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Quantification and Persistence of Recombinant DNA of Roundup Ready Corn and Soybean in Rotation

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The presence of the recombinant *cp4 epsps* gene from Roundup Ready (RR) corn and RR soybean was quantified using real-time PCR in soil samples from a field experiment growing RR and conventional corn and soybean in rotation. RR corn and RR soybean *cp4 epsps* persisted in soil for up to 1 year after seeding. The concentration of recombinant DNA in soil peaked in July and August in RR corn and RR soybean plots, respectively. A small fraction of soil samples from plots seeded with conventional crops contained recombinant DNA, suggesting transgene dispersal by means of natural process or agricultural practices. This research will aid in the understanding of the persistence of recombinant DNA in agricultural cropping systems.

KEYWORDS: Agriculture; DNA persistence; environment; field conditions; genetically modified; herbicide; real-time PCR; soil; transgenic corn; transgenic soybean

INTRODUCTION

The use of genetically modified (GM) crops is widespread in countries such as Canada, the United States, Brazil, and Argentina (*1*), where primarily herbicide-tolerant or insect-resistant GM cultivars are grown. About 15% of the corn and 60% of the soybeans grown in Ontario (Canada) contain the recombinant 5-enol-pyruvyl-shikimate-3-phosphate synthase gene from *Agrobacterium* spp. CP4 (*cp4 epsps*), which confers tolerance to glypohsate [*N*-(phosphonomethyl)glycine]. The cultivation of GM crops is associated with the concern of the introduction and dispersal of recombinant DNA in the environment, where it may or may not alter the fitness of indigenous plant species through gene flow (*2*, *3*). Examples of recombinant DNA dispersal by GM plants to plant relatives have been shown in field experiments (*4*–*6*).

Conversely, there is a paucity of information on the fate of recombinant DNA in agricultural soils. Of interest is soil recombinant DNA persistence from GM crops because naturally competent bacteria capable of integrating foreign DNA into their genomes may be present in soil (*7*, *8*). The impact of integration of new genes into competent bacteria on the structure and function of the soil microbial community is not known. Examples of horizontal gene transfer among bacterial genomes are numerous in the literature (*9*–*11*), and horizontal gene transfer from plants to bacteria is a suspected component of evolution (*12*). Moreover, studies performed in controlled conditions have shown the possibility for the transfer of recombinant DNA from GM plants to competent bacteria (*13*–*15*), although the transfer of transgenes from commercialized GM crops to soil bacteria has not been reported in field soil. However, horizontal transfer of transgenes in agricultural soil has not been extensively researched to date, and more field studies are required (*16*, *17*). To assess and model the possibility of horizontal gene transfer from GM crops to soil bacteria, knowledge of the availability and accessibility of the recombinant DNA in the soil is required.

DNA can persist in soil (*18*–*21*). In agricultural field conditions, recombinant DNA from decaying GM plant material was detected for months (*23*, *24*) or years (*25*). However, in these studies, the authors worked with plant material buried in the soil, and DNA that originated from living GM plants was not used. In soil, roots are an important reservoir of genetic material that constantly release DNA into the soil environment by way of exudation, cell sloughing, and/or decomposition. Furthermore, if plant-to-bacteria gene transfer occurs in field soil, it would be expected to occur during the growing season, as bacterial populations peak when soil temperatures and nutrient supply are optimal. Consequently, monitoring recombinant DNA

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Table 1. Description of the Treatments Applied during the Field Study as a Function of the Crop Line and Herbicide Used[a]

	2003		2004	
treatment	corn type	herbicide	soybean type	herbicide
1	Roundup Ready	Roundup	Roundup Ready	Roundup
2	Roundup Ready	conventional[b]	Roundup Ready	conventional[c]
3	conventional	conventional	conventional	conventional
4	Roundup Ready	Roundup	conventional	conventional
5	Roundup Ready	conventional	conventional	conventional
6	conventional	conventional	Roundup Ready	Roundup
7	conventional	conventional	Roundup Ready	conventional

[a] Each treatment was represented by 12 × 14 m plots randomly repeated in four rows. [b] Isoxaflutole/atrazine. [c] Quizalofop-*p*-ethyl/imazethapyr.

in soil over a full growing season in a GM cropping system allows the availability of transgenes to soil bacteria for genetic transformation to be determined.

Real-time PCR techniques can be used to detect and quantify recombinant DNA from genetically modified organisms (*26–30*). We have previously described a novel method for recovering and quantifying the transgenes for Roundup Ready (RR) corn and RR soybean in soil (*31*). The present research is part of a study on recombinant DNA persistence in agricultural soil. Data on quantification and persistence of RR transgenes in the soil of one cycle of a corn/soybean rotation are presented.

MATERIALS AND METHODS

Experimental Design. The experimental field was located at the Elora Research Station (Elora, ON, Canada; 43° 41′ N, 80° 26′ W). The site selected was a sward where no genetically modified crop had been grown previously. The soil was a Conostogo silt loam soil (sand, 26.1%; silt, 60.1%; clay, 13.8%; pH, 7.3; OM, 5%; CEC, 27.1 cmol kg^{-1}). The field was divided into 28 plots of 12 × 14 m corresponding to seven treatments (**Table 1**) randomly distributed in four blocked replications. To minimize cross-contamination, the experimental units were separated by a 3 m winter barley (*Hordeum vulgare* L.) border within replicates and by a 14 m border between replicates. The field was tilled 8 cm deep on May 5, 2003, and plots were sown (May 22, 2003) to RR corn line DKC35-51 (Monsanto, St. Louis, MO) or its nontransgenic counterpart, DK355, in 76 cm rows. Herbicides were applied as follows: Roundup (1.8 kg ha^{-1} glyphosate) on June 21 and 30, 2003, or conventional (79 g of ai ha^{-1} isoxaflutone + 800 g of ai ha^{-1} atrazine) on May 26, 2003 (**Table 1**). Corn was hand harvested on October 22, 2003, and mechanically combined on October 28, 2003. The same plots were seeded to soybean on May 30, 2004, with 19 cm row spacing under no-tillage cultivation. Soybean lines were DKB06-52 (RR) and OAC Bayfield (conventional). Herbicides were applied as follows: Roundup Transorb (1.8 kg of ai ha^{-1} glyphosate + 72 g of ai ha^{-1} quizalofop-*p*-ethyl + 75 g of ai ha^{-1} imazethapyr + 840 g of ai ha^{-1} bentazon) on June 22 and July 8, 2004, or conventional (72 g of ai ha^{-1} quizalofop-*p*-ethyl + 75 g of ai ha^{-1} imazethapyr + 840 g of ai ha^{-1} bentazon) on June 22, 2004 (**Table 1**). On October 8, 2004, the soybeans were harvested using a commercial combine.

Soil Sampling. Soil samples were collected four times per year. In 2003, soil sampling was performed on May 23 (presowing), July 14 (1 week after the second glyphosate application), August 15 (at corn silking, i.e., maximum biomass), and October 31 (after harvest). In 2004, soil samplings were performed on May 19 (presowing), July 17 (stage V6 of soybean), August 16 (stage R4), and October 18 (after harvest). To investigate the persistence of soybean DNA in the soil, it was sampled once more on May 3, 2005.

At each sampling date, soil samples 2 cm diameter and 15 cm deep (10 per plot, except for May 2005, for which eight per plot were taken) were collected into acetate sleeves using two corers, one for plots seeded with RR crops and one for plots seeded with conventional crops. Samples were taken at a distance of at least 3 m inward from the edges of the plots. The sampling method differed between dates: in May 2003, samples were taken systematically in a W pattern from each plot and nonplot area; in July 2003, they were taken about 15 cm away from the corn stalks, in the zone of growing roots. On later sampling dates, because roots (or root debris) were well distributed throughout the plots, cores were collected from the middles of adjacent rows. Soil samples were stored within the next 4 h at either −20 °C (2003) or 4 °C (2004–2005) until processing. In 2004 and 2005, it was decided to store samples at 4 °C to prevent freezing/thawing of samples and release of DNA from plant cells. DNA degradation in soil was minimal at 4 °C over a 24 h period (unpublished data).

In the laboratory, the soil cores were subsampled in a laminar flow hood. One subsample was taken from each core at a depth of 3 cm, and one subsample was taken at a depth of 12 cm. At the top (3 cm deep) or the bottom (12 cm deep) of the acetate tubes, "windows" (1.5 × 1.5 cm) were cut with a flame-sterilized scalpel. To further reduce the potential of contamination, the exposed surface of the soil core was scraped with a single-use autoclaved wooden spatula and discarded. A soil subsample (ca. 1 g) was taken from the inside of the core with a fresh, clean sterilized wooden spatula and transferred to a 1.5 mL sterile microtube.

DNA Isolation. Soil DNA was isolated from a 0.25 g subsample (moist soil) as previously described (*31*) using the UltraClean-htp 96-well Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) following the manufacturer's instructions with modifications. Briefly, 1.25 mg of aurintricarboxylic acid (a nuclease inhibitor), 50 μL of 200 mM $AlNH_4(SO_4)_2$, and one glass bead (6.35 mm diameter) were added to each well after addition of the inhibitor removal solution (IRS). The samples were then shaken at 20 strokes s^{-1} for 1 h. At the end of the extraction process, total soil DNA was eluted in a final volume of 100 μL.

The number of soil cores analyzed differed between dates of field sampling. For the May 2003 sampling, 5 cores per plot were analyzed. For the other samplings, 10 cores from plots seeded with RR crops and at least 5 cores from plots seeded with conventional crops were analyzed. The RR corn transgene was targeted in DNA extracts from soil samples collected in 2003 and in May and August of 2004, whereas the RR soybean transgene was targeted in DNA extracts from soil samples collected in 2004 and in May of 2005.

Real-Time PCR Conditions. Real-time PCR amplifications were performed on 1 μL of DNA extracts with the iCycler (Bio-Rad) in 96-well plate microtubes containing a final volume of 20 μL of PCR mixture. The detection and quantification of recombinant DNA of RR corn and RR soybean were based on the amplification of the junction between the chloroplast transit peptide element (CTP2 for RR corn and CTP4 for RR soybean) and the *cp4 epsps* gene (*31*). The real-time PCR mixture contained 1 × iQ Supermix [50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.8 mM dNTPs, 0.5 unit of *Taq* polymerase, 3 mM $MgCl_2$ (Bio-Rad)], 500 nM of forward (5′-GGCTCTGAGCTTCGTCCTCT-TAAGG-3′ for RR corn and 5′-ATCAGTGGCTACAGCCTGCAT-3′ for RR soybean) and reverse (5′-GAATGCGGACGGTTCCGGAAAG-3′) primers, 400 nM of molecular beacon (FAM-5′-CGCGAT-CATTTGCGGGCGGTTGCGGGCGATCGCG-3′-Dabcyl), and 10 ng/μL of T4 gene 32 protein (Roche, Laval, QC, Canada). Conditions for real-time PCR amplifications were a 3 min step at 95 °C followed by 45 cycles of 10 s at 94 °C and 20 s at 53 °C. Fluorescence was monitored during the annealing step (53 °C). The copy number of target DNA copies recovered from soil was quantified using the sigmoidal curve fitting method described by Gulden et al. (*32*).

To estimate the vertical distribution of target DNA within soil cores, the numbers of transgenes measured quantified in the top and bottom subsamples were compared. For each crop (corn and soybean), the sums of the total number of transgenes (nontransformed data) were calculated for each sampling date. The relative proportions of transgenes in the top and bottom subsamples were expressed as percentages of the total number of transgenes (top and bottom combined) quantified at a given

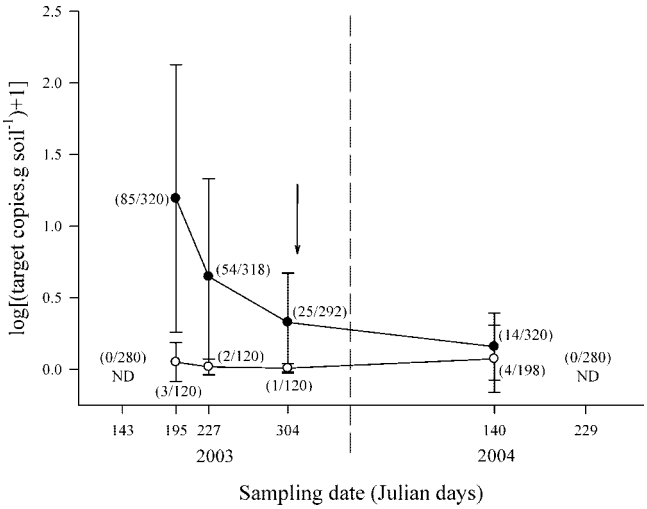

Figure 1. Quantification of recombinant DNA from Roundup Ready (RR) corn in soil samples collected in plots cultivated with RR (●) or conventional (○) corn, between May 2003 and August 2004. Data are means (±SD) of log-transformed numbers of copies of transgenes per gram of fresh soil. Numbers of positive observations from total numbers of samples processed are given in parentheses. ND indicates target DNA was not detected. Arrow represents day of harvest.

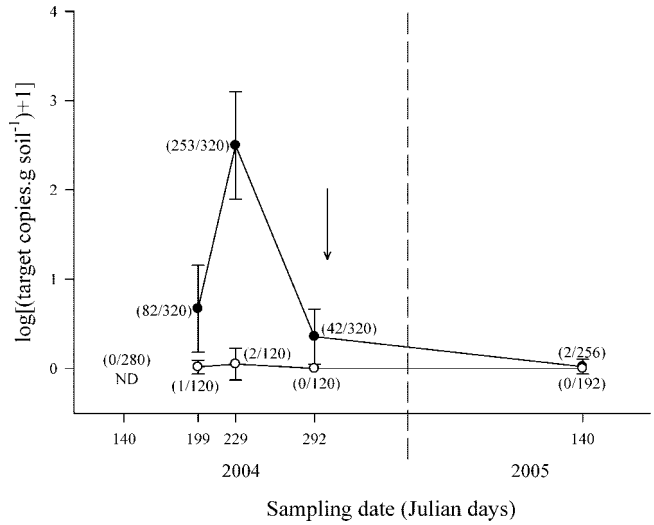

Figure 2. Quantification of recombinant DNA from Roundup Ready (RR) soybean in soil samples collected in plots cultivated with RR (●) or conventional (○) soybean, between May 2004 and May 2005. Data are means (±SD) of log-transformed numbers of copies of transgenes per gram of fresh soil. Numbers of positive observations from total numbers of samples processed are given in parentheses. ND indicates target DNA was not detected. Arrow represents day of harvest.

sampling date. The distributions of transgenic DNA between the 3 and 12 cm deep subsamples were compared for plots where target DNA was detected by performing paired *t* tests (on log-transformed values).

RESULTS

Target DNA of corn origin (*ctp2-epsps* junction) was successfully detected and quantified in soil samples collected between July 2003 and May 2004 (**Figure 1**). No transgene was detected in soil samples collected in May 2003 (prior to seeding) or in August 2004. In soil samples collected from plots seeded with RR corn, the average quantity of target DNA peaked in July 2003 and then decreased over time (**Figure 1**). The percentage of "positives" over the total number of samples showed a similar trend (**Figure 1**, numbers in parentheses). However, the presence of the target DNA was detected in a low percentage of soil samples from plots cultivated with RR corn (27% at the most in July 2003). The presence of the target DNA was also detected in a few soil samples collected in conventional corn. Herbicide treatments did not affect the quantity and persistence of transgenic DNA in soil (data not shown).

Similarly, target DNA of soybean origin (*ctp4-epsps* junction) was detected and quantified in soil samples collected between July 2004 and May 2005 (**Figure 2**). The transgenic target sequence was not detected in May 2004, prior to seeding. The average quantity of target DNA in plots cultivated with RR soybean peaked in August 2004. At that time, 79% of the soil subsamples were positive for target DNA. In conventional soybean, the presence of target DNA was detected in 1 and 2 subsamples (of 120) in July and August 2004, respectively. No difference between herbicide treatments was observed (data not shown).

The distributions of recombinant DNA between the 3 and 12 cm subsamples were different for the corn and soybean plots during the growing season (**Figure 3**). In corn, the proportion of recombinant target DNA in 3 cm subsamples was greater in August and October; however, in July and the following May, more recombinant DNA was found in the 12 cm deep subsamples. In contrast, about 80% of the recombinant soybean DNA was found in the 3 cm deep soil subsamples throughout the growing season. In the following May, no recombinant DNA was detected at either depth in soybean.

DISCUSSION

This study reports the quantification and persistence of recombinant DNA in soil in an RR corn and soybean rotation experiment. No soil samples collected prior to seeding of RR corn or RR soybean were positive for recombinant DNA, indicating the absence of natural background in the soil of the study field for the two targeted transgenes. The high specificity of the molecular tools used to detect these target sequences (*31*) precluded detection of false positives from other possible origins (e.g., other plant species or soil microbes). Small quantities of target DNA were detected in a few extracts from soil samples from conventional corn and soybean treatments. The origin of this DNA remains unknown. A subsample of seeds was tested in the greenhouse to confirm no contamination of the conventional corn or soybean with RR seed. Contamination was avoided in the laboratory during the handling of samples and extraction of DNA. To minimize this possibility, soil samples from plots cultivated with conventional crops were always processed prior to their RR counterparts. Contamination with recombinant DNA can also occur at the time of the field sampling. In a field study on the spread of recombinant DNA from plots grown with transgenic sugar beet (*Beta vulgaris* L.), Meier and Wackernagel (*33*) detected target DNA in soil samples collected prior to planting, although the study field had never been cultivated with transgenic plants before. The presence of this recombinant DNA was attributed to the spread of transgenic plant material from other fields. Pollen dispersal is the most probable method for the movement of plant material occurring during the growing season. Moreover, harvesting machinery can spread crop residue, particularly in corn. This, along with the closeness of the RR and conventional plots, could explain the occurrence of recombinant corn DNA in the soil

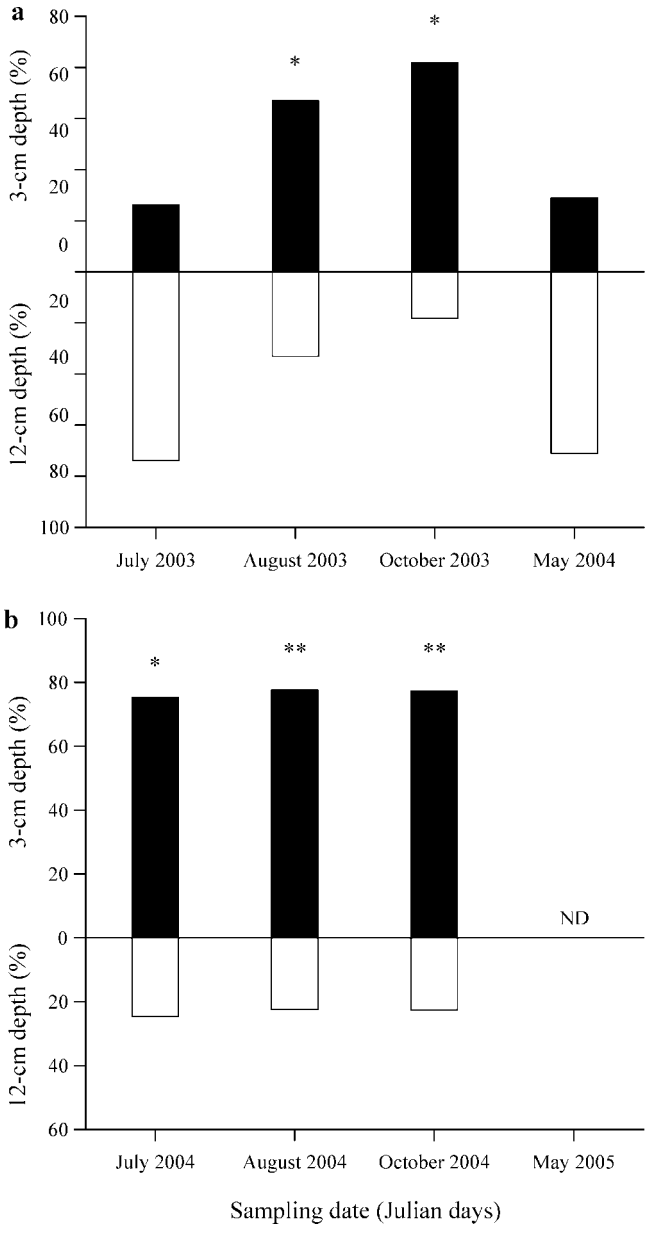

Figure 3. Relative proportions (percentages) of transgenes detected in the 3 cm deep (black bars) and the 12 m deep (white bars) subsamples of soil cores collected in plots cultivated with (**a**) Roundup Ready corn and (**b**) Roundup Ready soybean. Percentages were calculated from the total amount of target copies (in 1 g of soil) of all subsamples at a given date. ∗, $P < 0.05$; ∗∗, $P < 0.005$; ND, not determined.

samples collected in May 2004 in plots where conventional corn was grown in 2003 (**Figure 1**).

The present study confirmed that recombinant DNA from decaying plant material can persist in the soil for several months (*23*–*25*). Recombinant corn and soybean DNA were detected the following spring, 7 months after the crop had been harvested. However, nondetection of recombinant corn DNA beyond this time indicates that recombinant corn DNA may persist for less than a year. In a study conducted on transgenic sugar beet, Gebhard and Smalla (*25*) detected transgenic DNA from sugar beet in soil samples 2 years after their introduction in a disposal site. Hay et al. (*24*) concluded that recombinant DNA of transgenic poplar leaves buried in early August in soil does not persist for more than 4 months under natural conditions. These studies suggest that persistence in the soil of recombinant DNA of plant origin is variable. It may be governed by factors such as the amount and type of plant material, soil type, soil moisture content, temperature, activity of soil organisms, and climatic conditions.

Despite the low detection limit of the method employed in the present study (*31*, *32*), the transgene from RR corn was detected in less than one-third of the soil DNA samples at each sampling time in 2003. The absence of target DNA in soil DNA extracts does not necessarily signify that the target was absent from the soil samples. The quantification of target DNA in soil DNA extracts can be underestimated, and false negatives are possible. The failure to detect target DNA in false negatives can be due either to a strong PCR inhibition by humic acids or to a low DNA extraction efficiency. In the present work, the effect of humic substances was diminished by the addition of T4 gene 32 protein in the PCR mixture. The use of this protein is effective but in some cases cannot overcome PCR inhibition by humic acids (*31*). The efficiency of soil DNA extraction can also produce false negatives as poor extraction yields have a diluting effect on (target) DNA. Total DNA amount was quantified on the 320 DNA extracts of October 2004 from plots cultivated with RR soybean using PicoGreen (Molecular Probes, Eugene, OR), but no relationship was found between total DNA content and detection of transgenic soybean DNA (data not shown).

In soil DNA extracts from plots grown with RR soybean, the percentages of positives were higher than with the RR corn DNA extracts and reached up to 79% in August 2004. The differences observed between the two crops may be explained by the spatial distribution of roots in soil. Corn plants grow coarse sparse roots, while soybean plants produce numerous fine roots that grow horizontally near the surface. These morphological root traits are reflected by the distribution of the transgenes between the 3 and 12 cm subsamples. During the growing season, more recombinant soybean DNA was found in the top subsamples, whereas the distribution of recombinant corn DNA did not tend to differ between subsamples (**Figure 3**). Consequently, because of the low root density of corn plants in the soil, the ability to detect root material or free DNA released by roots was less with corn than with soybean. The following May, more corn subsamples were positive (3.47%) for target recombinant DNA than soybean subsamples (0.45%). Greater apparent persistence of recombinant DNA in corn may have been due to environment or quantitative or qualitative differences in plant residue between these crops.

The study of the vertical distribution of transgenes in the soil profile suggested that recombinant DNA follows a spatial cycle in the soil that differs between these crops. Whereas the proportions of transgenes between the 3 and 12 cm subsamples were generally equally distributed throughout the growing season of RR corn and RR soybean, this pattern was different in soil cores collected the next spring. Seventy-one percent of the transgenes were detected in the 12 cm subsamples in the spring following RR corn. Recombinant DNA was degraded over winter, and this process was accelerated in the surface subsamples. Surface soil is more exposed to biotic and abiotic factors than deeper soil horizons. As topsoil warms earlier in spring, DNA is exposed earlier to degradation by soil DNase activity and breaks down more quickly. DNA present in the upper soil horizon is also subject to climatic extremes of precipitation, drought, freezing, and thawing. Moreover, a recent study in a controlled environment showed that watering can leach recombinant DNA through soil (*34*). Leaching through precipitation and spring snowmelt is therefore likely to move recombinant DNA downward.

No differences were observed between herbicide treatments. This is not surprising because such differences were not expected to be detected on a short-term time scale. Glyphosate targets not only plants but also some bacteria and fungal species (*35*). Therefore, because soil organisms partially influence decomposition rates of plant material and DNA in the soil, the quantity and persistence of recombinant DNA in the soil of glyphosate-treated plots may or may not be altered. We are also conducting studies in the same field on the dynamics of bacterial- and fungal-based soil food webs.

The location of the transgenes in the soil was not investigated in this study. It is likely that the largest quantity of recombinant DNA was present in living and decaying plant tissues. This DNA is not necessarily directly accessible to competent bacteria for transformation because natural transformation requires naked DNA (*36*). Nevertheless, plant tissue in soil constitutes a pool of DNA that can supply competent bacteria with foreign DNA. Bacteria in the rhizosphere are considered to be the most likely to be transformed because decaying plant cells release free DNA in the immediate soil environment (*37*).

This paper is, to the best of our knowledge, the first field survey on the quantification and persistence of recombinant DNA in soil samples in a typical corn/soybean crop rotation over time. The methods employed here, using real-time PCR, proved to be reliable and allowed both qualitative and quantitative measurements of the presence of recombinant DNA in an agricultural system over time.

ABBREVIATIONS USED

CP4-EPSPS, 5-enol-pyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; CTP, chloroplast transit peptide; DNA, DNA; EPSPS, 5-enol-pyruvyl-shikimate-3-phosphate synthase; GM, genetically modified; PCR, Polymerase Chain Reaction; RR, Roundup Ready.

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