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Impact of Bt Corn on Rhizospheric and Soil Eubacterial Communities and on Beneficial Mycorrhizal Symbiosis in Experimental Microcosms

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A polyphasic approach has been developed to gain knowledge of suitable key indicators for the evaluation of environmental impact of genetically modified Bt 11 and Bt 176 corn lines on soil ecosystems. We assessed the effects of Bt corn (which constitutively expresses the insecticidal toxin from *Bacillus thuringiensis*, encoded by the truncated *Cry1Ab* gene) and non-Bt corn plants and their residues on rhizospheric and bulk soil eubacterial communities by means of denaturing gradient gel electrophoresis analyses of 16S rRNA genes, on the nontarget mycorrhizal symbiont *Glomus mosseae*, and on soil respiration. Microcosm experiments showed differences in rhizospheric eubacterial communities associated with the three corn lines and a significantly lower level of mycorrhizal colonization in Bt 176 corn roots. In greenhouse experiments, differences between Bt and non-Bt corn plants were detected in rhizospheric eubacterial communities (both total and active), in culturable rhizospheric heterotrophic bacteria, and in mycorrhizal colonization. Plant residues of transgenic plants, plowed under at harvest and kept mixed with soil for up to 4 months, affected soil respiration, bacterial communities, and mycorrhizal establishment by indigenous endophytes. The multimodal approach utilized in our work may be applied in long-term field studies aimed at monitoring the real hazard of genetically modified crops and their residues on nontarget soil microbial communities.

Crop plants genetically modified (GM) for resistance to pests represent a potential environmentally safe tool to decrease the amount of chemical pesticides used in agriculture. One of the most widespread GM crops is Bt corn, which constitutively expresses the insecticidal toxin from *Bacillus thuringiensis*, encoded by the truncated *Cry1Ab* gene. Bt corn is also often modified to express the PAT gene from *Streptomyces* spp., which confers tolerance to the herbicide glufosinate ammonium. Concerns have been raised about the environmental risks associated with the release of transgenic crops, including the potential impact on nontarget organisms, such as beneficial insects, soil bacteria, and fungi, which play a fundamental role in crop residue degradation and in biogeochemical cycles. In fact, many studies showed that soil microbes represent important key nontarget organisms able to highlight unforeseen collateral effects of transgenic plants on natural and agricultural ecosystems. For example, GM potato lines producing *Galanthus nivalis* agglutinin and *Brassica napus* resistant to the herbicide glyphosate modified the composition and diversity of soil and rhizospheric microbial communities (22, 63). Other works reported different effects of GM plants on soil microorganisms, mainly at the rhizosphere level, where root exudates directly affect the composition of microbial soil communities, in terms of both structure and function (5, 21, 37, 38, 50, 60, 61, 64, 66, 80).

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Laboratory and field studies have demonstrated that *B. thuringiensis* toxin is released in soil through three main pathways: (i) root exudates (55, 58, 59), (ii) plant residues plowed into the soil after crop harvest (29, 81), and (iii) pollen falling down (35). In soil, *B. thuringiensis* toxin does not change its conformation (34) and remains active, protected from microbial degradation by absorption to clays or linkage to humic acids (6, 8, 31). Moreover, *B. thuringiensis* toxin released through corn root exudates retains its activity for 180 to 234 days in both laboratory and soil experiments (56, 67, 71), thus representing a potential risk for nontarget organisms and microorganisms (34, 73, 79, 82). For example, some authors reported a reduction in the growth of bacteria occurring on feces of the crustacean *Porcellio scaber* fed with GM corn (13). Other studies showed no deleterious effects on soil microbial communities by *B. thuringiensis* toxin released into soil through root exudates and by residues of Bt corn on culturable bacteria and saprophytic fungi, both in vitro and in vivo (32, 56, 72). An unexpected effect—a higher lignin content compared to the nontransgenic isolines, which could affect microbial saprophytic communities—has been shown in some Bt transformants of canola, potato, maize, tobacco, and cotton (68).

The results available on the impact of GM plants on natural and agricultural ecosystems show that specific effects of single transformation events should be tested on a case-by-case basis, using different target and nontarget organisms and multimodal experimental approaches and taking into account biochemical, physiological, and molecular parameters.

We carried out a 2-year polyphasic experiment with the aim of gaining knowledge on suitable key indicators to be used for

the evaluation of environmental impact of genetically modified Bt corn on soil ecosystems. The experiments were aimed at assessing the effects of two Bt corn lines (Bt 11 and Bt 176) on (i) rhizospheric and bulk soil eubacterial communities, by means of denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes, a molecular fingerprinting technique widely used to study the modification induced by different factors on soil microbes (4, 14, 20, 27, 39, 40, 42, 46, 47, 54, 65, 80); (ii) the arbuscular mycorrhizal (AM) fungus *G. mosseae*, a nontarget microorganism, which establishes mutualistic symbioses with the roots of most plant species; and (iii) soil respiration.

To this aim, we evaluated the effects of root exudates on eubacterial communities by using biochemical and molecular parameters and on different stages of the *G. mosseae* life cycle. Moreover, we investigated the effects of plant residues on total and active eubacterial communities on *G. mosseae* and indigenous AM fungus symbionts.

MATERIALS AND METHODS

Experimental design. Bt corn plants (transformation events Bt 11 and Bt 176) genetically modified to express the *Cry1Ab* gene from *B. thuringiensis* and the nontransgenic corn line NK4640—the parental line of Bt 11, hereafter designated by Wt—were used to test their effects on the composition and activity of soil and rhizospheric bacterial communities and on the AM fungal species *G. mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe (IMA 1).

In the first year, research was carried out at the microcosm level by using corn plants grown in an experimental model system (73) and at the greenhouse level by growing corn plants in pots filled with nonsterile agricultural soil.

In the second year, the experiments were carried out at greenhouse level by growing corn plants in pots filled with nonsterile agricultural soil where residues were plowed under at harvest.

(i) Microcosm experiments. Sporocarps of the AM fungal species *G. mosseae*, maintained in the pot culture collection of the Department of Crop Plant Biology, University of Pisa, Pisa, Italy, were extracted from pot culture soil by wet sieving and decanting down to a mesh size of 100 μm (17). Fungal material retained on sieves was flushed into petri dishes, manually collected with forceps under a dissecting microscope (Wild; Leica, Milano, Italy), and placed on 47-mm-diameter cellulose ester Millipore membranes (0.45-μm-diameter pores). Each membrane, inoculated with 10 sporocarps, was covered by another membrane and the sandwich obtained was incubated in moistened sterile acid-washed quartz grit (2- to 5-mm diameter) in the dark for 15 days at 25°C. After sporocarp germination, the root system of both Bt and Wt corn plants was split in three parts, each sandwiched within the Millipore membranes bearing germinated sporocarps. Plants with sandwiched root systems were placed into 10-cm-diameter pots; buried with sterile, acid-washed quartz grit; and maintained under controlled conditions (18 to 24°C; 16- to 8-h photoperiod of irradiance at 100 μE m^{-2} s^{-1}; 60% relative humidity). Ten replicates were set up for each trial. Two root sandwiches from each plant were harvested after 8 and 35 days, respectively, for the bioassay of *G. mosseae*, the third sandwich was harvested after 35 days, and the roots were maintained at −20°C until utilized for bacterial community studies (five replicates).

(ii) Greenhouse experiments. After 35 days' growth in the microcosm, the plants were transferred into 40-cm-diameter pots filled with agricultural soil collected from arable fields of the Centro Interdipartimentale di Ricerche Agro-Ambientali "Enrico Avanzi", S. Piero a Grado, Pisa, Italy, with the following composition: sand (65%), silt (22.8%), clay (12.2%), organic matter (1.7%), pH 7.5. Corn plants were cultivated and maintained in a greenhouse for 10 weeks.

In the second year, plants were grown in pots for 12 weeks and then plowed under; leaves and stems of Bt 176, Bt 11, and Wt plants were cut into ≈2- to 3-cm pieces and mixed with the soil originating from the same pot where they were grown. The biomass of plant residues plowed per pot, calculated using the mean of three corn plants grown in the same experimental conditions, was 8.3 ± 0.7 g (dry weight).

Bioassay of *G. mosseae*. (i) First year; microcosm level. The sandwiches used for the bioassay of *G. mosseae* were carefully opened, and plant roots were cleared with 10% KOH, stained with 0.05% trypan blue in lactic acid, and assessed for mycorrhizal infection. Infected roots from the first harvest were selected under the dissecting microscope, mounted on microscope slides, and observed under a Reichert-Jung Polyvar light microscope to determine total number of fungal appressoria developing into functional infection units (i.e., those developing arbuscules). Roots from the second harvest were assessed for the percentage of infected root length, calculated by using the grid line intersect method (19). Percentage data were analyzed by analysis of variance (ANOVA) after arcsin square root transformation.

(ii) First year; greenhouse level. After 8 and 10 weeks' growth in a greenhouse, plant root systems were sampled and the percentage of mycorrhizal infection was calculated as described above. All data were submitted to one-way ANOVA.

(iii) Second year; greenhouse level. Soil samples containing plant residues were collected at time zero (July) and 2 months (September) and 4 months (November) after being plowed under; they were utilized to carry out the bioassay on the AM fungus *G. mosseae*. To test the effects of Bt plant residues on spore germination and hyphal growth of *G. mosseae*, 15 sporocarps were placed on membranes in a sandwich system without plant roots. Sandwiches were placed onto petri dishes, covered with sampled soil mixed (1:1) with Terra Green 18/40 (Oil Dri, Vernon Hills, Illinois), and maintained in the dark at 18 to 24°C at 60% RH. After 21 days, membranes were opened and stained with 0.05% trypan blue in lactic acid to assess spore germination and hyphal growth.

To evaluate the infectivity of indigenous AM fungal propagules, seeds of *Medicago sativa* were planted in soil samples collected from the experimental pots. After 6 weeks, plant roots were cleared and stained as described and the percentage of infected root length was calculated. The collected data were submitted to one-way ANOVA.

Analyses of eubacterial communities. (i) First year, microcosm level; DNA extraction from rhizospheres of microcosm-grown plants. Roots from sandwich membranes harvested after 35 days were shaken in Ringer's solution with glass beads for 30 min; the pellet obtained by centrifugation was used for DNA extraction by cetyltrimethylammonium bromide (CTAB) protocol (2).

(ii) First year, greenhouse level; rhizosphere and bulk soil DNA extraction. For the study of the composition of the bacterial communities, rhizosphere and bulk soil were sampled from the same experimental pots after 8 and 10 weeks. Five replicates for each plant line were mixed together, sieved at 2 mm, and then maintained at −20°C for molecular analyses. Rhizosphere samples consisted of the roots and the soil tightly adhering to roots.

DNA was extracted in triplicate from 500 mg of rhizosphere and bulk soil with the FastDNA Spin Kit for Soil (BIO 101 Systems Q-BIO Gene, Rome, Italy) in its own beadbeating system (FastPrep FP120; Savant, Rome, Italy), following the manufacturer's instructions. The three samples were then collected for molecular analysis.

DNA extraction from culturable heterotrophic aerobic fraction of rhizosphere eubacterial community. A total of 5 g of rhizosphere soil was suspended in 45 ml of sterile water and shaken for 30 min; serial dilutions were plated on 0.1% tryptic soy agar and incubated at 28°C for 5 days. All the colonies from the 10^{-3} dilution were collected with 2 ml of sterile water and centrifuged, and the pellets were frozen at −20°C. The DNAs were extracted from cumulative pellets by the CTAB method (2).

(iii) Second year, greenhouse level. The samples analyzed were the same utilized for the studies of *G. mosseae*, with the exception that at time zero rhizosphere soil was collected from three replicate plants for each line.

Rhizosphere and bulk soil DNA and RNA coextraction. The simultaneous coextraction of DNA and RNA from rhizosphere and bulk soil was performed according to Griffiths et al. (23), lysing cells by means of the Fast Prep Instrument FP 120 (Savant) and the DNA Spin Kit for Soil (BIO 101 Systems Q-BIO gene) lysing matrix; the only modification was a second extraction with half the initial volume of CTAB extraction buffer and phenol-chloroform-isoamilic alco-

TABLE 1. Primers used in this work for 16S rDNA sequencing

Primer (*E. coli* position)	Sequence 5′–3′	Reference or source
P0 (27f)	GAGAGTTTGATCCTGGCTCAG	33
P8 (342r)	CTGCTGCCTCCCGTAG	33
P4 (561r)	CTTTACGCCCAGTAATT	This work
P4A (561f)	AATTACTGGGCGTAAAG	This work
P2 (704f)	GTAGCGGTGAAATGCGTAGA	This work
P3B (765r)	CTGTTTGCTCCCCACGCTTTC	This work
P5 (930f)	AAGGAATTGACGGGGGC	33
P6 (1495r)	CTACGGCTACCTTGTTACGA	33

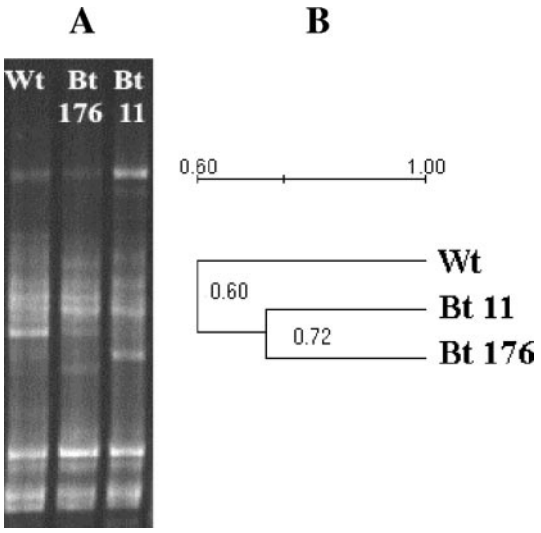

FIG. 1. (A) 16S rRNA gene DGGE profiles (V6-V8 region) of rhizospheric eubacterial microflora associated with Bt and non-Bt corn plants grown in microcosm. (B) Cluster analysis based on UPGMA of DGGE profiles shown in panel A. Scale bar numbers indicate similarities among profiles.

hol (25:24:1), pH 8.0. Nucleic acids were extracted from three subsamples for transgenic and nontransgenic plants and collected together for bulk soil, while the rhizospheric soil of three replicate plants for each line was analyzed separately. Subsequently, half of the final volume of resuspended nucleic acids was treated with RQ1 RNase-free DNase (Promega, Milano, Italy) or DNase-free RNase (Roche Diagnostics, Monza, Italy) alternatively, following the manufacturers' instructions. DNA was recovered after ethanol precipitation and resuspended in TE buffer, pH 8 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8), while RNA was immediately stored at −80°C.

DNA extraction from culturable heterotrophic aerobic fraction of bulk soil eubacterial community. Immediately after being sieved, 10 g of soil samples from the 4 months' harvest was suspended in 90 ml of sterile water and shaken for 30 min; serial dilutions were plated on 0.1% tryptic soy agar and incubated at 28°C for 5 days. Before collecting total aerobic heterotrophic culturable bacteria from soil of the second sampling, we randomly isolated nearly 100 colonies for each line, and all the colonies from the same dilution were collected with sterile water and centrifuged. The pellets were frozen at −20°C. The DNAs were extracted from isolates and cumulative pellets by the CTAB method (2).

PCR amplification of 16S rRNA gene fragments for DGGE analysis. For DGGE analysis, the V6 to V8 regions of 16S rRNA genes were amplified with primer pair GC986f and Uni1401r as described by Felske et al. (14). The reaction mixture (50 μl) contained 25 ng of DNA, 250 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 1× buffer, and 2.5 U of *Taq* DNA polymerase (Polymed, Florence, Italy). The buffer contained 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, and 5 mM $MgCl_2$. The reaction was performed in a PTC 200 thermocycler with the following thermal protocol: 1 initial cycle of 94°C for 1.5 min, 56°C for 30 s, and 72°C for 45 s. Subsequently, 33 cycles were carried out, each consisting of 95°C for 20 s, 56°C for 30 s, and 72°C for 45 s, followed by a final extension step at 72°C for 5 min. Each sample was amplified three times, and the amplicons were pooled together before DGGE analysis, according to previous reports (25, 64, 65).

Reverse transcription-PCR (RT-PCR) amplification of 16S rRNA fragments for DGGE analysis. To generate cDNA, 16S rRNA was reverse transcribed with RT enzyme ImProm II (Promega) with primer Uni1401r, following the manufacturer's instructions. Then, 5 μl of RT reaction mixture was used in a 50-μl amplification reaction mixture under the same conditions described for 16S rRNA gene fragments.

DGGE analysis of eubacterial community. The analysis was performed with the D-CODE System (Bio-Rad, Milan, Italy) on a 6% polyacrylamide gel (acrylamide/bis ratio, 37.5:1), under denaturation conditions (urea, 7 M; 40% formamide with a denaturing gradient ranging from 42 to 58%); the gels were run in 1× Tris-acetate-EDTA buffer at 75 V for 16 h at 60°C and were stained with 12

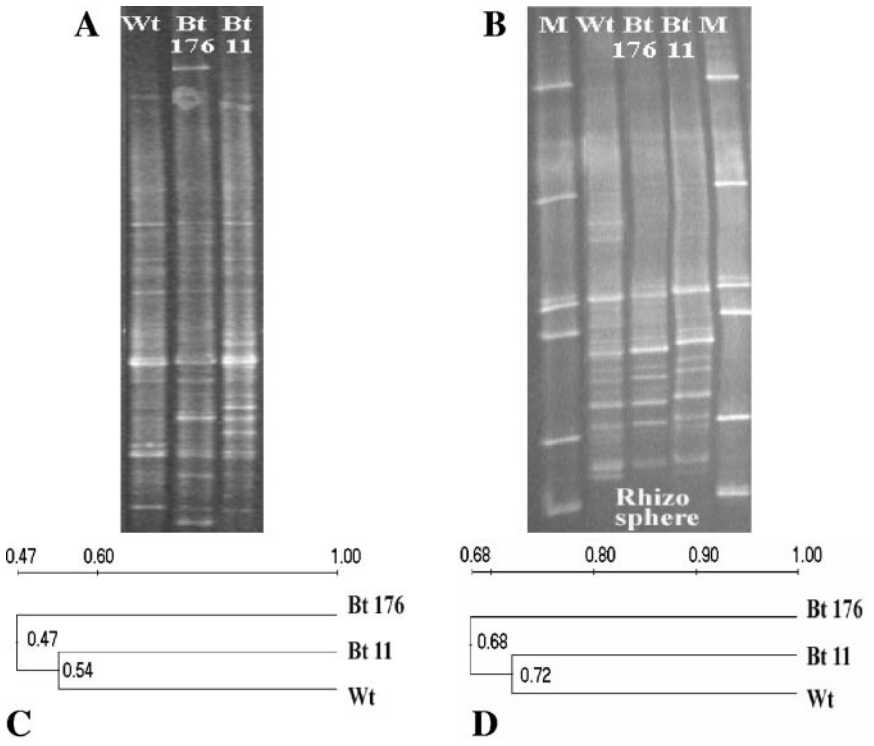

FIG. 2. 16S rRNA gene DGGE profiles (V6-V8 region) of rhizospheric eubacterial communities associated with Bt and non-Bt corn plants grown in pots for 8 (A) and 10 (B) weeks. (C and D) Cluster analyses based on UPGMA of DGGE profiles shown in panels A (C) and B (D). Scale bar numbers indicate similarities among profiles.

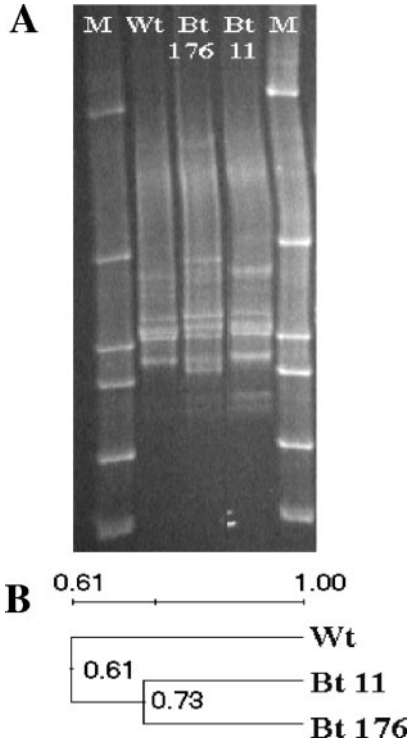

FIG. 3. (A) 16S rRNA gene DGGE profiles (V6-V8 region) of culturable rhizospheric bacteria of Bt and non-Bt corn plants grown in pots for 10 weeks. (B) Cluster analysis based on UPGMA of DGGE profiles shown in panel A. Scale bar numbers indicate similarities among profiles.

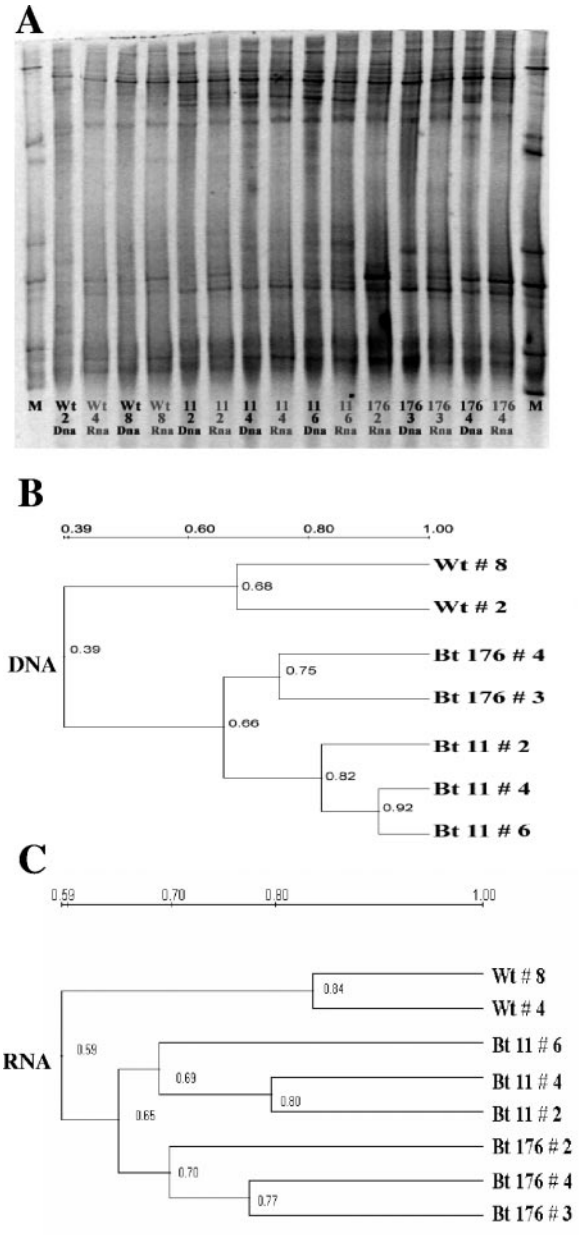

FIG. 4. (A) 16S rRNA gene and rRNA DGGE profiles (V6-V8 region) of rhizospheric eubacterial communities of individual Bt and non-Bt corn plants after 12 weeks' growth. (B and C) Cluster analyses based on UPGMA of DGGE profiles of rRNA genes and rRNA, respectively. Scale bar numbers indicate similarities among profiles.

ml of 1× Tris-acetate-EDTA buffer containing 1.2 μl of SYBR Green I (dilution, 1:10,000) for 30 min in the dark. Visualization and digital pictures were performed with a ChemiDoc System (Bio-Rad).

Dendrogram construction. Using fingerprinting pattern of each plot, genetic similarities of the populations in the different samples were determined by pairwise comparison of the presence and absence of bands and of the intensity of each band in different samples with Diversity Database Software (Bio-Rad). A matrix containing similarity values was obtained with the Dice coefficient. This matrix was used to construct a dendrogram according to the unweighted-pair group method, using arithmetic average (UPGMA) cluster analysis. Cophenetic correlation coefficients were determined to assess the significance of clusters obtained (agreement between similarity values implied by the phenogram and the original similarity matrix).

Amplified 16S rRNA gene restriction analysis (ARDRA). Amplification of nearly all the16S rRNA genes was performed directly on DNA of bacterial isolated strain with two universal primers 27f and 1495r (34) under the following reaction conditions: 25 ng of DNA in a 50-μl reaction mixture containing 250 μM each primer, 250 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 1× buffer, and 2.5 U Polytaq (Polymed Biotechnology Division, Florence, Italy). 16S rRNA genes were amplified in a 25-cycle touchdown PCR with 30 s of denaturation at 95°C, 30 s of annealing at temperature decreasing five grades from 60°C to 50°C every five cycles, 2 min of elongation at 72°C, and a final elongation of 10 min at the same temperature. An aliquot of each PCR, containing 200 ng of DNA, was digested with 10 U of the restriction enzymes AluI and MspI (Roche Diagnostics) separately in a total volume of 20 μl at 37°C for 3 h. The reaction products were analyzed on a 1× Tris-borate-EDTA agarose gel (2.5% [wt/vol]) with an electrophoretic run at 150 V-150 mA for 150 min. The combination of profiles obtained by the two digestions allowed isolates to be grouped into operative taxonomic units (OTUs) (45).

Sequencing of 16S rDNAs. Nearly all 16S rRNA genes from selected OTUs were sequenced with the primers listed in Table 1. Sequencing was carried out at the the Interdepartmental Centre for Agricultural, Chemical, and Industrial Biotechnology (CIBIACI) at the University of Florence using the ABI PRISM BigDyeTM Terminator Cycle Sequencing kit, version 1.1 (PE Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The parameters for cycle sequencing in the Primus 96 Plus thermocycler (MWG Biotech) were 18 s of delay at 96°C, followed by 25 cycles, each consisting of 18 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Electrophoresis was performed with an ABI Prism 310 CE system (PE Applied Biosystems).

Sequences were entered into the BLAST nucleotide search program of the National Center for Biotechnology Information to obtain closely related phylogenetic sequences. The dendrograms displaying phylogenetic positions of the seven sequenced isolates were based on alignments with similar 16S rRNA gene sequences, performed with CLUSTAL W software. The phylogenetic tree was then generated by the neighbor-joining method with TREECON software and bootstrap values based on 100 replicates (7).

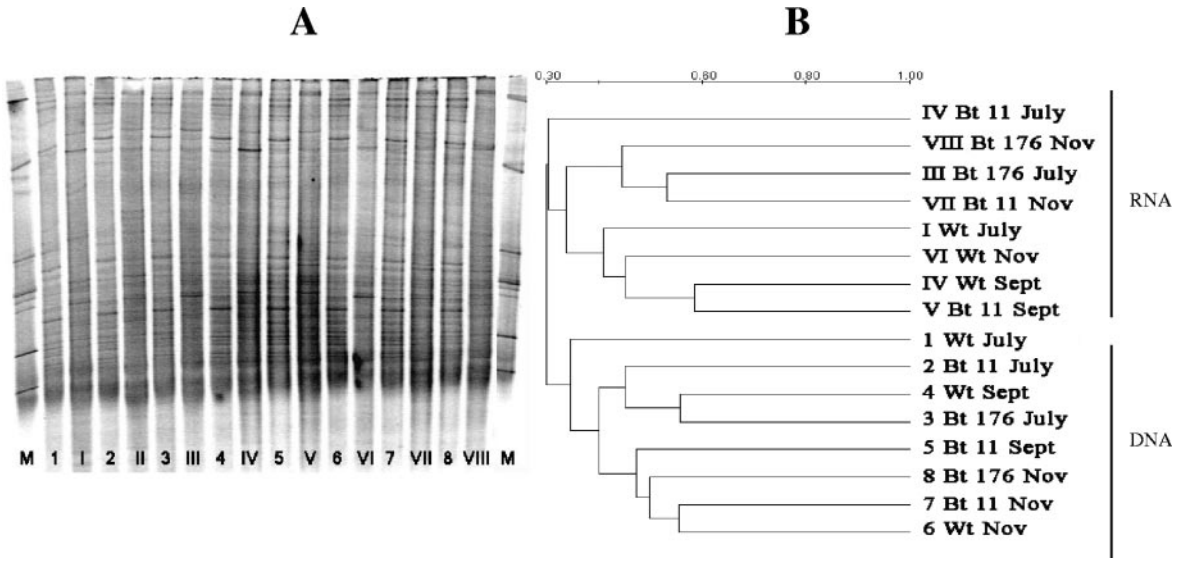

FIG. 5. (A) 16S rRNA gene and rRNA DGGE profiles (V6-V8 region) of soil eubacterial communities after Bt and non-Bt corn residues are plowed under. Arabic and Roman numerals indicate rRNA genes and rRNAs, respectively. (B) Cluster analysis based on UPGMA of DGGE profiles shown in panel A. Scale bar numbers indicate similarities among profiles.

Soil respiration. Biochemical analyses were carried out with samples collected in the first year after 8 and 10 weeks of plant growth and in the second year with samples collected 2 months after residues were plowed under. Soil was previously air-dried, sieved to 2 mm, rewetted to its −33-kPa field water tension, and incubated at 30°C.

Soil respiration was measured in a closed system by the method of Isermeyer (30), where NaOH solution was neutralized by CO_2 derived from soil organic matter oxidation. CO_2 evolution was measured after l, 2, 4, 7, 10, 14, and 17 days. Average values of the CO_2 daily evolution are given in milligrams per CO_2 carbon kg^{-1} (dry weight) of soil to obtain cumulative respiration curves. Multiple linear regression was performed to model relationships between CO_2 production and the different variables (time, treatment, time × treatment) by using SPSS software for Windows, version 12 (SPSS, Inc., Chicago, Ill.).

Nucleotide sequence accession numbers. GenBank accession numbers for the isolates sequences reported in this work are as follows: AY965246, AY965247, AY965248, AY965249, AY965250, AY965251, and AY965252.

RESULTS

Analyses of eubacterial community. DGGE analysis of rhizospheric eubacteria of seedlings grown in the sandwich system showed differences among the communities characterizing the three corn lines, and Bt plants clustered together after UPGMA analysis (Fig. 1), although the cophenetic correlation

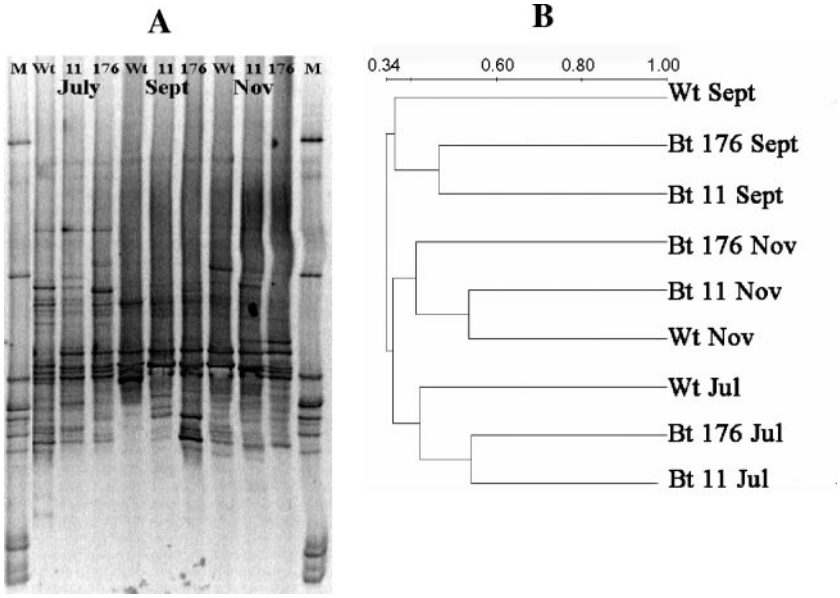

FIG. 6. (A) 16S rRNA gene DGGE profiles (V6-V8 region) of culturable heterotrophic aerobic soil bacteria after Bt and non-Bt corn residues were plowed under. (B) Cluster analysis based on UPGMA of DGGE profiles shown in panel A. Scale bar numbers indicate similarities among profiles.

TABLE 2. Distribution of ARDRA profiles of bacterial strains isolated from soil amended with Bt and non-Bt corn residues (4 months in September) into OTUs

Corn line	No. of OTUs																					Total no. of strains	No. of OTUs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
Wt	7	3				17	3	5	3	9	8	15	2									72	10
Bt_1 1	16	24	4	4										2	3	3	7					63	8
Bt_1 76	21	20	3	2	4													2	4	4	3	63	9
Total	44	47	7	6	4	17	3	5	3	9	8	15	2	2	3	3	7	2	4	4	3	198	

was very low ($r < 0.1$). DGGE patterns of rhizospheric eubacteria from plants grown in the soil for 8 and 10 weeks showed specific bands characterizing the three lines (Fig. 2A and B). UPGMA analyses clustered Bt 11 with the nontransgenic line (Fig. 2C and D), although the phenograms were supported by a very low cophenetic correlation ($r < 0.1$).

DGGE patterns of rhizospheric heterotrophic aerobic culturable bacteria from plants grown in the soil (10 weeks) yielded slight differences between transgenic and nontransgenic lines (Fig. 3A), and the UPGMA analysis, which grouped Bt 11 and Bt 176 in the same cluster, was not supported by cophenetic correlation ($r < 0.1$) (Fig. 3B).

Total (16S rRNA gene) and active (16S rRNA) rhizospheric bacterial communities of individual Bt and non-Bt corn plants grown in soil (12 weeks) and analyzed to verify the occurrence of intraline variability showed that the latter was lower than interline one (Fig. 4A). UPGMA analysis of rRNA gene samples detected large differences between transgenic and nontransgenic plants and clustered together patterns of plants belonging to the same line ($r = 0.777$) (Fig. 4B). The active communities, represented by rRNA profiles (Fig. 4C) showed a similar discrimination pattern, with Wt plants clustering separately, which was supported by a high cophenetic correlation ($r = 0.977$). Active communities showed 25% reduction in the number of bands with respect to total communities in all the samples analyzed (Fig. 4A).

Total (16S rRNA gene) and active (16S rRNA) soil bacterial communities, as affected by plant residues, were analyzed. DGGE profiles and UPGMA analysis of all the samples produced two distinct clusters, showing a clear-cut separation between patterns obtained from rRNA genes and rRNA, whereas no significant discrimination between Bt and non-Bt lines was shown ($r = 0.652$) (Fig. 5).

UPGMA analysis of DGGE patterns of soil aerobic heterotrophic culturable bacteria as affected by plant residues showed large differences among all the samples ($r = 0.638$) (Fig. 6).

By means of ARDRA, the isolates were gathered into OTUs (Table 2). The OTUs grouping with the highest isolate numbers were the same for the two GM plants and were different from those of the nontransgenic line. The 16S rRNA gene sequence of one isolate for each OTU yielding the highest strain numbers (Fig. 7) indicated that the most represented strains for GM plants were gram-positive bacteria with a low G+C content, strictly related to *Bacillus megaterium* (OTU 1 of Bt 11 and Bt 176; sequence similarity, >99%) and *Bacillus simplex* (OTU 2 of Bt 11 and Bt 176; sequence similarity, >99.6%) subgroups. Sequences from isolates of Wt lines showed that the most represented strains belonged to the β-proteobacterium *Delftia tsuruhatensis* subgroup (OTU 6; sequence similarity, 99.4%), the γ-proteobacterium *Xanthomonas retroflexus* subgroup (OTU 10; sequence similarity, 99.0%),and the *Pseudomonas fluorescens* subgroup (OTU 12; sequence similarity, 99.7%).

Bioassay of the AM fungus *G. mosseae*. The impact of Bt plants on the AM fungal species *G. mosseae* was assessed at different stages of symbiosis establishment by monitoring the development of fungal infection units and the colonization of host roots.

After 8 days' growth in microcosm, *G. mosseae* was able to produce entry points in the roots of all plant lines tested, but the number of infection units forming arbuscules in Bt 11 and Bt 176 roots was significantly lower than in the wild type (Table 3). After 35 days, the percentage of infected root length in Bt 176 plants was significantly lower ($P = 0.02$) than in wild-type plants (Table 3). No significant differences in infected root length were found with Bt 11 plants.

The analyses of Bt 11 and Bt 176 corn roots grown in soil for 8 and 10 weeks showed significant differences in the percentage of infected root lengths, compared with the wild type ($P = 0.0004$ and $P = 0.0002$, respectively) (Table 3).

To test the impact of plant residues on AM fungi, germination ability, hyphal growth of presymbiotic mycelium of *G. mosseae*, and the percentage of colonization by indigenous fungal propagules were analyzed. Sporocarp germination and mycelial length did not show significant differences between soil samples containing Bt and non-Bt corn residues (Table 4). Indigenous AM fungi were not significantly affected in their ability to colonize *M. sativa* roots grown in soils containing Bt and Wt plant residues, both at time zero and at the second sampling, 2 months after being plowed under (Table 5). On the contrary, at the third sampling 4 months after being plowed under, the percentage of infected root length of *M. sativa* grown in soils containing Bt 11 corn residues was significantly lower ($P = 0.05$) than that detected in wild-type plants.

Soil microbial respiration. Tests of between-subject effects showed no differences between soil samples from nontransgenic and Bt corn plants after 8 and 10 weeks of growth (Fig. 8).

A significantly lower soil respiration was detected in soil samples containing Bt corn residues with a significant difference for values of time × treatment ($P = 0.0001$), i.e., for comparison of regression slopes (Fig. 9).

DISCUSSION

In this work, we evaluated the impact of Bt 11 and Bt 176 corn plants and their residues on bulk soil and rhizospheric eubacterial communities on the arbuscular mycorrhizal fungus

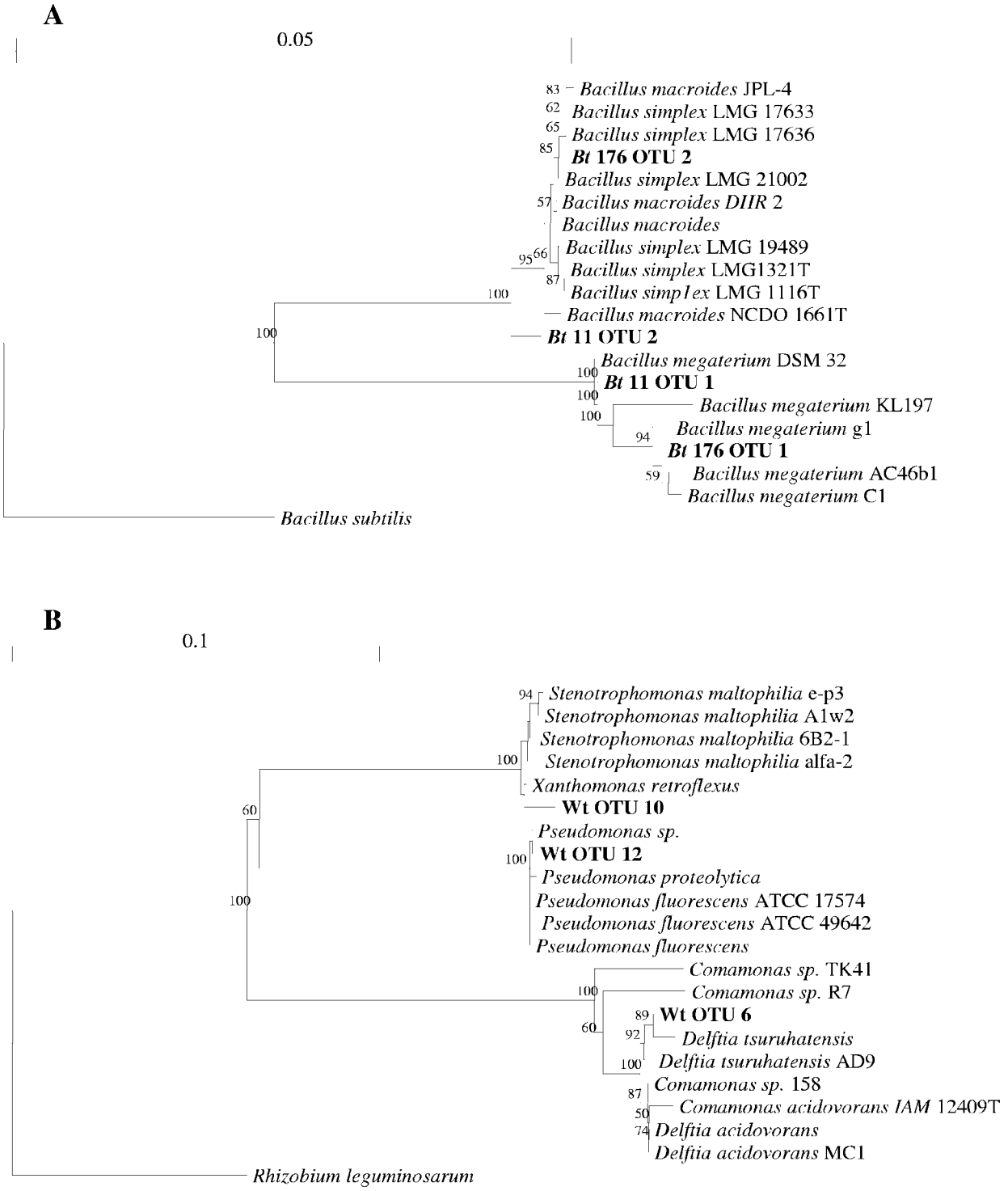

FIG. 7. Phylogenetic neighbor-joining trees based on the comparison of 16S rRNA gene sequences of the seven bacterial isolates from soil containing Bt and non-Bt residues (boldface type) and similar sequences from the National Center for Biotechnology Information database. *Bacillus subtilis* (A) and the α-proteobacterium *Rhizobium leguminosarum* (B) were used as the outgroups. Scale bars correspond to 0.05 (A) and 0.1 (B) substitution per nucleotide position. Bootstrap values of >50 are indicated at nodes (100 replications).

G. mosseae and on soil respiration. Microcosm experiments showed differences in rhizospheric eubacterial communities associated with the three corn lines; in addition, a significantly lower level of mycorrhizal colonization was detected in Bt 176 corn roots. Greenhouse experiments showed differences in rhizospheric eubacterial communities (both total and active), in culturable rhizospheric heterotrophic bacteria, and in mycorrhizal colonization between Bt and non-Bt corn plants. Plant

TABLE 3. Intraradical colonization of Bt and nontransformed corn plants at different times after inoculation with the arbuscular mycorrhizal fungus *G. mosseae*[a]

Corn line	No. of entry points developing arbuscules at 8 days	% of infected root length at:		
		35 days	8 wk	10 wk
Bt 11	6.2 ± 2.8*	6.7	9.5*	13.8***
Bt 176	7.4 ± 4.2*	5.3*	7.3**	9.4***
Wt	22.2 ± 4.8	10.9	21.1	23.5

[a] *, **, and ***, values statistically different from non-Bt corn line (Wt; NK4640) at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

TABLE 5. Percentage of root length colonized by indigenous arbuscular fungi inhabiting soil containing Bt and non-Bt corn residues, assessed in *Medicago sativa* plants[a]

Sampling time	% of infected root length		
	Wt	Bt 11	Bt 176
Residues plowed under	37.2	33.2	28.5
2 mo	26.7	25.6	24.7
4 mo	20.8	10.2*	19.5

[a] In rows, an asterisk indicates statistically different values ($P = 0.05$).

residues of transgenic plants, plowed under and mixed with soil for up to 4 months, affected soil respiration and mycorrhizal establishment by indigenous endophytes and soil bacterial communities.

Our data point out differences, both in the total amount (16S rRNA gene) and in the metabolically active fraction (16S rRNA) of the rhizospheric eubacterial communities between Bt and non-Bt corn when individual plants were analyzed ($r = 0.777$ and 0.977, respectively), whereas large differences among the three lines, not related to the genetic transformation, were shown in other experiments. Interestingly, a lower number of fragments was detected in DGGE of the active eubacterial communities than in total ones associated with both transgenic and nontransgenic plants. Other authors, by using phospholipid fatty acid profiles and community-level physiological profiles, reported that Bt plants did not produce large differences in soil microbial communities and that soil type represented the most important factor affecting community composition (3).

Our experiments aimed at assessing the variability of bacterial communities associated with individual plants of the same line showed that intraline variation was lower than interline variation (64). The intraline differences may represent an intrinsic characteristic of corn, since nontransgenic plants also showed variability in DGGE profiles, as previously reported (25, 61, 64, 80).

DGGE profiles of aerobic culturable bacteria showed some differences between Bt and non-Bt corn plants, in contrast with previous studies (32, 56). Our results are probably due to the culture-independent methods of analysis utilized in this work, i.e., DGGE, which is able to reveal the large diversity of soil microbial communities by separating different sequence variants.

DGGE profiles of bacteria from soil amended with plant residues of Bt and non-Bt corn clearly discriminated between active and total communities for all sampling times analyzed. Moreover, UPGMA analysis of profiles produced by aerobic culturable bacteria isolated from residue-amended soil showed clusters corresponding to sampling times, probably representing the result of the dynamics of degradation of different plant residues (12, 20, 28, 36, 64). Bacterial strains isolated from the soil amended with Bt corn residues belonged to a subgroup of the genus *Bacillus* (52), whereas bacterial strains isolated from soil containing residues of wild-type corn were related to plant growth-promoting rhizobacteria (64, 69).

The present results show that transgenic Bt 11 and Bt 176 corn plant lines affected the early events of mycorrhizal establishment and the development of symbiosis by *G. mosseae*. The experimental model system used allowed us to monitor the formation of functional infective structures, i.e., entry points developing arbuscules, which were significantly reduced in Bt 11 and in Bt 176 corn roots by 72% and 67%, respectively, compared with non-Bt corn plants. Root colonization in greenhouse experiments maintained this trend, since the percentage of infected root length of Bt plants was about 50% of that recorded for the nontransgenic line. These data are consistent with previous results showing that the Bt 176 corn line affected the successful development of infection structures by hindering the production of viable infection pegs (73). However, the cellular interactions between host roots and AM fungal hyphae leading to the failure of intraradical colonization in corn lines actively expressing the constitutive insecticidal *B. thuringiensis* toxin remain to be investigated. Previous works reported that transformed eggplant plants constitutively expressing the Dm-AMP1 antimicrobial defensin from *Dahlia merckii* releasing the protein in root exudates did not interfere with the host recognition system and symbiosis establishment by the AM fungus *G. mosseae* (74). Other authors have obtained similar findings, that transgenic *Nicotiana sylvestris*- and *Nicotiana tabacum*-expressing chitinases and pathogenesis-related proteins did not influence the establishment of mycorrhizal infection by *G. mosseae* (76, 77). These results may be explained by

TABLE 4. Germination percentages and mycelial growth of the arbuscular mycorrhizal fungus *G. mosseae* grown in soil containing Bt and non-Bt corn residues[a]

Sampling time	% Germination			Mycelial length (mm/germinated sporocarp)		
	Wt	Bt 11	Bt 176	Wt	Bt 11	Bt 176
Residues plowed under	64	35	55	18.61 ± 2.46	17.91 ± 4.94	14.26 ± 4.99
2 mo	66	55	55	25.10 ± 1.45	23.68 ± 2.51	20.36 ± 2.06
4 mo	60	57	54	38.98 ± 5.32	34.96 ± 3.78	40.56 ± 5.38

[a] Values reported in rows are not statistically different ($P = 0.05$).

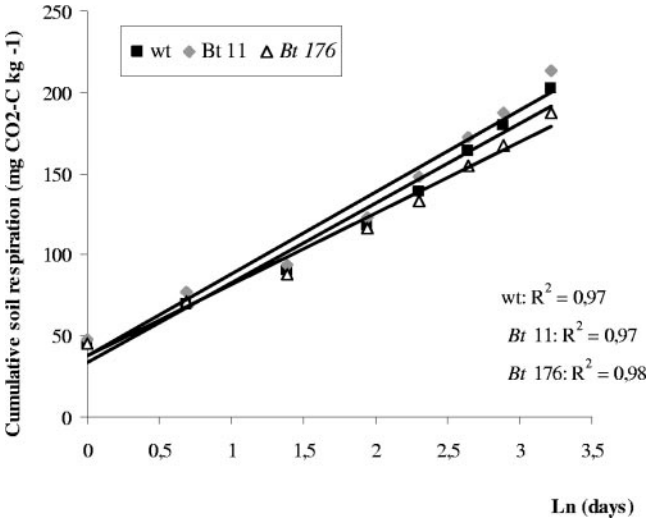

FIG. 8. Linear regression of cumulative respiration of soil collected from pots where Bt and non-Bt corn lines were grown for 10 weeks.

the ability of AM fungi to tolerate plant defense compounds during the early stages of mycorrhizal infection (11, 18, 44, 78).

Plant residues did not affect germination ability and hyphal growth of presymbiotic mycelium of *G. mosseae*, while a significantly lower percentage of colonization by indigenous AM fungi was found 4 months after Bt 11 corn residues were plowed under. It remains to be further investigated whether such lower levels of mycorrhizal infection are due to Bt corn litter or to factors independent of the presence of transgenic crop residues.

Soil respiration did not show changes during plant growth, while a lower evolution of CO_2 from the soils amended with Bt corn residues was found, in agreement with previous data (9, 15, 32, 57). In fact, in soil samples amended with Bt corn plant residues, the cumulative amount of carbon evolved as CO_2 decreased by 10% compared with CO_2 values obtained with

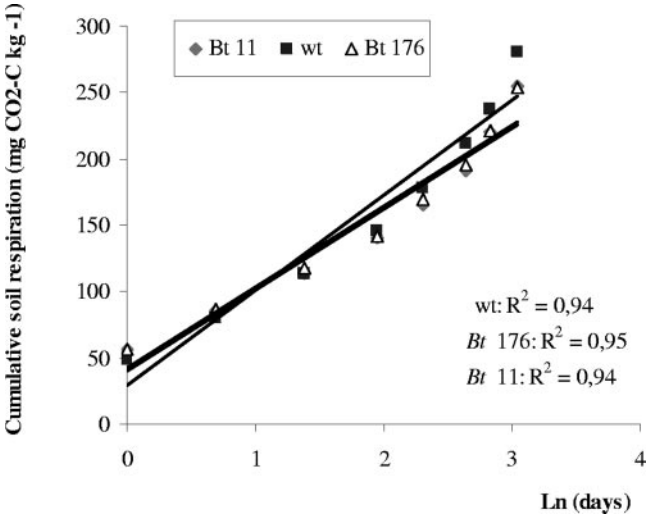

FIG. 9. Linear regression of cumulative respiration assessed on soil where Bt and non-Bt corn lines were grown 2 months after plant residues were plowed under.

soils amended with non-Bt corn plant residues, although such reduction is less than that reported by Flores et al. (15). Interestingly, the activity of rhizospheric bacterial communities may also be affected by changes in root exudation induced by mycorrhizal colonization (1, 39, 66, 75).

It is interesting to note that other authors reported a significantly higher lignin content in some Bt corn transformants than in their respective non-Bt near-isolines (15, 41, 57). Interestingly, Donegan et al. (10) reported that some Bt cotton lines affected total microbial population, whereas no effects were produced by the purified *B. thuringiensis* toxins, suggesting that the impact on soil microorganisms was due to transformation events or tissue culture practices rather than to the products of the inserted genes. Since lignin, a major plant structural component, is slowly degraded by soil microbes, its increase in plant residues may retard litter decomposition and produce a lower CO_2 evolution, as indicated in our work.

Even though the limitations of molecular investigation in microbial soil ecology have been pointed out (26), in particular concerning direct nucleic acid extraction (16, 23, 43, 62), PCR (51, 53, 70), RT-PCR (24, 49), and DGGE (48), our molecular data show that different bacterial communities are associated with the different corn lines and that both actively growing Bt corn plants and their plowed-under residues did not reduce soil and rhizospheric bacterial species richness, as assessed by DGGE fragments. Further studies in the field are necessary to evaluate the impact of GM crops on soil microbial communities, since soil is a complex system where the numerous trophic and nontrophic interactions among organisms are difficult to monitor by short-term researches. In particular, the persistence of GM plant residues should be investigated to assess whether they represent a real hazard to nontarget beneficial soil microbes, whose variation might produce long-term effects on crops successively cultivated in the same soil in the years to come.

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