tamura et al. 2013).

Antimicrobial activities of the crude extracts of plants and endophytic bacteria. The *in vitro* antimicrobial activity of crude extracts of plants and secondary metabolites derived from endophytic bacteria were assayed by the agar well diffusion method described by Pongtharangkul and Demirci (2004). The endophytic bacteria were cultivated in tryptic soy broth (TSB) for three days. The cell-free supernatant of bacterial isolates was extracted by the solvent extraction procedure using ethyl acetate (Elissawy et al. 2019). The horseradish plants were dried at room temperature and powdered. 10 g plant powder was extracted twice with 200 mL ethanol (80%) for 2 h at 80 °C.

Pathogens such as gram-positive bacterium *Staphylococcus aureus* Rosenbach (MRSA16), gram-negative bacterium *Escherichia coli* (Migula) Castellani and Chalmers (NCTC9001) and the yeast *Candida albicans* (C.P. Robin) Berkhout (NCPF3179) were obtained from the National Collection of Type Cultures (Porton Down, UK). The pathogenic microbial strains were plated on nutrient agar (Sigma

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Table 1. Sequence similarities of endophyte bacteria isolated from the root system of horseradish with sequences registered in GenBank

Strain	Length (bp)	Isolated strains deposited to Gen Bank	Closest match among bacteria (16S rRNA genes) (GenBank)		
		accession number	species	accession number	Id (%)
KRN1	1 460	MH165369	*Paenibacillus typhae*	NR_109462.1	98.8
KRN2	1 459	MH165370	*Raoultella ornithinolytica*	NR_114736.1	98.6
KRT1	1 454	MH165371	*Stenotrophomonas maltophilia*	NR_119220.1	98.5
KRT3	1 445	MH165372	*Pseudomonas baetica*	NR_116899.1	98.8
KRT5	1 450	MH165373	*Serratia ficaria*	NR_041979.1	99.4
KRT11	1 440	MH165374	*Pseudomonas kilonensis*	NR_028929.1	99.4
KRT14	1 464	MH165375	*Paenibacillus tundrae*	NR_044525.1	99.1
KRT17	1 438	MH165376	*Microbacterium oxydans*	NR_044931.1	99.5
KRT21	1 448	MH165377	*Pseudomonas umsongensis*	NR_025227.1	99.0
KRT22	1 452	MH165378	*Enterobacter ludwigii*	NR_042349.1	99.6
KLT23	1 437	MH165384	*Achromobacter kerstersii*	NR_152015.1	99.4

Aldrich, St. Louis, USA), and wells were made using a well borer. 60 µL of cell-free suspension from endophytic bacteria and plant extract were added to these wells, and the plates were incubated at 37 °C for 24 h for bacteria, and 72 h for fungi. Ampicillin sodium salt (1 mg/mL) and Amphotericin B (5 mg/mL) were used as a standard. The antimicrobial activities were assessed by the presence or absence of inhibition zones in millimeters. The antifungal activity of plant extract and cell-free suspensions of endophytic bacterial isolates against plant pathogenic fungi *Fusarium culmorum* (Wm. G. Sm.) Sacc., *F. solani* (Mart.) Sacc., and *Rhizoctonia solani* J.G. Kühn were studied as described by Egamberdieva et al. (2017).

The cell wall degrading enzymes. The production of β-1,3-glucanase was determined following the method of Walsh et al. (1995) using the substrate lichenan (Sigma-Aldrich, St. Louis, USA) in top agar plates. The cellulase activity of endophytic bacteria was evaluated by using the substrate carboxymethylcellulose in top-agar plates (Hankin and Anagnostakis 1977). The chitinase activity of bacterial isolates was studied as described by Malleswari and Bagyanarayana (2013), and lipase activity using the tween lipase indicator assay (Howe and Ward 1976).

Data analyses. The mean values of indole-3-acetic acid (IAA) production, antimicrobial activity, the standard deviation (SD), and the estimated standard error of the mean (SEM), and sample size (N) were extracted for each observation using the analysis of variance package included in Microsoft Excel 2013 (Redmond, USA).

Table 2. Sequence similarities of endophyte bacteria isolated from leaves of horseradish with sequences registered in GenBank

Strain	Length (bp)	Isolated strains deposited to Gen Bank	Closest match among bacteria (16S rRNA genes) (GenBank)		
		accession number	species	accession number	Id (%)
KLT2	1 462	MH165379	[*Brevibacterium*] *frigoritolerans*	NR_117474.1	99.7
KLT3	1 457	MH165380	*Stenotrophomonas tumulicola*	NR_148818.1	98.6
KLT4	1 451	MH165381	*Pantoea agglomerans*	NR_041978.1	99.2
KLT8	1 455	MH165382	*Erwinia rhapontici*	NR_119365.1	98.6
KLT22	1 449	MH165383	*Pseudomonas reinekei*	NR_042541.1	98.9
KLT23	1 437	MH165384	*Achromobacter kerstersii*	NR_152015.1	99.4
KRT3	1 445	MH165372	*Pseudomonas baetica*	NR_116899.1	98.8

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Table 3. Antimicrobial activity of bacterial endophytes from horseradish against some human pathogenic bacteria and fungi

Treatment	*Candida albicans* Berkhout NCPF3179	*Escherichia coli* Castellani and Chalmers NCTC9001	*Staphylococcus aureus* Rosenbach MRSA16
Ampicillin sodium salt (1 mg/mL)	–	–	15 ± 1.0
Amphotericin B (5 mg/mL)	12 ± 0.4*	–	–
Paenibacillus typhae KRN1	–	4.5 ± 0.3	7.0 ± 0.5
Raoultella ornithinolytica KRN2	–	–	–
Stenotrophomonas maltophilia KRT1	4.0 ± 0.3	6.0 ± 0.4	3.5 ± 0.4
Pseudomonas baetica KRT3	–	–	6.5 ± 0.5
Serratia ficaria KRT5	7.5 ± 0.5	6.0 ± 0.5	7.0 ± 0.5
Pseudomonas kilonensis KRT11	5.5 ± 0.4	–	–
Paenibacillus tundrae KRT14	–	–	6.5 ± 0.5
Microbacterium oxydans KRT17	–	–	–
Pseudomonas umsongensis KRT21	–	5.5 ± 0.4	7.0 ± 0.5
Enterobacter ludwigii KRT22	4.0 ± 0.3	–	6.5 ± 0.5
Brevibacterium frigoritolerans KLT2	–	6.0 ± 0.5	9.0 ± 0.6
Stenotrophomonas tumulicola KLT3	4.5 ± 0.3	–	–
Pantoea agglomerans KLT4	5.6 ± 0.4	6.2 ± 0.5	7.0 ± 0.5
Erwinia rhapontici KLT8	–	–	–
Pseudomonas reinekei KLT22	7.0 ± 0.5	4.0 ± 0.4	–
Achromobacter kerstersii KLT23	–	–	–
Plant extract	7.0 ± 0.6	6.5 ± 0.5	4.5 ± 0.4

*growth inhibition zone (mm)

RESULTS

Isolation and identification of cultivable endophytic bacteria. In total, 11 species originated from roots (Table 1), and seven species from leaves of horseradish were isolated (Table 2). The isolates were 98.5–99.7% identical to their closest relatives registered in GenBank®. The length of the identified nucleotides sequences of 16S rRNA genes of the isolates varied from 1 437 to 1 464 bp and was considered as sufficient for a reliable identification based on 16S rRNA genes analysis using BLAST tool. As shown in Table 1, the roots of horseradish harboured 11 species belonging to four phyla: Gammaproteobacteria (KRN2, KRT1, KRT3, KRT5, KRT11, KRT21, and KRT22), Firmicutes (KRN1 and KRT14), Actinobacteria (KRT17) and Betaproteobacteria (KLT23). Table 2 contains seven strains isolated from leaves of horseradish and represents the phyla Gammaproteobacteria (KLT3, KLT4, KLT8, KLT22, and KRT3), Betaproteobacteria (KLT23) and Firmicutes (KLT2). Notably, the strains *Achromobacter kerstersii* KLT23 and *Pseudomonas baetica* KRT3 inhabited both roots and shoots of horseradish.

Antimicrobial activity of endophytic bacterial isolates. Among all tested bacteria, only *Stenotrophomonas maltophilia* KRT1, *Serratia ficaria* KRT5, and *Pantoea agglomerans* KLT4 exhibited strong antimicrobial activity against *C. albicans*, *E. coli*, and *S. aureus*. *Pseudomonas reinekei* KLT22, *P. umsongensis* KRT21, *Enterobacter ludwigii* KRT22, and *Paenibacillus typhae* KRN1 exhibited antimicrobial activity against two tested human pathogenic bacteria (Table 3). The methanol extract of plants showed strong growth inhibition against *S. aureus* and *E. coli*, but not against *C. albicans*.

Among the bacterial isolates, *Paenibacillus typhae* KRN1, *S. maltophilia* KRT1, *P. kilonensis* KRT11, *P. umsongensis* KRT21, *Brevibacterium frigoritolerans* KLT2, and *P. agglomerans* KLT4 showed moderate or strong inhibition against three tested plant pathogenic fungi *F. culmorum*, *F. solani*, and *R. solani* (Table 4, Figure 2). Three isolates, *P. typhae* KRN1,

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Table 4. Antifungal activity of bacterial endophytes from horseradish against plant pathogenic fungi and production of cell wall degrading enzymes

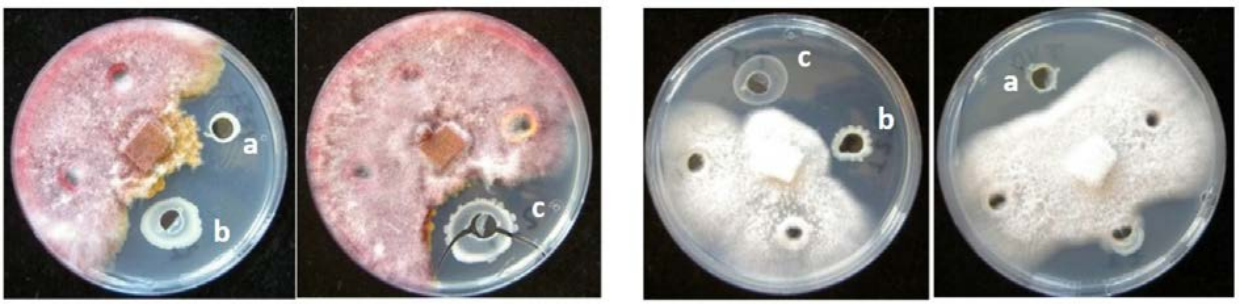
Bacterial isolates	Phytopathogenic fungi			Cell wall degrading enzymes			
	Fusarium culmorum	*F. solani*	*Rhizoctonia solani*	lipase	protease	chitinase	glucanase
Paenibacillus typhae KRN1	0.9 ± 0.1*	0.7 ± 0.1	0.9 ± 0.1	+	+	+	–
Raoultella ornithinolytica KRN2	–	–	–	–	+	–	–
Stenotrophomonas maltophilia KRT1	1.0 ± 0.1	0.8 ± 0.2	1.2 ± 0.1	–	+	+	+
Pseudomonas baetica KRT3	1.1 ± 0.2	0.9 ± 0.1	–	–	+	–	+
Serratia ficaria KRT5	1.2 ± 0.1	1.5 ± 0.2	–	+	–	–	–
Pseudomonas kilonensis KRT11	1.2 ± 0.1	1.3 ± 0.2	1.5 ± 0.2	–	+	+	–
Paenibacillus tundrae KRT14	–	0.7 ± 0.1	0.5 ± 0.1	–	+	–	+
Microbacterium oxydans KRT17	–	0.6 ± 0.1	–	–	–	–	–
Pseudomonas umsongensis KRT21	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	–	+	–	+
Enterobacter ludwigii KRT22	–	–	–	–	–	–	–
Brevibacterium frigoritolerans KLT2	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	–	+	+	–
Stenotrophomonas tumulicola KLT3	–	–	–	–	+	–	–
Pantoea agglomerans KLT4	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	+	+	–	+
Erwinia rhapontici KLT8	–	–	–	+	+	–	–
Pseudomonas reinekei KLT22	–	0.8 ± 0.1	–	–	+	–	+
Achromobacter kerstersii KLT23	–	–	–	–	–	–	–
Plant extract	0.5 ± 0.1	0.7 ± 0.1	–				

*growth inhibition zone (cm)

S. maltophilia KRT1, and *P. agglomerans* KLT4, were able to produce three out of four cell wall degrading enzymes. Only three bacterial isolates did not synthesise lipase, protease, cellulase, and chitinase enzymes.

DISCUSSION

Endophytic bacteria associated with plants play an essential role in plant health, producing various beneficial metabolites and thus have been considered as a source of valuable biologically active compounds (Köberl et al. 2013, Musa et al. 2020). To the best of our knowledge, this is the first report where endophytic bacteria associated with horseradish grown in the arid Chatkal Biosphere Reserve, Uzbekistan have been analysed. Only one report is available where Szücs et al. (2018) identified several endophytic fungi, i.e., *Fusarium*, *Macrophomina*, *Setophoma*, *Paraphoma*, and *Oidiodendron* from the roots of horseradish grown in Hungary. We found that 18 isolates isolated from the plant tissue of horserad-



F. culmorum *F. solani*

Figure 2. Antagonistic activity of *Pseudomonas kilonensis* KRT11 (a); *Stenotrophomonas maltophilia* KRT1 (b); *Pantoea agglomerans* KLT4 (c), against *Fusarium culmorum* and *F. solani*

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ish represented four phyla and six orders. Profiling of endophytic bacteria isolated from the roots of horseradish demonstrated that these included 11 strains belonging to the genera *Paenibacillus* (2), *Raoutella*, *Stenotrophomonas* (2), *Pseudomonas* (3), *Serratia*, *Microbacterium*, *Enterobacter* and *Achromobacter* from the root system. Seven isolates were isolated from the leaves belonging to the genera *Brevibacterium*, *Stenotrophomonas*, *Pantoea*, *Erwinia*, *Pseudomonas* (2) and *Achromobacter*. All of these bacterial genera were also found in other medicinal plants as well, e.g., *R. ornithinolytica* from *Panax ginseng* C. A. Mey. (Chowdhury et al. 2017), *S. maltophilia* from *Equisetum arvense* L. (Lata et al. 2019), *P. baetica* from *Zingiber montanum* (J. König) Link ex A. Dietr. (Nongkhlaw and Joshi 2014), and *M. oxydans* from *Aster tripolium* L. (Szymańska et al. 2016).

We found that the methanol extract of horseradish showed strong antimicrobial activity against *S. aureus*, *C. albicans*, and *E. coli*. In previous studies, the antimicrobial activities of horseradish plant extracts were effective against *E. faecalis* (Jang et al. 2020). The biologically active compounds of medicinal plants can strongly affect endophytic microbes living inside the plant tissue and also their physiological functions (Köberl et al. 2013). In our study, thirteen bacterial isolates showed moderate or strong inhibition against one or more human pathogenic bacteria, i.e., *E. coli* and *S. aureus* and against the yeast *C. albicans*. Nongkhlaw and Joshi (2015) reported that *Pseudomonas baetica* ENIB7 isolated from medicinal plant *Zingiber montanum* (J. König) Link ex A. Dietr. possessed antagonistic activity against human pathogenic strains *S. aureus* and *E. coli*. According to Akinsanya et al. (2015), endophytic bacteria associated with medicinal plants produce bioactive compounds with antimicrobial activities similar to their host.

In our study, bacterial endophytes of horseradish demonstrated antagonistic activities against the plant pathogenic fungi *F. culmorum*, *F. solani*, and *R. solani* and, moreover, produced lipase, cellulase, chitinase, and glucanase enzymes. It is known that secretion of cell wall degrading enzymes by microbes is one of the main mechanisms in the suppression of plant pathogens. Furthermore, high proportions of endophytes with antifungal properties were also reported among plant-associated bacteria with *H. perforatum* L. (Egamberdieva et al. 2017).

This is the first study on culturable endophytic bacterial diversity of horseradish (*A. rusticana* G. Gaertn., B. Mey. & Scherb.) from an arid region in Uzbekistan. The endophytic bacteria associated with horseradish and plant extracts possessed antimicrobial activity against plant pathogenic fungi but also against human pathogenic bacteria. These findings indicate that the biological activity of plants plays an important role in shaping or regulating the physiological properties of their endophytes, and horseradish could be suggested as a valuable source of biologically or pharmaceutically active compounds.

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