Occurrence and Location of Endophytic Bacteria in Garden and Wild Strawberry

S. Kukkurainen, A. Leino, S. Vähämiko, H.R. Kärkkäinen, K. Ahanen, and S. Sorvari¹

MTT Agrifood Research Finland, Horticulture, Toivonlinnantie 518, FIN-21500 Piikkiö, Finland

R. Rugienius

Lithuanian Institute of Horticulture, Babtai, LT-4335, Kaunas Distr., Lithuania

O. Toldi

Agricultural Biotechnology Center, H-2100, Gödöllő, Szent-Györgyi Albert út 4, P.O. Box 411, Hungary

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Abstract. The occurrence of bacteria in different tissues was studied using field-grown strawberries, in vitro-grown strawberries, wild strawberries, and aseptically germinated strawberry seedlings. Strawberry has a number of endophytic bacteria in its the internal tissue, most of which appear to be nonpathogenic. In the in vitro-grown strawberries, all identified isolates were in the genus *Pantoea*. In field-grown garden and wild strawberries the most common genera were *Pantoea* and *Pseudomonas*. Location of eubacterial inhabitants within strawberry tissue sections was studied by in situ hybridization. Bacteria were detected in flower stalks, leaf stalks, leaves, stolons, berries and aseptically germinated seedlings. The existence of bacteria in seeds and seedlings suggests that bacteria are able to move up to the generative tissue and, ultimately, to the next generation, forming a symbiosis-like chain of plant-bacteria coexistence.

Plants are not microbial vacuums. Intercellular spaces, vascular systems and even single cells are inhabited by several kinds of bacteria and other microbes. The asymptomatic existence of endophytic bacteria has been reported for many species, e.g., strawberry (Berg et al., 2000; Ferguson et al., 2000; Guinebreiere et al., 1999, Tanprasert and Reed, 1997), tomato (Samish et al., 1961), pea (Elvira-Recueno and van Vuurde, 2000), potato (Sturz et al., 1999), cucumber (Mahaffee and Kloepper, 1997), cotton (Chen et al., 1995), and globe artichoke (Peñalver et al., 1994). Both nonpathogenic and pathogenic bacteria can be present, causing no apparent effect on the plant (Bacon and Hinton, 1997). Endophytic bacteria have also been reported to promote plant growth and development (Frommel et al., 1991; Nejad and Johnson, 2000; Pillay and Nowak, 1997; Sturz et al., 1998) through production of phytohormones (Frommel et al., 1991; Holland, 1997), through induction of hormone synthesis by plants (Lazarovits and Nowak, 1997), or through fixation of atmospheric nitrogen (Barraquio et al., 1997). Endophytes may suppress disease by inducing systemic resistance in plants (Cameron et al., 1994), or by producing antibiotic secondary metabolites (Wright et al., 2001), fungal cell wall-degrading enzymes (Pleban et al., 1997) or Fe³⁺-chelating siderophores (Davison, 1988).

The aim of this study was to find out which

bacterial taxa are present in the different tissues of strawberry, what kind of endophytic populations occur within strawberries grown in different conditions, and whether endophytic bacteria are transferred, within seeds, to the next generation.

Materials and Methods

Plant material. Experimental material was collected from healthy looking plants. 'Hella', 'Pocahontas', and 'Ydun' were field-grown, stolon-propagated garden strawberries that have never been micropropagated. 'Jonsok', 'Bounty', 'Korona', and 'Kent' were field-grown, previously micropropagated garden strawberries. The in vitro cultures of garden strawberry included 'Hella', 'Jonsok', 'Bounty', and 'Pocahontas'. Wild strawberry plants were collected both from the field and the edge of the forest. All plant materials were collected in summertime in 2000 and 2001 at the Institute of Horticulture of Agrifood Research Finland (MTT) in Piikkiö.

The plants used for in situ hybridization included greenhouse grown 'Jonsok', in vitro-grown 'Jonsok', and aseptically germinated seedlings of 'Bounty' and 'Kent'. The occurrence of bacteria was studied by using leaf stalks, leaves, flower stalks, flowers and berries of greenhouse grown 'Jonsok', and leaf stalks of in vitro-grown 'Jonsok'. Aseptically germinated seedlings were obtained from surface-disinfected and surface sterility-checked seeds. The seeds were germinated on cellulose paper moistened with sterile distilled water, and the seedlings were transferred to a solid, hormone-

free Murashige and Skoog (1962) medium of half strength, without casein hydrolysate. Leaf stalks of the seedlings were used for the in situ hybridization.

Isolation of bacteria. Leaf stalks, leaves, roots, flower stalks, flowers, berries, seeds and internodal sections of stolons of field-grown garden strawberry (*Fragaria xananassa* Duch.) and wild strawberry (*Fragaria vesca* L.) were washed 1 min in running tap water and 1 min in running deionized water. The material was surface sterilized 2 min in 50% EtOH, 2–6 min (depending on the plant tissue) in 5.25% sodium hypochlorite, with 1% Triton X-100 (BDH Laboratory Supplies, Poole, U.K.), 2 min in 50% EtOH, and finally rinsed 3 × 3 min in sterile distilled water. Surface sterilized leaves, stalks, roots and stolons were cut into 4- to 5-mm pieces, berries were peeled, pistils remained uncut, and stamens either cut in half or left uncut.

Surface sterility was tested by imprinting the pieces on agar plates. After 30 minutes, the pieces were removed and transferred onto agar plates of the same type and incubated at 28 °C up to 3 weeks. For surface sterility check and bacterial culture, the following media were used: tryptic soy broth (Sigma Co., St. Louis, Mo.) with 1.7% agar (Sigma type A), potato dextrose broth (Sigma) with 1.7% agar, malt extract broth (Sigma) with 1.7% agar, nutrient peptone medium [10 g·L⁻¹ glucose, 5 g·L⁻¹ malt extract, 1 g·L⁻¹ yeast extract, 8 g·L⁻¹ nutrient broth (Sigma), 1 g·L⁻¹ Bacto peptone (Difco Laboratories Inc., Detroit, Mich.), 20 g·L⁻¹ agar, pH 6.8 to 7.0], cornmeal-malt extract agar (Bacon et al., 1977), SC medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, DSMZ medium 751) and M9 medium (DSMZ medium 382). Bacterial growth associated with the plant pieces was purified by repeated plating on agar medium. If sterility check was positive, the culture was discarded. Plant parts from the in vitro cultures of 'Jonsok', 'Pocahontas', 'Hella', and 'Bounty' were sterility checked, although they were not surface disinfected.

Bacterial cultures from berries and wetted seeds were established by crushing the plant material using mortar and pestle with phosphate-buffered saline solution (PBS: 8.0 g·L⁻¹ NaCl, 0.2 g·L⁻¹ KCl, 1.1 g·L⁻¹ Na₂HPO₄, and 0.2 g·L⁻¹ KH₂PO₄, pH 7.4), and plating the slurry. Apart from small plant pieces, extraction of vascular fluid from 10- to 15-cm-long stalks and stolons was used for isolation of bacteria. Surface sterilized stalks and stolons were placed into plastic tubes filled with sterile distilled water through an aseptically cut hole in the cap. A disposable plastic pipette was aseptically sealed with the aid of Parafilm to the end of the stalk or stolon and pressed in order to obtain a vacuum in the pipette. After 12 h, the fluid conveyed to the pipette was spread onto plates. Alternatively, fluid was extracted from stalks and stolons by centrifugation (Hallmann et al., 1997).

Identification of bacteria. Isolates were Gram stained using the Gram stain kit (Sigma HT90-A). Gram-negative rod-shaped bacteria were tested using the API 20 E test system (bioMérieux sa, Marcy-l'Etoile, France). The seven-digit API profile was complemented with

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¹To whom reprint requests should be sent; e-mail seppo.sorvari@mtt.fi.

additional tests for nitrate and nitrite reduction, motility and growth on McConkey broth with 1.7% agar (Sigma). In a few cases, a closer identification of pseudomonads was made by observing fluorescent pigment production on King agar B (Fluka Chemie AG, Buchs, Switzerland, according to King et al., 1954). Identification of some Gram-negative and Gram-positive isolates was confirmed through gas-liquid chromatography, in combination with API 20 NE, API 20 E or ID 32 STAPH plates (bioMérieux), at Y-Lab, University of Turku, Finland.

Bacterial lysates were prepared from broth cultures according to Lu et al. (2000). PCR with ERIC1R [5'-ATGTAAGCTCCTGGGGATTCAC-3'] and ERIC2 [5'-AAGTAAGTGACTGGGGTGAGCG-3'] primers (Versalovic et al., 1991) was carried out using PCR Master Mix (Cat. no. M7502, Promega Corporation, Madison, Wis.) and a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Mass.). Primers were purchased from the DNA Synthesis and Sequencing Laboratory, University of Helsinki, Finland. Strains from the microbial culture collections HAMBI (University of Helsinki, Finland) and DSMZ were used as standards.

In situ hybridization. Endophytic bacteria was located using *in situ* hybridization with the digoxigenin 3'-labelled EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') probe, which is according to Amann et al. (1992) complementary to the eubacterial 16S rRNA sequence.

The EUB338 sequence was obtained from the DNA Synthesis and Sequencing Laboratory of the University of Helsinki and the DIG Oligonucleotide 3'-End Labeling Kit (Cat. no. 1 362 372) from Roche Diagnostics GmbH, Mannheim, Germany.

Plant material was washed and surface-sterilized, fixed according to Pirttilä et al. (2000), and embedded (Ruzin, 1999) in paraffin (Cat. no. 1.07164, Merck KGaA, Darmstadt, Germany). Sections of 10 to 12 µm thick were attached to Bind-Silane-coated (Promega Q421A) glass slides and baked overnight at +42 °C. The sections were deparaffinized, rehydrated (Rolighed and Lindeberg, 1996), permeabilized 10 min with 30 µg·mL⁻¹ Proteinase K (Sigma P6556) in TES buffer [50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl], and fixed in 0.4% formaldehyde (Rolighed and Lindeberg, 1996).

Slides were prehybridized for 1 h at 38 °C in hybridization standard buffer with formamide, according to Eisel et al. (2000), where formamide concentration was modified to 20%. The probe was diluted with hybridization buffer up to the concentration of 0.35 ng·µL⁻¹, and added to the slides in volumes of 100 µL/slide. The slides were placed on a heating tray at 80 °C for 10 min to denature the DNA, and then cooled on ice. The probe was hybridized to the sections overnight at 38 °C in a humid chamber. The unhybridized probe was removed from the slides by washing 2 × 5 min in 2× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulphate (SDS) at RT, and

2×15 min in 0.5× SSC, 0.1% SDS at 38 °C, and the slides were cooled to RT. A negative control was prepared by applying hybridization buffer without a probe on one slide. Hybrids were visualized with the DIG Nucleic Acid Detection Kit (Roche Cat. no. 1 175 041) and slides were mounted in Aquamount (BDH).

Results

Microbial endophytes were found and isolated from field-grown garden strawberry, in vitro-grown garden strawberry, and wild strawberry. In aseptically germinated seedlings, however, the existence of bacteria could be shown by *in situ* hybridization only.

The distribution of bacteria in the different plant types was similar (Table 1). Gram-negative rods were the most predominant group among the endophytic isolates, ranging from 75% to 93%. The proportion of Gram-positive rods ranged from 0% to 21%, and Gram-positive cocci from 1% to 7%.

Pantoea spp. and *Pseudomonas fluorescens* were the most frequently isolated and identified bacteria in the different strawberry types (Table 2). *Pantoea* was present in all strawberry types, whereas pseudomonads were not discovered in the in vitro-grown or nonmicropropagated field-grown garden strawberry.

A summary of bacterial isolates from different plant tissues is presented in Table 3. Bacteria identified as *Pantoea* spp. were discovered from all vegetative tissues. Pseudomonads,

Table 1. Distribution of endophytic bacteria in different strawberry types, according to their Gram staining ability and morphology.

	Garden type, field-grown, not micropropagated	Garden type, field-grown, micropropagated	Garden type, in vitro-grown	Wild type	Wild type, field-grown
Gram-negative rods	84% (70 ^a)	90% (147)	92% (33)	93% (26)	75% (18)
Gram-positive rods	11% (9)	9% (15)	6% (2)	0% (0)	21% (5)
Gram-positive cocci	5% (4)	1% (2)	3% (1)	7% (2)	4% (1)
Total of bacterial isolates	100% (83)	100% (164)	100% (36)	100% (28)	100% (24)

^aThe numbers of isolates are given next to the percentages in parentheses.

Table 2. Distribution of endophytic bacterial taxa and groups in different strawberry types. The frequencies of bacteria are given as absolute numbers and percentages of the total number of bacterial isolates (in parentheses).

Bacterial taxa and groups	Garden type, field-grown, not micropropagated	Garden type, field-grown, micropropagated	Garden type, in vitro-grown	Wild type	Wild type, field-grown
<i>Bacillus cereus</i>		1 ^{az} (1%)			
<i>Bacillus</i> sp.	1 ^{az} (1%)	2 ^a (1%)			
<i>Bacillus subtilis</i>		2 ^{az} (1%)			
<i>Brevundimonas diminuta</i>		1 ^{az} (1%)			
<i>Chryseomonas luteola</i>		2 ^a (1%)			
<i>Enterobacter</i> sp.		2 (1%)			
<i>Klebsiella</i> sp.	1 (1%)	1 (1%)			
<i>Kocuria kristinae</i>		1 ^{az} (1%)			
Nonfermenter sp.	1 (1%)	4 (2%)		2 (7%)	
<i>Pantoea</i> spp.	28 (34%)	27 (16%)	6 (17%)	2 ^{ay} (7%)	3 (13%)
<i>Pantoea</i> spp. group A		1 (1%)	21 (58%)		1 (4%)
<i>Pseudomonas fluorescens</i>		11 (7%)		4 ^{ay} (14%)	
<i>Pseudomonas</i> sp.		3 (2%)		1 (4%)	
Fluorescent pseudomonads		5 (3%)		1 (4%)	1 (4%)
<i>Sphingomonas paucimobilis</i>		2 ^{az} (1%)			
<i>Stenotrophomonas maltophilia</i>		4 (2%)			
Unidentified rods, Gram-	40 (48%)	84 (51%)	6 (17%)	16 (57%)	13 (54%)
Unidentified rods, Gram+	8 (10%)	10 (6%)	2 (6%)		5 (21%)
Unidentified cocci, Gram+	4 (5%)	1 (1%) 1 (3%)	2 (7%)	1 (4%)	
Number of isolates	83 (100%)	164 (100%)	36 (100%)	28 (100%)	24 (100%)

^aIdentified by Y-Lab, University of Turku.

^{ay}Some of the isolates were identified by Y-Lab, University of Turku.

Table 3. Endophytic bacteria isolated from different plant types grouped by identification and plant tissue origin; A = garden strawberries 'Bounty', 'Jonsok', 'Korona' and 'Kent'; B = garden strawberries 'Hella', 'Pocahontas', and 'Ydun'; I = in vitro garden strawberries 'Bounty', 'Jonsok', 'Pocahontas', and 'Hella'; V = wild strawberry from the field; and W = wild strawberry from the edge of the forest.

Bacterial taxa and groups	Plant tissue							
	Root	Stolon	Leaf stalk	Leaf	Flower stalk	Flower	Berry	Seed
<i>Bacillus cereus</i>	A ^z							
<i>Bacillus</i> sp.	A ^z *B ^z *							
<i>Bacillus subtilis</i>	A ^z *							
<i>Brevundimonas diminuta</i>			A ^z *					
<i>Chryseomonas luteola</i>	A ^z *				A			
<i>Enterobacter</i> sp.							A	
<i>Klebsiella</i> sp.		B					A	
<i>Kocuria kristinae</i>			A ^z *					
Nonfermenter sp.		A	ABW		A			
<i>Pantoea</i> spp.	A	BI	ABIVW	BIW ^z *	AB	A	AB	
<i>Pantoea</i> spp. group A	A	I	I	IV ^{**y}				
<i>Pseudomonas fluorescens</i>	A ^z *W	A	AW ^z *	W	A ^z *	W ^z *		A ^z *
<i>Pseudomonas</i> sp.		W			A	A		A
Fluorescent pseudomonads		A	A	AVW	A			
<i>Sphingomonas paucimobilis</i>	A ^z *	A ^z *						
<i>Stenotrophomonas maltophilia</i>		A			A			
Unidentified rods, Gram-	A	ABIW	ABIVW	ABIVW	ABW	AW	ABW	A
Unidentified rods, Gram+	A	IV	BV	A	B		AB	A
Unidentified cocci, Gram+			AB	BIV	W			

^zIdentified by Y-Lab, University of Turku.

^yA mixture of leaves and stalks was used for isolation of bacteria.

Fig. 1. Fragment patterns created in ERIC-PCR. (M) molecular weight marker (*Pst*I-digested λ -DNA, Promega), (1a,b) *Pseudomonas aeruginosa* HAMBI 25, (2a,b) *P. chlororaphis* HAMBI 1977, (3a,b) *P. fragi* HAMBI 28, (4a,b) *P. fluorescens* HAMBI 2167, (5a,b) *P. putida* HAMBI 17, (6a,b) *P. syringae* pv. *syringae* HAMBI 1436, (7a,b) *P. syringae* pv. *phaseolicola* HAMBI 1435, (8a,b) *Ralstonia pickettii* HAMBI 2158, (9a,b) *Xanthomonas campestris* HAMBI 104, (10a,b) *Pantoea agglomerans* HAMBI 1899, (11a,b) *Erwinia carotovora* HAMBI 1429, (12a,b) *Burkholderia cepacia* HAMBI 1976, (13a,b) *Burkholderia gladioli* HAMBI 2157, and *Pantoea* isolates (14a,b) K57, (15a,b) K64, (16a,b) K61a, and (17a,b) negative control lacking template DNA are shown.

Pseudomonas fluorescens in particular, were also found distributed in all plant parts, except berries. *Bacillus* was detected only in roots, whereas *Chryseomonas* and *Sphingomonas* were found inhabiting both roots and aerial tissues. The bacteria isolated from seeds were *Pseudomonas fluorescens* and *Pseudomonas* sp., which indicates that seeds function, not only as carriers of genetic information, but also as carriers of symbiotic-like or other bacteria between generations.

Seventy-five percent of the isolates from the in vitro-grown strawberry were identified as *Pantoea* spp. by API 20 E. By ERIC-PCR, most of these isolates were shown to form a group that was later named group A. The grouping was made on the basis of a uniform amplification product that resembled the strongly amplified fragments of *Pantoea agglomerans* (HAMBI

1899) and *Erwinia carotovora* (HAMBI 1429) (Fig. 1). In in vitro-grown strawberries *Pantoea* was found in 27 cases of 36 isolates, and 21 of them belonged to *Pantoea* group A. For all field-grown garden strawberries, and wild strawberries *Pantoea* was in 56 cases of 247 isolates and 6 cases of the 52 isolates respectively, but for these plant types there were observed only single isolates of *Pantoea* group A bacteria.

Pseudomonas species, identified as *Pseudomonas aeruginosa*, *P. fluorescens/putida* or *Pseudomonas* sp. by API 20 E, were present in all plant tissue types, except berries. In ERIC-PCR, similarities, but not completely matching band patterns, were found with the reference strains *P. fluorescens* (HAMBI 2167), *P. putida* (HAMBI 17), *P. aeruginosa* (HAMBI 25), *P. fragi* (HAMBI 28), *P. syringae* pv. *syringae* (HAMBI 1436), *P. syringae* pv. *phaseolicola*

(HAMBI 1435), and *P. chlororaphis* (HAMBI 1977). Some subgroups of *Pseudomonas* species, originating from stolon, leaf stalk, leaf, flower and root, were found to fluoresce at 365 nm.

By means of in situ hybridization with the eubacterial probe EUB338, endophytes were detected in all plant tissues tested (Fig. 2). Endophytes were detected single, in pairs or in small microcolonies, especially in parenchymal tissues of stalks (Fig. 2b and c) and stolons. There were two main types of microcolonies: strings of loosely associated cells attached to plant cell wall (Fig. 2a, thick arrows) and irregular aggregates of either small rod-shaped cells (Fig. 2a, thin arrows) or larger rounded cells. Rod-shaped bacteria attached to host cell walls were detected in the in vitro 'Jonsok' (Fig. 2d). Endophytes were also detected in

Fig. 2. In situ hybridization of strawberry (*Fragaria xananassa* Duch.) with a DIG-labelled oligonucleotide probe complementary to eubacterial 16S rRNA sequence. (a) A string of larger rod-shaped cells (thick arrows) is attached to the host cell wall in berry. Thin arrows point to a microcolony of smaller rods. (b) Leaf stalk parenchyma with bacteria between cells. (c) Flower stalk parenchyma. Hybridization signal was detected at cell walls and in intercellular spaces. (d) Leaf stalk parenchyma of in vitro strawberry with bacteria attached to cell walls. (e and f) Vascular tissue of in vitro seedling. Arrows point at cells presenting strong hybridization signal.

aseptically germinated seedlings (Fig. 2e and f) but the isolation of bacteria from them was not successful.

Discussion

Isolation of a complete endophytic population is problematic because of the adhesion of bacteria to plant cell structures and because surface disinfection is always a compromise between elimination of surface contaminants and survival of cortex endophytes (Hallmann et al., 1997). In

addition, there can be bacteria (e.g., phytoplasma) that are nonculturable, or population densities can be below the detection level.

In the in vitro strawberry, Gram-negative bacteria were the most frequent. This supports the results of Reed et al. (1995) who mostly found Gram-negatives as contaminants of micropropagated mint. *Pantoea* genus, especially *Pantoea* group A, seems to be favored by in vitro culture. Conditions of in vitro culture have been reported to make plants susceptible to potential bacterial colonizers (Leifert et al., 1994). Such factors as

weak plant epidermis, poorly developed stomata and open wound surfaces facilitate the entrance of bacteria into tissue, and special nutritive and physical conditions can change or favor certain types of endophytic bacterial populations (Leifert et al., 1994). However, bacteria belonging to the *Pantoea* genus are commonly found in endophytic habitats in leaf tissues of citrus rootstock (Araújo et al., 2001) and stems of pea (Elvir-Recuenco and van Vuurde, 2000).

Because of rare contacts with humans, wild strawberry, unlike garden strawberry, was not

expected to contain human-associated bacteria. However, human-associated bacteria, such as Gram-positive cocci, were detected in wild strawberry with about the same frequency as in field-grown garden strawberry. The main difference was that *Bacillus* spp. could not be identified in wild strawberry. In field-grown garden strawberry, in contrast, some identified *Bacillus* spp. were isolated from roots. According to Hallmann (2001), roots are an important gateway for colonization by endophytic bacteria. In the postcolonization process, however, *Bacillus* spp. bacteria are possibly not able to penetrate or are prevented from penetrating into vascular tissue, which is the route to aerial parts of the plant (Hallmann, 2001).

Another major group of bacteria were *Pseudomonas* species, some of which were identified as *P. fluorescens*. Parallel results have been obtained for artichoke, where the amount of endophytic isolates was 75% (Peñalver et al., 1994). The major genera in pea were *Pseudomonas*, *Pantoea* and *Bacillus* (Elvira-Recuenco and van Vuurde, 2000). Seeds, which are important carriers of genetic information, are generally considered free of contamination. Bacterial isolates from seeds have previously been argued not to be truly endogenous, but rather strains introduced from the environment or latent phytopathogens (Araújo et al., 2001; Mundt and Hinkle 1976). However, in our study pseudomonads were found in seeds, as well as in roots and aerial parts of the plant. Mundt and Hinkle (1976) have made similar observations in ovules and seeds of diverse species where they found *Bacillus*, *Erwinia*, *Flavobacterium* and *Pseudomonas*. These observations support the idea that seeds function as carriers of endophytic bacteria to the next generation. Further, the presence of bacteria in tissues of aseptically germinated seedlings was confirmed by *in situ* hybridization.

According to Hallmann (2001) and Kobayashi and Palumbo (2000), the effects of endophytes to plants can be categorized into growth regulating effects and disease control. Endophytic bacteria can also prevent plant growth (Schippers et al., 1990). Sturz et al. (1999) found that the bacteria located in the inner plant tissue is able to contribute different disease resistance than the bacteria of outer tissue. They observed that disease control of antibiosis of bacteria is most effective in potato tubers in the outer layers, while the bacteria found in the inner layers have weaker effects. In silver birch (*Betula pendula*), *Pseudomonas fluorescens* was found to be a promising biocontrol agent for seedling nurseries (Björklöf et al., 2002).

In this study, endophytic bacterial populations of garden strawberry and wild strawberry were investigated. A *Pantoea* spp. group A was predominant in the *in vitro* strawberry, but rarely detected in field-grown strawberry. *Pseudomonas* species, *Pseudomonas fluorescens* in particular, were found widely distributed in different strawberry tissues.

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