Plant and Soil **266:** 11–21, 2004.

Bacterial communities associated with the rhizosphere of transgenic Bt 176 maize (*Zea mays*) and its non transgenic counterpart

L. Brusetti[1], P. Francia[1], C. Bertolini[1], A. Pagliuca[1], S. Borin[1], C. Sorlini[1], A. Abruzzese[2], G. Sacchi[2], C. Viti[3], L. Giovannetti[3], E. Giuntini[4], M. Bazzicalupo[4] & D. Daffonchio[1,5]
[1]*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (DISTAM), Università degli Studi di Milano, via Celoria 2, 20133, Milano, Italy.* [2]*Dipartimento di Produzione Vegetale (DIPROVE), Università degli Studi di Milano, via Celoria 2, 20133, Milano, Italy.* [3]*Dipartimento di Biotecnologie Agrarie (DIBA), Università degli Studi di Firenze, P. le delle Cascine 27, 50144, Firenze, Italy.* [4]*Dipartimento di Biologia Animale e Genetica (DBAG), Università degli Studi di Firenze, via Romana 17, 50125, Firenze, Italy.* [5]*Corresponding author**

Received 3 February 2003. Accepted in revised form 29 October 2003

Key words: ARISA, bacterial community genetic profile, transgenic Bt maize 176, Community Level Catabolic Profiling, plant exudates, rhizosphere

Abstract

The effect of transgenic Bt 176 maize on the rhizosphere bacterial community has been studied with a polyphasic approach by comparing the rhizosphere of Bt maize cultivated in greenhouse with that of its non transgenic counterpart grown in the same conditions. In the two plants the bacterial counts of the copiotrophic, oligotrophic and sporeforming bacteria, and the community level catabolic profiling, showed no significant differences; differences between the rhizosphere and bulk soil bacterial communities were evidenced. Automated ribosomal intergenic spacer analysis (ARISA) showed differences also in the rhizosphere communities at different plant ages, as well as between the two plant types. ARISA fingerprinting patterns of soil bacterial communities exposed to root growth solutions, collected from transgenic and non transgenic plants grown in hydroponic conditions, were grouped separately by principal component analysis suggesting that root exudates could determine the selection of different bacterial communities.

Introduction

Transgenic plants resistant to insects like those expressing *Bacillus thuringiensis* Cry proteins (Bt plants), offer several advantages over their corresponding non transgenic cultivars. For example, Bt maize is self-protected against European corn borer (*Ostrinia nubilalis*), so it reduces the specific insecticide treatments, decreasing the risk of pollution due to chemical treatment. However transgenic plants may have unintended modified properties, as is the case of Bt maize where the lignin content is higher than in the non transgenic counterpart (Saxena and Stotzky, 2001). Also transgene expression can vary, depending on the transgene insertion site (van Leeuwen et al., 2001), and the genes at or around the insertion site can be affected in expression rate. Furthermore transgene rearrangement can occur (Windels et al., 2001), with a potential to cause changes in gene expression and plant phenotype.

Plant root microflora is very sensitive to environmental factors, evident in the small differences found between cultivars that often select for different microbial rhizosphere populations (Chiarini et al., 1998; Gomes et al., 2001; Smalla et al., 2001; Smit et al., 2001). The effects of transgenic plants on the rhizosphere community have been the subject of many recent studies. In several cases differences in the microbial community structure, with the non transgenic plants, have been observed, as in the cases of transgenic canola (*Brassica napus*) (Dunfield and Germida,

*FAX No: +39-0250316694.
E-mail: daniele.daffonchio@unimi.it

2001; Gyamfi et al., 2002; Siciliano and Germida, 1999), and alfalfa (Di Giovanni et al., 1999). Instead other studies have revealed no significant differences, some examples being transgenic potato expressing GUS and Barnase (Lukow et al., 2000) and some genetic lines expressing phage T4 lysozyme (Heuer et al., 2002; Lotmann et al., 1999, 2000). For other lines of transgenic potato expressing phage T4 lysozyme there was a significant increase in the death rate of *Bacillus subtilis* in contact with potato roots, and it was deduced that phage T4 lysozyme is released in plant root exudates (Ahrenholtz et al., 2000).

Root exudates play an important role in microflora selection, and selectively influence microbial growth. In fact qualitative and quantitative differences in root exudation can strongly affect the structural and functional diversity of the rhizosphere population (Mansouri et al., 2002; Oger et al., 1997, 2000; Savka and Farrand, 1997). It has been shown that the maize Bt exudation pattern differs from that of non Bt cultivars, due to the release of Cry protein that maintains the insecticidal activity (Saxena et al., 1999; Saxena and Stotzky, 2000). The phenomenon has been demonstrated to be widespread in transgenic maize, and several transgenic lines have been shown to release Cry protein in root exudates (Saxena et al., 2002). The release of Cry protein in the root exudates could potentially affect the prevalence of insect larvae in the rhizosphere by controlling potential root pathogens (a beneficial effect) or by killing saprophitic insects living in the rhizosphere (an unwanted deleterious effect) (Saxena et al., 1999). Similarly the presence of an additional protein in the root exudate could also influence the microbial community in the rhizosphere.

From the abundance of data available it is clear that the effect of transgenic plant on the microbial rhizospheric community, and consequently the evolution of its potential adverse effects, strongly depends on the particular plant, transgene and condition considered. It is therefore important to have reliable tests to evaluate these effects. These should include both cultivation and molecular methodologies, including PCR-based population fingerprinting methods which allows to describe the complexity of a microbial community. To better represent the complexity of a microbial community, a suitable PCR-fingerprinting method must be as much informative as possible giving a high number of amplified products.

In this study we aimed to evaluate whether the bacterial community structure in the rhizosphere of Bt maize 176 is different respect to the non transgenic control plants. We used a polyphasic approach, including cultivation based methods and molecular techniques based on DNA analysis, to evaluate potential effects towards cultivable as well as total eubacteria in two growth phases of maize grown in pots in the greenhouse. Moreover, in order to evaluate the effects of transgenic and nontransgenic plant exudates on bacterial community, the same soil used for plant growth was exposed in axenic condition to a sterile maize root growth solution. After incubation the exudates-enriched bacterial community was analysed by ARISA.

Materials and methods

Plant growth conditions and rhizosphere sampling

Transgenic Maize Bt 176, Novartis (maize Bt) and the conventional non transgenic parental cultivar Tundra, Novartis (maize Tun) were used in all the experiments. The maize plants were grown in a greenhouse (with a 14 h photo-period and day-night temperatures of 26–20 °C) in circular-section pots (40 cm in diameter and 35 cm high) containing an organic soil compost Technic n° 7 (selected sphagnum, 25% dry matter, 12% organic C, total organic N 0.22%, N (NO_3+ NH_4) 125 ppm, P_2O_5 175 ppm, K_2O 175 ppm, pH in H_2O 5.5–6) from Potgrondbedrijf Vrienzenveen B.V., The Netherlands. The control treatment consisted of pots containing the same soil, but without plants, kept under the same conditions. All the pots were arranged at random and were moved daily to ensure uniform conditions. Water adjustment to 70% soil water holding capacity were performed daily.

The sampling of the bulk soil and rhizosphere was performed before planting (0 day only for bulk soil), and after 30 and 100 days of plant growth. At each sampling there was the withdrawal and analysis, of three pots for each condition (bulk soil without plants, maize Bt and maize Tun rhizospheres), totalling 21 samples. Rhizosphere soil samples were taken from three individual plants (one per pot) of each genotype. Rhizosphere soil was defined as the soil still attached to the roots after shaking the roots by hand, thus separating off soil not adhering tightly to the roots; the rhizosphere soil samples were prepared from the soil fraction tightly attached to the roots as described (Baudoin et al., 2002; Gomes et al., 2001). Bulk soil samples were taken from the plant-free pots by coring the first 15 cm of top soil at the center of the pot.

Plate counts and analysis

Plate counts were determined for culturable aerobic copiotrophic bacteria, aerobic oligotrophic bacteria and aerobic sporeforming bacteria. Aerobic copiotrophic bacteria plate counts were determined on Plate Count Agar medium (Difco, Milan, Italy) supplemented with 100 g mL^{-1} of actydione, after two days of incubation at 30 °C. Aerobic oligotrophic bacteria plate counts were determined on 1/10 diluted Tryptic Soy broth (Difco) agarised with 15 g L^{-1} of Bacto Agar (Difco), after four days of incubation at 30 °C. Aerobic sporeforming bacteria plate counts were determined, pasteurising the soil sample at 80 ∘C for 15 min, on Tryptic Soy Agar (Difco) after two days of incubation at 30 °C. Means, standard deviation and analysis of variance of bacterial counts were made, and individual means in the ANOVA were compared using Tukey's test.

Community level catabolic profiling (CLCP) in Biolog GN microplates

For each experiment the rhizosphere soil (soil attached with the roots, gently removable) and rhizoplane fraction (it was that which was obtained when the roots, washed with sterile H_2O, was transferred in a sterile saline solution and shacked with sharp pebbles vigorously) were prepared from three individual plants of each genotype, as described by Zafar et al. (1986). Bulk soil from pots without plants was used as the control. Each fraction was transferred to a centrifuge tube and centrifuged at 1500 × *g* for 2 min. Supernatants were transferred to new tubes and diluted with sterile 0.85% NaCl to a final transmittance of 85% using a Biolog turbidimeter (Biolog Inc., Hyward, CA) (Di Giovanni et al., 1999). One hundred and fifty μl of each suspension were used to inoculate Biolog-GN microplates (two replicates for each extract). The microplates were incubated in the dark at 23 °C. Colour development was measured approximately every 12 h until metabolic profiles were stabilised, using a DV990BV4 computer-assisted microplate reader (GDV, Rome, Italy) at 590 nm.

The optical density values in each microplate were corrected for the background value of the control wells. In order to normalise for minor differences in inoculum density, the microplates were compared at a standardised references point in colour development (average well colour development, AWCD) (Garland and Mills, 1991). The statistical analysis was made on microplates having AWCD values of $1.0 \pm 5\%$ (Insam et al., 2001). The 95 variables were transformed to principal components (PCs) using the covariance of the variables. The first two PCs, that explained more than 75% of the total variance, were used to test the significant differences between genotypes and between plant genotypes and bulk soil (multivariate analysis of variance, MANOVA, $P < 0.05$). Statistical analysis was performed with the program Statistica 5.0 (StatSoft Inc., USA).

Molecular analysis of the rhizosphere bacterial community by ARISA fingerprinting

For the ARISA experiments, DNA was extracted from 2.5 g of rhizosphere and bulk soil at each sampling time, and eluted in 400 μL of TE pH 8 as described by Zhou et al. (1996).

DNA was purified from humic acids by fractioning the crude extract onto Sepharose 4B (Sigma, Milan, Italy) as described by Jackson et al., (1997). The purity of the DNA obtained after treatment with Sepharose 4B was tested by PCR amplification of 16S rRNA gene as previously described (Zucchi et al., 2003). After the PCR amplification tests, fractions 2, 3, 4 and 5 obtained from Sepharose 4B purification were pooled for each soil sample. DNA was precipitated with ethanol, resuspended in TE pH 8 and used for further PCR experiments.

For the ARISA experiments (Fisher and Triplett, 1999), the intergenic transcribed spacers (ITS) between 16S and 23S rDNA were amplified using primers S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 as previously described (Daffonchio et al., 1998), except that primer S-D-Bact-1494-a-S-20 was $5'$ end labelled with the phosphoramidite dye 5-FAM. Aliquots of the PCR products (1 to 5 μL) were mixed with 1 μL of the GeneScan-1000 [ROX] internal size standard (Applied Biosystems), 20 μL of deionized formamide was added, the mixture was denatured at 95 °C for 5 min and cooled in an ice bath. The PCR products were then analysed in an ABI Prism 310 genetic analyser (Applied Biosystems), using a 47 cm × 50 μm capillary filled with POP-4 polymer (Applied Biosystems). The samples were run under standard ABI 310 denaturing electrophoresis conditions for 40 min. The data were analysed using the GeneScan 3.1 software program (Applied Biosystems).

The peak matrices corresponding to the ARISA profiles were subjected to principal component analysis (PCA [Ranjard et al., 2001]) by using Jaccard coefficient in the NTSYS pc 2.01 software (Applied

Biostatistics Inc., USA). Cluster significance in the PCA plot was checked by a Least Significance Difference test, $P < 0.05$.

Effect of root growth solution on rhizosphere bacterial community

Maize seeds were imbibed for 1 h in distilled H2O and then surface sterilised for 20 min in 6% Na hypochloride. The seeds were then germinated in the dark at 26 °C in Petri dishes on filter paper in sterile conditions. The coleoptile of the seedlings, with a main root length of about 4 cm, was sealed into a sterile 100 mL growth container (rhizotron) containing 75 mL of Hoagland solution and incubated in the dark under continuous sterile air flow through a 0.22 μm filter. The plants were grown in the phytotron under the following conditions: 14 h day-length with a PAR of 250 μmol m^{-2} s^{-1}, 26–20 °C day-night cycles, 70% relative humidity, 250 μE s^{-1} m^{-2} photon flux density. The volume of growth solution was checked periodically and maintained constant by adding sterile distilled H_2O in order to avoid an over-concentration of mineral nutrients. After each addition the solution was analysed for sterility using Plate Count Agar (Difco) medium, and any non sterile rhizotrons were discharged. After 14 days growth the sterile solutions were collected, frozen rapidly and stored at −80 °C. The root and shoot weight was recorded. Total organic carbon content was measured by wet oxidation with 0.25 keq m^{-3} $K_2Cr_2O_7$ and subsequent titration with 0.1 kmol m^{-3} $(NH_4)_2Fe(SO_4)_2$ $6H_2O$, as described in Sacchi et al. (2000).

Fifty grams of the same soil used for plant cultivation was mixed carefully and homogenised. One gram aliquots of this soil were placed aseptically in 12 sterile glass tubes that had two drainage layers (each about 2 cm thick), sterile glass wool lying on sterile glass beads (average diameter 3 mm) at the bottom of the glass tube. A set of four of these microcosms was watered with 1 mL per microcosm of Bt maize root growth solution. A second microcosm set was watered with Tun maize root growth solutions. A third set, acting as the control, was watered with sterile Hoagland solution. The twelve microcosms were incubated for 48 h at 28 °C. After incubation the entire soil content of the microcosm underwent DNA extraction and purification, as previously described. ARISA was done as already described.

Table 1. Bacterial plate counts of rhizosphere samples from Bt maize and non transgenic maizes (Bt and Tun) and control root free soil (N)

Bacterial group	Plant growth (Days)	3 Plate counts		
		N[a]	Bt[b]	Tun[b]
Copiotrophic	0	7.27	n.a.[c]	n.a.
	30	6.83A	6.62B	6.57B
	100	6.31B	7.12A	6.50B
Oligotrophic	0	7.30	n.a.	n.a.
	30	6.97A	6.85B	6.73C
	100	6.37B	6.96A	7.15A
Sporeforming	0	3.87	n.a.	n.a.
	30	4.20A	4.00B	3.85B
	100	4.22A	4.14A	4.31A

[a] Average (determination on 3 samples) of $\log_{10}$ CFU g^{-1} (fresh weight) of soil.
[b] Average (determination on 3 plants) of $\log_{10}$ CFU g^{-1} (fresh weight) of root. Significant differences at Tukey's test (ANOVA; $P < 0.05$) within a sampling time are indicated by different letters.
[c] not applicable.

Results

Bacterial plate counts and community level catabolic profiling

A count was made of the number of aerobic copiotrophic, oligotrophic and sporeforming bacteria recovered from the bulk soil and rhizosphere at three different sampling times. The bacterial count in rhizosphere soil was determined after 30 and 100 days of plant growth, while in the bulk soil the bacteria were counted at 0, 30 and 100 days of plant growth (Table 1). The counts of copiotrophic and oligotrophic bacteria were always similar, between 3×10^6 to 3×10^7 per g of fresh weight, and the same range was found in the bulk soil and rhizosphere of both Bt and Tun maize. A lower number of sporeforming bacteria, ranging between 8×10^3 to 3×10^4 per g, was found in both the bulk soil and the two rhizospheres. ANOVA analysis of the bacterial counts revealed significant differences ($P < 0.05$) between Bt and Tun maize rhizospheres only for copiotrophic bacteria at 100 days and for oligotrophic bacteria at 30 days. Significant differences were found between the communities of the two rhizospheres and the bulk soil, these differences being evident for all three bacterial groups analysed at day 30, and for the oligotrophic bacteria also at day 100.

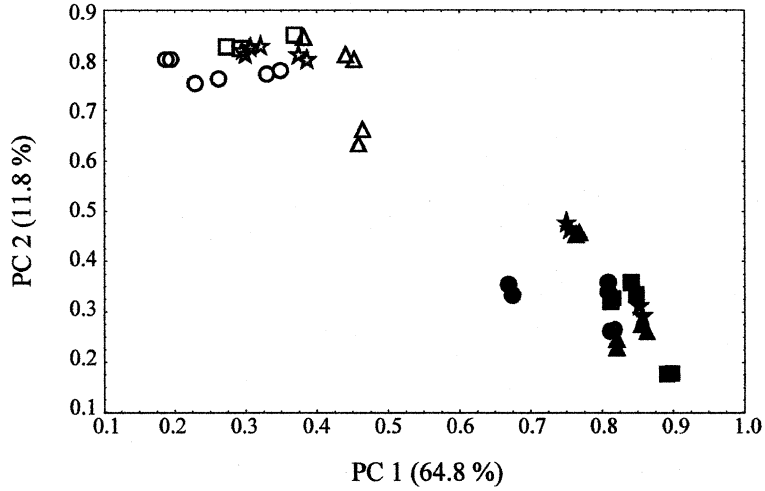

Figure 1. Principal components analysis (PCA) of community-level catabolic profile (CLCP) of rhizoplane and rhizosphere soil samples from transgenic Bt maize and non transgenic Tun maize at 30 (white filled symbols) and 100 (black filled symbols) days; dots = rhizoplane Bt maize, squares = rhizosphere soil Bt maize, triangles = rhizoplane Tun maize, stars = rhizosphere soil non transgenic maize).

The differences in the bacterial counts were confirmed by the CLCP analysis of the rhizosphere of the two plants and the bulk soil for the three sampling times. The multivariate data set obtained from the respiration rates of the 95 sole carbon source obtained from the Biolog GN microplates were reduced to two PCs that explained more than 76% of the total variance, and were compared by MANOVA (Figure 1). Significant differences ($P < 0.05$) in CLCP were detected between the rhizosphere as well as the bulk soil communities at both sampling times, while no significant differences were found between the Bt and Tun maizes, neither in the rhizosphere nor in the rhizoplane (Table 2).

ARISA of rhizosphere and bulk soil bacterial community

The total eubacterial communities in the rhizosphere of Bt and Tun maize plants and in bulk soil were analysed by ARISA fingerprinting. Of 21 samples only 18 could be analysed, the three not-analysed bulk soil samples, (two at time 0 and one at 30 day sampling) not giving any detectable PCR product, despite several attempts to improve the DNA purification (data not shown). The number of detectable peaks in the electropherograms ranged between 42 to 80 and the fragment length was between 215 and 970 bp.

Experiments were performed to evaluate the reproducibility of the ARISA by comparing independent amplifications of the same DNA sample. The experiment showed good reproducibility with a 95.6% overlap of the peaks (data not shown). ARISA profiles of three independent rhizosphere samples obtained from three Tun maize plants at the same sampling time showed very similar, profiles with an 87% overlap of the peaks, confirming the suitability of the method and the reproducibility of the microcosms used.

Figure 2 shows example of the ARISA profiles of Bt and Tun maize rhizosphere and bulk soil at sampling times 0 (only bulk soil), 30 and 100 days. In total, at each sampling time (from 0, 30 and 100 days of plant growth), three replicate samples (from three different pots) were analyzed by ARISA of the eubacterial community in the bulk soil (taken from pots without plants), and in the rhizosphere of Bt and Tun maize (only at times 30 and 100 days). Note that the soil and rhizosphere samples at 30 days are characterised by major peaks grouped around 650 bp, indicating that bacterial population is dominated by certain bacterial groups; instead, in samples taken after 100 days, the major peaks are scattered in a wider range of fragment sizes. The peak data were used for PC analysis as described in Methods. The scatter plot resulting from the analysis of the first two PCs explaining 39.4% of the total variance is shown in Figure 3. The variability among some replicates of the same type of plant is greater than the variability observed among replicates of different plant types, especially at 100 days. However, the 18 samples are clustered into two main groups, separated along the second principal component (PC2). The first group (positive coordinates on PC2) includes all the samples taken at 0 and 30

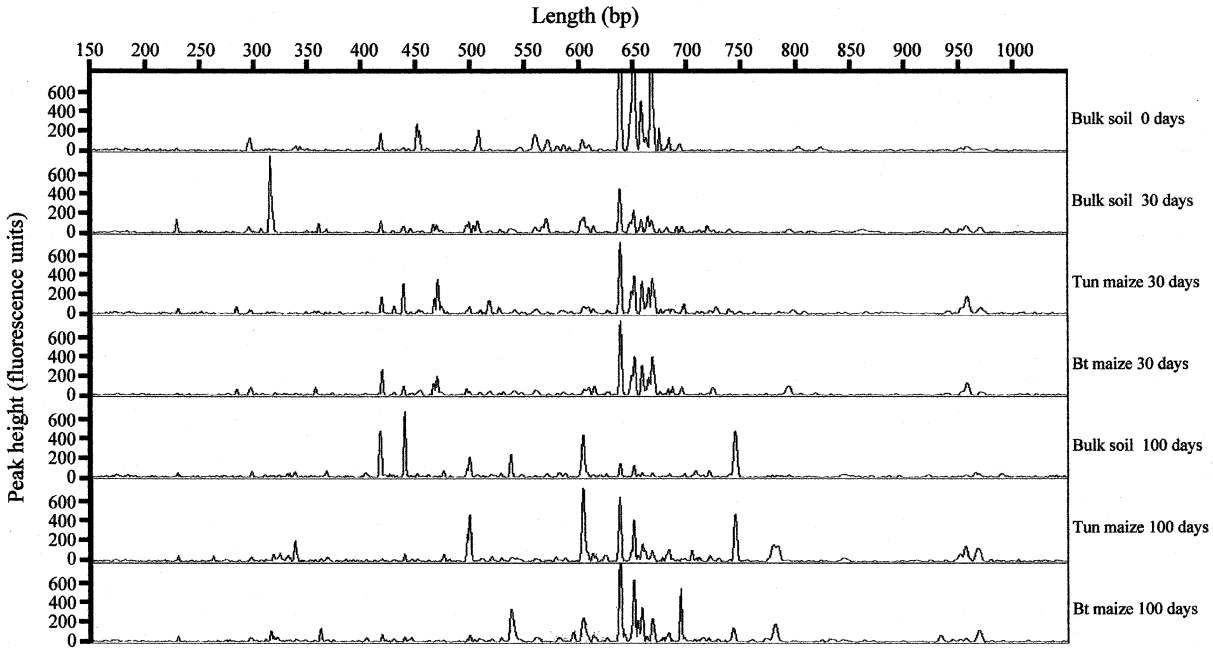

Figure 2. Example of ARISA profiles at three different times of sampling from bulk soil and rhizosphere of Bt maize and its non transgenic counterpart. In total, at each sampling time (from 0, 30 and 100 days of plant growth), three replicate samples (from three different pots) were analyzed by ARISA of the eubacterial community in the bulk soil (taken from pots without plants), and in the rhizosphere of Bt and Tun maize (only at time 30 and 100 days).

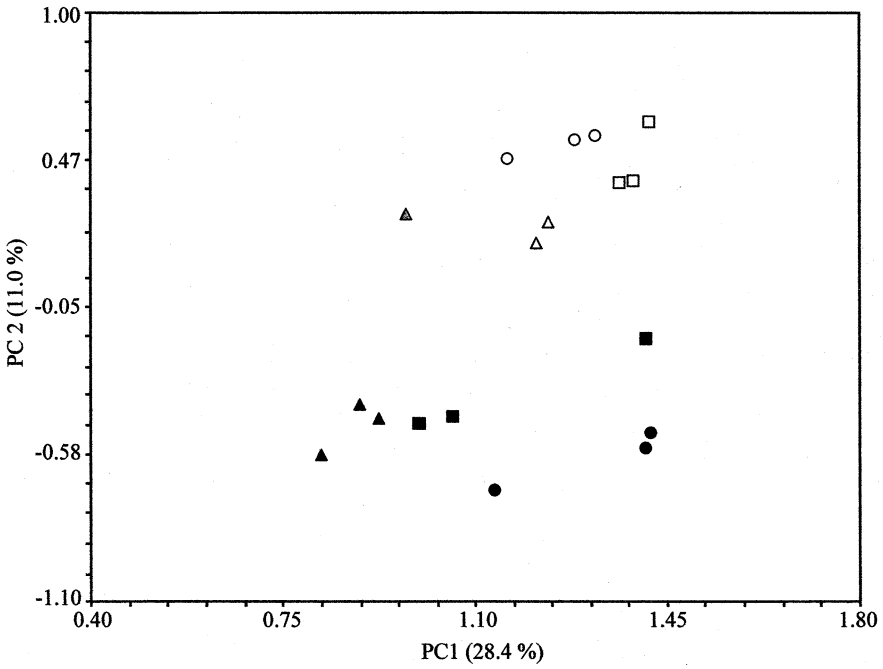

Figure 3. Principal component analysis showing the distribution of ARISA profiles at 0 (grey filled symbol), 30 (white filled symbols) and 100 (black filled symbols) days. Bt maize (■), non transgenic maize (●), bulk soil (▲).

Table 2. Pairwise statistical comparisons of community-level catabolic profile (CLCP) of rhizosphere samples from Bt maize and Tun maize and control root free soil (N)

Plant growth (Days)	Significant differences ($P < 0.05$) in CLCP of rhizospheres and bulk soil[a]					
	RTun[b]/RBt[c]	PTun[d]/PBt[e]	RTun/N	RBt/N	PTun/N	PBt/N
30	No	No	Yes	Yes	Yes	Yes
100	No	No	Yes	Yes	Yes	Yes

[a]CLCP determinations made on 6 replicates. MANOVA on the first two PCs.
[b]Rhizosphere soil of maize Tun.
[c]Rhizosphere soil of maize Bt.
[d]Rhizoplane of maize Tun.
[e]Rhizoplane of maize Bt.

days, the second group those taken at 100 days. In the first group the sample at time 0 was separated from the samples taken at 30 days along PC1. MANOVA analysis made over PCA indicated that the bacterial communities of the rhizosphere and bulk soils were significantly discriminated as a function of sampling time (i.e., plant age for the rhizosphere samples) ($P < 0.05$). In the 30 days samples MANOVA analysis showed significant difference along the first principal component axis between the Bt maize and both the Tun maize and control soil eubacterial microbial populations ($P < 0.05$). In the samples taken after 100 days the microbial population of the bulk soil is well separated from the rhizosphere populations both of Bt and Tun maize ($P < 0.05$). PCA showed at 100 days a more scattered distribution of the data points than at 30 days, indicating a higher variability of the eubacterial population between plants than at 30 days.

Effect of Bt 176 maize exudates on the soil bacterial community

An experiment was performed to compare the effects of root growth solution, of Bt and Tun maize grown in hydroponic condition, on the soil bacterial community. The amount of total carbon in the root growth solutions from Bt and Tun maize plants was comparable being 260 ± 30 and 310 ± 40 μg ml^{-1}, respectively. Figure 4A shows examples of the ARISA pattern obtained. The number of detectable peaks in the electropherograms ranged between 70 and 120 and the fragment length was between 210 and 926 bp. The samples had similar major peaks but showed differences in the minor peak populations. By analysing ARISA patterns with PCA it was possible to place the bacterial communities according to treatment type, three dimensional plotting along the three principal components (Figure 4b). The first (PC1), second (PC2) and third (PC3) principal components explained, respectively, 42.0%, 9.3% and 5.5% of the variance in the data. The PCA showed that the root growth solutions of the two maize cultivars selected different bacterial communities. Three main groupings could be identified on the plot: one included the soil community of the four control soil samples, a second the soil bacterial communities from the four samples exposed to the Tun maize exudates while the third grouping in the plot, separated from the other samples on the PC2, included the ARISA profiles of bacterial communities enriched by the root growth solution of Bt maize. MANOVA analysis showed significant differences ($P < 0.05$) along the second principal component axis only between the Bt maize and both the Tun maize and control soil eubacterial populations.

Discussion

There has been strong debate on the safety of genetically modified plants ever since the introduction onto the market of plant products deriving from transgenic crops. This debate is still very much alive, and several issues have been raised, including the safety of transgenic food and the environmental impact of transgenic plants. The fires of the debate are mainly fed by the insufficient knowledge available on biological systems, and by the potential danger that specific genetic manipulations could give unexpected effects (Schubert, 2002). In fact, Bt maize has been found to have unexpected modifications that could exert some effect on the soil environment; it is known that Bt maize has a higher lignin content than its non transgenic counterpart (Saxena and Stotzki, 2001), potentially affecting the rate of maize tissue degradation in soil, and transgenic Cry protein has been found to be released in the root exudates (Saxena et al., 1999, 2002; Saxena

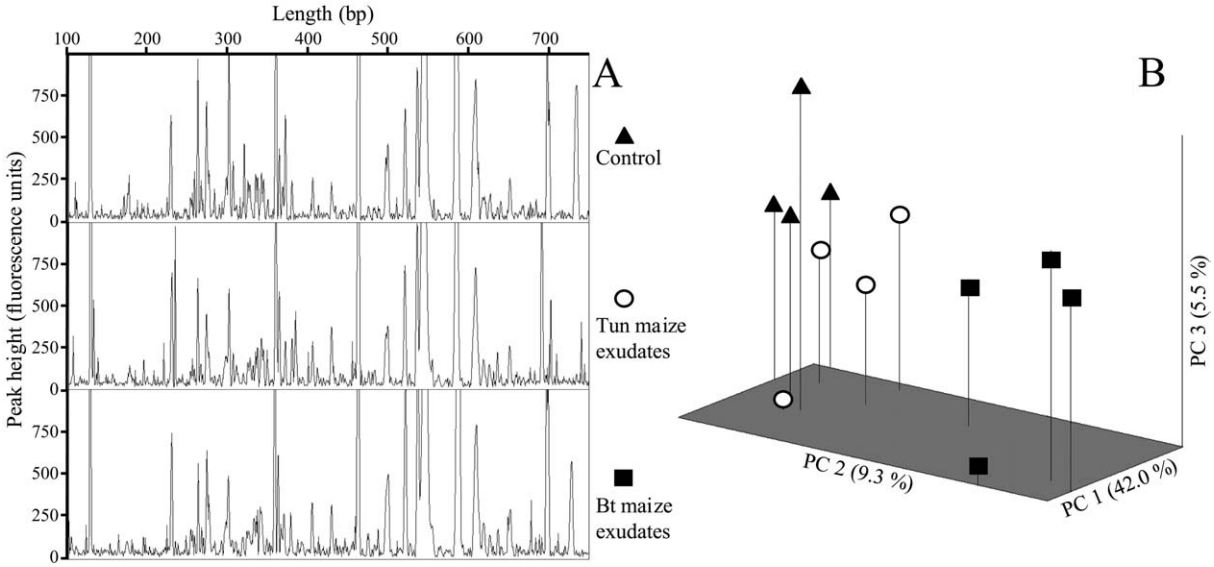

Figure 4. Effects of exudates on soil bacterial community. (A) Example of ARISA profiles from soil exposed to control solution and Bt and non Bt maize root growth solutions. (B) PCA of the ARISA fingerprinting.

and Stotzky, 2000). Indeed the rhizosphere environment hosts a complex microorganism network which interacts very closely with plant roots, the outcome of such interaction being seen in the strong influence plant type has on the microbial ecosystem of the soil. In fact, it is known that soil bacterial communities are more strongly influenced by plant species and different cultivars than by other environmental factors like soil type and agricultural practices (Gomes et al., 2001; Heuer et al., 2002).

On considering the different exudate pattern of Bt maize with respect to non transgenic maize, we were prompted to evaluate whether this unexpected characteristic could affect the diversity of the rhizosphere microflora. For this study we used a polyphasic approach that allowed us to examine both the cultivable microflora, in terms of abundance and functional diversity, and the total bacterial population of rhizosphere and bulk soil using cultivation-independent molecular methods.

Analysis of the abundance of cultivable copiotrophic, oligotrophic and sporeforming bacteria showed major differences between the bulk soil and rhizosphere bacterial communities, as expected, but only sporadic differences could be detected between the different plant growth phases or between the transgenic and the non transgenic cultivars. These results were also confirmed by an analysis of the functional diversity of the community performed through CLCP using the Biolog GN microplates, that did not detect differences between the two cultivars. The only significant differences were between the rhizosphere/rhizoplane system and the bulk soil. These data are in agreement with a previous study of Saxena and Stotzky (2001) who did not find any significant differences in the colony-forming units of culturable bacteria, actinomycetes, fungi, protozoa and nematodes in the rhizospheres of transgenic Bt and non transgenic maize. Koskella and Stotzky (2002) confirmed that several Cry toxins from *B. thuringiensis* have no microbicidal or microbiostatic activity against selected bacteria.

Molecular techniques are highly sensitive to even small changes in the structure of microbial communities, and can detect minor variation in bacterial composition. We further investigated the bacterial community structure of the Bt maize rhizosphere using ARISA (Fisher and Triplett, 1999), a population fingerprinting approach. Among the molecular techniques for the soil bacterial community analysis ARISA proved to be very effective. The ARISA (Fisher and Triplett, 1999) is a PCR-based fingerprinting approach targeting the 16S-23S intergenic transcribed spacers a region considered hypervariable respect to the adjacent genes and hence useful to discriminate bacteria at the subspecies level (Daffonchio et al., 1998, 2000). The advantage of ARISA respect to the traditional analysis of fingerprinting patterns in

agarose/polyacrylamide gels is the separation of labelled fragments in an automated sequencer which guarantees high sensitivity also for poorly amplified fragments and high resolution even of hundreds of peaks per profile (Ranjard et al., 2001). The high number of peaks which this system allows to detect is very informative and makes ARISA more suitable for comparison of whole community diversity than other methods such as DGGE and TGGE analysis.

A relatively low level of variability (39.4%) was explained by the first two principal components of PCA of rhizosphere ARISA patterns (Figure 3), confirming that soil is a complex and heterogeneous environment.

Despite the relatively low level of variability explained by PCA, the ARISA patterns allowed to argue some indication: (i) rhizosphere-induced bacterial population shift, with respect to bulk soil populations, which reflecting differences already observed with cultivation methods. (ii) plant age-dependent population patterns. (iii) bacterial community differences between Bt maize and its non transgenic counterpart.

With regarding to time-dependent community shifting, the PCA of the ARISA fingerprinting patterns indicated a separation between the community colonising the maize after 30 days of growth and the population in the senescent plants (after 100 days of growth). The relative low variability observed by ARISA in the rhizosphere samples of each plant type at 30 days, showing few major peaks around 650 bp, could be due to the active root exudation of young plants that could have selected a bacterial community with few dominant strains, possibly r-strategists, typical of uncrowded, nutrient-rich environments. On the contrary reduced exudation by senescent roots (sampling time 100 days) would lead to other environmental factors, such as localised root cell lysis, or heterogeneous microniche soil composition, exerting a more marked influence on the bacterial community, resulting in greater sample variability. In this environmental conditions at low nutrient level, K-strategists, colonising niches that have reached their carrying capacity, would became prevalent, resulting in an increase of the observable diversity. An r/K-strategist succession has already been observed by Chiarini et al. (1998) in maize roots, the r-strategists dominating the early stages of maize development and the K-strategists in mature roots. Gomes et al. (2001) found, in tropical soils, a more pronounced rhizosphere effect in young roots of maize (20 days after sowing) than in the mature plant (106 days after sowing). They found by TGGE, as we did with ARISA, that the bacterial community in the rhizosphere of young plants is dominated by very few genotypes, represented by intense TGGE bands.

ARISA allowed the differentiation of the bacterial communities of transgenic maize and its non transgenic counterpart, indicating that the two plants tend to select different rhizosphere communities, an effect revealed by PCA analysis of ARISA profiles to be more pronounced in the samples obtained after 30 days of plant growth. The differences seen in the ARISA patterns for the transgenic and non transgenic cultivars could to be due to the root exudation pool which in maize Bt is known to be different respect to its non-transgenic counterpart at least for the release of the Cry protein (Saxena et al., 1999, 2002; Saxena and Stotzky, 2000). To investigate this aspect, we performed an experiment using a more simplified experimental model, exposing root- free soil to root growth solutions recovered from transgenic and non transgenic plants.

The bacterial community ARISA profiles obtained from the total DNA extracted after exposing soil to root growth solutions, clearly confirmed that the exudate of the transgenic plant led to a different bacterial community selection than that of the non transgenic plant. The major peaks of the ARISA profiles, presumably representing the dominant bacterial communities, remained stable, however the minor peaks varied greatly, reflecting the shifting of the less dominant communities. This observation led us to suppose that the Bt maize exudate differs in several ways from the non transgenic plant, not just in the Cry protein. However these differences need confirmation by a careful chemical characterisation of the exudate of transgenic Bt maize and its non transgenic counterpart. It is interesting to note that other genetic maize modifications, such as the glufosinate herbicide (syn. L-phosphinothricin) resistance conferred by the *pat* gene, did not affect the bacterial community structure significantly, as evidenced by 16S rRNA gene PCR-SSCP community profiling (Schmalenberger and Tebbe 2002). In fact it would be interesting to evaluate whether this lack of effect on the rhizosphere bacterial community is correlated to an unaltered exudation pattern in *pat* gene-tagged maize. The physiological reason of the different exudation patterns between transgenic and non transgenic Bt maize are not clear. However this characteristic is common to Bt maize other than Bt 176. Infact modification of the plant genotype with the Cry1Ab gene affects the exuda-

tion pattern, releasing the transgenic Cry protein also in other transgenic Bt cultivars such as Bt11 and MON810, a modification already tested in 12 different transgenic hybrids (Saxena et al., 2002). Other authors reported no significant changes in the rhizosphere of Cry expressing cotton (Donegan et al., 1995) and potato (Donegan et al., 1996). However these investigations have been based on the cultivable microflora analysis, that was, at least in our work, less sensitive to small shifts of a community than the molecular fingerprinting methods based on total soil DNA.

The environmental significance of the differences remains to be determined. Extensive studies made on another plant model, the phage T4 lysozyme-expressing transgenic potato, have shown that general environmental parameters such as cultivar and soil type, seasonal climate changes and plant age have a more marked effect on the rhizosphere microflora than the presence of the transgenic character (Heuer et al., 2002; Lottmann et al., 1999, 2000, 2001). Note however that the T4 lysozyme expressing potato seems to alter its exudation pattern by secreting, into the exudate, the lysozyme that could have some effect on non-target bacterial strains such as *B. subtilis* (Ahrenholtz et al., 2000). To address the question of the general environmental effects of transgenic Bt maize on rhizosphere bacteria it would be interesting to evaluate whether other components of the exudate are affected in the transgenic plant, and which bacteria respond specifically to these changes.

Acknowledgements

The work was supported by the Italian Ministry for University and Scientific Research within the project 'Risposta della comunità microbica del suolo a differenti pressioni antropiche: effetti su struttura, dinamica e diversità della microflora' (Cofin 2000). Partial support comes also from the EU project TRANSBAC QLK3-CT-2001-02242 (5th RTD Program). The manuscript was edited by Barbara Carey.

References

Ahrenholtz I, Harms K, de Vries J and Wackernagel W 2000 Increased killing of *Bacillus subtilis* on the hair roots of transgenic T4 lysozyme-producing potatoes. Appl. Environ. Microbiol. 66, 1862–1865.

Baudoin E, Benizri E and Guckert A 2002 Impact of growth stage on the bacterial community structure along maize roots as determined by metabolic and genetic fingerprinting. Appl. Soil Ecol. 19, 135–145.

Chiarini L, Bevivino A, Dalmastri C, Nacamulli C and Tabacchioni S 1998 Influence of plant development, cultivar and soil type on microbial colonisation of maize roots. Appl. Soil Ecol. 8, 11–18.

Daffonchio D, Borin S, Frova G, Manachini P L and Sorlini C 1998 PCR fingerprinting of whole genomes, the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveals a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int. J. Syst. Bacteriol. 48, 107–116.

Daffonchio D, Cherif A and Borin S 2000 Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the '*Bacillus cereus* group'. Appl. Environ. Microbiol. 66, 5460–5468.

Di Giovanni G D, Watrud L S, Seidler R J and Widmer F 1999 Comparison of parental and transgenic alfalfa rhizosphere bacterial communities using Biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). Microb. Ecol. 37, 129–139.

Donegan K K, Palm C J, Fieland V J, Porteous L A, Ganio L M, Schaller D L, Bucao L Q and Seidler R J 1995 Changes in levels, species and DNA fingerprinting of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. Appl. Soil Ecol. 2, 111–124.

Donegan K K, Schaller D L, Stone J K, Ganio L M, Reed G, Hamm P and Seidler R J 1996 Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var. *tenebrionis* endotoxin. Transgenic Res. 5, 25–35.

Dunfield K E and Germida J J 2001 Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiol. Ecol. 38, 1–9.

Fisher M M and Triplett E W 1999 Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. 65, 4630–4636.

Garland J L and Mills A L 1991 Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. 57, 2351–2359

Gyamfi S, Pfeifer U, Stierschneider M and Sessitsch A 2002 Effects of transgenic gluphosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiol. Ecol. 41, 181–190.

Gomes N C M, Heuer H, Schönfeld J, Costa R, Medonça-Hagler L and Smalla K 2001 Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232, 167–180.

Heuer H, Kroppenstedt R M, Lottmann J, Berg G and Smalla K 2002 Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. Appl. Environ. Microbiol. 68, 1325–1335.

Insam H, Feurle J and Rangger A 2001 Community level physiological profiles (Biolog substrate use tests) of environmental samples. *In* Molecular Microbil Ecology Manual. Eds. Akkermans A D L, van Elsas J D and de Bruij F J. pp. 1–6. Kluwer Academic, Dordrecht, The Netherlands.

Jackson C R, Harper J P, Willoughby D, Roden E E and Churchill P F 1997 A simple, efficient method for the separation of humic substances and DNA from environmental samples. Appl. Environ. Microbiol. 63, 4993–4995.

Koskella J and Stotzky G 2002 Larvicidal toxins from *Bacillus thuringiensis* subsp. *kurstaki, morrisoni* (strain *tenebrionis*), and *israelensis* have no microbicidal or microbiostatic activity against selected bacteria, fungi, and algae in vitro. Can. J. Microbiol. 48, 262–267.

Lottmann J and Berg G 2001 Phenotypic and genotypic characterisation of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants. Microbiol. Res. 156, 75–82.

Lottmann J, Heuer H, de Vries J, Mahn A, Düring K, Wackernagel W, Smalla K and Berg G 2000 Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. FEMS Microbiol. Ecol. 33, 41–49.

Lottmann J, Heuer H, Smalla K and Berg G 1999 Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. FEMS Microbiol. Ecol. 29, 365–377.

Lukow T, Dunfield P F and Liesack W 2000 Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol. Ecol. 32, 241–247.

Mansouri H, Petit A, Oger P and Dessaux Y 2002 Engineered rhizosphere: the trophic bias generated by opine-producing plants is independent of the opine type, the soil origin, and the plant species. Appl. Environ. Microbiol. 68, 2562–2566.

Oger P, Mansouri H and Dessaux Y 2000 Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. Mol. Ecol. 9, 881–890.

Oger P, Petit A and Dessaux Y 1997 Genetically engineered plants producing opines alter their biological environment. Nature Biotech. 15, 369–372.

Ranjard L, Poly F, Lata J -C, Moughel C, Thioulouse J and Nazaret S 2001 Characterisation of fungal and bacterial soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and methodological variability. Appl. Environ. Microbiol. 67, 4479–4487.

Sacchi G A, Abruzzese A, Lucchini G, Fiorani F, Cocucci S. 2000 Efflux and active re-absorption of glucose in roots of cotton plants grown under saline conditions Plant Soil 220, 1–11.

Savka M A and Farrand S K 1997 Modification of rhizobacterial populations by engineering bacterium utilisation of a novel plant-produced resource. Nature Biotech. 15, 363–368.

Saxena D and Stotzki G 2001 *Bt* corn has a higher lignin content than non-*Bt* corn. Am. J. Botany 88, 1704–1706.

Saxena D and Stotzky G 2000 Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn *in vitro* and *in situ*. FEMS Microbiol. Ecol. 33, 35–39.

Saxena D and Stotzky G 2001 *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol. Biochem. 33, 1225–1230.

Saxena D, Flores S and Stotzky G 1999 Insecticidal toxin in root exudates from Bt corn. Nature 402, 480.

Saxena D, Flores S and Stotzky G 2002 Bt toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events. Soil Biol. Biochem. 34, 133–137.

Schmalenberger A and Tebbe C C 2002 Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar *Bosphore*. FEMS Microbiol. Ecol. 40, 29–37.

Schubert D 2002 A different perspective on GM food. Nature Biotech. 20, 969.

Siciliano S D and Germida J J 1999 Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. FEMS Microbiol. Ecol. 29, 263–272.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H and Berg G 2001 Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. 67, 4742–4751.

Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S, Wernars K 2001 Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a weath field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. 67, 2284–2291.

Van Leeuwen W, Ruttnik T, Borst Vrenssen A W M, van der Plas L H W and van der Krol A R 2001 Characterisation of position-induced spatial and temporal regulation of transgene promoter activity in plants. J. Exp. Bot. 52, 949–959.

Windels P, Taverniers I, Depicker A, Van Bockstaele E and De Loose M 2001 Characterisation of the Roundup Ready soybean insert. Eur. Food Res. Technol. 213, 107–112.

Zafar Y, Ashraf M and Malik K A 1986 Nitrogen fixation associated with roots of Kallar grass (*Leptochloa fusca* L. Kunth). Plant Soil 90, 93–105.

Zhou J, Bruns M A and Tiedje J M 1996 DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62, 316–322.

Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, Gigliotti C, Barbieri P, Sorlini C and Daffonchio D 2003 Response of bacterial community during bioremediation of an oil-polluted soil. J. Appl. Microbiol. 94, 248–257.