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ENDOPHYTIC BACTERIA FROM *CAPSICUM ANNUUM* VAR. *GROSSUM* CULTIVARS AND THEIR INHIBITORY EFFECTS ON *LISTERIA MONOCYTOGENES*

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Endophytic microorganisms living inside plant tissues might have numerous positive effects on the host plants. Endophytes can promote the growth and yield of the plant, help to remove contaminants from the tissues, and can suppress growth of pathogens; however, some enteric human pathogenic bacteria have also been isolated as endophytes. The aims of our study were the characterisation and identification of endophytic coliform bacteria isolated from different cultivars of sweet pepper (*Capsicum annuum* var. *grossum*) using a selective (VRBL) agar medium, and determination of antagonistic interactions between these endophytes and *Listeria monocytogenes*. The bacterial isolates showed heterogeneity based on their phenotypic and genotypic properties. Results of identification by molecular biological methods also confirmed the presence of different genera/species. When the antagonistic effect of the isolated endophytic bacteria was tested it was found that one isolate – identified as *Pseudomonas putida* – showed significant inhibition on the growth of *Listeria monocytogenes*.

Keywords: endophytic bacteria, antagonism, *Listeria monocytogenes*, *Pseudomonas putida*

Food-borne pathogenic bacteria have notable significance in the food industry as they can contribute to economical losses and – what is more important – they can cause severe food-borne diseases (EFSA, 2013).

Contamination of the fresh produces by wide range of pathogenic microbes can occur at any point in the food production chain. Incidences of connected outbreaks are increasing and the majority of food-borne diseases has been linked to leafy greens, sprouts, herbs, and different fruits and vegetables. The increasing number of outbreaks can be attributed to the elevated consumption of fresh, minimally processed, ready-to-use/ready-to-eat products. However, scientific publications increasingly support the hypothesis that certain human pathogens have adapted to the plants in a way being able to persist on or within the plants as part of their natural lifecycle between the infected hosts (Warriner & Namvar, 2010). It is known that plants harbour internal (endophytic) bacteria that are essential to their health, but human pathogens have also been detected as endophytes. Among natural endophytic bacteria *Salmonella*, *Staphylococcus*, *Mycobacterium*, *Klebsiella,* and *Burkholderia* species have already been identified, hereby supporting the hypothesis that human pathogens can internalise the healthy plant tissue (Warriner & Namvar, 2010).

Based on the severity and numbers of the outbreaks caused by consumption of contaminated fresh produces, the most common human pathogenic bacteria are *Listeria*

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monocytogenes, various serogroups of *Salmonella* (e.g., Typhimurium, Newport, Reading, Saintpaul, etc.) and enterohaemorrhagic *Escherichia coli* strains (Manios et al., 2013).

Listeria monocytogenes is one of the most dangerous pathogenic bacteria causing relatively rare but serious illness with high morbidity, hospitalisation, and mortality rate. In 2011 the numbers of food-borne cases caused by *L. monocytogenes* in Hungary and in the European Union were 11 and 1476, respectively. Despite these relatively low numbers of infections mediated by *L. monocytogenes*, this food safety problem is considered significant (EFSA, 2013).

Effective protection of processed food or raw materials against pathogenic bacteria could be promoted by the application of antagonistic bacteria that are able to inhibit the growth of these harmful microorganisms (Teplitski et al., 2010). Endophytic microorganisms live inside the plant tissues without causing symptoms of diseases (Zinniel et al., 2002) and have positive effects on the plants. Endosymbiotic bacteria of plants can enhance the nutrient uptake by the hosts and protect them from pests and pathogens, thus they could contribute to the increased tolerance of the host plants against biotic and abiotic stresses (Clay & Schardl, 2002). Nevertheless, as it was mentioned above, pathogenic bacteria can internalise plant tissues and survive within the plant cells as endophytes (Berg et al., 2005; Ansingkar & Kulkarni, 2010; Warriner & Namvar, 2010).

Species belonging to the *Pseudomonas* genus inhabit a broad variety of niches including plants, soil, water, insects, and humans. Their ubiquitous nature is attributed mainly to the unusually divergent physiological, metabolic, and stress-tolerant characteristics. *P. putida* is especially important as a potential model organism in biotechnology and synthetic biology (Nikel et al., 2014), and it is one of the most frequent soil and rhizosphere bacteria armed with several antagonistic traits (Berg et al., 2002).

The aims of our study were the characterisation and identification of endophytic coliform bacteria isolated from different cultivars of sweet peppers using coliform selective VRBL agar medium, and determination of the antagonistic interactions between the endophytes and *L. monocytogenes*.

1. Materials and methods

1.1. Isolation of potential endophytic bacteria from sweet pepper cultivars

Bacteria were isolated from different tissues of hydro- and soil-cultures of *Capsicum annuum* var. *grossum* (sweet pepper) KPA and HO cultivars using coliform selective violet red bile lactose (VRBL) agar (Biokar Diagnostics). The tissues were disinfected with 70% ethanol for 1 min, sodium hypo-chlorite solution (mixture of hypo-chlorite and sterile distilled water in a ratio of 1:4 supplemented with 0.1% Tween 80) for 10 min, with 70% ethanol again for 1 minute, and finally rinsed with sterile tap water for five times. The sterile plant tissues were placed in tubes containing 2 ml of Peptone-Glucose-Yeast extract (PGY) broth (peptone 5 g l^{-1}; glucose 1 g l^{-1}; yeast extract 2.5 g l^{-1}) supplemented with Nystatine (0.1 g l^{-1}) and NaCl (5 g l^{-1}), and incubated in a rotary shaker at 25 ºC for 7 days. A loopful broth from positive (turbid) tubes was inoculated onto VRBL agar plates, which were incubated at 30 ºC for 3 days. Colonies of potential endophytic coliform bacteria were isolated, sub-cultured on PGY agar plates and analysed further.

1.2. Phenotypic characterisation of the potential endophytic bacterial isolates

Bacterial isolates were subjected to phenotypic trials. Cell morphology was tested by light microscopy, while Wallerstein Laboratory – WL – Nutrient Agar (ATLAS, 1995), Chromocult coliform agar (Merck), and Pseudomonas P and Pseudomonas F agars (Merck) were applied for macro-morphological analyses. Oxidase-, catalase-, KOH-, and OF-tests were used as biochemical and physiological assays.

1.3. Molecular biological analyses

Molecular analyses were used for characterisation and identification of the isolates. PCR typing using the M13 minisatellite primer (VASSART et al., 1987) and amplified rDNA restriction analysis (ARDRA) using two different restriction endonucleases (*Msp*I and *Hae*III) were applied for genotyping purposes. *Pseudomonas* isolates were detected by a genus-specific PCR method developed in our laboratory applying the Ps-f (5'-ACGATCCGTAACTGGTCTGAGA-3') and Ps-r (5'-CCACTGGTGTTCCTTCTT-ATATC-3') primer pairs. Positive isolates were identified at species level by direct sequencing of the *rpoB* gene (TAYEB et al., 2005). The non-*Pseudomonas* isolates were identified by sequencing the 16S rDNA PCR products generated by the 27f – 1492r primer pair (MAIWALD, 2004) and the nucleotide sequences were aligned with those deposited in the GenBank.

1.4. Determination of the interactions between L. monocytogenes *and bacterial isolates*

Antagonistic effect of the isolated bacteria on L. *monocytogenes* CCM 4699 was determined by interaction studies using co-culturing of the strains and testing the inhibitory effect of the cell free supernatant of the isolated cultures. For co-culturing test 10^4 cells of L. *monocytogenes* were massively inoculated onto Tryptone Soya Agar (TSA) plates (Biokar Diagnostics), and after drying, 10 µl of cell suspensions (containing 10^6–10^7 cells) of overnight bacterium cultures were dropped on the surface of the agar plates. The plates were incubated at 20, 25, and 30 °C for 3 days. Growth inhibition of *L. monocytogenes* was detected by the formation of clearing zones around macro-colonies of the tested isolates.

The inhibitory effect of the culture supernatants was tested as follows. Cell-free supernatants were prepared from overnight cultures of the isolates in PGY broth incubated at 20, 25, and 30 °C. Cell suspensions were centrifuged at 14 000 r.p.m. for 15 min and the supernatants were filtered by 0.2 µm pore-size membrane filters. The inhibitory effect of the supernatants on *L. monocytogenes* was tested in micro-titre plates using MultiSkan Ascent (Thermo Electron Corporation) equipment. Wells of the plates were filled with 300 µl liquid composed of PGY broth (four-fold strength, 75 µl), *L. monocytogenes* cell suspension (75 µl, 3×10^5 cells ml^{-1}), and cell-free supernatant of the test strain in four different volumes (150, 100, 50, and 25 µl) that were adjusted to the final volume by sterile distilled water. Consequently, the concentration of the cell-free supernatants corresponded to 1/2, 1/3, 1/6, and 1/12 dilutions and to the cell density of 10^6 cells ml^{-1}. Inoculated plates were incubated at 20 °C and the OD values at 595 nm were recorded in every 30 min during 24 h. Growth curves were generated from the OD values versus time data.

1.5. Detection of siderophores

Cell-free supernatant was prepared from 24-hour-culture of HP-PR-H-4 *P. putida* isolate as described in section 1.4., and siderophores were detected by spectrum analysis in the range of 380–420 nm (Manninen & Mattila-Sandholm, 1994).

2. Results and discussion

2.1. Results of phenotypic and genotypic analyses

Altogether 42 bacteria were isolated from different organs (roots, stem, leaf, and fruit) of hydro- and soil-cultures of *Capsicum annuum* var. *grossum* KPA and HO cultivars on VRBL agar plates. VRBL is a selective medium for detection and enumeration of coliform bacteria, and typical *Escherichia coli* colonies should have violet-red colour on this medium (Biokar, 2009), nevertheless appearance of other coliforms are not defined in the data sheet. Colonies of the isolates were mainly pink, pinkish-beige, beige and brown, while zones with different shade were also observed in case of some colonies after 48 h of incubation. None of the isolated colonies had typical *E. coli* appearance. In spite of the lactose content of this medium, the lactose negative *Pseudomonas aeruginosa* has a good growth on it, and its colonies appear in pinkish-beige colour. Several smaller and larger colonies of this colour developed during 3 days of incubation.

Different morphological, physiological, and biochemical properties of the isolates were determined as described in section 1.2. Phenogram of the isolates was constructed by the application of StatSoft® - STATISTICA 10 software (Fig. 1). The isolates showed extended phenotypic heterogeneity, as the 42 isolates were clustered into 25 groups, but phenotypically identical isolates could also be found between bacteria isolated from different parts of the plants.

Using the M13 minisatellite primer for PCR typing, the isolates were clustered into genotypically related groups, in which – in contrast to the phenotypic alignment – only isolates from the same tissues and culturing conditions showed clonal identity (Fig. 2/A). Results of the restriction enzyme analysis of the 16S rDNA amplicons using *Msp*I and *Hae*III restriction endonucleases (Fig. 2/B) indicated that this fingerprinting technique had lower discrimination ability compared to the M13 minisatellite targeted PCR typing, although it proved to be suitable for differentiation at species or genus level.

A genus-specific PCR was used for the detection of *Pseudomonas* isolates. Amplicons were generated in case of 14 bacteria, which indicated that 1/3 of the isolates belonged to the *Pseudomonas* genus. Identification of these *Pseudomonas* isolates was done by sequencing the *rpoB* gene, while in case of twenty-eight non-*Pseudomonas* bacteria the 16S rRNA gene was used as the target for sequence analysis. Results of molecular identification (Table 1) showed that the isolated bacteria represented members of *Enterobacteriaceae* (species of *Enterobacter*, *Erwinia*, *Leclercia, Pantoea* and *Serratia* genera), *Pseudomonadaceae* (species of *Pseudomonas* genus), *Rhizobiaceae* (*Agrobacterium*/*Rhizobium* genera), *Comamona-*

daceae (*Delftia* genus), and *Alcaligenaceae* (*Achromobacter* genus) families, which refers to the presence of a diverse *Enterobacteriaceae* and non-*Enterobacteriaceae* Gram-negative bacterium biota in/on the sweet pepper plants.

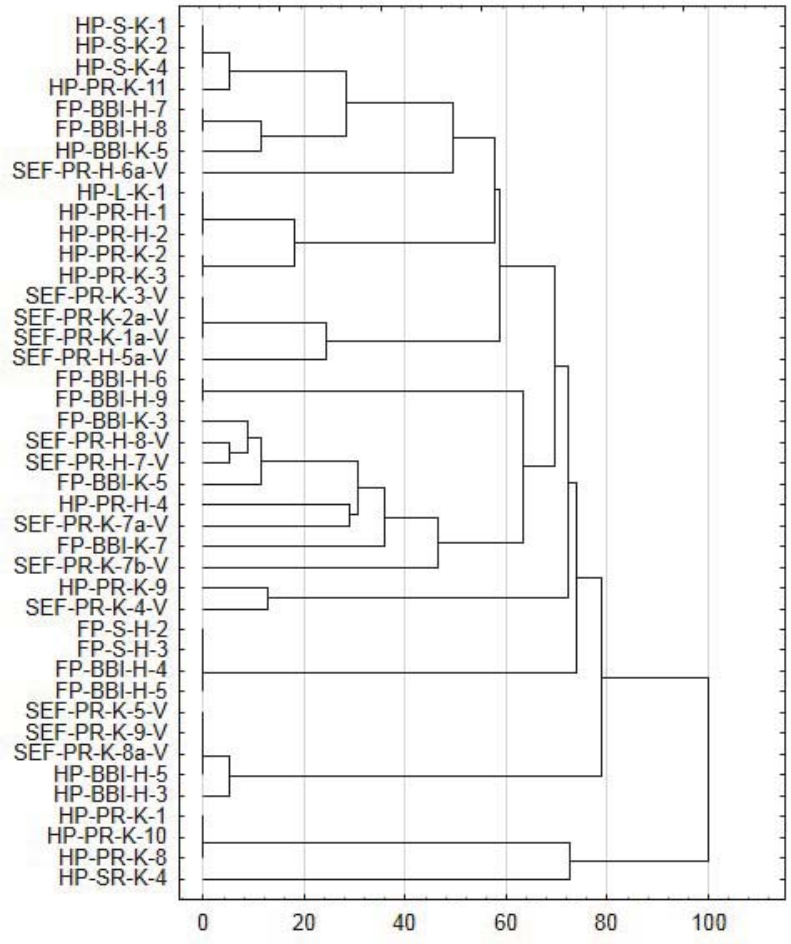

Fig. 1. Phenogram of the bacterium isolates originated from different tissues of HO and KPA cultivars of hydro- and soil-cultured *Capsicum annuum* var. *grossum* plants
(HP: hydro-cultured plant; FP: soil-cultured plant; SEF: Soil-cultured seedling; S: stem; PR: primary root; SR: secondary root; BBI: bibulous part of the berry; L: leaf; H: *Capsicum annuum* var. *grossum* HO cultivar; K: *Capsicum annuum* var. *grossum* KPA cultivar)

A

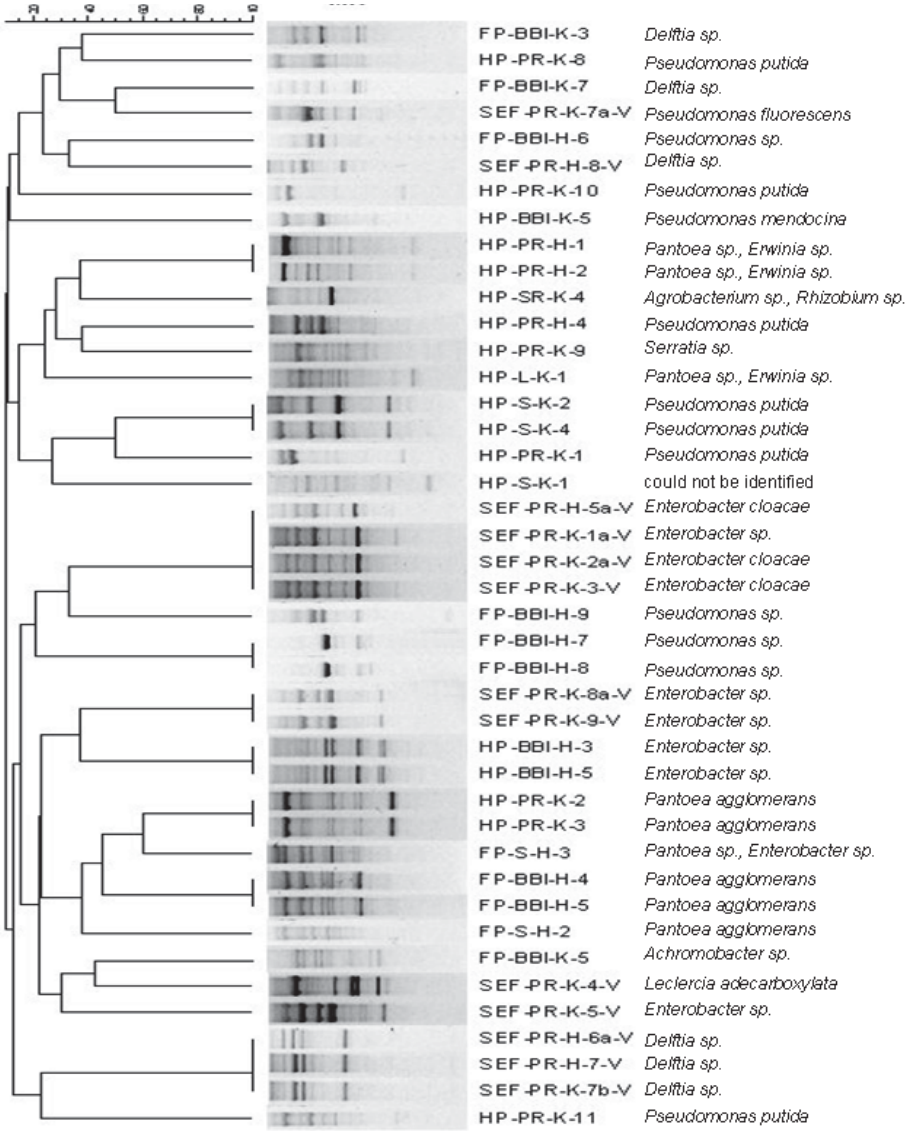

Fig. 2. Results of molecular genotyping by A) PCR typing using the M13 minisatellite primer and B) ARDRA fingerprinting using the *Hae*III and *Msp*I restriction endonucleases. Identity of the isolates at species or genus level is also shown

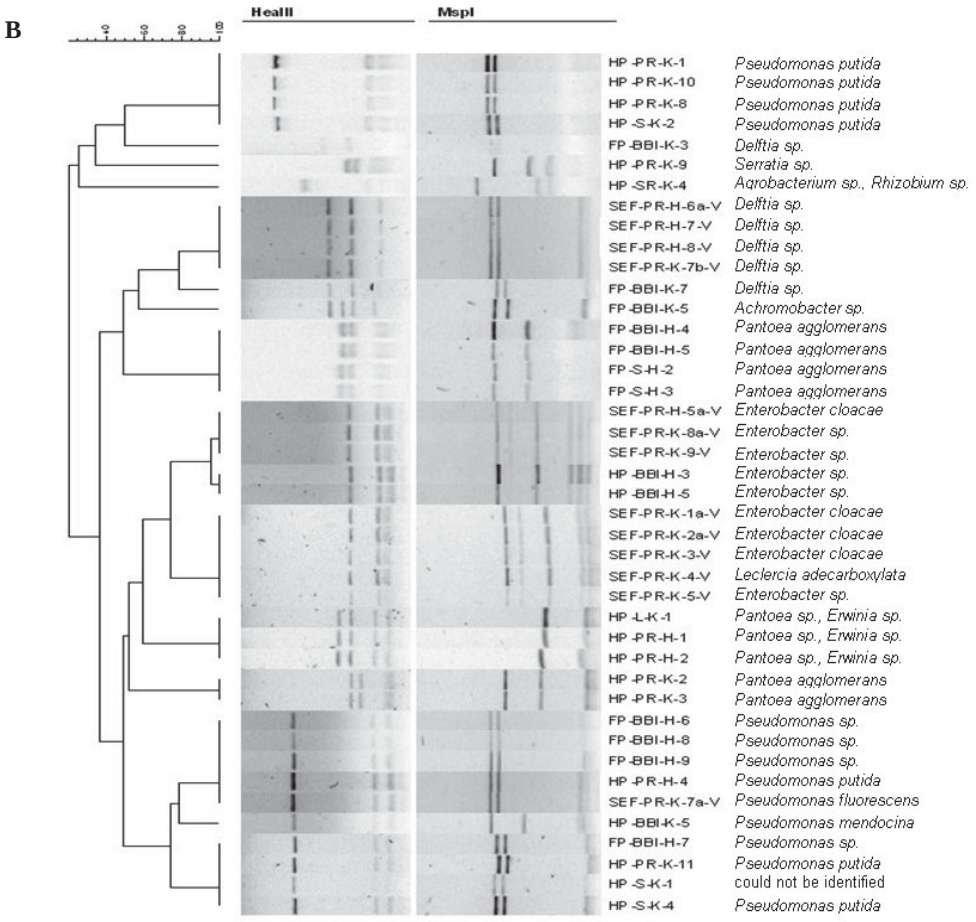
B
HaeIII
MspI
HP-PR-K-1 Pseudomonas putida
HP-PR-K-10 Pseudomonas putida
HP-PR-K-8 Pseudomonas putida
HP-S-K-2 Pseudomonas putida
FP-BBI-K-3 Delftia sp.
HP-PR-K-9 Serratia sp.
HP-SR-K-4 Agrobacterium sp., Rhizobium sp.
SEF-PR-H-6a-V Delftia sp.
SEF-PR-H-7-V Delftia sp.
SEF-PR-H-8-V Delftia sp.
SEF-PR-K-7b-V Delftia sp.
FP-BBI-K-7 Delftia sp.
FP-BBI-K-5 Achromobacter sp.
FP-BBI-H-4 Pantoea agglomerans
FP-BBI-H-5 Pantoea agglomerans
FP-S-H-2 Pantoea agglomerans
FP-S-H-3 Pantoea agglomerans
SEF-PR-H-5a-V Enterobacter cloacae
SEF-PR-K-8a-V Enterobacter sp.
SEF-PR-K-9-V Enterobacter sp.
HP-BBI-H-3 Enterobacter sp.
HP-BBI-H-5 Enterobacter sp.
SEF-PR-K-1a-V Enterobacter cloacae
SEF-PR-K-2a-V Enterobacter cloacae
SEF-PR-K-3-V Enterobacter cloacae
SEF-PR-K-4-V Leclercia adecarboxylata
SEF-PR-K-5-V Enterobacter sp.
HP-L-K-1 Pantoea sp., Erwinia sp.
HP-PR-H-1 Pantoea sp., Erwinia sp.
HP-PR-H-2 Pantoea sp., Erwinia sp.
HP-PR-K-2 Pantoea agglomerans
HP-PR-K-3 Pantoea agglomerans
FP-BBI-H-6 Pseudomonas sp.
FP-BBI-H-8 Pseudomonas sp.
FP-BBI-H-9 Pseudomonas sp.
HP-PR-H-4 Pseudomonas putida
SEF-PR-K-7a-V Pseudomonas fluorescens
HP-BBI-K-5 Pseudomonas mendocina
FP-BBI-H-7 Pseudomonas sp.
HP-PR-K-11 Pseudomonas putida
HP-S-K-1 could not be identified
HP-S-K-4 Pseudomonas putida

Fig. 2. Cont.

Table 1. Results of molecular identification based on sequence analysis of 16S rDNA or *rpoB* genes

Type of plant culture	Genera or species names	Number of isolates	Classification of the isolates	
			Family	Class
Hydro culture	*Enterobacter* sp.	2	*Enterobacteriaceae*	Gammaproteobacteria
	Erwinia sp. / *Pantoea* sp.	3		
	Pantoea agglomerans	2		
	Serratia sp.	1		
	Pseudomonas mendocina	1	*Pseudomonadaceae*	
	Pseudomonas putida	6		
	Agrobacterium sp. / *Rhizobium* sp.	1	*Rhizobiaceae*	Alphaproteobacteria
	NI[a]	1		
Soil culture	*Enterobacter* sp.	4	*Enterobacteriaceae*	Gammaproteobacteria
	Enterobacter cloacae	3		
	Enterobacter sp. / *Pantoea* sp.	1		
	Leclercia adecarboxylata	1		
	Pantoea agglomerans	3		
	Pseudomonas sp.	5	*Pseudomonadaceae*	
	Pseudomonas fluorescens	1		
	Delftia sp.	6	*Comamonadaceae*	Betaproteobacteria
	Achromobacter sp.	1	*Alcaligenaceae*	
	Altogether	42		

[a]: could not be identified

Results of molecular identification indicated that not only coliforms, but lactose-negative *Enterobacteriaceae* and non-*Enterobacteriaceae* strains were also isolated from VRBL agar plates. Significant differences between colonies of *Enterobacter* and some *Pseudomonas* isolates were not found, while colonies of the other bacterium genera could be differentiated from each other on the whole. Out of the 42 isolates 20 proved to be members of the *Enterobacteriaceae* family, while ten isolates belonged to the coliform group.

2.2. Antagonistic effect of the isolates on L. monocytogenes

Testing the antagonistic effect of the 42 isolates on *L. monocytogenes* CCM 4699 by co-culturing of the strains as described in section 1.4, partial or total inhibition was observed in case of seven isolates at 25 and 30 °C, while additional three isolates showed partial inhibitory

effect at 20 °C on the growth of the pathogen (Table 2). The most significant inhibitory effect was attributed to one of the *Pseudomonas putida* isolates (HP-PR-H-4), which originated from the primary root of a hydro-cultured HO pepper plant, and performed the best growth inhibition at 25 °C.

Table 2. Identity and origin of bacterium isolates having partial or total inhibition against *L. monocytogenes* CCM 4699 at different incubation temperatures

Code of the isolates	Genera or species name	Origin of the isolates	Inhibitory effect against *L. monocytogenes* at		
			20 °C	25 °C	30 °C
HP-PR-H-4	*Pseudomonas putida*	Hydro-culture, HO cultivar, primary root	+	+	+
HP-PR-K-1	*Pseudomonas putida*	Hydro-culture, KPA cultivar, primary root	+	+	+
HP-PR-K-8	*Pseudomonas putida*		+	+	+
HP-PR-K-10	*Pseudomonas putida*		+	–	–
HP-S-K-1	NI[a]	Hydro-culture, KPA cultivar, stem	+	+	+
HP-S-K-2	*Pseudomonas putida*		+	+	+
HP-S-K-4	*Pseudomonas putida*		+	+	+
HP-SR-K-4	*Agrobacterium* sp./ *Rhizobium* sp.	Hydro-culture, KPA cultivar, secondary root	+	+	+
FP-BBI-H-4	*Pantoea agglomerans*	Soil-culture, HO cultivar, fruit	+	–	–
FP-BBI-H-6	*Pseudomonas* sp.		+	–	–

[a]: could not be identified

Growth curve analysis of *L. monocytogenes* CCM 4699 in the presence of cell-free supernatants of *P. putida* HP-PR-H-4 at 25 ºC showed that extracellular substance(s) even in relatively low proportions (in 1/6 and 1/12 dilutions) could inhibit the growth of the pathogen (Fig. 3/B), which indicated the potential biocontrol activity of this isolate. Supernatant of the cells cultivated at 20 ºC had inhibitory effect as well, although the supernatant even in the highest proportion (in 1/2 dilution) could not suppress the growth of the pathogen totally (Fig. 3/A). When the supernatant was generated by cultivating the *P. putida* strain at 30 ºC, the inhibitory effect could only be observed in case of higher supernatant proportions (in 1/2 and 1/3 dilutions). Interestingly, stimulation of the pathogen was noticed at lower proportions of the supernatant (Fig. 3/C).

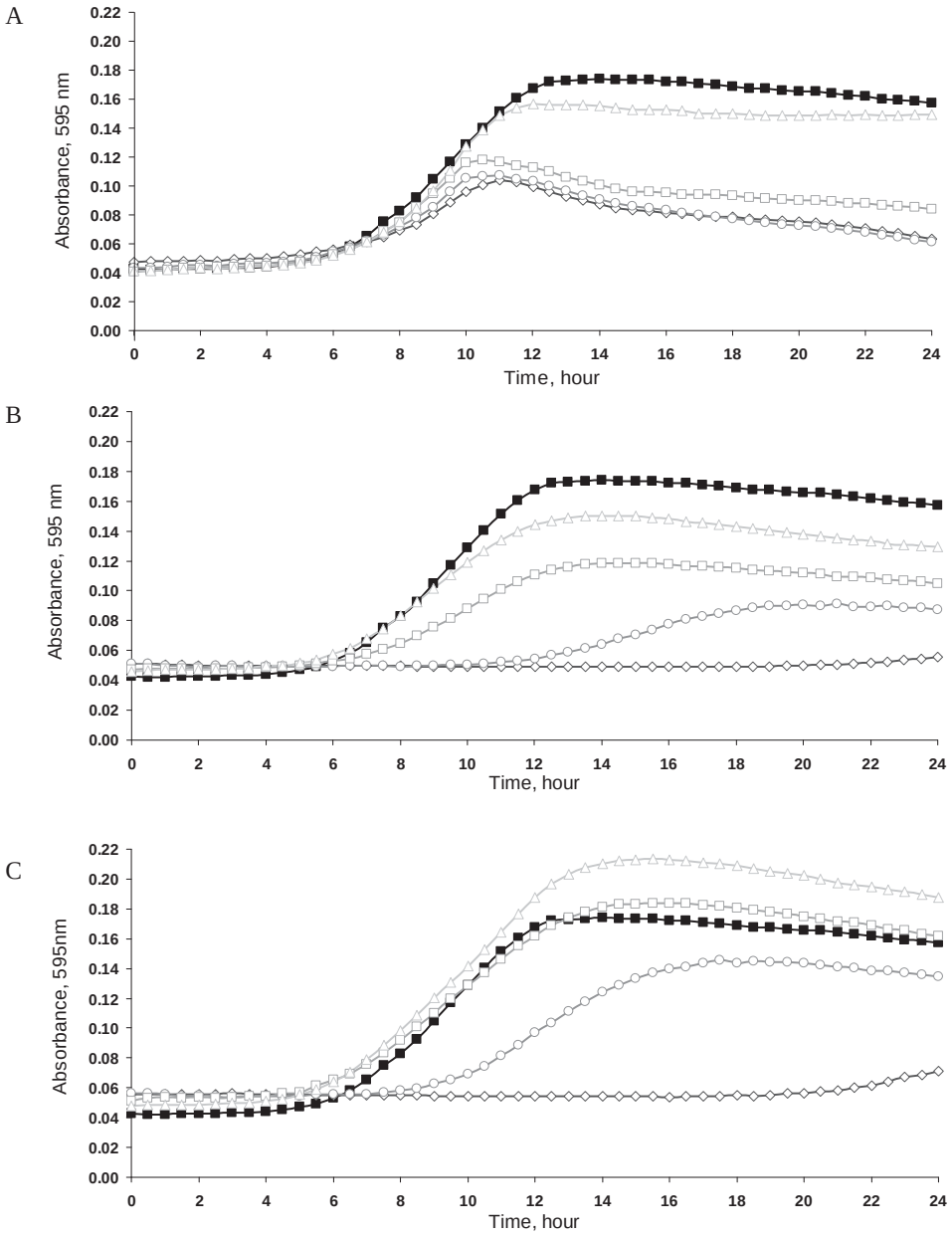

Fig. 3. Effect of cell-free supernatants of *P. putida* HP-PR-H-4 on the growth of *L. monocytogenes* CCM 4699 at 20 ºC. Cell-free supernatants of HP-PR-H-4 culture cultivated at A: 20 ºC; B: 25 ºC, and C: 30 ºC were prepared as described in section 1.4.
—■—: Control; —◇—: 150 µl (1/2); —○—: 100 µl (1/3); —□—: 50 µl (1/6); —△—: 25 µl (1/12).

It was published by HAAS and DÉFAGO (2005) that fluorescent pseudomonads can inhibit the growth of plant pathogenic bacteria by antibiotic production, induced systemic resistance, and/or specific pathogen–biocontrol strain interactions. *Pseudomonas* species occurring in natural environments can produce numerous antibiotics (RAAIJMAKERS et al., 1997), although in antibiosis siderophors have also important role. Pyoverdin (or pseudobactin) and ferripyoverdin are iron-carriers, while pyochelin is a good Cu^{2+} and Zn^{2+} chelator. These siderophores are sought to deprive pathogens of iron, copper, and/or zinc, thus suppress their growth (HAAS & DÉFAGO, 2005). *Pseudomonas putida* WCS358 was found to be able to produce pseudobactin, and via the siderophore-mediated competition for iron it could suppress soil-borne plant diseases (BAKKER et al., 1993; MEZIANE et al., 2005). MANNINEN and MATTILA-SANDHOLM (1994) detected *Pseudomonas* siderophores by analysing the absorption spectrum, and observed that both *P. putida* strains tested had an absorbance peak at 400 nm. Absorption maximum of approximately 400 nm was typical for the pyoverdine class of siderophores (MANNINEN & MATTILA-SANDHOLM, 1994). According to our results, *P. putida* HP-PR-H-4 strain – isolated in this study – produced an extracellular siderophore – probably pseudobactin – with an absorption peak at 415 nm (data not shown), which can be responsible for the antagonistic effect of the cell-free supernatant of this strain. Exact identification of the siderophore, however, needs further analysis.

3. Conclusions

Sweet pepper plants cultivated under different conditions (in hydro- or soil culture) harbour diverse *Enterobacteriaceae* populations as it was determined by molecular typing, fingerprinting, and identification of the isolates originated from VRBL coliform selective agar plates. This culture medium, however, did not prove selective enough for coliforms and *Pseudomonas aeruginosa* in case of these types of samples. Antagonistic effect of an endophytic *Pseudomonas putida* isolate against *Listera monocytogenes* illustrated that antagonists of food-borne human pathogens among the endophytic bacteria can be expected, therefore further investigations may lead to the development of protective cultures in the future.

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