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Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere

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Abstract

A containment experiment was carried out in order to evaluate possible shifts in eubacterial and *Pseudomonas* rhizosphere community structures due to the release of genetically modified Basta-tolerant oilseed rape and the associated herbicide application. Treatments included cultivation of the transgenic plant as well as of the wild-type cultivar in combination with mechanical removal of weeds and the application of the herbicides Basta (active ingredient: glufosinate) and Butisan S (active ingredient: metazachlor). Rhizosphere soil was sampled from early and late flowering plants as well as from senescent plants. A culture-independent approach was chosen to characterize microbial communities based on denaturing gradient gel electrophoresis of 16S rRNA gene fragments amplified from rhizosphere DNA using eubacterial and *Pseudomonas*-specific PCR primers. Dominant pseudomonads in the rhizosphere were analyzed by sequence analysis. Whole community and *Pseudomonas* electrophoresis fingerprints revealed slightly altered microbial communities in the rhizosphere of transgenic plants; however, effects were minor as compared to the plant developmental stage-dependent shifts. Both herbicides caused transient changes in the eubacterial and *Pseudomonas* population structure, whereas differences due to the genetic modification were still detected at the senescent growth stage. The observed differences between transgenic and wild-type lines were most likely due to unintentionally modified plant characteristics such as altered root exudation.

Keywords: Transgenic oilseed rape; Denaturing gradient gel electrophoresis; Basta; Butisan S; *pat* gene; Rhizosphere

1. Introduction

Herbicides are chemical additives frequently used in agriculture and several of them cause considerable environmental pollution and human health problems due to their persistence and toxicity. In order to improve weed control, transgenic plants that allow non-selective herbicide application at any time have been constructed. One product which is commercially available is transgenic oilseed rape that contains the *pat* gene from *Streptomyces* encoding phosphinothricin acetyltransferase and tolerates glufosinate (phosphinothricin). The genetically modified plant is supposed to be used in combination with this herbicide, whereas in conventional agriculture other herbicides such as Butisan S (active ingredient: metazachlor) are applied for weed control. Butisan S is used as pre-emergence and early post-emergence control of rapeseed and annual grasses. Lysimeter and outdoor studies indicated that Butisan S and its metabolites are rapidly degraded in soil [1]. Effects on the soil microflora have not been reported. Glufosinate is produced naturally by *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus* [2,3], and showed weak antibacterial and antifungal activity [2]. A few studies also indicated that several bacteria are sensitive towards this herbicide, which may lead to decreased soil fertility [4,5]. Kriete and Broer [5] demonstrated a negative effect due to glufosinate application on the growth of nitrogen-fixing rhizobia, nodule formation and nitrogen fixation. Nevertheless, many bacteria are resistant to glufosinate or are even able to degrade the herbicide by deamination and decarboxylation [4,6–10].

One of the major concerns in the use of transgenic crops

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is the true potential for unwanted side effects on key soilborne microorganisms. Furthermore, altered agricultural practices such as replacing conventional cultivation of oilseed rape by herbicide-tolerant plants in combination with the appropriate herbicide may lead to changes of microbial diversity.

The genus *Pseudomonas* includes bacteria with a broad range of functions and its members are known for their metabolic versatility. Many strains are able to degrade a broad range of environmental pollutants [11,12] and also play an important role in the degradation of herbicides [13,14]. Some *Pseudomonas* species are potential pathogens for humans, animals or plants [15,16], whereas others are prominent rhizosphere organisms and have frequently been reported to exhibit plant growth-promoting and biocontrol functions. This is mainly achieved by the production of siderophores [17] or antimicrobial compounds [16,18].

Population analysis of pseudomonads has been mainly based on cultivation and subsequent characterization [19–21]. As the majority of bacteria is not accessible to cultivation, various cultivation-independent techniques have been developed to characterize microbial communities, including denaturing gradient or temperature gradient gel electrophoresis (DGGE/TGGE) [22,23], terminal restriction fragment length polymorphism [24,25], and polymerase chain reaction (PCR)–single strand conformation polymorphism [26]. Most of them are based on sequence differences in the 16S rRNA gene and target all eubacteria rather than particular bacterial groups. Although members of the genus *Pseudomonas* are in general easy to cultivate, they may enter a viable but non-culturable state induced by stress [27–29]. Troxler et al. [29] tested the persistence of a *Pseudomonas fluorescens* biocontrol strain in large outdoor lysimeters and found that the majority of cells were viable but non-culturable. Furthermore, various *Pseudomonas* spp. colonizing potato endophytically were identified by cultivation-independent analysis which were not detected by isolation (Reiter, personal communication). Therefore, various genus-specific PCR primers and oligonucleotide probes targeting 16S rRNA genes have been developed in order to detect and quantify pseudomonads in natural environments [30–33].

The objective of this study was to investigate possible effects of the genetically modified glufosinate-tolerant oilseed rape or the associated herbicide application on the rhizosphere microflora. Therefore, eubacterial and *Pseudomonas* populations were monitored by using a cultivation-independent approach at different plant growth stages in the rhizospheres of wild-type and transgenic oilseed rape cultivated in combination with the appropriate herbicides.

2. Materials and methods

2.1. The containment experiment

Seeds of the transgenic winter oilseed rape, variety Liberator C/6AC (DSV, Salzkotten-Thüle, Germany), containing the *pat* gene for Basta tolerance as well as the non-transgenic variety were sown on TKS2 soil substrate (Frux ED 63 not pasteurized soil substrate; Gebr. Patzer, Sinntal-Jossa, Germany; 100–250 mg l^{-1} nitrogen, 100–250 mg l^{-1} potassium oxide, and 100–200 mg l^{-1} phosphor pentoxide) in the greenhouse. After germination plantlets were vernalized for 68 days at 3–5°C.

Pots (50 l) were filled with soil from agricultural fields around the ARC Seibersdorf research Center (44% sand, 34% silt, 22% clay, 0.15% N, 4.73% C, pH 7.8). Two weeds that commonly co-occur with oilseed rape, *Capsella bursa-pastoris* and *Descurainia sophia*, were sown and six rapeseed plantlets were transplanted into each pot. The experiment was performed in containment to conform with Austrian regulations and simulated agricultural conditions. Pots were arranged in a completely randomized block design and the following treatments were replicated eight times: (i) non-transgenic rapeseed plants with Butisan S (treatment M), (ii) non-transgenic rapeseed plants with mechanical removal of weeds (treatment MM), (iii) transgenic rapeseed plants with Basta (treatment G), and (iv) transgenic rapeseed plants with mechanical removal of weeds (treatment GM). Butisan S (active ingredient: metazachlor, 2-chloro-*N*-(*N*-2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-ylmethyl)acetamide) was applied by spraying in a concentration of 0.01 mg cm^{-2} (recommended concentration), whereas the concentration of Basta (active ingredient: glufosinate, ammonium-DL-homoalanin-4-yl(methyl)phosphinate) was 0.03 mg cm^{-2} (recommended concentration). Herbicide application and sampling time points are summarized in Table 1. On each sampling

Table 1
Overview of the containment experiment showing planting time, times of herbicide applications and sampling points

Treatment	Oilseed rape variety	Herbicide	Planting	Herbicide application	Sampling
M	Liberator C/6AC	Butisan S	11 May	25 May	6 June[a], 6 July[b], 6 September[c]
MM	Liberator C/6AC	–	11 May	–	6 June, 6 July, 6 September
G	Liberator C/6AC–*pat*	Basta	11 May	4 June, 4 July	6 June, 6 July, 6 September
GM	Liberator C/6AC–*pat*	–	11 may	–	6 June, 6 July, 6 September

[a]Early flowering stage.
[b]Late flowering stage.
[c]Senescent stage.

date two plants per pot were harvested. Bulk soil was removed by shaking the plants, and the tightly adhering soil, which was defined as rhizosphere soil, was sampled. Subsequently, the soil was sieved using a 1-mm sieve, thoroughly mixed and stored at −20°C.

2.2. DNA isolation and PCR conditions

DNA was isolated according to previously published protocols [25,34]. Rhizosphere soil (~0.15 g) and 3.8 mg of lysozyme were resuspended in 0.75 ml sodium phosphate buffer (pH 8.0), and this mixture was incubated at 37°C for 15 min. Subsequently, the samples were cooled on ice, 750 mg of acid-washed glass beads (Sigma; 0.09–0.13 mm) were added, and bead-beating was performed three times for 90 s at full speed in a mixer mill (type MM2000; 220V, 50 Hz; Retsch, Haam, Germany) with intervals of 30 s. After 45 μl of a 20% sodium dodecyl sulfate solution had been added, the mixture was added at room temperature for 15 min. Then, one volume of phenol was added, and the mixture was mixed and centrifuged for 5 min at 10 000 × *g*. The organic phase was extracted again with 0.12 M sodium phosphate solution, and the aqueous phases were pooled and extracted with one volume of chloroform. Samples were centrifuged for 5 min at 10 000 × *g*, and 0.1 volume of 5 M potassium acetate was added to the aqueous phase to precipitate humic acids. After incubation for 15 min at room temperature, samples were centrifuged for 5 min at 10 000 × *g*, and the resulting pellet was washed with 70% ethanol, dried and resuspended in 80 μl of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). For further purification, spin columns that contained Sepharose CL-6B (Amersham Pharmacia Biotech, Uppsala, Sweden) and polyvinylpyrrolidone (20 mg of Sepharose CL-6B ml^{-1}) were prepared. Passage through two columns was needed to remove all PCR-inhibiting substances.

The primer pair fD1 and rD1 [35] was used to amplify eubacterial 16S rRNA genes by PCR. Reactions were carried out in a PT-100™ thermocycler (MJ Research, Watertown, USA), applying an initial denaturation step of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 1 min at 52°C and 2 min at 72°C and a final extension step at 72°C for 4 min. The reaction mixtures (50 μl) contained 1× reaction buffer (Invitrogen, Carlsbad, CA, USA), 200 μM (each) dATP, dGTP, dTTP and dCTP (Invitrogen), 2.5 mM $MgCl_2$, 0.2 μM (each) primer, 10 ng template DNA and 2 U *Taq* DNA polymerase (Invitrogen). For the amplification of *Pseudomonas* 16S rRNA genes PCR products were purified using a NucleoTraPCR kit (Macherey-Nagel, Düren, Germany) and used as a template for *Pseudomonas*-specific PCR, which was described by Johnsen et al. [32]. In order to separate PCR amplicons by DGGE the forward primer 8f [35] was amended with a GC-clamp and used in combination with the *Pseudomonas*-specific primer PSM_G [30].

2.3. DGGE

DGGE analysis was performed according to Muyzer et al. [22] using the DCode™ universal mutation detection system (Bio-Rad). PCR samples (7 μl) were loaded onto 6% w/v polyacrylamide gels (ratio acrylamide to bisacrylamide, 37:1) submerged in 1×TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA, pH 7.4). For the separation of eubacterial 16S rRNA genes polyacrylamide gels were made with a denaturing gradient ranging from 45 to 70% (where 100% denaturant contains 7 M urea and 40% formamide), whereas a gradient from 30–70% was used for the DGGE analysis of pseudomonads. Gels were electrophoresed at 70 V for 16 h at 60°C. After electrophoresis, DNA bands were visualized by silver staining. In order to determine similarities between DGGE profiles, a binary matrix was established recording the absence or presence of bands. Cluster analysis was performed based on similarities calculated according to Nei and Li [36] and using the UPGMA (unweighted pair group with mathematical averages) method. Tree generation was performed applying the TREECON software package [37] with 100 bootstrap replications.

2.4. Cloning of Pseudomonas 16S rRNA genes

Rhizosphere soil from the first and third sampling was used to generate clone libraries of *Pseudomonas* partial 16S rRNA genes. PCR products obtained with the primers 8f and PSM_G (see above) were purified using the NucleoTraPCR kit (Macherey-Nagel). PCR amplicons were ligated into the pGEM-T plasmid vector (Promega, Mannheim, Germany) and transformed into NovaBlue Singles competent cells (Novagen, Madison, WI, USA) as recommended by the manufacturer. Recombinants, appearing as white colonies on indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), were harvested, resuspended in 80 μl TE buffer and boiled for 10 min. Subsequently, cells were centrifuged for 5 min at 10 000 × *g* and supernatants (1 μl) were used in PCR reactions using the primers PSM_G and 8f-GC and the conditions described above to amplify cloned inserts. Subsequently, amplicons were electrophoresed on denaturing gradient gels as described above. Clones that showed the same running distance as members of the *Pseudomonas* community were used for sequence analysis.

2.5. Sequence analysis

For sequencing, clone inserts were amplified by PCR using the primers M13for and M13rev as described for the amplification of eubacterial 16S rRNA genes. PCR products were purified with the NucleoTraPCR kit (Macherey-Nagel) according to the manufacturer's instructions and used as templates in sequencing reactions. Sequencing

was performed by the dideoxy chain termination method with the primers M13for and M13rev, respectively, using an ABI 373A automated DNA sequencer and the ABI Prism big dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA). Sequences were subjected to a BLAST analysis [38] with the NCBI database.

2.6. Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers AY081748–AY081758.

3. Results

3.1. DGGE analysis of rhizosphere eubacterial communities

All rhizosphere samples produced positive PCR signals with the primer pair applied. Community analysis of the dominant oilseed rape rhizosphere bacteria by DGGE revealed highly complex population structures in all treatments and at all sampling points. The DGGE fingerprints of the rhizosphere communities obtained from late flowering plants are shown in Fig. 1. DGGE patterns obtained from eight replicate pots were indistinguishable, indicating a high level of reproducibility in the DNA isolation, PCR and DGGE procedures. This also shows that the treatment variation is greater than plant-to-plant variation. At the early flowering stage the number of detectable bands ranged from 24 to 25, whereas at the late flowering stage 26 (MM treatment) to 29 (GM treatment) bands were detected. At the senescent stage 32 (MM treatment) to 36 bands (M treatment) were found. Most bands were present in all treatments; however, some bands were specifically detected in particular treatments. Some operational taxonomic units (OTUs) colonized exclusively the rhizospheres of transgenic oilseed rape, whereas others were enriched or inhibited due to herbicide application (Fig. 1).

Cluster analysis indicated a great impact of the sampling time on the eubacterial community structures as the DGGE fingerprints obtained from the individual sampling points always grouped together. At the early and late flowering stages the herbicide Basta had a pronounced effect on rhizosphere bacteria, whereas at the senescent stage differences between transgenic and wild-type rhizosphere eubacterial communities were found (Fig. 2).

3.2. DGGE analysis of rhizosphere Pseudomonas communities

Dominant *Pseudomonas* communities in the oilseed rape rhizosphere were investigated by specific PCR amplification of *Pseudomonas* 16S rRNA genes and subsequent DGGE profiling (Fig. 3). Similar to the eubacterial DGGE fingerprints, little variation was generally found between profiles obtained from replicate pots (Fig. 3). The number of dominant *Pseudomonas* OTUs ranged at the early flowering stage from five (M and MM treatment) to six (G and GM treatment). At the late flowering and senescent stages three or four dominant DGGE bands were detected. Some OTUs were specifically found in the

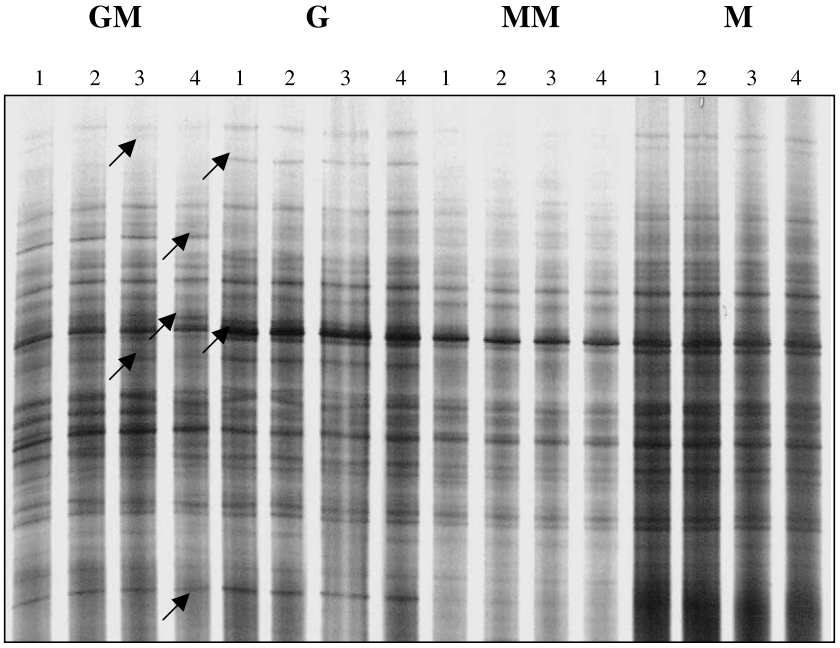

Fig. 1. DGGE profiles of bacterial oilseed rape rhizosphere communities at the late flowering growth stage. Different lanes (1–4) represent rhizosphere samples from different pots. GM, transgenic plants without herbicide; G, transgenic plants with Basta application; MM, wild-type plants without herbicide application; B, wild-type plants with Butisan S application. Arrows indicate bands that were affected by the herbicide application or the genetic modification.

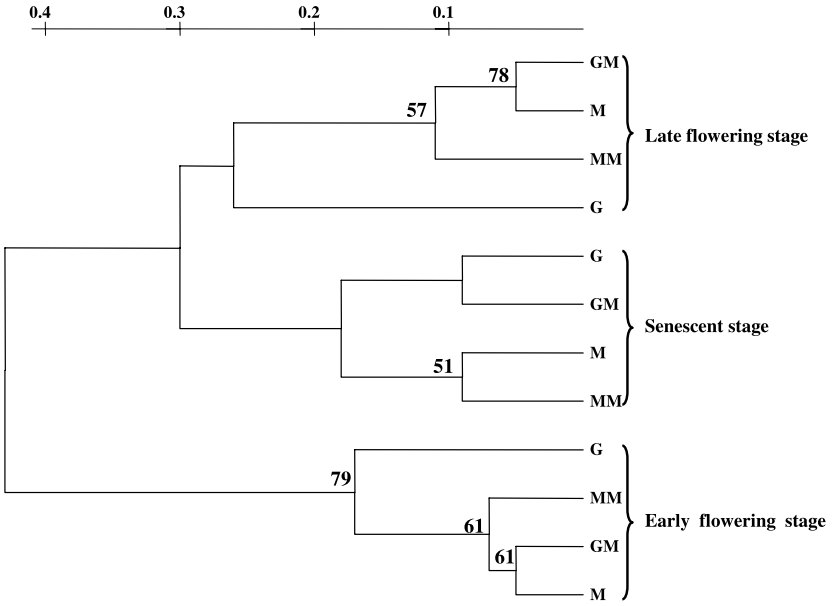

Fig. 2. UPGMA tree representing the genetic similarity of representative eubacterial community profiles obtained by DGGE. The scale indicates the dissimilarity level. Bootstrap values greater than 50 are given at nodes.

rhizospheres of transgenic oilseed rape, such as the bands designated as 1c or 3b in Fig. 3. Other bands, such as 1f or 3e, corresponded to pseudomonads that only colonized the rhizospheres of herbicide-treated plants.

Cluster analysis indicated that at the early flowering stage the rhizosphere was colonized by populations that were different from those found at later growth stages (Fig. 4). At the late flowering and senescent stage, the same *Pseudomonas* populations were found in the rhizospheres of all treatments except the treatment with Butisan S application. Butisan S had an impact on the *Pseudomonas* community structure only at the late flowering stage. At the early flowering stage, as well as at the senescent growth stage, wild-type plants with and without Butisan S application hosted similar populations of this bacterial group. The application of Basta led to altered community structures at all growth stages sampled (Figs. 3 and 4). In general, the genetic modification had the least impact on the *Pseudomonas* populations in the rhizosphere (Fig. 4), as compared to seasonal and herbicide effects.

3.3. Sequence analysis of amplified Pseudomonas 16S rRNA genes

In order to identify the dominant *Pseudomonas* community members at the early flowering and senescent

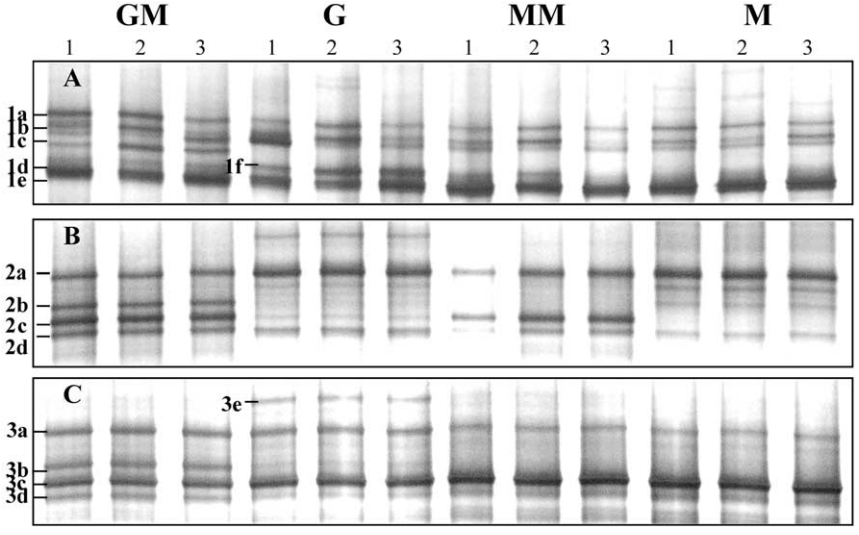

Fig. 3. DGGE profiles of *Pseudomonas* rhizosphere communities at the early (A) and late (B) flowering stage and at the senescent growth stage (C). Different lanes (1–3) represent rhizosphere sample from different pots. GM, transgenic plants without herbicide; G, transgenic plants with Basta application; MM, wild-type plant without herbicide application; B, wild-type plants with Butisan S application. Bands 2a, 2b, 2c and 2d had the same mobilities as bands 1a (3a), 3b, 1f and 1d.

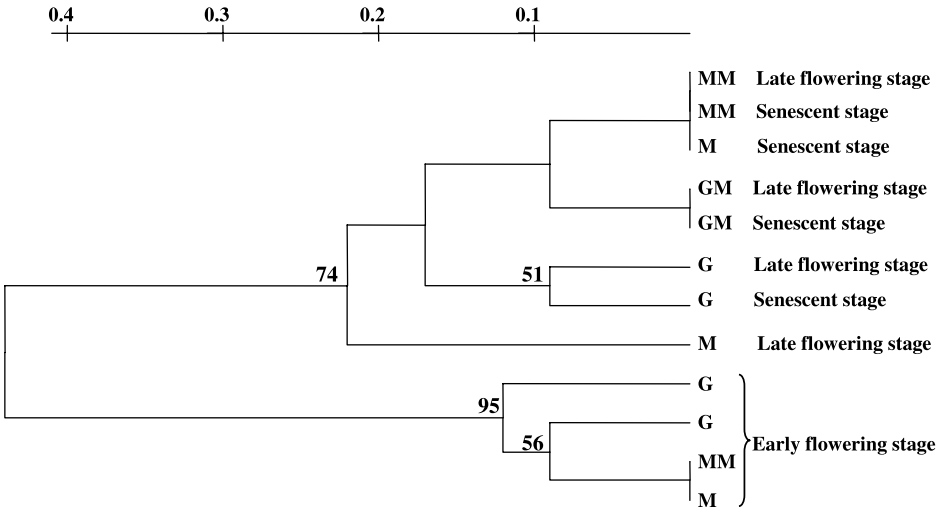

Fig. 4. UPGMA tree representing the genetic similarity of representative *Pseudomonas* community profiles obtained by DGGE. The scale indicates the dissimilarity level. Bootstrap values greater than 50 are given at nodes.

growth stages, clone libraries of amplified *Pseudomonas* partial 16S rRNA genes were established. In general, clones that were highly represented in the 16S rRNA gene library were also dominant in DGGE gels. Ten percent of the clones showed different mobilities in DGGE gels than the dominant bands detected in DGGE community fingerprints, and their sequences showed highest similarities to *Pseudomonas* species. Two clones derived from *Burkholderia* species and one sequence had 90% similarity to a *Bradyrhizobium* 16S rRNA gene. In order to identify the highly dominant *Pseudomonas* species in the DGGE fingerprints, two cloned 16S rDNA fragments that co-migrated with bands of the corresponding community profiles were selected and sequenced. The results of the sequence analysis and their tentative phylogenetic affiliations are shown in Table 2. All sequences showed high similarities (97–100%) to known *Pseudomonas* 16S rDNA sequences deposited in the NCBI database.

Six sequences were obtained from the early flowering stage that showed the same mobilities in DGGE gels as the dominant bands of the *Pseudomonas* community fingerprints. Clones with the mobilities of bands 1a and 1b, which were found in all treatments, showed highest similarity to *P. gingeri* and *P. marginalis*, respectively. Sequence 1c was identical to 16S rDNA sequences of *P. corrugata*, *P. tolaasii* and *P. fluorescens*, indicating that the amplified 16S rRNA gene region is not sufficiently variable to distinguish these species. This band was exclu-

Table 2
Sequence analysis of clones containing partial *Pseudomonas* 16S rRNA genes (*E. coli* position 8–468) obtained from oilseed rape rhizosphere

DGGE clone	Closest NCBI database match	% Similarity	Accession number
Early flowering stage			
1a, 3a	*P. gingeri*	100	AF320991
1b	*P. marginalis*	98	AY071916
	P. putida	98	AF307869
1c	*P. corrugata*	100	AF348508
	P. tolaasii	100	AF348507
	P. fluorescens	100	AJ011331
1d, 3d	*P. frederiksbergensis*	99	AJ105379
	P. borealis	99	AJ012712
1e	*P. frederiksbergensis*	99	AJ105379
	P. borealis	99	AJ012712
1f	*P. frederiksbergensis*	99	AJ105379
	P. borealis	99	AJ012712
Senescent stage			
3a, 1a	*P. gingeri*	100	AF320991
3b	*P. synxantha*	97	D84025
3c	*P. synxantha*	99	D84025
3d, 1d	*P. frederiksbergensis*	99	AJ105379
	P. borealis	99	AJ012712
3e	*P. gingeri*	99	AF320991
	P. putida	99	AF094736
	P. asplenii	99	AB021397

sively detected in the rhizospheres of transgenic rapeseed rhizospheres that were not treated with any herbicide. Three sequences corresponding to bands 1d, 1e and 1f exhibited 99% homology to *P. frederiksbergensis* and *P. borealis*. However, their sequences were different in up to four nucleotides and they also showed different mobilities in DGGE gels. Bands 1d and 1e were highly abundant in all treatments, whereas band 1f was mainly found in Basta-treated rapeseed rhizospheres.

Five sequences corresponding to dominant DGGE bands of the rhizosphere soils sampled at the senescent growth stage were obtained. Similarly to the early flowering stage, sequence 3a, which proved to be identical to sequence 1a, was found in all treatments and showed 100% homology to *P. gingeri*. In addition, sequence 3d that was present in all rhizospheres was identical to sequence 1d and showed 99% similarity to *P. frederiksbergensis* and *P. borealis*. At this growth stage band 3c was highly abundant in the oilseed rape rhizospheres, and its corresponding sequence showed the greatest similarity to a *P. synxantha* 16S rRNA gene. Similarly, sequence 3b was highly related to *P. synxantha*, however, this DGGE band appeared only in transgenic plant rhizospheres that did not receive herbicide application. Sequence 3e, which was specifically detected in the Basta-treated rhizospheres, showed 99% similarity to *P. gingeri*, *P. putida* and *P. asplenii*.

4. Discussion

The release of transgenic plants is still a controversial issue due to potential negative effects on ecologically important organisms. The rhizosphere is a hot spot of microbial activity and hosts a range of bacteria that are beneficial for plant growth and health. Microbial populations in the rhizospheres of various transgenic plants such as those producing antibacterial proteins have been analyzed [39–42]. However, the effect of herbicide tolerant plants and the associated altered herbicide application on the plant-associated microflora has rarely been addressed. Total microbial community analysis indicated that the plant growth stage had, among the parameters tested, the most pronounced effect on the rhizosphere populations of oilseed rape (Fig. 2). Similar findings were obtained by Gomes et al. [43], who found strong seasonal shifts in the bacterial rhizosphere communities of two maize cultivars. However, contrasting results were found by Duineveld et al. [44], who monitored the presence and activity of dominant bacterial populations in relation to plant development by using culture-independent methods. In that study rhizosphere communities showed a high level of similarity regardless of the developmental stage of the plant. In our experiment, minor differences that were represented by the absence or presence of individual bacterial OTUs were found between rhizosphere communities of wild-type and transgenic plants (Fig. 1). The presence of the *pat* gene does not suggest a priori effects on the microflora, and we speculate that the observed differences were due to changes in exudation patterns. Root exudates represent an important source of substrates available for rhizosphere and rhizoplane microorganisms and they therefore selectively influence the microbial community structure [45,46]. Recently, Schmalenberger and Tebbe [47] compared bacterial communities in rhizospheres of transgenic maize containing the *pat* gene to those found in association with the isogenic, non-transgenic cultivar. In that study, no differences were detected. Other reports showed different microbial communities associated with the roots of field-grown transgenic and non-transgenic glyphosate-tolerant oilseed rape [48,49]. However, in these studies three different cultivars were tested and one of them was genetically engineered. Therefore, the authors could not exclude the possibility of additional genotypic differences besides the genetic insertion conferring herbicide resistance. Becker et al. [50] studied the diversity of *Rhizobium leguminosarum* bv. *viciae* in fields cultivated with transgenic, Basta-tolerant and wild-type rapeseed cultivars. Various strains were found in soils around the transgenic lines that were not detected in control treatments. In addition, fields of some transgenic lines possessed a higher *Rhizobium* diversity. However, no significant effects on rhizobial numbers or soil basal respiration were observed [50]. Transient but significant effects on microbial communities due to unintentionally altered plant characteristics by genetic engineering were also reported by Donegan et al. [51]. In that study, total bacterial numbers, bacterial species and DNA fingerprints of microorganisms associated with cotton expressing the *Bacillus thuringiensis* endotoxin were compared to those of the wild-type plant.

Herbicide application caused minor shifts in the rhizosphere eubacterial community structures that were possibly due to the enrichment of microbes being involved in the herbicide degradation and to the inhibition of sensitive organisms (Fig. 1). The herbicide Basta had a rather strong impact at the early and late flowering stages, whereas Butisan S affected the rhizosphere microflora to a lower extent (Fig. 2). Weak antibacterial activity of Basta was reported in previous studies [2,4]; however, no long-term effects on the microbial activity were expected [52]. The observed effects due to Basta can also be explained by the application of this herbicide two days before sampling. At the senescent growth stage, the herbicides had the least impact on the rhizosphere microbial community structure, confirming the transient nature of any effects due to the application of herbicides Basta or Butisan S.

Pseudomonads are prominent rhizosphere microbial community members and frequently show beneficial effects on plant growth and health. We therefore analyzed the effect of the genetic modification conferring herbicide resistance, the herbicide application and the plant develop-

mental stage on the rhizosphere *Pseudomonas* populations. Our results showed that the plant growth stage had a rather strong impact on the *Pseudomonas* communities (Fig. 4). However, unlike the effect of the plant growth stage on eubacterial populations, the pseudomonads were only influenced at early growth stages. Most treatments showed highly similar *Pseudomonas* communities at the late flowering and the senescent stages (Fig. 3). Seasonal shifts in the rhizosphere have also been reported for other bacterial taxa such as the α- and β-proteobacteria [43]. Recently, Picard et al. [53] found a significant effect of the plant developmental stage on the abundance and diversity of 2,4-diacetylphloroglucinol-producing pseudomonads colonizing maize roots. The herbicide Basta negatively affected a *Pseudomonas* population whose partial 16S rDNA sequence was identical to that of *P. corrugata*, *P. tolaasii* and *P. fluorescens*. Plant growth-promoting activity was reported for these organisms ([54], see GenBank entry AF348507), suggesting that Basta application may at least transiently affect plant beneficial bacteria. Inhibition and promotion of *Pseudomonas* species by Basta was observed at all plant growth stages. Butisan S caused a reduced richness of pseudomonads at the late flowering stage; however, at the senescent growth stage *Pseudomonas* populations were not affected by this herbicide. Possibly Butisan S was already completely degraded or the break-down products did not have any impact on pseudomonads.

At all growth stages, transgenic and wild-type rhizospheres hosted slightly different *Pseudomonas* populations (Fig. 3). At the early flowering stage a pseudomonad that had the identical partial 16S rDNA sequence as three plant beneficial strains of *P. corrugata*, *P. tolaasii* and *P. fluorescens* only colonized roots of transgenic plants, suggesting that unintentionally altered plant characteristics may also promote the growth of plant beneficial bacteria. At the later growth stage, a *P. synxantha*-like population was detected in the rhizosphere of transgenic plants, and this organism did not colonize wild-type plants.

In conclusion, the main objective of this study was to determine possible effects of the transgenic Basta-tolerant oilseed rape or the associated herbicide application on the rhizosphere microflora. Slightly altered microbial communities were detected in the rhizospheres of transgenic plants. However, effects were minor as compared to shifts caused by the plant developmental stage. Comparison of the agricultural practices associated with the cultivation of Basta-resistant and wild-type oilseed rape indicated that the herbicide Basta had a more pronounced impact on the rhizosphere microflora than Butisan S. However, the nature of this effect was only transient.

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