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Activity of microorganisms in the rhizosphere of herbicide treated and untreated transgenic glufosinate-tolerant and wildtype oilseed rape grown in containment

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Abstract

An experiment was carried out with plants grown in containment in order to determine potential effects on metabolically active microbial populations as well as on soil enzyme activities in the rhizosphere of genetically modified Basta-tolerant oilseed rape. Transgenic as well as isogenic wild-type plants were grown in combination with the associated herbicide Basta (active ingredient glufosinate) or the herbicide Butisan S (active ingredient metazachlor), respectively. In control treatments, weeds were mechanically removed. Rhizosphere soil was sampled at the early and late flowering stage as well as at senescence. RNA was isolated and 16S rRNA was amplified by reverse transcription and PCR. Amplicons were subjected to denaturating gradient gel electrophoresis in order to generate community fingerprints of metabolically highly active bacteria. Additionally, RNA was hybridized with group-specific probes. Furthermore, bacterial biomass and activities of invertase, alkaline phosphatase, urease and arylsulfatase were determined. Results showed that oilseed rape rhizosphere bacteria were affected by the genetic modification as well as by the herbicide application, however, these effects were minor compared to the influence of the plant growth stage. At senescence, invertase, urease and alkaline phosphatase activities were significantly enhanced in the rhizospheres of transgenic plants as compared to wild-type plants propably due to an altered root exudation in comparison to the wildtype plant. Dot blot hybridizations indicated altered activities and/or abundances of various phylogenetic groups at all sampling times. The transformation process may have unintentionally altered the physiology of plants (e.g. root exudation) leading to changes in rhizosphere community structures and bacterial activities.

Introduction

Genetically modified plants tolerating non-selective broad-spectrum herbicides such as glyphosate or glufosinate ammonium have been recently developed. These varieties allow improved weed control with a reduced number of herbicides that have to be applied. One crop that is commercially available is transgenic oilseed rape (*Brassica napus*) that contains the *pat* gene from *Streptomyces* encoding phosphinothricin acetyltransferase and therefore tolerates glufosinate (phosphinothricin). The cultivation of the genetically modified oilseed rape allows application of the broad-spectrum herbicide at any time, whereas in conventional agriculture other herbicides such as Butisan S (active ingredient: metazachlor) and used for pre-emergence and early post-emergence weed control. Glufosinate is naturally produced by soil *Streptomyces* spp. and shows weak antimicrobial activity (Bayer et al., 1972; Wohlleben et al., 1992). However,

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various reports demonstrated that in soil, glufosinate degrades within 7–21 days to a non-toxic intermediate that is rapidly mineralized (Domsch, 1992; Faber et al., 1997; Gallina and Stephenson, 1992; Ismail and Ahmad, 1994; Tebbe and Reber, 1991). Furthermore, many soil bacteria are resistant to glufosinate (Allen-King et al, 1995; Bartsch and Tebbe, 1991; Tebbe and Reber, 1988). On the other hand, some microbes were reported to be sensitive towards this herbicide (Ahmad and Malloch, 1995). Kriete and Broer (1996) showed that the growth of nitrogen-fixing rhizobia, nodule formation and nitrogen fixation are effected by glufosinate. The half life-time of Butisan S in soil ranges from 10 to 35 days (Domsch, 1992).

There are concerns regarding the release of transgenic crops because of the potential unwanted impact on soil functioning due to altered microbial communities. Although the cultivation of herbicide-tolerating plants does not suggest a priori effects on the soil microflora, it has been reported that both glyphosate- and glufosinate-tolerant oilseed rape varieties host different rhizosphere and endophytic bacterial populations as compared to the wild-type varieties (Siciliano and Germida, 1999; Dunfield and Germida, 2001; Gyamfi et al., 2002). On the other hand, no effect due to the genetic modification on rhizosphere microbial communities associated with glufosinate-resistant maize was found (Schmalenberger and Tebbe, 2002). In addition to the genetic modification the altered weed control may influence soil-borne and plant-associated microorganisms.

The objective of this study was to investigate potential effects of genetically modified glufosinate-tolerant oilseed rape or the associated herbicide application on active members as well as on the function of the rhizosphere microflora. Frequently, the 16S rRNA gene is used as a molecular marker for cultivation-independent community analysis. However, analysis based on genomic DNA detects the numerically most abundant bacteria, but gives no information on the activity or the viability of the corresponding cells. Ribosomal RNA was reported to be roughly proportional to growth activity in pure culture (Wagner, 1994) and is therefore more suitable to study active bacterial populations. Consequently, in this investigation RNA was isolated from the oilseed rape rhizosphere and used for reverse transcription-PCR (RT-PCR) using eubacterial primers targeting 16S rRNA genes. Amplicons were subjected to denaturating gradient gel electrophoresis (DGGE) in order to generate fingerprints of the metabolically most active members of the rhizosphere microflora. Furthermore, RNA was bound on membranes and hybridized with group-specific probes in order to allow a quantitative comparison of the abundance and activity of major bacterial taxa in individual treatments. In addition, differences in function of oilseed rape rhizosphere microorganisms were determined by analysing the activities of several key enzymes.

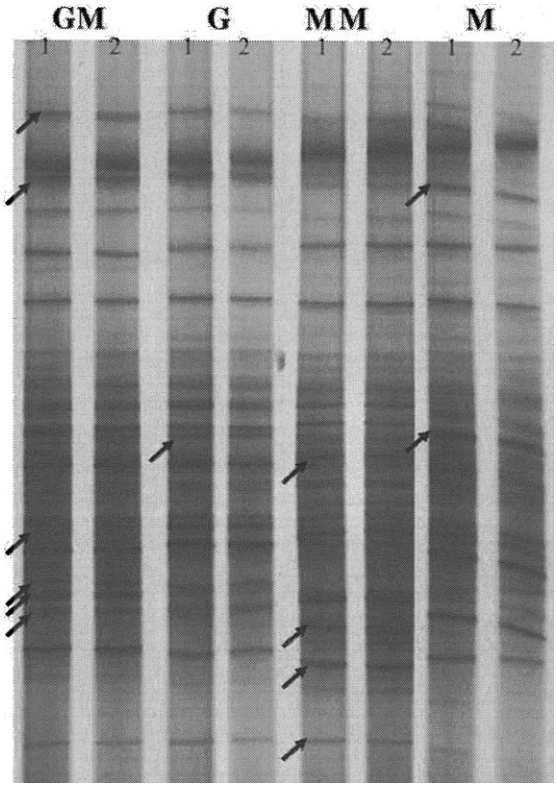

Figure 1. DNA- and RNA-based DGGE profiles of bacterial oilseed rape rhizosphere communities at the early flowering stage. Different lanes (1, 2) represent rhizosphere samples from two different pots. GM, transgenic plants without herbicide application; G, transgenic plants with Basta application; MM, wild-type plant without herbicide application; M, wilt-type plants with Butisan S application. Arrows indicate bands that were affected by the herbicide application or the genetic modification.

Materials and methods

The containment experiment

Transgenic winter oilseed rape (*Brassica napus*), variety Liberator C/6AC (DSV, Salzkotten, Thüle, Germany) containing the *pat* gene for Basta tolerance, as well as the isogenic parental line were grown in TKS2 soil subtrate (Frux ED63 not pasteurized soil

substrate; Gebr. Patzer, Sinntal-Jossa, Germany; 100–250 mg L^{-1} nitrogen, 100–250 mg L^{-1} potassium oxide, and 100–250 mg L^{-1} phosphor pentoxide) in the greenhouse. After germination plantlets were vernalized for 68 days at 3–5 °C.

Soil from agricultural fields around the Seibersdorf laboratory (Calcic Chernozem) (44% sand, 34% silt, 22% clay, 0.15% N, 4.73% C, pH 7.8) were used to fill 50 l pots. Six rapeseed plantlets were transplanted into each pot. In addition, two weeds commonly occuring with oilseed rape, *Capsella bursapastoris* and *Descurainia sophia*, were sown. In order to conform with Austrian regulations and to simulate the agricultural condition the experiment was performed in a containment and included the following treatments: (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). Pots were arranged in a completely randomized block design and each treatment was replicated eight times. Herbicides were applied by spraying in a concentration of 0.01 mg cm^{-1} (Butisan S, active ingredient metazachlor) and 0.03 mg cm^{-1} (Basta, active ingredient glufosinate) according to the manufacturer's recommendations. Herbicide application and sampling times are shown in Table 1. Weeds could not be eliminated completely by the first herbicide application. Therefore, Basta was applied a second time, which is possible due to the genetic modification, and which is also according to the agricultural practise. On each sampling date rhizosphere soil was taken from two plants, sieved through a 1-mm sieve, thoroughly mixed and stored at −20 °C.

RNA isolation and RT-PCR conditions

RNA was isolated according to a protocol that was originally applied to isolate RNA from pine trees (Chang et al., 1993) and adapted as described by Sessitsch et al. (2002). Briefly, soil was resuspended in extraction buffer (2% CTAB, 2% PVP K30, 100 m*M* Tris-HCl, pH 8.0, 25 m*M* EDTA, pH 8.0, 0.5 g L^{-1} spermidine), which was freshly amended with 2% β-mercaptoethanol. Cells were lysed by bead-beating and proteins were removed by extraction with chloroform. RNA was precipitated overnight with LiCl. RNA was dissolved in RNase-free water. The resulting RNA solution was extracted with chloroform and RNA was again precipitated. Contaminating DNA was removed by digestion with DNase and finally, passage through CL-6B spin columns was performed in order to remove PCR-inhibiting substances.

RT-PCR was performed using the Supercript^{TM} One-Step^{TM} RT-PCR System (Gibco, BRL) according to the manufacturer's instructions. Reactions were performed with 0.15 μM each of the primers U968-GC and L1401 (Nübel et al., 1998) and 0.1–1 μL extracted RNA. Reverse transcription was carried out for 30 min at 50 °C. Amplifications were carried out with a thermocycler (PTC-100^{TM}, MJ Research, Inc.) using an initial denaturation step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min annealing at 52 °C and 2 min extension at 72 °C. The PCR reaction mixture (50 μL) used to check RNA for the presence of contaminating DNA contained 1 × reaction buffer (Gibco, BRL), 200 μM each dATP, dCTP, dGTP and dTTP, 2 m*M* $MgCl_2$ and 2 U Taq DNA polymerase (Gibco, BRL), and 0.15 μm of the primer set U968-GC/L1401. RT-PCR and PCR amplification products were examined by gel electrophoresis on 1% agarose gels.

Denaturating gradient gel electrophoresis (DGGE)

DGGE analysis was performed according to Muyzer et al. (1993) using the DCode^{TM} Universal Mutation Detection System (Bio-Rad). PCR samples (7 μL) were loaded onto 6% (wt/vol.) polyacrylamide gels (ratio acrylamide to bisacrylamide, 37:1) submerged in 1 × TAE buffer (40 m*M* Tris, 40 m*M* acetate, 1 m*M* 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). Gels were electrophoresed at 70V for 16 h and 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.

Dot-blot hybridization

Soil RNAs from 4 replicate pots were pooled and 2 μg were blotted in duplicates on Hybond N+ membranes (Amersham, Buckinghamshire, England) by using standard techniques (Sambrook and Russell, 2001). The probes ALF1b (Manz et al., 1992), BET42a (Manz et al., 1992), GAM42a (Manz et al., 1992), HGC69a (Roller et al., 1994), LGC353b (Meier et al., 1999) and CF319a (Manz et al., 1996) were used

Table 1. Overview of the containment experiment showing planting time, times of herbicide application 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.

to specifically detect α-, β-, and γ-Proteobacteria, high GC- and low GC-Gram-positives and the phylum *Cytophaga/Flavobacterium/Bacteroides* (CFB), respectively. Oligonucleotide probes were 5′ labeled by using phage T4 polynucleotide kinase (Promega) and 30 μCi of -^{32}P]ATP (3000 Ci/mmol; Amersham). For hybridization 5 μL of labeled probes were used. Prehybridization, hybridization and stringent washing steps were performed as described previously (Manz et al., 1992, 1996; Meier et al., 1999; Roller et al., 1996). Hybridized membranes were incubated with a detection screen (Molecular Dynamics, Sunnyvale, California) and signals were detected and quantified with a PhosphorImager (Gel and Blot Imaging System Storm 860, Amersham Biosciences).

Microbial biomass determination and analysis of enzyme activities

Ninhydrin reactive N as an indicator for microbial biomass nitrogen (N_{mic}) was extracted after fumigation with chloroform for 24 h (Amato and Ladd, 1988), and microbial N compounds in the 2 *M* KCl extracts were colorimetrically determined at 570 mm.

Invertase activity was determined as described by Schinner and von Mersi (1990) using sucrose as substrate (3 h at 50 °C) followed by colorimetric determination of the reducing sugars. Urease activity was assayed as described by Kandeler and Gerber (1988) using urea as substrate. After incubation at 37 °C for 2 h, the NH_4^+-N formed was extracted with 1 *M* KCl and measured by a colorimetric technique. Alkaline phosphomonoesterase was assayed by using buffered disodium phenylphosphate (20 m*M*) solution (3 h at 37 °C, pH 10). The released phenol was estimated colorimetrically at 400 nm (Hoffmann, 1968). Arylsulfatase activity was measured according to Tabatabai and Bremner (1970) using p-nitrophenylsulfate solution as the substrate. This enzyme assay measures *p*-nitrophenol colorimetrically as the reaction product after incubation for 1 h at 37 °C.

Data handling and statistical analysis

Microbial biomass (N_{mic}), and soil enzyme activity (invertase, urease, phosphatase, arylsulfatase) were calculated on an oven-dry weight (105 °C) basis. The data distribution passed the normality test (Kolmogorov-Smirnov test). Differences of the means of microbiological variables between all treatments were tested by univariate analysis of variance, followed by the multiple range test (Duncan test). The effect of the genetic modification on enzyme activity was tested by comparing the measured mean of the MM and M treatment with the G and GM treatment, respectively. The effect of two different plant protection measures on enzyme activity was tested by comparing the means of the M and G treatment with the MM and GM treatment, respectively. The classification of the treatments based on eubacterial community profiles obtained by RNA-based DGGE, and the subsequent relation of the classification to the treatment was carried out by cluster analysis based on similarities calculated according to Nei and Li (1979) and using the UPGMA (unweighted pair group with mathematical averages) method. Tree generation was performed applying the TREECON software package (van de Peer and de Wachter, 1994). Discriminant function analysis was applied in order to determine whether the treatments can be identified by their microbial variables (dot blot hybridizations, biomass, invertase, urease, phosphatase, arylsulfatase) and evaluate the discriminatory importance of each variable. The groups were defined according to the treatment. Multivariate Wilks' Lambda was used for the stepwise selection of the variables. Significance was accepted at the $P < 0.05$ level of probability.

Results

RNA-based DGGE community profiles

Reserve transcription and subsequent amplification of partial 16S rRNA generated amplification products from all isolated RNA samples. No amplification products were obtained without reverse transcription indicating that the isolated RNA was free of contaminating DNA. DGGE analysis of metabolically highly active bacterial populations in the oilseed rape rhizospheres revealed highly complex fingerprints in all treatments and at all sampling points. Rhizosphere samples from eight replicate pots showed almost identical community fingerprints demonstrating a low plant-to-plant variation and a high level of reproducibility in the RNA isolation, RT-PCR and DGGE procedures. At each plant growth stage similar community profiles were found, however, some bands were specifically detected in individual treatments (Figure 1). Some DGGE bands were exclusively found in the rhizospheres of transgenic plants, whereas other bacteria showed high activities in the rhizospheres of wildtype plants. Similarily, some microbes were activated or inhibited by the herbicide application (Figure 1).

Cluster analysis demonstrated that the plant growth stage had the most pronounced impact on active microbial populations in the oilseed rape rhizospheres (Figure 2). Particularly at the early flowering stage bacterial communities were highly different compared to those observed at later plant growth stages. At the late flowering and at the senescent growth stage the rhizosphere microflora was more affected by the herbicide application than by the genetic modification.

Dot-blot hybridization

Probes specific for the α-, β- and γ-Proteobacteria, for the high GC and low GC Gram-positives and for the *Cytophaga/Flavobacterium/Bacteroides* (CFB) phylum were used to compare the abundances of rRNA genes derived from these groups in individual treatments and sampling points. As the degree of signal intensity depends on the probe and the target region only results within a given phylogenetic group were compared. At the early flowering stage, α-Proteobacteria and high GC Gram-positives showed comparable hybridization signals in all treatments, whereas the rRNA abundances of the other phylogenetic groups were increased by herbicide application (Figure 3). In addition, the rhizosphere of transgenic oilseed rape without herbicide application contained lower amounts of rRNA derived from CFB and β-Proteobacteria. At the late flowering stage, all phylogenetic groups showed higher amounts of rRNA in the rhizospheres of transgenic plants as compared to those of wild-type plants. Application of the herbicide Basta led to slightly increased abundances of CFB and high GC Gram-positives and slightly reduced abundances of low GC Gram-positives. At that plant growth stage Butisan S caused increased rRNA contents of all phylogenetic groups except CFB (Figure 3). At the senescent growth stage, Butisan S caused inhanced rRNA abundances of all phylogenetic groups, whereas the opposite effect was observed with Basta (Figure 3). Microorganisms from the rhizosphere of plants that had been treated with Basta showed lower hybridization signals with all probes. All phylogenetic groups contained higher rRNA amounts in the rhizosphere of transgenic plants than in those of control plants.

Microbial biomass and enzyme activities

At the late flowering stage, higher microbial biomass was detected than at other sampling stages, although no treatment effects were observed at the early and late flowering stage (Figure 4). At the senescent growth stage, microbial biomass was higher in the rhizosphere of transgenic oilseed rape without herbicide application than in other treatments, although this effect was not significant. Similarly, no effect due to the herbicide application or the genetic modification on enzyme activities was observed at the early and late flowering stage (Table 2, Figure 4). However, at the senescent growth stage invertase, urease and alkaline phosphatase activity were significantly enhanced in the rhizospheres of transgenic plants as compared to wild-type plants (Table 2, Figure 4). Basta caused significantly decreased invertase, urease and phosphatase activities. Enzyme activities of rhizospheres treated with Butisan S were enhanced, however, significant effects were only observed with urease (Table 2). No treatment or time effect was observed with arylsulfatase.

Discriminant analyses of various microbial properties (dot blot hybridization signals, microbial biomass, urease, alkaline phosphatase, arysulfatase activity) revealed a clear grouping of samples taken at the individual plant growth stages (Figure 5). Discriminant function (DF) 1 explained 77% of the total variance

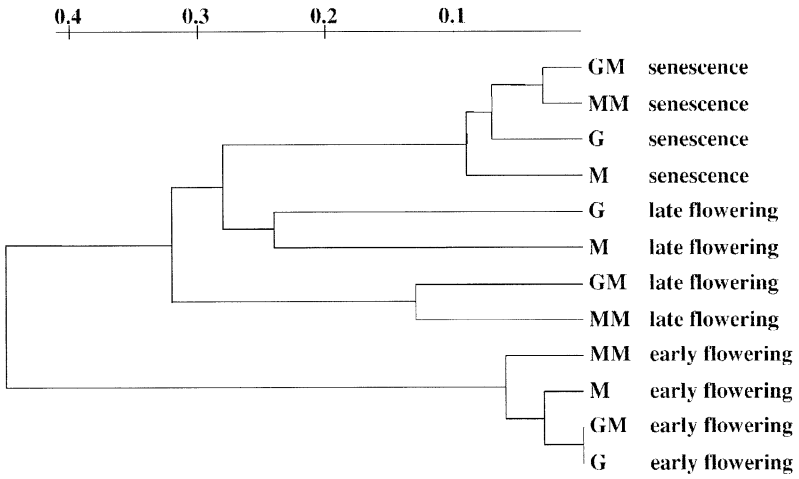

Figure 2. UPGMA tree representing the genetic similarity of representative eubacterial community profiles obtained by RNA-based DGGE. The scale indicates the dissimilatory level. Abbreviations as in Figure 1.

of the data set and was dominated mainly by urease and phosphatase activity, which had the highest standardised coefficient in the linear combination (Table 3). Because of the high eigenvalue of DF 1, this variable was the most important in relation to all other discriminant functions. DF 2 explained only 23% of the total variance (Figure 5, Table 3). Early and late flowering as well as senescent stages discriminated along axis 2 mainly due to differences in microbial biomass and urease activity (Figure 5, Table 3). These multivariate analyses clearly showed that the stage of plant development had a higher impact on microbial properties than either genetic modification or type of plant protection.

Discussion

Recent studies showed that transgenic, glufosinate-tolerant oilseed rape may host altered plant-associated microbial communities in comparison to the wild-type (Dunfield and Germida, 2001; Gyamfi et al., 2002). Microbial community shifts were observed by 16S rRNA-based population analysis of eubacteria and pseudomonads (Gyamfi et al., 2002) as well as by fatty acid analysis and carbon substrate utilization (Dunfield and Germida, 2001). However, no differences could be detected between bacteria colonizing the rhizospheres of glufosinate-tolerant maize and its non-transgenic cultivar (Schmalenberger and Tebbe, 2002). Our study aimed at analyzing potential effects of transgenic glufosinate-resistant oilseed rape and the application of the associated herbicide on metabolically active rhizosphere bacterial populations. These were addressed by isolating RNA from rhizosphere soils and subsequently analyzing 16S rRNA by either DGGE analysis or by dot-blot hybridization with group-specific probes. This approach was chosen as the rRNA content roughly correlates with the metabolic activity of a cell (Wagner, 1994). Community analysis of metabolically active bacteria clearly showed that among all parameters tested the plant growth stage had the most pronounced effect. This is in agreement with a previous study demonstrating that DNA-based (eubacterial and *Pseudomonas*) community fingerprints were influenced by the introduction of the *pat* gene, by the herbicide application and by the plant growth stage, however, the latter parameter had the most severe effect (Gyamfi et al., 2002). Active (based on RNA analysis) and dominant (based on DNA analysis) populations were highly similar. Apart from the plant growth stage, the application of either Butisan S or Basta had a more pronounced effect on active bacterial populations than the genetic modification. In contrast, DNA-based community analysis revealed that at the senescent growth stage the impact by the herbicides was minor as compared to that of the insertion of the *pat* gene (Gyamfi et al., 2002).

Canonical discriminance analysis was used to evaluate the results of dot-blot hybridizations, microbial biomass and enzyme activities in combination. It could be clearly demonstrated – in agreement with the community analysis of active bacteria – that the plant growth stage had by far the most severe effect on the activity of rhizosphere bacteria. Similarly, Heuer et al.

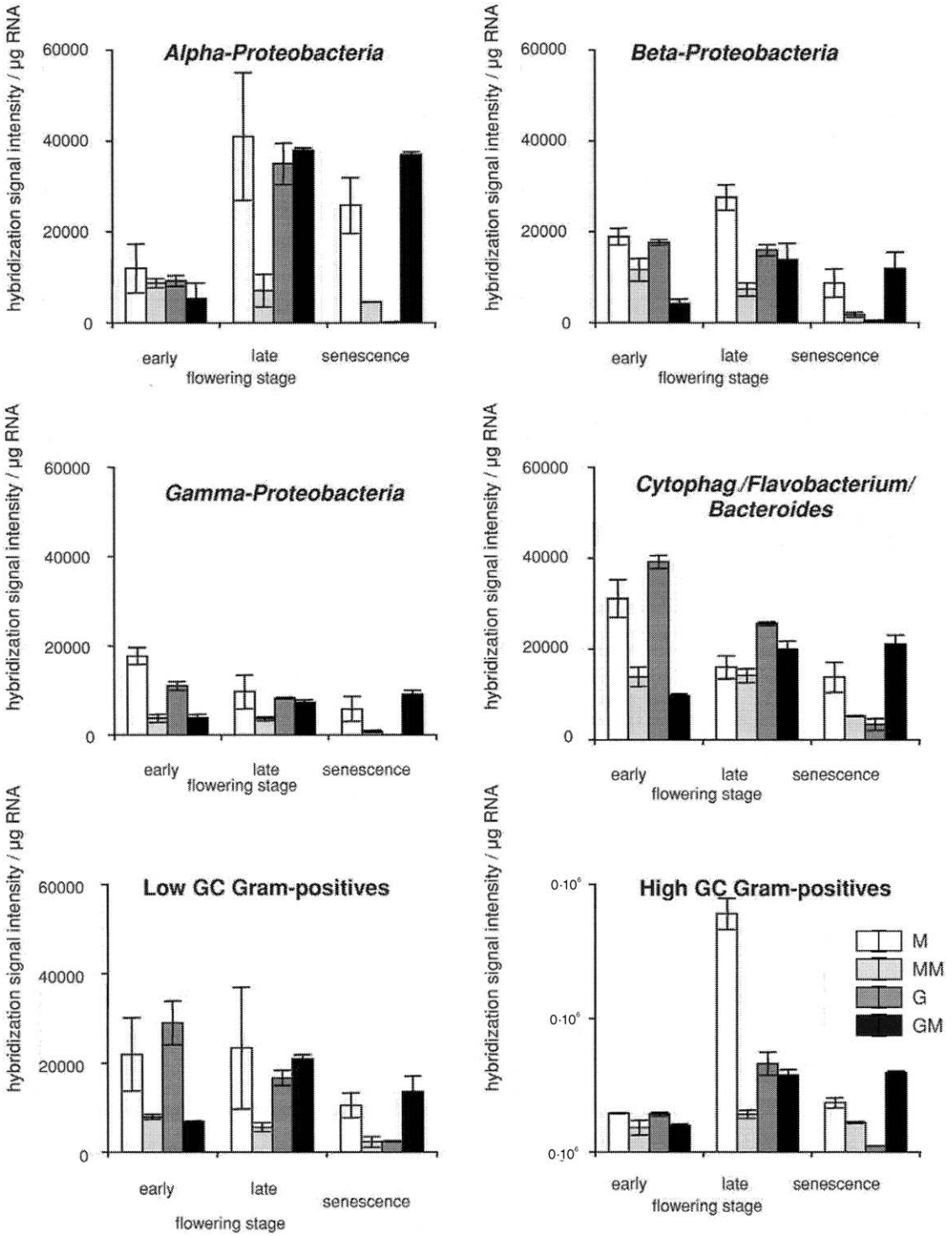

Figure 3. Results obtained by dot-blot hybridization of rhizosphere RNA obtained from different plant growth stages and treatments with group-specific probes. Values shown represent radiodecay signals obtained by PhosphorImager analysis and are the mean of two replicates. Whiskers indicate maximum and minimum values. Abbreviations as in Figure 1.

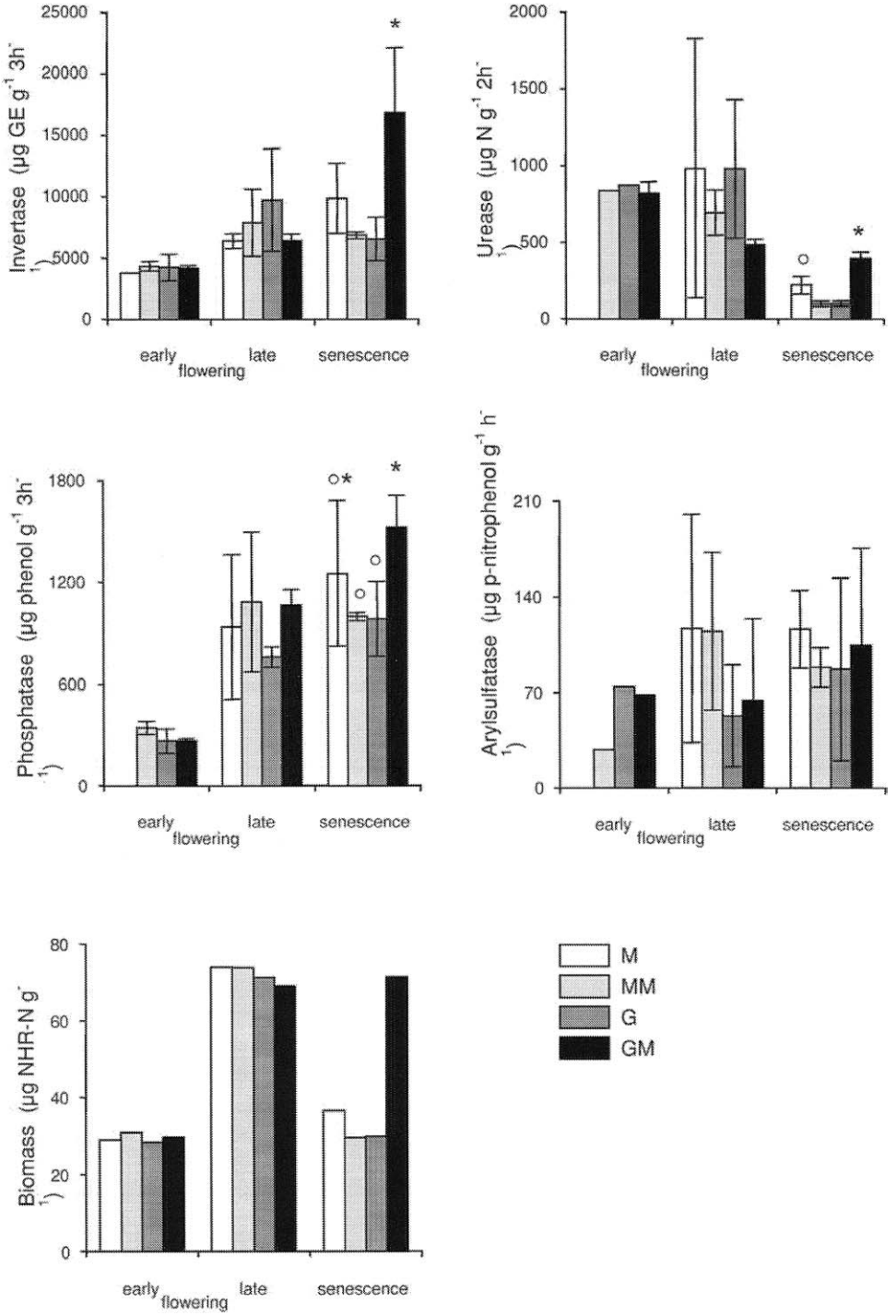

Figure 4. Microbial biomass and enzyme activity. Values shown are the mean of three replicates, whiskers indicate standard deviation. Bars without whiskers show means of two replicates within each plant growth stage. Means which are significantly different from each other at $P<0.05$ (Duncan's test) are indicated by asterisks or circles. Abbreviations as in Figure 1.

Table 2. Effect of the genetic modification and the type of plant protection on soil enzyme activities at the early (1) and late (2) flowering growth stage as well as at the senescent growth stage (3). Given are t-values and levels of significance. GM, transgenic plants without herbicide application; G, transgenic plants with Basta application; MM, wild-type plants without herbicide application; M, wild-type plants with Butisan S application. $^{*}P < 0.05$, **P<0.01, $^{***}P < 0.01$; n.d., not determined

	Plant growth stage	Genetic modification		Plant protection	
		G-M	GM-MM	M-MM	G-GM
Invertase	1	n.d.	0.74	n.d.	0.15
	2	−1.83	0.93	−0.93	1.38
	3	1.70	−3.28*	1.81	−3.21*
Phosphatase	1	n.d.	3.25*	n.d.	0.04
	2	0.72	0.72	−0.43	−4.91**
	3	0.96	−4.78**	1.03	−3.21*
Urease	1	n.d.	0.28	n.d.	0.72
	2	0.01	2.39	0.58	1.90
	3	3.39*	−11.98***	3.47*	−11.75***
Arylsulfatase	1	n.d.	n.d.	n.d.	n.d.
	2	1.21	1.06	0.03	−0.27
	3	0.70	−0.38	1.52	−0.31

(2002) reported that the influence of transgenic T4-lysozyme expressing potatoes on the associated microflora is minor in comparison to seasonal fluctuations. Seasonal shifts in the bacterial rhizosphere communities seem to be a common phenomenon and were also demonstrated by other authors (di Cello et al., 1997; Gomes et al., 2001; Sessitsch et al., 2003). However, Duineveld et al. (2001), who monitored the presence and activity of dominant bacterial populations in the rhizosphere of chrysanthemum in relation to plant development, found contrasting results. Root-associated microbial communities showed a high degree of similarity regardless of the developmental stage of the plant.

Our results suggest that the transgenic oilseed rape may have an altered root exudate composition and/or concentration as compared to the isogenic wildtype plant. As no a priori effects on microbial populations can be expected from the presence and expression of the *pat* gene, we assume that the observed differences were due to changes in exudation patterns. Furthermore, high invertase activities in the rhizospheres of transgenic plants at senescence strongly indicated the presence of higher saccharose concentrations in the root zone most likely resulting from altered root exudation. The presence of this carbohydrate caused a massive growth of microorganisms of various phylogenetic groups and probably led to phosphorus depletion. In addition, a high urea content and therefore high urease activity was observed in the rhizosphere of glufosinate-tolerant rape, which were probably derived from dying microbial or plant cells. Root exudates selectively influence root-associated microbial populations as they represent an important source of substrates available for microorganisms (Grayston et al., 1998; Yang and Crowley, 2000). It is likely that during the transformation process plant characteristics such as root exudation were unintentionally altered leading to changes in rhizosphere community structures and bacterial activities. Previously, Sessitsch et al. (2003) reported transient but significant effects on culturable populations of *Bacillus* spp. associated with roots of potato expressing a lytic peptide. However, the impact could not be attributed to the production of the antimicrobial peptide, and was rather explained by altered plant characteristics. Similarly, Donegan et al. (1995) found significantly but transiently altered microorganisms in the rhizosphere of cotton expressing the *Bacillus thuringiensis* endotoxin in comparison to the wildtype plant. Differences were explained by unintentional changes caused by the transformation event.

Herbicides affected microbial activities already at early plant growth stages. However, early effects were only observed by dot-blot hybridization but not by analysis of enzyme activities. Schäffer (1993) described in his review paper only few consistent patterns of soil enzyme inhibition by herbicides, even at concentrations greatly exceeding recommended application rates. Since herbicides are not designed to inhibit soil enzymes (Speir and Ross, 2002), direct inhibition seems to be unlikely, but at a later stage of plant development enzyme-producing microbes and therefore enzyme production may be restrained. The application of Basta initially activated members of the β and γ-Proteobacteria, the CFB and the low GC Gram-positives, that probably were either not sensitive to the herbicide or were even able to metabolize the herbicide. The second application of Basta did not cause an activation detectable by dot-blot hybridization. However, at the senescent growth stage all phylogenetic groups were severely inhibited leading to low microbial biomass and reduced activities of invertase and alkaline phosphatase. Eventually, a late break-down product of Basta was toxic for microorganisms and therefore responsible for the observed effects. The application of Butisan S caused the activation of several phylogenetic groups at all sampling times. This is surprising as it was reported that this herbicide degrades in soil within 35 days (Domsch, 1992), whereas our results indicate a long-term effect of Butisan S or of its break-down products on microbial populations and activities. The lag before changes in enzyme activities were detected may indicate that degradation products of Butisan S were the actual inhibitors or that the rate of interference was controlled by its diffusion to the site of action (Schäffer, 1993). An alternative explanation is that the disturbance of microbial equilibrium by the toxin produced an environment with selective elimination of susceptible genotypes, concurrent with a temporary increase of extracellular enzyme activities by cell lysis.

Results obtained by analysis of metabolically active microbial populations as well as of enzyme activities were in agreement with those obtained by population analysis of dominant bacteria. Abundant and active microbial communities were affected by the genetic modification and the herbicide application, however, effects were minor compared to the influence of the plant growth stage. The different enzyme activities found in the rhizosphere of transgenic plants suggest that altered root exudation was eventually responsible for the observed effects. We, therefore, believe that unintentionally altered characteristics such as root exudation of genetically modified plants merit further investigation. Dot-blot hybridization and particularly enzyme analysis allowed quantitative examination of treatment effects and would be suitable for long-term monitoring of potential ecological effects associated with the release of transgenic plants.

Table 3. Results of discriminant analyses of various microbial variables (dot blot hybridization signals, microbial biomass, invertase, urease, alkaline phosphatase, arylsulfatase activity) including data from the early and late flowering stage as well as from the senescent stage

	Discriminant function	
	DF 1	DF2
Wilks's lambda	0.001	0.067
Eigenvalue	45.48	13.92
Degrees of freedom	22	10
Cumulative variance %	77	100
Canonical correlation coefficient	0.99	0.97
Correlation coefficient[a]		
Microbial biomass	−0.08	0.38
Urease	0.26	0.28
Phosphatase	−0.18	0.03
Invertase	−0.08	−0.04
Arylsulfatase	−0.04	−0.01
Probe ALF	−0.04	0.14
Probe BET	0.05	0.18
Probe GAM	0.06	0.08
Probe LGC	0.08	0.10
Probe HGC	−0.01	0.18
Probe CFB	0.11	0.07

[a]Denotes pooled within-group correlation between the discriminating variables and the canonical discriminant functions.

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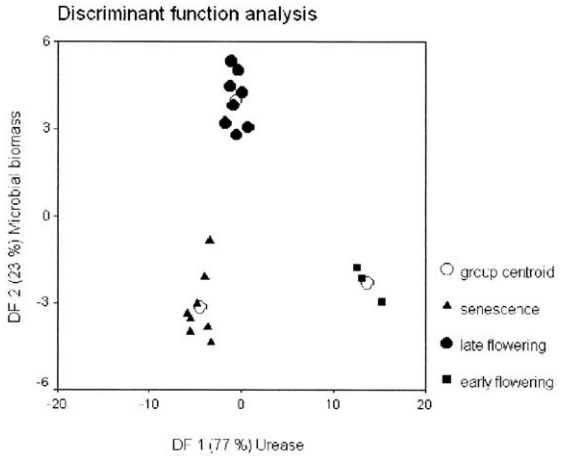

Figure 5. Two-dimensional plot (DF1, DF2) of the discriminant analysis of the microbial variables (dot-blot hybridizations, microbial biomass, enzyme activity) including data of all treatments. Data are labelled by the time of sampling.

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