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Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria

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Abstract

A field release of genetically engineered potato plants that produce bacteriophage T4-lysozyme for enhanced bacterial resistance was monitored for changes in plant-associated bacterial populations, in the functions of potentially beneficial bacteria and in the diversity of antagonistic bacterial species. These parameters have been analyzed for two T4-lysozyme-expressing lines, a transgenic control and a non-transgenic line, over a period of 2 years at different stages of plant development and at two different locations. Two microenvironments, the rhizo- and geocaulosphere, were investigated. No significant differences in aerobic plate counts were observed between the four plant lines. In addition, no significant differences in the functions of potentially beneficial bacteria (percentage of auxin [indole-3-acetic acid = IAA]-producing isolates) and antagonistic bacteria (antagonists to *Erwinia carotovora* and *Verticillium dahliae*) were found. The diversity of antagonistic species isolated from each plant line and microenvironment was investigated to determine if the diversity and composition of potentially beneficial bacteria were influenced. Altogether, 28 different potato-associated species with antagonistic effects to phytopathogens were detected. Antagonistic strains of seven species were found only on control plants. The observed effect was minor relative to the natural variability observed during the monitoring period. This is the first study including plant-associated bacteria responsible for plant growth and health and provides an example for performing risk assessment studies for transgenic plants under a variety of environmental conditions.

Keywords: Risk assessment; Transgenic potato; T4-lysozyme; Potentially beneficial plant-associated bacteria

1. Introduction

In recent years, molecular techniques for modifying the genome of a plant have become common, but little is known of the consequences of these genetically engineered plants on plant-associated bacteria. There have been numerous field releases of transgenic plants but the majority of these field tests have been evaluated only for efficacy of the plant engineered traits or outcrossing effects. Practical approaches for testing the effects of transgenic plants on key groups of soil microorganisms have been

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suggested [1,2]. Risk assessments for transgenic plants with respect to plant-associated microorganisms have been reported in the last few years. Wilson et al. [3] analyzed the colonization of mannityl opine-producing transgenic tobacco plants with a mannityl opine-catabolizing strain of *Pseudomonas syringae*. They observed enhanced colonization of the phyllosphere by the catabolizing strain compared to that of the near isogenic (non-catabolizing) strain of *P. syringae*. Opine-producing potato plants exerted an influence on the naturally occurring root-associated microflora [4] and on introduced bacteria [5]. Donegan et al. [6] observed an influence of cotton plants which were genetically engineered to produce the *Bacillus thuringiensis* (Bt) endotoxin, on both abundance and diversity of indigenous soil bacteria and fungi. The authors suggested that the observed effects may not have resulted from the foreign gene product, but from a change in plant characteristics due to the genetic manipulation. In contrast, the environmental release of Bt-producing potato plants had no significant or persistent impact on the microbial species associated with plant leaves [7]. Minor differences in the species composition of bacteria in the phyllosphere of T4-lysozyme-producing potatoes and control plants were detected by Heuer and Smalla [8].

Potato plants were engineered to produce T4-lysozyme to enhance their resistance to *Erwinia carotovora* ssp. *atroseptica*. This is a broad-host-range pathogen causing seed piece decay, blackleg and aerial soft rot, especially of potatoes [9]. Lysozymes are a widespread family of enzymes. Most of the investigated soil- and plant-associated bacteria such as *Pseudomonas stutzeri, Acinetobacter calcoaceticus, Bacillus subtilis* and *Rhizobium* spp. were susceptible to T4-lysozyme at very low concentrations [10]. In these experiments the sensitivity of bacteria was also observed in the absence of the chelating agent EDTA [10]. Bacteriophage T4-lysozyme is the most active member of a class of bacteriolytic enzymes, which have been detected in several plant species [11,12]. The secretion of foreign T4-lysozyme into the intercellular spaces of transgenic potato plants affects resistance against *Erwinia carotovora* already at relatively low levels of expression [13]. T4-lysozyme was the first trait to be exploited to produce plants with resistance against bacteria. Recently, this strategy was also applied for other plants, e.g. oilseed rape and tomato [14].

Plant-associated bacteria are subdivided into beneficial, deleterious and neutral groups on the basis of their effects on plant growth and health [15]. Beneficial bacteria are responsible for plant growth promotion and biological control of soil-borne pathogens [15,16]. If this group of bacteria is disturbed due to the influence of T4-lysozyme, the positive effect of the constructed plant would be cancelled. Alternatively, if beneficial bacteria were not influenced, a synergistic effect could occur. Due to the non-selective effects of T4-lysozyme, it is necessary to assess the influence on the potato-associated beneficial bacteria. For this study we investigated the effects on bacteria in the rhizosphere [15,17] and in the geocaulosphere (tuber surface) [18].

The objective of our investigations was to evaluate the effects of transgenic T4-lysozyme-producing potatoes on plant-associated bacteria, and especially on species that are potentially beneficial bacteria in the rhizo- and geocaulosphere, under field conditions. These potentially beneficial bacteria were characterized according to their ability to produce indole-3-acetic acid (IAA); to show antagonistic activity to the bacterial target pathogen *E. carotovora* ssp. *atroseptica*; or antagonistic activity to the phytopathogenic fungus *Verticillium dahliae*. All of the active strains were identified to monitor the possible influence of T4-lysozyme on the diversity of species with antagonistic properties.

2. Materials and methods

2.1. Plants and experimental design

The transgenic potato lines, DL4, DL5 constitutively expressing and secreting T4-lysozyme, and a transgenic control line, DC1, carrying the same construct [13], including the *npt*II gene, but without T4-lysozyme gene were derived from variety Désirée. All plant lines, including the wild-type DESI, were provided by Dr. K. Düring (Federal Centre for Breeding Research on Cultivated Plants [BAZ], Quedlinburg, Germany). In 1996 and 1997 potato plants were grown in field trials in Groß Lüsewitz (GL) and Quedlinburg (QB) (Germany, areas of the BAZ).

Trials at both sites consisted of 32 completely randomized plots; eight plots were planted with DESI, eight plots were planted with DC1, eight plots were planted with DL4, and eight plots were planted with DL5. Each of the 32 plots contained 15 plants. Soil parameters at both locations were analyzed by the Institute for Agricultural Analysis and Research (LUFA) Rostock (Germany) and are described in Table 1. The potatoes were planted as seed pieces on 28 May 1996 and harvested on 1 October 1996. In the second year the potatoes were planted on 16 April 1997 and harvested on 8 September 1997. Samples were collected on three occasions during the vegetation period corresponding to the growth stages of plants: young plants (growth stages 11–19); flowering plants (growth stages 61–69); senescent plants (growth stages 91–97), according to Hack et al. [19]. For sampling the potato plants, different combinations of experiments were performed as described in Table 2.

2.2. Determination of culturable bacterial counts

Plant roots with adhering soil taken from five plants and from one plot were aseptically sampled into sterile Stomacher bags and treated as one sample. Prior to cell extraction, 5 g of each pooled sample was transferred into a new Stomacher bag. Samples were extracted in a Stomacher laboratory blender (BagMixer, Interscience, St Nom, France) with different sterile solutions: (a) 20 ml PBS contained the following constituents per liter demineralized water: 8 g NaCl, 0.2 g KCl, 1.4 g Na_2HPO_4, 0.24 g KH_2PO_4 (all from Merck, Darmstadt, Germany); (b) 15 ml SDP contained per liter demineralized water: 1 g sodium deoxycholate (Merck), 25 g polyethylene glycol 6000 (Merck); (c) 0.5 g Chelex 100 resin (Bio-Rad, Hercules, CA, USA); (d) 15 ml demineralized water. The extraction protocol was based on the method of Herron and Wellington [20]. Five tubers from five plants from one plot were sampled into sterile Stomacher bags and treated as one sample. From each tuber surface, a thin layer was rasped under aseptic conditions with a commercial grater and 5 g of fresh tuber material was extracted in a Stomacher blender as described above. For each sample the three combined solutions (= 50 ml) were serially diluted and plated on R2A medium (Difco Laboratories, Detroit, MI, USA). Plates were incubated for 5 days at 20°C and bacterial colonies were counted to calculate the means of colonies ($\log_{10}$ CFU) based on fresh weight. Bacterial plate counts of the rhizosphere population were analyzed to determine differences between transgenic and non-transgenic plants and temporal effects over the growing season. The aerobic plate counts, for the geocaulosphere populations, were also analyzed for differences between the four plant lines and geocaulosphere populations from different soil types were compared. For further testing, bacterial isolates at each sampling time and microenvironment were randomly selected and subcultured in culture tubes on nutrient agar (Gibco, Paisley, UK) resulting in 2304 rhizosphere isolates and 1344 geocaulosphere isolates (Table 3). The culture tubes were stored at 4°C.

2.3. Screening for bacteria isolated from the rhizo- and geocaulosphere antagonistic against E. carotovora and V. dahliae

The in vitro inhibition of *E. carotovora* was determined in a dual culture assay on Luria-Bertani-agar (LB, Difco). 100 μl of an overnight culture of *E. carotovora* ssp. *atroseptica* was plated on LB agar and bacteria were streaked as broad bands. Zones of inhibition were measured after incubation at 20°C at 24 and 48 h. Two different test strains were investigated: *E. carotovora* ssp. *atroseptica* DSM 30168 and *E. carotovora* ssp. *atroseptica* (strain no. 459, National Collection of Plant Pathogenic Bacteria, Harpenden, UK).

The in vitro inhibition of *V. dahliae* was determined in a dual culture assay on Waksman agar (WA), according to Berg and Ballin [21]. Zones of inhibition were determined after 5 days of incubation at 20°C. The strain used, *V. dahliae* V16 (culture collection of the University of Rostock, Germany), was originally isolated from *Solanum tuberosum*.

2.4. Screening for IAA-producing bacteria isolated from the rhizo- and geocaulosphere

The microplate method used was a modification of the method developed by Sawar and Kremer [22].

All isolates were grown on half-strength tryptic soy agar (TSA, Gibco, Paisley, UK). For the colorimetric IAA assay, a 24-h culture of each isolate was suspended in sterile PBS to an optical density of approximately 0.5 at 500 nm. The suspension (500 μl) was added to 5 ml of growth medium, which contained the following per liter demineralized water: 5 g glucose, 0.025 g yeast extract, 0.204 g L-tryptophan. Controls were prepared by substituting bacterial suspension with sterile water. Tubes were incubated in the dark at 20°C for 72 h. Prior to analyses for auxin (IAA equivalents), 1 ml of growth medium of each isolate was centrifuged at 4°C for 10 min. The supernatant of each sample (150 μl) was dispensed into wells of 96-well microplates followed by addition of 100 μl of Salkowski reagent (2 ml 0.5 M $FeCl_3$ mixed with 98 ml 35% perchloric acid). Following reaction for 30 min, the color intensity was measured at 530 nm on a microplate reader (Spectramax-250, Molecular Devices, Sunnyvale, CA, USA).

2.5. Identification of bacterial antagonists

All isolates with antagonistic properties against *E. carotovora* and/or *V. dahliae* were identified on the basis of whole-cell cellular fatty acids, derivatized to methyl esters, i.e., FAME (fatty acid methyl esters), and analyzed by gas chromatography using the MIDI system (Microbial Identification System, Inc., Newark, NJ, USA). Strains which could not be identified by FAME analysis were additionally tested by Biolog (Biolog, Hayward, CA, USA) or using the analytical profile index API 20E or 20NE (bioMérieux, France).

2.6. Determination of root biomass

At the end of the growing season, the dry weight of roots was determined for all plant lines. For this analysis, eight replicates of pooled root samples (based on three roots) were taken for each plant line (DESI, DC1, DL4 and DL5), weighed and dried for 24 h at 105°C. For each plant line, the data of the calculated mean per root were analyzed and the results were tested for significances between the four lines.

2.7. Statistics

All statistical analyses were performed at $P < 0.05$ level with the SAS software package (Proc GLM or Proc GENMOD in SAS release 6.12; SAS Institute, Cary, NC, USA). Bacterial counts were transformed to $\log_{10}$ values before two-factor analysis of variance (rhizosphere: plant lines and growth stages [sampling times]; geocaulosphere: plant lines and field sites [GL, QB]). Similarly, both the percentages of IAA-producing bacteria and of antagonistic bacteria were studied by two-factor analysis of variance. Root dry weight data of DESI, DC1, DL4 and DL5 were analyzed by multiple comparison of the means of one root per plant line.

3. Results

3.1. Determination of culturable bacterial counts

The rhizosphere was sampled at three different times during the monitoring period according to different growth stages of potato plants. The number of aerobic bacteria that could be recovered from the rhizosphere on R2A was not significantly different between the four plant lines and remained at levels of ca. $\log_{10}$ 7.7 CFU g^{-1} fresh weight (fw) of root (Fig. 1). In the second year (1997) significantly more bacteria could be recovered at the second (flowering plants) and third (senescent plants) sampling time for all four plant lines.

The geocaulosphere was only sampled at the end of growing season (senescent potato plants) when tubers were fully developed. The number of aerobic

Table 1
Characteristics of soils used in this study; GL (Groß Lüsewitz), QB (Quedlinburg)

Parameter[a]	GL	QB
Texture	sandy loam	silt loam
pH	5.9	5.9
Organic matter (%)	1.1	2.2
P_2O_5 (mg 100 g^{-1} soil)	20	36
K_2O (mg 100 g^{-1} soil)	14	31
Mg (mg 100 g^{-1} soil)	10	15

[a]Analyzed by the Institute for Agricultural Analysis and Research (LUFA), Rostock (Germany).

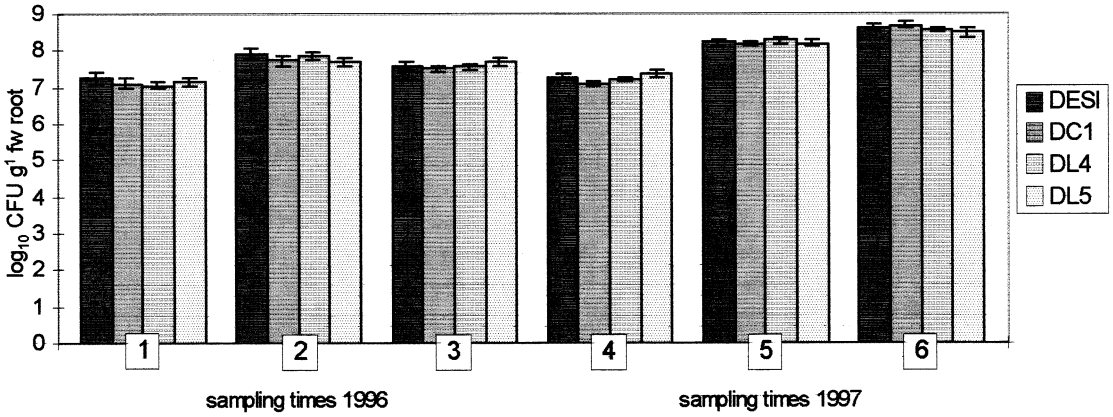

Fig. 1. Mean values ± S.E.M. of bacterial plate counts of the rhizosphere for the field site in Groß Lüsewitz. Log_{10} of colony-forming units (CFU) per gram of root fresh weight of rhizosphere population densities of the four plant lines DESI (non-transgenic), DC1 (transgenic control), DL4 and DL5 (transgenic test lines). Populations at each sampling time (1 and 4: young plants; 2 and 5: flowering plants; 3 and 6: senescent plants) represent the average bacterial population densities of eight replicates. No significant difference was observed between the means of the four plant lines ($P = 0.7704$) over all sampling times. Significant differences ($P < 0.0001$) in bacterial plate counts in the rhizosphere were determined between the means of the different stages of plant development over all plant lines in 1997.

bacteria that could be recovered from the geocaulosphere (tuber surface) on R2A was not significantly different between the four plant lines and remained at ca. log_{10} 7.1 CFU g^{-1} fresh weight of tuber (Fig. 2). Significantly fewer ($P < 0.0001$) bacteria could be detected from the caulosphere from plants from Quedlinburg (log_{10} 6.93 CFU g^{-1} tuber fw) than from the field site of Groß Lüsewitz (log_{10} 7.37 CFU g^{-1} tuber fw). Overall, during the monitoring period, the number of culturable bacteria from the rhizosphere was significantly larger ($P = 0.0001$) than from the geocaulosphere.

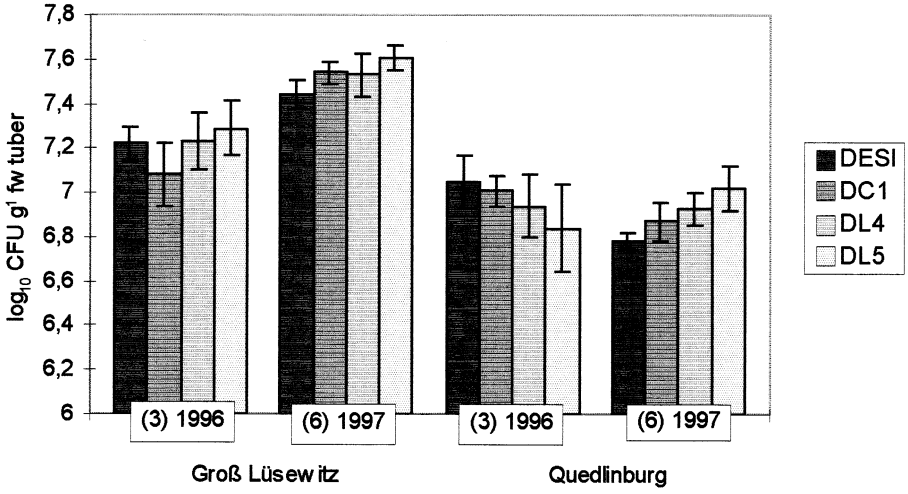

Fig. 2. Mean values ± S.E.M. of bacterial plate counts of the geocaulosphere for the field sites in Groß Lüsewitz and Quedlinburg. Log_{10} of colony-forming units (CFU) per gram of tuber fresh weight of geocaulosphere population densities of the four plant lines DESI (non-transgenic), DC1 (transgenic control), DL4 and DL5 (transgenic test lines). No significant difference was observed between the means of the four plant lines over both years and locations ($P = 0.6007$). Significant differences in bacterial plate counts in the geocaulosphere were determined between the means of both locations over all plant lines and both years ($P < 0.0001$).

Table 2
Monitoring experiments to evaluate the effects of T4-lysozyme-producing potato plants on plant-associated bacteria and root biomass

Sampling times 1996 and 1997	Sample material	Monitoring experiments
1: young plants	fresh roots	CFU[a] of culturable bacteria
2: flowering plants		screening for bacteria antagonistic against *E. c. a.*[b]
		screening for bacteria antagonistic against *V. d.*[b]
		screening for IAA-producing bacteria[c]
		identification of antagonists[d]
3: senescent plants	fresh roots and tubers	CFU[a] of culturable bacteria
		screening for bacteria antagonistic against *E. c. a.*[b]
		screening for antagonistic bacteria against *V. d.*[b]
		screening for IAA-producing bacteria[c]
		identification of antagonists[d]
	whole fresh roots	determination of root dry weight per plant

[a]Colony-forming units (CFU) based on the total number of culturable bacteria on R2A medium containing cycloheximide (100 mg l^{-1}).
[b]Dual culture assay on Luria-Bertani agar (*E. carotovora* ssp. *atroseptica*) and Waksman agar (*V. dahliae*).
[c]Microplate method according to Sawar and Kremer [22].
[d]Fatty acid methyl ester (FAME) profiles, Biolog (Biolog, Hayward, CA), API 20E/API 20NE (bioMérieux, France).

3.2. *Screening for bacteria isolated from the rhizo- and geocaulosphere antagonistic against E. carotovora and V. dahliae*

A total of 3648 isolates (Table 3) were analyzed for their ability to suppress the two phytopathogens *E. carotovora* and *V. dahliae* in a dual culture assay (Table 4). Ninety-six bacterial isolates (Table 3) were randomly picked and investigated per plant line and sampling time. The percentage of antifungal isolates in the rhizosphere of all plant lines varied strongly between the different samplings and ranged from 1 to 11. There were no significant differences in the percentage of antifungal bacteria isolated from the rhizosphere between the four plant lines at any of the samplings (Table 4). No antibacterial isolate was observed from the rhizosphere in 1996, whilst 40 were isolated in 1997, suggesting temporal variation. In 1997 the percentage of rhizosphere isolates with antibacterial activity ranged from 0 to 7.

In the geocaulosphere, antifungal bacteria represented up to 8% of the population (Table 4). Significant differences between the lines were found in 1997 for the field site in GL. On average 8% of the bac-

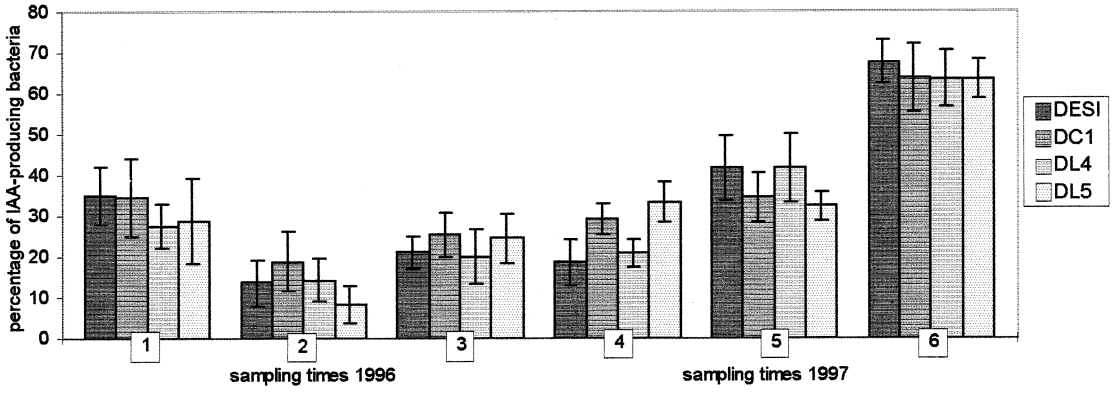

Fig. 3. Mean values ± S.E.M. of IAA-producing bacteria in the rhizosphere for the field site in Groß Lüsewitz. Percentage of IAA-producing bacteria in the rhizosphere of potato (mean ± S.E.M.) of the four plant lines DESI (non-transgenic), DC1 (transgenic control), DL4 and DL5 (transgenic test lines). In the rhizosphere no significant difference in IAA-producing bacteria was observed among the means of the four plant lines over all sampling times ($P = 0.9475$). Statistical significances were determined between the means of the six sampling times (1 and 4: young plants; 2 and 5: flowering plants; 3 and 6: senescent plants) over all plant lines ($P < 0.0001$).

Table 3
Composition of selected bacteria for investigating IAA-producing bacteria and antagonists

Number of *rhizosphere*[a] isolates							
Plant line	Sampling times 1996			Sampling times 1997			Total
	1	2	3	4	5	6	
DESI	96	96	96	96	96	96	576
DC1	96	96	96	96	96	96	576
DL4	96	96	96	96	96	96	576
DL5	96	96	96	96	96	96	576

Number of *geocaulosphere*[a,b] isolates					
Plant line	Sampling time 1996		Sampling time 1997		Total
	GL	QB	GL	QB[c]	
DESI	96	96	96	48	336
DC1	96	96	96	48	336
DL4	96	96	96	48	336
DL5	96	96	96	48	336

[a]Isolates were randomly selected from R2A and subcultured in culture tubes on nutrient agar. The culture tubes were stored at 4°C.
[b]Investigated only at the end of the growing season at both field sites (GL, Groß Lüsewitz; QB, Quedlinburg).
[c]Reduced sample size due to partial destruction of the field.

teria isolated from the transgenic line DL5 were antagonistic against fungi whilst from the control plants only 0–2% of bacteria were antifungal. But these differences were not observed either in the other samplings or for the field site in QB. The percentage of antibacterial isolates ranged from 0 to 2. Only one isolate of the geocaulosphere with antibacterial properties against *E. carotovora* ssp. *atroseptica* was isolated in the first year.

Overall, the percentage of antifungal bacteria in the rhizosphere (GL: 5.8%) was significantly higher ($P = 0.0187$) than in the geocaulosphere (GL: 3.4%, and QB: 4.0%) over all plant lines. In general, significantly more antifungal isolates were found in the rhizo- and geocaulosphere than isolates with antibacterial activity.

3.3. Screening for IAA-producing bacteria isolated from the rhizo- and geocaulosphere

The same 3648 isolates (Table 3) from the two investigated microenvironments were analyzed for their ability to produce the plant growth hormone auxin (IAA equivalents) in vitro. A large number of bacterial isolates from both microenvironments were able to excrete this plant growth hormone. The percentage of positive isolates in the rhizosphere

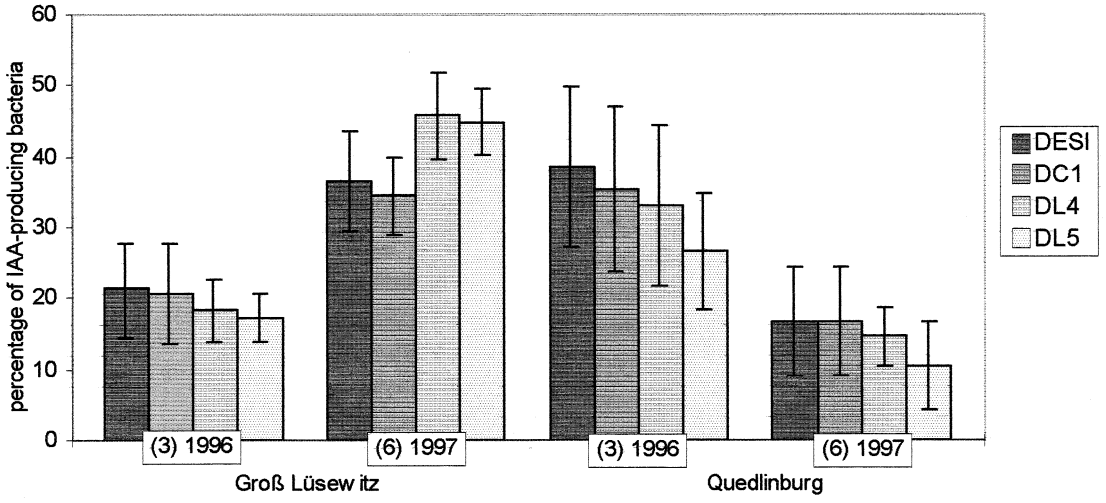

Fig. 4. Mean values ± S.E.M. of IAA-producing bacteria in the geocaulosphere for the field sites in Groß Lüsewitz and Quedlinburg. Percentage of IAA-producing bacteria in the geocaulosphere of potato (mean ± S.E.M.) of the four plant lines DESI (non-transgenic), DC1 (transgenic control), DL4 and DL5 (transgenic test lines). In the geocaulosphere no significant difference in IAA-producing bacteria was observed among the means of the four plant lines over both locations ($P = 0.9204$). No significant difference was found between the means of the two locations ($P = 0.5262$) over all plant lines.

Table 4
Percentage of bacteria antagonistic against *V. dahliae* and *E. carotovora* in the rhizosphere and geocaulosphere of transgenic and non-transgenic potatoes

Rhizosphere							Geocaulosphere				
Line	Sampling times 1996			Sampling times 1997			Line	Groß Lüsewitz		Quedlinburg	
	1	2	3	4	5	6		1996	1997	1996	1997
Percentage of bacteria antagonistic against *V. dahliae*											
DESI	6.25 ± 5.98	8.33 ± 17.81	9.37 ± 12.14	2.08 ± 3.60	8.33 ± 9.31	3.12 ± 4.03	DESI	6.25 ± 5.89	0	2.08 ± 3.85	6.25 ± 7.14
DC1	5.20 ± 8.83	2.38 ± 4.06	7.14 ± 10.12	5.20 ± 8.26	8.33 ± 11.02	9.37 ± 8.77	DC1	4.16 ± 11.78	2.08 ± 3.60	4.16 ± 6.29	6.25 ± 5.51
DL4	6.25 ± 7.38	4.16 ± 6.29	1.04 ± 2.94	8.33 ± 7.21	6.25 ± 5.51	4.16 ± 5.89	DL4	2.08 ± 3.85	2.08 ± 5.51	4.16 ± 6.29	4.16 ± 5.79
DL5	6.25 ± 9.70	2.08 ± 3.85	4.16 ± 6.29	2.08 ± 3.60	11.45 ± 7.14	7.29 ± 6.50	DL5	2.08 ± 3.85	8.33 ± 9.31	1.04 ± 2.94	4.16 ± 5.89
Percentage of bacteria antagonistic against *E. carotovora*											
DESI	0	0	0	0	3.12 ± 5.79	1.04 ± 2.75	DESI	0	0	0	2.08 ± 3.60
DC1	0	0	0	3.12 ± 5.79	3.12 ± 5.79	6.25 ± 5.51	DC1	0	0	0	0
DL4	0	0	0	2.08 ± 3.60	5.20 ± 10.97	4.16 ± 8.33	DL4	0	0	0	0
DL5	0	0	0	2.08 ± 3.60	4.16 ± 5.89	7.29 ± 8.77	DL5	0	1.04 ± 2.75	0	2.08 ± 3.60

Bacteria antagonistic against *V. dahliae* in the rhizosphere of potato. DESI (non-transgenic), DC1 (transgenic control), DL4 and DL5 (transgenic test lines). On the basis of 3648 tested bacterial isolates (1996: 1–3; 1997: 4–6) the average of antifungal bacteria was determined. In the rhizosphere no significant difference in antifungal bacteria was observed between the means of the four plant lines ($P = 0.8642$). No statistical significance was determined between the means of the six sampling times over all lines ($P = 0.3484$). In the geocaulosphere no significant difference in antifungal bacteria was observed between the means of the four plant lines ($P = 0.9353$) and between the means of the two locations ($P = 0.8288$). Bacteria antagonistic against *E. carotovora* in the rhizosphere of potato. In the rhizosphere no significant difference in antibacterial isolates was observed between the means of the four plant lines ($P = 0.3599$). No statistical significance was determined between the means of the three (4–6) sampling times over all lines ($P = 0.2122$). In the geocaulosphere no significant difference in antibacterial isolates was observed between the means of the four plant lines ($P = 0.8078$) and between the means of the two locations ($P = 0.7533$).

Table 5
Biodiversity of antagonistic bacteria associated with transgenic and non-transgenic potatoes

Species	Number of isolates identified[a]							
	rhizosphere				geocaulosphere			
	DESI	DC1	DL4	DL5	DESI	DC1	DL4	DL5
Actinobacillus lignieresii	–	1	–	–	–	–	–	–
Agrobacterium tumefaciens	1	–	–	–	–	–	1	1
Bacillus amyloliquefaciens	–	–	–	–	–	1	–	–
Bacillus cereus	–	–	–	–	–	–	1	–
Bacillus megaterium	–	–	–	–	–	1	–	–
Bacillus mycoides	–	–	–	–	–	–	1	–
Bacillus thuringiensis	–	–	–	–	–	–	1	–
Burkholderia cepacia	1	1	–	–	1	–	–	–
Comamonas acidovorans	–	–	–	–	1	2	1	–
Enterobacter intermedius	–	–	–	–	–	–	1	–
Francisella philomiragia	–	1	–	–	–	–	–	–
Paenibacillus macerans	–	–	–	–	–	–	–	1
Paenibacillus polymyxa	–	–	–	1	–	–	–	–
Pantoea agglomerans	–	2	3	3	–	–	–	–
Proteus vulgaris	–	–	1	–	–	–	–	–
Pseudomonas chlororaphis	5	3	3	5	1	1	–	–
Pseudomonas corrugata	1	–	–	1	–	–	–	–
Pseudomonas fluorescens	5	4	3	2	1	1	–	2
Pseudomonas faecalis	–	–	–	1	–	–	–	–
Pseudomonas marginalis	2	2	–	–	–	1	1	–
Pseudomonas putida	1	7	2	4	3	4	2	5
Pseudomonas savastoni	–	–	1	–	–	–	–	1
Pseudomonas syringae	2	3	1	1	–	–	–	–
Serratia grimesii	3	1	–	–	–	–	–	–
Serratia plymuthica	–	1	–	1	–	–	–	–
Serratia proteamaculans	–	–	–	1	–	–	–	–
Sphingomonas paucimobilis	–	–	–	–	–	1	–	–
Stenotrophomonas maltophilia	1	–	–	–	1	–	1	2

[a]Identification of 28 species based on FAME analysis or API 20E/20NE and Biolog.

ranged from 8.4 to 67.7 (Fig. 3). In 1996, for all plant lines, the percentage of IAA-producing bacteria in the rhizosphere decreased from young to flowering plants, and increased from flowering to senescent plants. No significant difference could be detected between the four plant lines DESI, DC1, DL4 and DL5 at any of the sampling times. In 1997 flowering and senescent plants had significantly higher levels of IAA-producing bacteria than young plants (Fig. 3).

In the geocaulosphere the percentage of positive isolates ranged from 10.4 to 45.8. In 1996 higher levels of IAA-producing bacteria in the geocaulosphere were found in the QB field, whereas in the second year the reverse result was obtained with higher levels found at the GL site (Fig. 4).

In the rhizosphere significantly ($P = 0.0035$) higher percentages of IAA-producing isolates (32.6%) were found than in the geocaulosphere (GL: 29.9%, QB: 24.0%) for all four plant lines.

3.4. Identification of bacterial antagonists

All isolates with antagonistic properties against *E. carotovora* and/or *V. dahliae* were characterized according to their fatty acid patterns (FAME). Isolates which could not be identified by FAME analysis were additionally tested by Biolog and/or API. The relative abundances of the common, recurrently isolated bacterial antagonists are shown in Table 5. From the 124 antagonistic bacteria characterized from the six sampling dates, 28 species could be

Table 6
Biomass of roots of the four plant lines at the end of growing season at the field sites of Groß Lüsewitz (GL) and Quedlinburg (QB)

Line	Root dry weight (g root^{-1})[a]			
	1996		1997	
	GL	QB	GL	QB[b]
DESI	4.4	4.4	5.9	n.i.
DC1	3.6	4.1	5.5	n.i.
DL4	2.8[c]	2.3[c]	2.9[c]	n.i.
DL5	4.0	3.8	6.4	n.i.

[a]Three roots from one plot were sampled into Cryovac bags (Grace Multiflex GmbH, Flensburg, Germany) and treated as one sample; samples (eight replicates per plant line) were weighed and dried for 24 h at 105°C.
[b]Not investigated (n.i.).
[c]Significantly lower than root dry weights of the other four plant lines.

identified. Most (71.4%) of the identified species were Gram-negative, belonging to the group of fluorescent pseudomonads. *Pseudomonas putida* was the most common species comprising 22.6% of all antagonistic isolates. *Pseudomonas chlororaphis* and *P. fluorescens* each comprised another 14.5% of the antagonistic isolates. Non-fluorescent pseudomonads which could be identified were *Burkholderia cepacia, Sphingomonas paucimobilis* or *Stenotrophomonas maltophilia*. In contrast, only 6.5% of the antagonistic bacteria (28.6% of the identified species) were Gram-positive. All of them belonged to the genera *Bacillus* or *Paenibacillus*. For 14 other species, only one antagonistic isolate could be detected.

Seven species, *Actinobacillus lignieresii, Bacillus amyloliquefaciens, Bacillus megaterium, Burkholderia cepacia, Francisella philomiragia, Serratia grimesii* and *Sphingomonas paucimobilis*, were isolated only from control plants (DESI, DC1). These isolates represented 9.7% of all identified antagonistic isolates in this work.

3.5. Determination of root biomass

At the end of the growing season the dry weight of roots was determined. Over the monitoring period, the root biomass of the transgenic line DL4 was significantly ($P = 0.0001$) lower than the biomasses of the three other lines DESI, DC1 and DL5 (Table 6). No significant difference was found between the root dry weights of these three plant lines. The mean dry weight of DESI, DC1 and DL5 was 4.7 g per root.

4. Discussion

The secretion of the T4-lysozyme into the intercellular spaces changes the composition of common root exudates or of common compounds of the whole plant tissues of transgenic potato plants. Over the period of field experiments in 1996 and 1997, expression of the foreign T4-lysozyme gene was monitored by Western blotting analysis (data not shown) using an affinity-purified polyclonal anti-T4-lysozyme antibody from rabbit [13]. T4-lysozyme could be detected in protein extracts in each sample (leaves) over the two growing seasons. The percentage levels of total soluble protein varied from 3 to 6×10^{-4} (1996) and 7 to 10×10^{-4} (1997) [23]. The expression level of T4-lysozyme was higher for the transgenic line DL4 than for the line DL5 [23].

The plant species-dependent [24] or cultivar-dependent [25] colonization of the rhizosphere by bacteria has been reported. Oger et al. [4] demonstrated that the increased number of mannopine (produced by transgenic potatoes)-utilizing bacteria detected in the rhizosphere of transgenic plants appears to be related to the production of mannopine by the plants. All these reports support the thesis of Rovira [26] that root exudates play a key role in the selective stimulation of microorganisms. If there is any effect of T4-lysozyme in potato, it should be detected upon investigation of root- and tuber-associated bacteria. The results obtained from all the experiments in this study indicated that T4-lysozyme produced by transgenic potatoes did not cause any detectable effect on the counts of culturable aerobic bacteria. There was no significant difference in total bacterial counts between the four plant lines in both microenvironments. The detected aerobic plate counts in the rhizosphere ($\log_{10}$ 7.7 CFU g^{-1} root fw) were comparable with counts determined on other host plants, e.g. $\log_{10}$ 7–8 CFU g^{-1} fw for barley and wheat [27], $\log_{10}$ 8 CFU g^{-1} fw for maize [28], and $\log_{10}$ 8.17 CFU g^{-1} fw for oilseed rape [29]. Although only 1–10% of bacteria can be recovered

[30], the widely used methodology (dilution plates), despite its faults and problems, is still much used [15]. However, the effect on the larger group of unculturable bacteria could not be detected with this method. Therefore, culture-independent methods, such as temperature or denaturing gradient gel electrophoresis, should be used [31,32].

No difference in the percentage of potentially beneficial bacteria between different plant lines was observed. With respect to the distribution of beneficial bacteria, the abundance of IAA-producing isolates was significantly higher than that of isolates which were antagonistic. These two parameters, IAA production and antagonistic activity, were chosen to monitor the main functions of potentially beneficial bacteria. Microbially derived IAA has been implicated in the stimulation of growth or pathogenesis of plants [33]. Since auxins are also produced by plants, one way to prove the involvement of bacterial IAA in the stimulation of plant growth would be to test either Tn*5* mutants deficient in IAA production or mutants overproducing these substances. Barbieri and Galli [34] showed that mutants of *Azospirillum brasiliense* deficient in IAA production did not promote the formation of lateral roots as compared to the wild-type. Conversely, an IAA-overproducing mutant of *P. fluorescens* stimulated the root development of cherry [35]. These results indicate a concentration-dependent effect of IAA. Concentrations of IAA produced by the investigated isolates were comparable with those of other plant growth-promoting rhizobacteria [21,36]. Only a small number of bacteria were antagonistic to our model pathogens. The proportion of the isolates which were antagonistic ranged from 1 to 10%. Hebbar et al. [37] reported that 1–7% of the rhizobacteria of maize were antifungal. Kremer et al. [38], who studied the ecology of the rhizosphere of different weeds, reported that antifungal bacteria represented up to 18% of culturable bacteria.

The results do not indicate any influence of transgenic T4-lysozyme on the function of potentially beneficial bacteria. However, it is possible that this function was performed by other bacterial species because of the varying sensitivity of rhizobacteria to T4-lysozyme [10]. To analyze the composition and biodiversity of these beneficial bacteria the active isolates were identified by various methods. The species composition of the antagonistic bacteria allowed us to estimate potential changes in the bacterial populations between transgenic and non-transgenic plants. Antagonistic strains of seven species were only found on control plants. Most of them were found once, with the exception of *Serratia grimesii* which was found four times. However, the composition of the bacterial community in the rhizosphere is influenced by various abiotic and biotic conditions and the specificity of each plant is also very high [28]. Considering this, the differences found were relatively low. Further approaches, with the inoculation of seed potatoes with *S. grimesii* strains or other T4-lysozyme-sensitive isolates, could clarify possible effects of transgenic T4-lysozyme. The release of both a modified *P. fluorescens* and the wild-type resulted in significant, but transient, perturbations of some of the culturable components of the indigenous microbial community of wheat [39,40].

Twenty-eight species with antagonistic effects were identified. The group of fluorescent pseudomonads associated with potatoes included *P. chlororaphis, P. fluorescens* and *P. putida*. They were reported to be the most beneficial and antifungal rhizobacteria of potato [41,42]. The non-fluorescent pseudomonads, e.g., *Stenotrophomonas maltophilia* or *Burkholderia cepacia*, were also reported as members of the naturally occurring rhizosphere community [43,44]. Other qualitatively important species were members of the genus *Serratia*. They were also isolated as antagonistic strains from roots [45], but the detected species *S. grimesii* has not been previously reported as a plant-associated bacterium.

Determination of the root weight allows indirect conclusions concerning the influence of T4-lysozyme produced by transgenic potato plants on plant growth-promoting rhizobacteria. If the root development is disturbed, the plant growth and health may be clearly reduced. Differences in root dry weight were observed for both years among the transgenic line DL4 and the three other lines (DESI, DC1 and DL5). The reason for this difference is unclear. The two transgenic T4-lysozyme-expressing lines differ in expression level to the effect that DL4 shows higher levels than DL5 [23]. In several laboratory and field studies there have been unintentional changes in plant characteristics due to genetic manipulation

and position effects from insertion site(s) [6]. We suppose the phenotypic deviation of DL4 is caused by a genotypic position effect or by a somaclonal variation.

The results suggest that the methods used were suitable because they were statistically valid: e.g., significant differences in culturable geocaulosphere bacteria between different soil types, in the percentage of strains antagonistic against *V. dahliae* between the two locations, and in the percentage of IAA-producing strains between the sampling times.

The monitoring of plant-associated bacteria and comparison of the collected data with established information should be included as a risk assessment procedure regarding plant-associated bacteria [7]. Our results provide an example for performing risk assessment studies for transgenic plants and for studying the effects on potentially beneficial bacteria under a variety of environmental conditions, e.g., different soil types.

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