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Epiphytic microorganisms on strawberry plants (*Fragaria ananassa* cv. Elsanta): identification of bacterial isolates and analysis of their interaction with leaf surfaces

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Abstract

Epiphytic bacteria were isolated from strawberry plants cultivated in the field or in the greenhouse in order to investigate their interaction with leaf-surface transport properties. Colonization of lower leaf sides was higher on field-grown plants, whereas upper leaf sides were more densely colonized on plants cultivated in the greenhouse. Fungal isolates significantly contributed to total microbial biomass on leaf surfaces of greenhouse-grown strawberry plants, whereas these organisms were rarely abundant on field-grown plants. Microscopic investigations of bacteria in the phyllosphere revealed that the highest densities of bacteria were observed on living trichomes, which obviously provide a source of nutrients. Isolated strains were characterized by colony morphology, microscopy and histochemistry. About 324 isolated bacterial strains were grouped into 38 morphotypes. Of the morphotypes, 12 were identified by 16S rRNA gene sequencing. Dominating bacteria belonged to the genus *Pseudomonas*, *Stenotrophomonas*, *Bacillus* and *Arthrobacter*. Cuticular water permeability of isolated cuticular membranes and intact leaf disks was measured before and after treatment with one of the most prominent epiphytic bacteria, *Pseudomonas rhizosphaerae*. Results showed that cuticular transpiration was significantly increased by *P. rhizosphaerae*. This shows that leaf-surface properties, such as cuticular water permeability, can be influenced by bacteria, leading to improved habitable conditions in the phyllosphere.

Keywords: Phyllosphere; *Fragaria ananassa* cv. Elsanta; Epiphytic bacteria; 16S rRNA; Leaf–bacteria interaction

1. Introduction

Surfaces of most plants are characterized by an associated epiphytic microflora living in the phyllosphere [1]. Although plant surfaces have to be considered as a hostile environment to bacteria, more than 85 different species of microorganisms in 37 genera have already been reported in the phyllosphere of rye, olive, sugar beet and wheat [2]. Leaf surfaces are hydrophobic and thus water availability is limited and strong UV radiation as well as limitations in nutrient availability contribute to stressful conditions. Additionally, steep gradients in temperature can occur on leaf surfaces, varying from 5–10 °C during the night to 40–55 °C during the day [3]. Nevertheless, microbial biofilms have been demonstrated in the phyllosphere of terrestrial plants [4]. It is assumed that biofilms promote metabolic and genetic exchange between microorganisms [5], influence phenotypic plasticity of epiphytic microorganisms and influence stress resistance of plants [6]. Microbial ecologists have made a huge effort investigating microbial diversity

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in the phyllosphere and studying biological interactions between microbial species, but interactions between leaf surfaces, forming the habitat, and microorganisms have rarely been analysed. It was the aim of this study to analyse the interaction between epiphytic bacteria and transport properties of the plant cuticle, using cultivable bacteria isolated from the phyllosphere of strawberry leaves and fruits. The specific effects of the isolated strain *Pseudomonas rhizosphaerae*, representing the most prominent isolate from leaf and fruit surfaces of strawberry, on the rates of cuticular transpiration of isolated cuticular membranes and intact leaves was analysed in detail.

2. Material and methods

2.1. Plant material

Epiphytic microorganisms were isolated from leaves, flowers and fruits of *Fragaria* × *ananassa* cv. Elsanta. Four plants were used in this study. The plants were grown in greenhouses (Institute of Botany II, University of Würzburg, Germany) and 3 plants were transferred to the field (in March 2000). All samples were taken from apparently healthy strawberry plants in August 2000.

2.2. Epifluorescense microscopy of leaf surfaces

Samples of upper and lower leaf sides from plants grown in the field and greenhouse were analysed by microscopy. Leaf pieces were stained with 0.2% (w/v) acridine orange in H_2O or 0.01% (w/v) DAPI in H_2O. Areas of approximately 25 mm^2 were cut from the leaves and stained on the surface. After 5–10 min, leaf samples were carefully rinsed with deionised water, placed on microscope slides and viewed by epifluorescense microscopy (Axioplan, Zeiss, Germany) using a HBO 50 W mercury lamp and the filter sets 01 (BP 365/12 nm, LP 397 nm) and 09 (BP 450–490 nm, LP 515 nm) (Zeiss).

2.3. Isolation of bacterial strains

Epiphytic microorganisms from leaflets and flowers were obtained by imprinting the intact plant samples gently on LB-agar plates (Fluka, Germany). Fruits were rolled over the agar surface. Agar plates were incubated at room temperature (RT, 22 °C). After 24–30 h, agar plates were sealed with parafilm and stored at 4 °C in the dark until further use. Single colonies were picked and cultivated on new agar plates. In order to distinguish between bacteria and fungi, agar plates contained either actidion (30 mg l^{-1}) or ampicillin (50 mg l^{-1}). Isolated fungi were not further investigated in this study. Isolated and separated colonies were classified according to differences in their morphology (colour, texture, appearance, colony size) and "morphotypes" were defined. From each morphotype, one typical strain was selected and used for further analysis. Glycerol cultures (LB broth:glycerol, 3:1) were prepared from the selected strains and stored at −80 °C.

2.4. Morphological description of isolated bacteria

Morphology of colonies was determined from cultures grown for at least 1 day at RT and stored at 4 °C for another 2 days. Production of fluorescent pigments was tested by cultivation of isolated strains on King B agar (Fluka, Germany), and bacteria were examined under UV-light. Bacteria grown overnight (in LB broth at RT) were examined for size, form and mobility by light microscopy. Bacteria were stained with 0.2% (w/v) acridine orange; Gram staining was carried out as described by Bast [7].

2.5. Pathogenicity test for isolated bacteria

For the tobacco hypersensitivity reaction [8], overnight cultures of the isolates were injected into intercellular spaces of intact tobacco leaves. Leaves were examined 24 and 48 h later and the number of hypersensitive necrosis was counted.

2.6. Identification of isolated bacterial strains by 16S rRNA gene sequences

PCR templates were prepared from 1–3 ml of a liquid culture grown in LB-broth overnight. Bacteria were centrifuged at 16,000*g* for 2 min using a tabletop centrifuge (Hermle, Germany) and pellets were resuspended in 1 ml sterile H_2O. Samples were centrifuged again for 2 min, and resuspended again in 500 μl sterile H_2O, sonicated in a sonication bath (Bandelin, Germany) for 7 min and heated at 93 °C for 15 min. After cooling, the solution was centrifuged for another 2 min and the supernatant was either directly used for PCR or, if necessary, for DNA purification by phenol/chloroform-extraction and precipitation in ethanol [9].

Amplification of 16S rRNA gene was carried out using the primer pair SL and SR, annealing to conserved regions of bacterial 16S rRNA genes (Table 1). The following PCR conditions were used: 300 ng genomic DNA, 5 μl of 10-fold reaction buffer, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Gibco BRL, USA), 200 μM of each of the four dNTPs (Promega, USA) and 250 pM of each primer (MWG-Biotech AG, Germany), dissolved in a total volume of 50 μl. Positive and negative controls with and without genomic DNA were regularly run in parallel. A thermocycler (Primus 25, MWG-Biotech AG, Germany) with the following programme was used: initial denaturation at 95 °C for 5

Table 1
Oligonucleotides corresponding to conserved regions of the 16S rRNA gene used for PCR (SL and SR) and sequencing (all)

Oligonucleotide	Sequence	Positions in *Escherichia coli* 16S rRNA gene	Reference
SL[a]	TTGGGATCCAGAGTTTGATCATGGCTCAGAT[c]	8–29	[38]
SR[b]	CACGAATTCTACCTTGTTACGACTTCACCCC[c]	1486–1507	[38]
Bacteria 338[a]	ACTCCGACGGGAGGCAGC	338–355	[39]
Universal 522[b]	GTATTACCGCGGCTG	522–536	[39]
Bacteria 785[a]	GGATTAGATACCCTGGTAG	785–803	[39]
Bacteria 1055[b]	CACGAGCTGACGACAGCCAT	1055–1074	[39]

[a] Used as sense primer.
[b] Used as antisense primer.
[c] The box marks additional nucleotides that do not bind with *E. coli*, but can be used as restriction site for cloning.

min, 33 cycles of 95 °C for 60 s, 48 °C for 60 s, 72 °C for 90 s and a final extension step at 72 °C for 10 min.

PCR products were either sequenced either directly or, if necessary, purified with the PCR-purification-kit QIAquick (Quiagen, Germany) according to the instructions of the producer. Sequencing was done on a DNA sequencer 4200 (LI-COR Bioscience GmbH, Germany) with all oligonucleotides listed in Table 1; each strain was sequenced independently at least twice. Partial sequences were aligned using Lasergene (DNASTAR, USA), producing an approximately 1500-bp fragment of the 16S rRNA gene. Identification was carried out aligning the 16S rRNA gene sequences obtained in this study with sequences of known strains using BLAST (NCBI) [10].

2.7. Transpiration experiments

Cuticular transpiration was measured using either a gravimetric method or radiolabelled water as described in detail by Schreiber et al. [11]. Isolated *Prunus laurocerasus* L. cuticular membranes (CM) [11] were mounted on transpiration chambers containing 900 μl sterile H_2O. The chambers were kept in polyethylene boxes on silicagel at 25 °C. The weight loss was measured once a day with a balance (Sartorius) to obtain the initial water permeability (*P1*). After 7 days, the cuticles were treated with 200 μl of the following solutions: (i) sterile H_2O, (ii) M9 salt medium without glucose but with 1 mM NaN_3, (iii) PBS with 1 mM NaN_3, (iv) washed cells of *P. rhizosphaerae* in PBS and (v–vii) supernatant from cultures of different cell densities with 1 mM NaN_3. The chambers were incubated at 25 °C and approximately 100% humidity for 24 h. After the treatment, solutions were carefully removed with tissue paper and the cuticles were rinsed with 300 μl PBS, respectively, 300 μl H_2O for the H_2O control. The chambers were treated as described above to obtain the final water permeability (*P2*).

F. ananassa leaf disks (2 cm diameter) were mounted on sterile transpiration chambers filled with 900 μl of a phosphorous buffered donor solution (PBS: 8 g NaCl, 1.44 g Na_2HPO_4, 0.2 g KCl, 0.24 g KH_2PO_4, and 1 l $H_2O_{deion.}$), each containing approximately 4×10^6 DPM of radio-labelled H_2O (specific activity 925 MBq g^{-1}, Hartmann Analytic, Germany). Stomatous abaxial sides were facing the inner volume compartments of the cambers containing radio-labelled water. Transpiration chambers were incubated upside down on scintillations tubes at 100% humidity and 25 °C. Within the first 24 h, initial water permeability across the outer astomatous leaf cuticle was measured at regular time intervals. The astomatous leaf surfaces were treated with 300 μl of the following solutions: (i) M9 salt medium without glucose but with 1 mM NaN_3, (ii) washed cells of *P. rhizosphaerae* in PBS and (iii) supernatant. Treated leaf disks were stored at 25 °C and 100% humidity. Twenty-four hours later, the bacterial solution and sterile supernatant were carefully removed from the leaf surfaces using tissue paper and rinsing leaf surfaces with 300 μl sterile PBS. Water permeability across the cuticles of leaf disks prepared as described above was measured again for another 48 h (*P2*).

Solutions for treating cuticles were prepared as follows: For treatment of astomatous leaf surfaces and cuticles *P. rhizosphaerae*, which was precultivated in LB medium at 25 °C and 180 RPM for 24 h, was selected. Preculture (50 μl) was transferred into mineral salt medium (100 ml of 10-fold M9 salt medium [9]) amended with 10 mM glucose as the sole carbon source and incubated on a rotary shaker at 25 °C and 180 RPM until the required cell densities were obtained. Cell density was determined measuring optical density at 600 nm. Cells were washed by centrifuging the culture at 10 °C and 10,000*g* for 15 min. The supernatant was carefully decanted and the pellet resuspended in PBS. This step was repeated and the pellet was resuspended in the initial volume. The supernatant for treatment was filtrated (sterile).

Linear regression lines were fitted to the transpiration kinetics of water across the cuticle. Coefficients of determination were always better than 0.99. Permeances were calculated from the slopes of the regression lines according to the following equation:

$$P = F/A * \Delta c. \quad (1)$$

Permeance P [m s^{-1}] is proportional to the flow F [DPM m^{-2} s^{-1}; g m^{-2} s^{-1}] divided by the driving force c [DPM m^{-3}; g m^{-3}]. Permeances before (*P1*) and after treatment with bacteria (*P2*) can be compared, and the effect is given by the ratio of the permeances *P2*/*P1*. Effects were calculated for each parallel separately. Since water permeability follows a log normal distribution [12], permeances were logarithmically transformed before calculating means with 95% confidence intervals. Significance of the mean effect on transpiration was tested by Student's *t*-test.

3. Results

3.1. Microscopy of leaf surfaces

Strawberry leaves were densely covered with two types of living trichomes: trichomes and glandular trichomes. Glandular trichomes could be found distributed all over the surface of both leaf sides, although higher densities were observed along the veins of adaxial leaf sides. On abaxial leaf sides, epiphytic bacteria were mostly found on both types of trichomes (Fig. 1(a) and (b)). On adaxial leaf surfaces, bacteria were either found to form aggregates (Fig. 1(c)) or as single cells (Fig. 1(d)).

3.2. Isolated strains

From each of the imprints of 8 leaflets, 1 blossom and 7 fruits, at least 15 colonies were selected, thus collecting 360 microbial strains. Around 281 strains were isolated from field-grown plants and 79 from the greenhouse. About 28% of the non-investigated strains were eukaryotic. The remaining 324 bacterial strains were grouped into 38 morphotypes (Fig. 2), except for 5%, which did not fit into any of these morphotypes. For further investigation of 18 of the morphotypes, 1 to 3 strains were selected of each type, resulting in 29 strains (Fa1 to Fa29). These 18 morphotypes covered 74% of all bacterial strains isolated, 40% were Gram-positive and 60% Gram-negative (Table 2). None of the identified strains caused hypersensitive necrosis (data not shown).

3.3. Identification

Of the 29 strains selected, 12 were identified by 16S rRNA gene analysis (Table 3). The identified strains represent 44% of all isolated strains. Results of the morphological and histochemical characterization were confirmed by the 16S rRNA gene analysis (Tables 2 and 3). Since is it known that chimeric sequences are accumulating in public databases [13], the sequences were analysed using the Chimera-Check-Program,

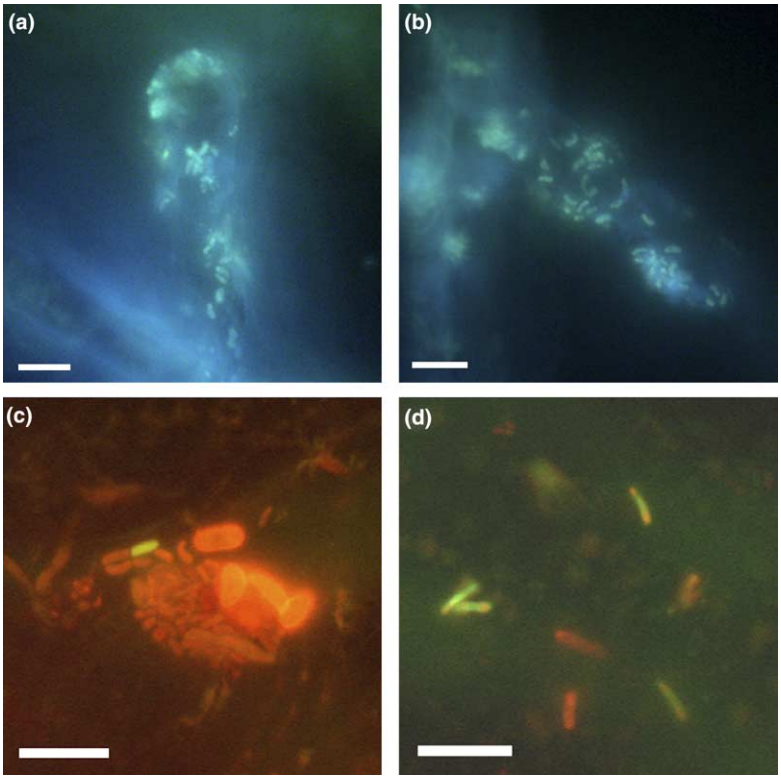

Fig. 1. Epifluorescense microscopy of *F. ananassa* leaf surfaces. Marker = 10 μm. (a) Microorganisms on a glandular trichome on the lower leaf side; (b) microorganisms on a trichome on the lower leaf side; both DAPI staining, (c) aggregate of cocci and different-sized rods on the upper leaf side; (d) single rods on the upper leaf side; both acridine orange staining.

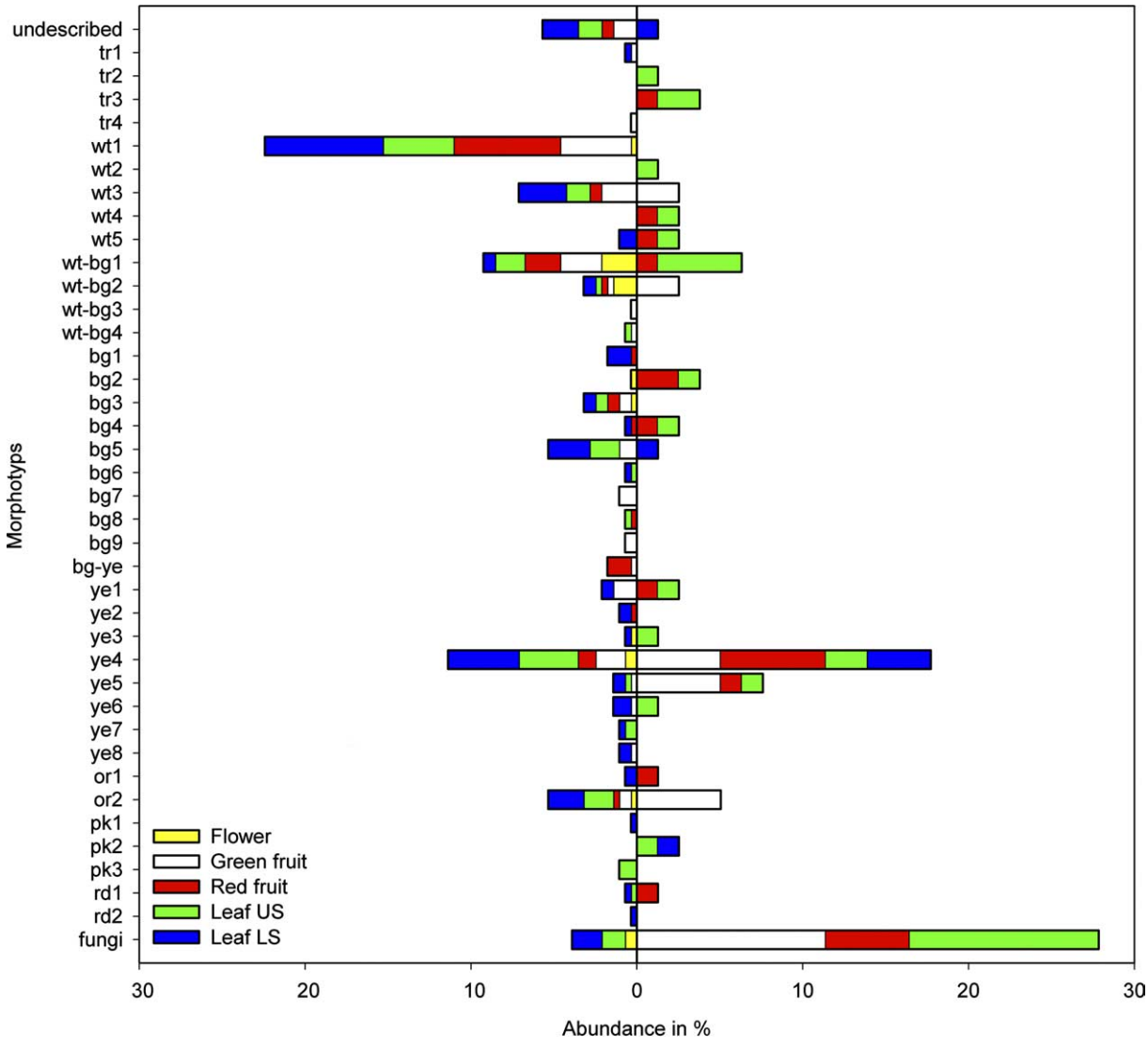

Fig. 2. Distribution of the morphotypes in the field (281 strains) and the greenhouse (79 strains) on plant parts. Abbreviations: tr, translucent; wt, white; bg, beige; ye, yellow; or, orange; pk, pink; rd, red; US, upper side; LS, lower side.

which is provided by RDP [14]. None of the sequences obtained in this study showed any signs of being chimeric.

3.4. Transpiration experiments

The initial permeance of the *Prunus* CM was 8.73×10^{-10} m s^{-1} ($+3.71 \times 10^{-10}$, -2.60×10^{-10}). The treatment of *Prunus* CM with approximately 3×10^{10} washed cells of *P. rhizosphaerae* resuspended in PBS had no effect on water permeability, neither had the treatment with the control solutions (water, M9 salt medium and PBS) (Fig. 3). Treatment, however, of the CMs with supernatant obtained from liquid cultures with increasing cell densities had an increasing effect on water permeability, ranging from 1.16 ± 0.17 up to 1.62 ± 0.32 (Fig. 3).

The permeance of the untreated *Fragaria* leaf disk was 1.46×10^{-9} m s^{-1} ($+2.19 \times 10^{-10}$, -1.90×10^{-10}). Transpiration kinetics of all treated leaf disks significantly increased after treatment with sterile filtrated supernatant of *P. rhizosphaerae* (Fig. 4). The average effect of increase was 1.34 ± 0.47, whereas treatment with M9 salt medium or washed cells of *P. rhizosphaerae* resuspended in PBS did not significantly increase cuticular transpiration (Fig. 5).

4. Discussion

4.1. Identification of the strains

Bacterial contaminants of strawberry explants have been recently isolated and characterized. The most

Table 2
Description of the isolates: source, colour, texture, autofluorescense, Gram-reaction, form, size and mobility

Isolate	Source[a]	Colour	Texture	Auto-fluorescense	Gram	Form	Size μm × μm	Mobility	Abbreviations according to Fig. 2
Fa1	B, LLS	White	Dull	n.d.	n.d.	n.d.	n.d.	n.d.	wt4
Fa2	A, LLS	White–beige	Shiny	–	–	Rods	1 × 2–6	n.d.	wt-bg2
Fa3	A, RF	White–beige	Shiny	+	–	Rods	1 × 3–4	+	wt-bg1
Fa4	B, LLS	Yellow	Shiny	–	–	Rods, slightly curved	1 × 3–5	n.d.	ye4
Fa5	A, LLS	Yellow	Shiny	–	+	Rods, slightly curved	1 × 2–4	n.d.	ye7
Fa6	D, LUS	Yellowish	n.d.	–	–	Rods	1 × 2–3	n.d.	ye6
Fa7	B, LUS	White	Dull	–	+	Rods	1.5 × 3–5	+	wt3
Fa8	D, LUS	Beige	Wrinkled, dull	+	–	Rod, pointy ends	1 × 3–6	n.d.	bg2
Fa9	B, FL	White–beige	Shiny	–	–	Rods	1 × 3–5	n.d.	wt-gb1
Fa10	B, LUS	Pink	Shimmering	n.d.	n.d.	n.d.	n.d.	n.d.	pk3
Fa11	B, FL	Orange	Shiny	–	–	Rods	1–1.5 × 2–3	+	or2
Fa12	D, RF	White	Waxy, shiny	+	–	Rods	1–1.5 × 4–7	n.d.	wt5
Fa13	D, LLS	Yellow	Shiny	–	–	Rods	1 × 2–2.5	n.d.	ye4
Fa14	C, LLS	White	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	wt3
Fa15	A, LUS	Beige	Dull	–	–	Rods	1 × 3–6	+	bg5
Fa16	D, RF	Beige	Wrinkled, dull	–	–	Rods	1 × 3–5	n.d.	bg2
Fa17	C, LUS	Orange	Shiny	–	–	Rods	1.5 × 2–3	n.d.	or2
Fa18	C, LLS	White	Threadlike	n.d.	n.d.	n.d.	n.d.	n.d.	wt1
Fa19	C, GF	Transparent	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr1
Fa20	C, LUS	Yellow	Shiny	–	–	Rods	1 × 2–3	n.d.	ye4
Fa21	A, LUS	Red	Shiny	–	+	Cocci	1	n.d.	rd1
Fa22	A, LUS	White	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	wt3
Fa23	D, RF	White	Waxy	–	n.d.	Rods, slightly curved	1.5 × 3–7	+	wt5
Fa24	C, LLS	Yellow	Rough	+	–	Rods	1 × 2–3	+	ye3
Fa25	C, LLS	White	Threadlike	–	+	Rods	1.5–2 × 3–8.5	n.d.	wt1
Fa26	A, LUS	Beige	Domed	–	+	Rods	1.5 × 4–6	n.d.	bg4
Fa27	D, LLS	Orange	Translucent	–	–	Rods	1 × 1.5–2	+	or1
Fa28	B, FL	White–beige	Shiny	n.d.	n.d.	n.d.	n.d.	n.d.	wt-bg2
Fa29	D, LUS	Beige	Domed	–	+	Rods	2–2.5 × 6–11	n.d.	bg4

[a] A, B, C: field-grown plants; D: plant from the greenhouse; FL: flower; GF: green fruit; RF: red fruit; LLS: leaf lower side; LUS: leaf upper side.

Table 3
Identified strains

NCBI Accession Number	Isolate	Frequency in %	Best hit	Identity in %	NCBI Accession Number
AY131214	Fa2	3.4	*Pseudomonas* sp. NZ099	99.7	AF388207
AY131215	Fa3	9.6	*Pseudomonas lurida*	99.9	AJ581999
AY131223	Fa4	14.2	*Pseudomonas rhizosphaerae*	99.4	AY152673
AY131216	Fa6	1.5	*Stenotrophomonas maltophilia* LMG 11087	99.1	X95924
AY131217	Fa7	6.8	*Bacillus cereus* Delaporte	99.9	AF155958
AY131218	Fa8	1.2	*Pseudomonas orientalis*	99.7	AF064457
AY131224	Fa20	14.2	*Pseudomonas rhizosphaerae*	99.5	AY152673
AY131225	Fa21	0.9	*Arthrobacter agilis*	99.3	X80748
AY131219	Fa24	0.9	*Pseudomonas fulva*	100	AB047001
			Pseudomonas parafulva	100	AB060133
AY131220	Fa25	19.4	*Bacillus weihenstephanensis*	100	AB021199
			Bacillus mycoides	100	AB021192
AY131221	Fa27	0.9	*Pseudomonas fulva*	100	AB047001
			Pseudomonas parafulva	100	AB060133
AY131222	Fa29	1.2	*Bacillus* sp. No. 49	99.7	AB066347

Frequency of the isolates, results of the 16S rRNA gene analysis, strains that showed the best results with BLAST (NCBI) [10].

common bacterium found was *Pseudomonas fluorescens*. In addition *Pseudomonas corrugata*, *Pseudomonas tolaasii* and *Pseudomonas paucimobilis*, one *Xanthomonas campestris*, two *Xanthomonas* spp., *Enterobacter* spp. and *Enterobacter cloacae* were also identified [15]. In the present study, 8 of the 12 identified strains were Gram-negative (Table 3), belonging to the *Gammaproteobacteria* [16]. With the exception of Fa6, which was identified as *Stenotrophomonas maltophilia* (*Xanthomonas* group), all other Gram-negative isolates belonged

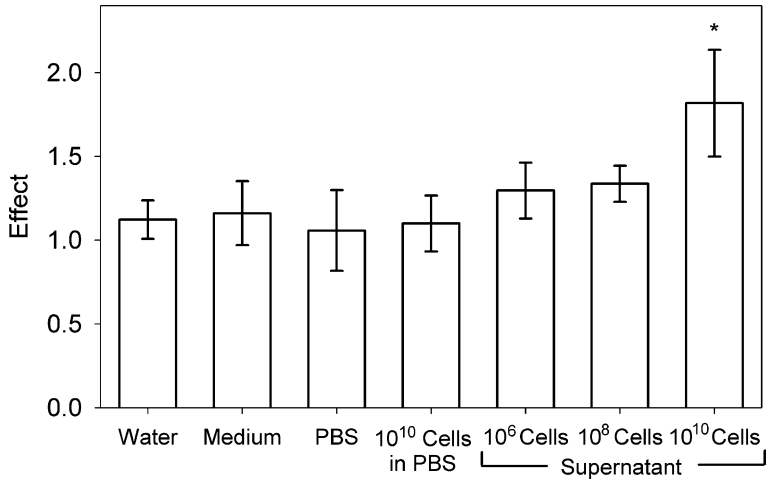

Fig. 3. Effect of treatment on *Prunus laurocerasus* cuticular membranes (CM). Results are arithmetic means with 95% confidence intervals as error bars. The effect of the treatment with the culture supernatant increased with increasing cell numbers. The asterisk indicates a statistically significant difference (*t*-test, $p = 0.05$) from the controls (water, M9 salt medium and PBS).

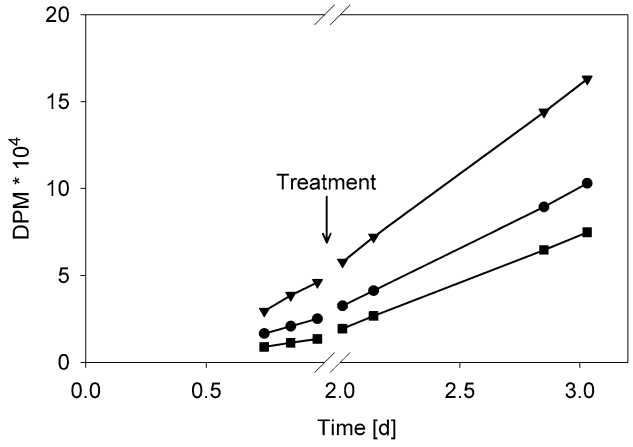

Fig. 4. Flow of ^{3}H-labelled water across the selected *Fragaria ananassa* leaf disks before and after treatment with a sterile culture filtrate of *P. rhizosphaerae*. The single effects of the treatment, resulting in an increased flow, were 1.34 (▼), 1.76 (●) and 2.58 (■).

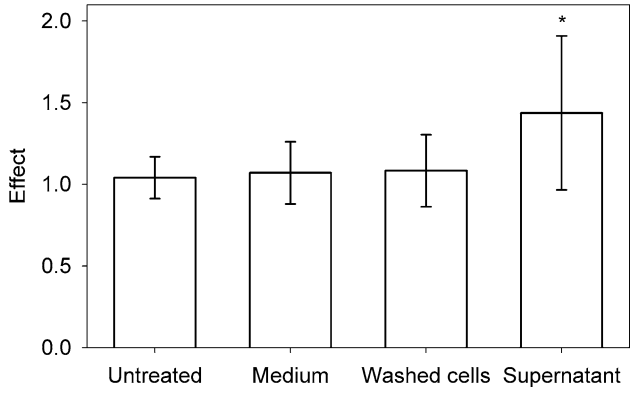

Fig. 5. Effect of treatment on *Fragaria ananassa* leaf discs with M9 salt medium, washed cells of *P. rhizosphaerae* in PBS and sterile supernatant of the same culture. In addition, leaf disks without any treatment are shown as a control for viability of the intact leaf disks during the experiment. Results are given as arithmetic means with 95% confidence intervals as error bars. The asterisk indicates a statistically significant difference (*t*-test, $p = 0.05$) from the controls (untreated leaf disks, M9 salt medium and washed cells).

to the genus *Pseudomonas* (Table 3). The other 4 isolates (Fa7, Fa25, Fa21 and Fa29) were Gram-positive (Table 3). Within this group, the isolates Fa7, Fa25 and Fa29 formed an extra branch that stands for the endospore-forming *Bacillus* group (Table 3). Gram staining supported these results (Table 2).

Isolate Fa2 showed 99.7% identity with a *Pseudomonas* species that was previously isolated from mushroom and causes bacterial blotch on *Agarius bisporus* [17]. Fa3 showed 99.9% identity with *Pseudomonas lurida*, a new fluorescent species that was isolated from grasses (accession number AJ581999). The 16S rRNA gene of isolate Fa4 and Fa20 was almost identical (99.9%), and the identity of both strains with *P. rhizosphaerae* was 99.4% and 99.5%, respectively. *P. rhizosphaerae* is a novel species, that was first isolated from the rhizosphere of grasses [18]. This species belongs to the non-fluorescent Pseudomonas group and its closest relative is *Pseudomonas graminis*, a new species that was isolated from the phyllosphere of grasses [19]. For Fa8, 99.7% identity was obtained with *Pseudomonas orientalis* that was first isolated from spring water [20]. Fa24 and Fa27 had identical 16S rRNA gene, although the strains are morphologically different. They had 100% identity with *Pseudomonas fulva* (accession number AB047001) and *Pseudomonas parafulva* (accession number AB060133), respectively. In contrast to Fa27, Fa24 produced fluorescent pigments while growing on King B agar. Isolate Fa6 showed 99.1% identity with *S. maltophilia*. *S. maltophilia* is widely known as a human pathogen occurring in hospitals [21], but was also isolated from leaves of *Solanum tuberosum* [22,23]. For Fa21, we gained 99.3% identity

with *Arthrobacter agilis*. *A. agilis* has not been described in the phyllosphere of any plant hitherto, but Heuer and Smalla [22] reported a not yet classified *Arthrobacter* species in the phyllosphere of *S. tuberosum*.

Fa7 showed 99.9% identity with *Bacillus cereus*. *B. cereus* has been reported in the phyllosphere of *Quercus ilex* [24]. Fa25 had 100% identity with *Bacillus weihenstephanensis*, a psychrotolerant species in alpine soil [25] and a *Bacillus mycoides* strain. *B. mycoides* was found in the phyllosphere of *Malus* × *domestica* [26], *S. tuberosum* [22] and *Q. ilex* [24]. Fa29 showed 99.7% identity with *Bacillus* sp. No.49 and No.54, which have been isolated from the composting of animal feces (accession number AB066345). Thus, the results of 16S rRNA gene analysis showed that most of the strains isolated have already been described in the phyllosphere of various plant species. One of the most prominent bacteria was *P. rhizosphaerae*, a newly described species that was first isolated from the rhizosphere of grasses [18]. Many of the organisms in the phyllosphere are not or difficult to cultivate, and the composition of the isolates strongly depends on conditions for isolation and cultivation (e.g. media). This might be the reason for the lack of *Xanthomonas* and *Erwinia* species, that are very common in the phyllosphere [27].

4.2. Morphology and microscopy

Microscopic investigations of the leaf surfaces (Fig. 1), the leaf imprints on the agar plates (data not shown) and the distribution of the morphotypes (Fig. 2) revealed that there was a clear difference between the greenhouse plants and the field-grown plants. On the leaves from the greenhouse plants, the majority (81%) of the isolates was found on the upper side, whereas from the field-grown plant leaves more strains could be isolated from the lower side (61%). Another difference was the observation that fungal isolates amounted to 28% on plants grown in the greenhouse, whereas only 4% of the isolates were fungi on plants grown in the field (Fig. 2). Under stress conditions in the field, such as high irradiation and low water availability, bacteria can better survive on the lower leaf surface, offering more protected sites [28], than on the upper leaf side. This is probably less relevant for epiphytic bacteria living on strawberry plants cultivated in the greenhouse, with constant and favourable growth conditions. In addition, higher degrees of humidity and constant temperatures favour the growth of fungi in the greenhouse compared to the field.

Furthermore, it is evident that some of the strains were pigmented (Table 2 and Fig. 2). Pigmentation is interpreted as a protection for high irradiation [6]. In the greenhouse, 40% of the strains showed a yellow, orange, pink or red pigmentation. In the field, 29% of the strains were pigmented. This lower percentage of pigmentation in the field was due to the high percentage of white and beige strains, which are likely soil-borne. One of the most abundant white morphotypes on field-grown strawberry plants (Table 3) was identified as a typical soil bacterium (*B. weihenstephanensis*) [25]. Many of these morphotypes without pigmentation did not occur in the greenhouse, because the plants investigated did not have contact with the soil. The strawberry plants in the field, however, were very close to the soil and microorganisms from the soil could easily be splashed onto plants by raindrops [29,30].

Microscopic investigations revealed that most of the bacteria were located on trichomes. This observation indicates that the nutrient availability on the surface of trichomes must have been larger than in other areas of the leaf surface, thus suggesting a pronounced heterogeneity of nutrient availability in the phyllosphere, as was also described for fructose availability [31]. Furthermore, it was demonstrated that the bacterial isolates described here were able to metabolize volatile compounds from strawberry trichomes [32].

4.3. Bacterial interactions with leaf surfaces and the effects on CM permeability

The permeance of untreated *Fragaria* leaf disc was 1.46×10^{-9} m s^{-1} ($+2.19 \times 10^{-10}$, -1.90×10^{-10}), which is in the scope of permeances that have been obtained for deciduous species, ranging from 1.55×10^{-9} to 4.02×10^{-9} m s^{-1} for *Gingko biloba* and *Juglans regia* [33]. The permeance of the *Prunus* cuticle membranes was in the scope of older leaves, as reported by Schreiber et al. [11]. In the past, it has been shown that colonization of the leaf surface with bacteria may significantly change the wettability of the cuticle [34,35]. In a recent study, we could show that the treatment of cuticular membranes from different plant species with 9 different bacterial isolates led to significant increases of cuticular transpiration [36]. The highest effect was observed for a bacterial strain isolated from *Hedera helix*, which was identified as *P. graminis*, a bacterium known to be specific for the phyllosphere [19] *P. graminis* was able to increase the water permeability by 50%. In this study, we isolated a close relative of *P. graminis*, *P. rhizosphaerae*, which was also one of the most abundant strains isolated from the phyllosphere of strawberry. To test its capability to change the properties of the cuticle, we first used *Prunus* CM as a model system. The cuticle was treated with washed cells and supernatants of the cultures in order to test the hypothesis that bacterial metabolites in the culture medium are causing the effect. The results showed that the bacteria obviously secrete metabolites able to change the cuticular water permeability; in addition, this effect increased with increasing cell densities of the cultures (Fig. 3). Similar results were

obtained with intact strawberry leaf discs (Figs. 4 and 5). At present, the nature of these metabolites is not known, although it was shown several times that a series of different compounds (e.g. surfactants) can act as plasticizers on plant cuticles, thus increasing cuticular permeabilities [37].

Plotting the initial resistances of water permeability of the untreated cuticle (1/*P1*) versus the effect of the treatment with sterile supernatant yields a linear relation (Fig. 6). This indicates that cuticles having the lowest initial permeabilities were most sensitive towards an increase in cuticular permeability induced by *P. rhizosphaerae*. However, both the cuticular permeability for water as well as the effects increasing the cuticular permeability showed large variations (Figs. 3–5). This is a characteristic property of cuticular membranes, described several times in the past [37], indisputably indicating the observed lateral heterogeneity of leaf surfaces as described above. Variability in barrier properties will result in a large variation in nutrient availability and therefore, an inhomogeneous distribution of microorganisms in the phyllosphere will be the consequence.

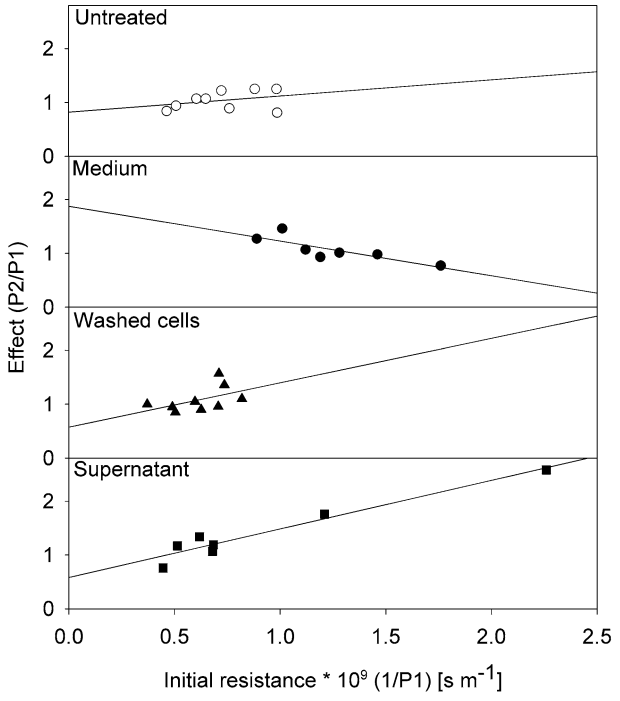

Fig. 6. Effect of treatment on cuticular transpiration as a function of the initial resistance of *Fragaria ananassa* leaf discs. The effects represent the ratio of water permeabilities *P2*/*P1* before and after treatment of leaf disks. The initial resistance is the reciprocal of the permeance measured before the treatment. For untreated leaves and leaf disks treated with M9 salt medium or washed cells, only weak correlations could be found ($r^2 = 0.11$, 0.68, respectively, 0.25), whereas treatment with sterile supernatant resulted in a strong correlation ($r^2 = 0.94$).

The results presented here convincingly evidence that bacteria living in the phyllosphere have the ability of interacting with leaf surfaces and, as a consequence, of altering their functional properties. Besides effects on leaf surface wetting [34,35], effects on barrier properties are described. Both increased wetting of the hydrophobic cuticle and transport across the hydrophobic leaf surface will lead to a larger availability of water and nutrients in the phyllosphere, and thus living conditions of bacteria in the phyllosphere will significantly improve.

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References

[1] Kinkel, L.L., Wilson, M. and Lindow, S.E. (2000) Plant species and plant incubation conditions influence variability in epiphytic bacterial population size. Microb. Ecol. 39, 1–11.
[2] Yang, D.-H., Crowley, D.E., Borneman, J. and Keen, N.T. (2001) Microbial phyllosphere populations are more complex than previously realized. Proc. Natl. Acad. Sci. USA 98, 3889–3894.
[3] Hirano, S.S. and Upper, C.D. (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*, a pathogen, ice nucleus and epiphyte. Microbiol. Mol. Biol. Rev. 64, 624–653.
[4] Morris, C.E., Monier, J.-M. and Jaques, M.-A. (1998) A technique to quantify the populations size and composition of the biofilm component in communities of bacteria in the phyllosphere. Appl. Environ. Microbiol. 64, 4789–4795.
[5] Davey, M.E. and O'Toole, G.A. (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64, 847–867.
[6] Lindow, S.E. and Brandl, M.T. (2003) Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883.
[7] Bast, E. (2001) Mikrobiologische Methoden. Spektrum Akademischer Verlag GmbH, Heidelberg.
[8] Klement, Z., Rudolph, K. and Sands, D. (1990) Methods in Phytobacteriology. Akademia Kiado, Budapest.
[9] Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratoy Press, Cold Spring Harbor.
[10] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[11] Schreiber, L., Skrabs, M., Hartmann, K., Diamantopoulos, P., Simanova, E. and Santrucek, J. (2001) Effect of humidity on cuticular water permeability of isolated cuticular membranes and leaf disks. Planta 214, 274–282.
[12] Baur, P. (1997) Lognormal distribution of water permeability and organic solute mobility in plant cuticles. Plant Cell Environ. 20, 167–177.
[13] Hugenholtz, P. and Huber, T. (2003) Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int. J. Syst. Evol. Microbiol. 53, 289–293.
[14] Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. and Tiedje, J.M. (2003) The Ribosomal Database Project

(RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31, 442–443.
[15] Tanprasert, P. and Reed, B.M. (1998) Detection and identification of bacterial contaminants of strawberry runner explants. Plant Cell, Tissue Organ Culture 52, 53–55.
[16] Madigan, M.T., Martinko, J.M. and Parker, J. (2000) Brock Biology of Microorganisms. Prentice Hall International, London.
[17] Godfrey, S.A.C., Harrow, S.A., Marshall, J.W. and Klena, J.D. (2001) Characterization by 16S rRNA sequence analysis of *Pseudomonads* causing blotch disease of cultivated *Agaricus bisporus*. Appl. Environ. Microbiol. 67, 4316–4323.
[18] Peix, A., Rivas, R., Mateos, P., Martinez-Molina, E., Rodriguez-Barrueco, C. and Velazquez, E. (2003) *Pseudomonas rhizosphaerae* sp. nov., a novel species that actively solubilizes phosphate in vitro. Int. J. Syst. Evol. Microbiol. 53, 2067–2072.
[19] Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. and Seyfath, W. (1999) A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov. Int. J. Syst. Bacteriol. 49, 297–308.
[20] Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D. and Leclerc, H. (1999) Taxonomic study of bacteria isolated from Lebanese spring waters: proposal for *Pseudonomas vedrella* sp. nov. and *P. orientalis* sp. nov. Res. Microbiol. 150, 303–316.
[21] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994) Bergey's Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore.
[22] Heuer, H. and Smalla, K. (1997) Evaluation of communtity-level catabolic profiling using BIOLOG GN microplates to study microbial community changes in potato phyllosphere. J. Microbiol. Meth. 30, 49–61.
[23] Wilson, M., Hirano, S.S. and Lindow, S.E. (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential growth within the leaf. Appl. Environ. Microbiol. 65, 1435–1443.
[24] Brunel, B., Perissol, C., Fernandez, M., Boeufgras, J.M. and Le Petit, J. (1994) Occurrence of *Bacillus* species on evergreen oak leaves. FEMS Microbiol. Ecol. 14, 331–342.
[25] von Stetten, F., Mayr, R. and Scherer, S. (1999) Climatic influence on mesophilic *Bacillus cereus* and psychrotolerant *Bacillus weihenstephanensis* populations in tropical, temperate and alpine soil. Environ. Microbiol. 1, 503–515.
[26] Kucheryava, N., Fiss, M., Auling, G. and Kroppenstedt, R.M. (1999) Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic in vitro to *Venturia inaequalis*, the causal agent of apple scab. Syst. Appl. Microbiol. 22, 472–478.
[27] Blakeman, J.P. (1993) Pathogens in the foliar environment. Plant Pathol. 42, 479–493.
[28] Andrews, J.H. and Harris, R.F. (2000) The ecology and biogeography of microorganisms on plant surfaces. Ann. Rev. Phytopathol. 38, 145–180.
[29] Blakeman, J.P. (1991) Foliar bacterial pathogens: epiphytic growth and interactions on leaves. J. Appl. Bacteriol. 70, 49S–59S.
[30] Lovell, D.J., Parker, S.R., van Peteghem, P., Webb, D.A. and Welham, S.J. (2002) Quantification of raindrop kinetic energy for improved prediction of splash-dispersed pathogens. Phytopathology 92, 497–503.
[31] Leveau, J.H.J. and Lindow, S.E. (2001) Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. Proc. Natl. Acad. Sci. USA 98, 3446–3453.
[32] Abanda-Nkpwatta, D., Krimm, U., Coinera, H.A., Schreiber, L. and Schwab, W. (2005) Plant volatiles can minimize the growth suppression of epiphytic bacteria by the phytopathogenic fungus *Botrytis cinerea* in co-culture experiments. Environ. Exp. Bot. (in press).
[33] Niederl, S., Kirsch, T., Riederer, M. and Schreiber, L. (1998) Co-permeability of ^{3}H-labeled water and ^{14}C-labeled organic acids across isolated plant cuticles. Plant Physiol. 116, 117–123.
[34] Knoll, D. and Schreiber, L. (1998) Influence of epiphytic microorganisms in leaf wettability: wetting of the upper leaf surface of *Juglans regia* and of model surfaces in relation to colonization by microorganisms. New Phytol. 140, 271–282.
[35] Knoll, D. and Schreiber, L. (2000) Plant–microbe interactions: wetting of ivy (*Hedera helix* L.) leaf surfaces in relation to colonisation by epiphytic microorganims. Microb. Ecol. 41, 33–42.
[36] Schreiber, L., Krimm, U., Knoll, D., Sayed, M., Auling, G. and Kroppenstedt, R.M. (2005) Plant–microbe interactions: identification of epiphyllic bacteria and their effects on barrier properties of isolated cuticles. New Phytol. doi:10.1111/j.1469-8137.2005.01343.X (in press).
[37] Schreiber, L., Bach, S., Kirsch, T., Knoll, D., Schalz, K. and Riederer, M. (1995) A simple photometric device analysing cuticular transport physiology: surfactant effect on permeability of isolated cuticular membranes of *Prunus laurocerasus* L. J. Exp. Bot. 46, 1915–1921.
[38] Schröder, D., Deppisch, H., Obermayer, M., Krone, G., Stackebrandt, E., Hölldobler, B., Goebel, W. and Gross, R. (1996) Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): systematics, evolution and ultrastructural characterization. Mol. Microbiol. 21, 479–489.
[39] Amann, R.I., Ludwig, W. and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59m, 143–169.