JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitation of Transgenic Plant DNA in Leachate Water: Real-Time Polymerase Chain Reaction Analysis

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Roundup Ready (RR) genetically modified (GM) corn and soybean comprise a large portion of the annual planted acreage of GM crops. Plant growth and subsequent plant decomposition introduce the recombinant DNA (rDNA) into the soil environment, where its fate has not been completely researched. Little is known of the temporal and spatial distribution of plant-derived rDNA in the soil environment and in situ transport of plant DNA by leachate water has not been studied before. The objectives of this study were to determine whether sufficient quantities of plant rDNA were released by roots during growth and early decomposition to be detected in water collected after percolating through a soil profile and to determine the influence of temperature on DNA persistence in the leachate water. Individual plants of RR corn and RR soybean were grown in modified cylinders in a growth room, and the cylinders were flushed with rain water weekly. Immediately after collection, the leachate was subjected to DNA purification followed by rDNA quantification using real-time Polymerase Chain Reaction (PCR) analysis. To test the effects of temperature on plant DNA persistence in leachate water, water samples were spiked with known quantities of RR soybean or RR corn genomic DNA and DNA persistence was examined at 5, 15, and 25 °C. Differences in the amounts and temporal distributions of root-derived rDNA were observed between corn and soybean plants. The results suggest that rainfall events may distribute plant DNA throughout the soil and into leachate water. Half-lives of plant DNA in leachate water ranged from 1.2 to 26.7 h, and persistence was greater at colder temperatures (5 and 15 °C).

KEYWORDS: Agriculture; Biotechnology; corn; DNA half-life; environment; genetically modified; glyphosate; leachate; method; real-time PCR; roots; soil; soil columns; soybean; temperature; transgenic plants; water

INTRODUCTION

Since their introduction in the mid 1990s, genetically modified (GM) crops have been adopted by many producers. Of these crops, GM soybean [*Glycine max* (L.) Merr.] and GM corn (*Zea mays* L.) are the most abundantly grown, comprising 61 and 23% of the global GM crop acreage (67.7 million ha in 2003), respectively (*1*). The introduction of transgenic crops was accompanied by a number of concerns including the escape and persistence of transgenes in the environment (*2*). Although we are beginning to understand the extent of transgene movement above the soil surface (*3*), little is known about the movement of recombinant plant DNA (rDNA) in the soil environment. A more complete understanding of the plant DNA cycle in the soil environment is necessary to assess the true ecological impact of GM crops.

During their life cycle, plants synthesize and replicate DNA, which is released into the soil environment. Lysis of root cap cells and root turnover may contribute to plant DNA release into the soil environment during vegetative growth, whereas during anthesis, substantial quantities of plant DNA can be added to the soil environment in the form of pollen (up to 10^7 pollen grains m^{-2}) in species such as corn (*4*). In annual crops, decomposition following physiological maturity is also a source of plant DNA in the soil environment. During decomposition, the majority of plant DNA seems to be degraded in the plant tissue and only a small portion of the total plant DNA is released into the soil environment (*5*). Although the sources are recognized, little is known about the temporal or spatial dispersal of plant-derived DNA in the soil environment.

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10.1021/jf0504667 CCC: $30.25
Published on Web 06/29/2005

There are several possible fates for free plant DNA in soil. DNA may be degraded by soil DNases and other nucleases present in the soil environment (*6*, *7*). Free DNA may bind to clay particles (*8*, *9*), where it may be partially resistant to degradation (*10*). Adsorption of DNA is influenced by clay particle characteristics (*9*), the charge of the DNA molecule, which is primarily regulated by pH (*11*, *12*), the size of the DNA molecule (*13*), and the divalent cation content of the soil (*14*, *15*). As a result, plant DNA may persist for an unknown period of time in the soil environment. For example, sugar beet (*Beta vulgaris* L.) DNA can be detected in soil for at least 1 year (*16*).

Soil microbes interact with free DNA. Unbound DNA may be used as a source of nutrients (*17*) or may be incorporated into genomes of competent bacteria via natural genetic transformation (*18*). For either to occur, free DNA must come in contact with soil bacteria. Movement of plant DNA in soil water facilitates this process. Poté et al. (*19*) demonstrated transport of supercoiled and open circular forms of antibiotic resistance genes through saturated soil columns in the laboratory; however, in situ transport of plant DNA by leachate water has not been adequately researched. The factors influencing movement of soil bacteria with soil water are well established in the literature (*20*−*22*).

Temporal and spatial dispersal of plant DNA in the soil environment is influenced by the persistence of plant DNA in leachate water. In sea water, free DNA persistence appears to be short and is influenced by temperature. For example, Duprey et al. (*23*) reported that *Salmonella* DNA persists in natural sea water for 3−8 days when incubated at 10 °C, but for only 2−4 days at 20 °C. The persistence of extracellular *Salmonella* DNA was shorter than that of DNA in intact cells. No studies on DNA persistence in leachate water in agriculture have been conducted to date.

The objectives of this study were to quantifiy the amount of DNA released by plant roots into leachate water during growth and early plant decomposition and to determine the influence of temperature on the persistence of corn and soybean DNA in leachate water using Polymerase Chain Reaction (PCR) analysis.

MATERIALS AND METHODS

DNA Leaching Study. The leaching study was a two-way factorial (crop × herbicide), completely randomized experimental design. Eight Roundup Ready (RR) corn (39T67) and eight RR soybean (DKB06-52) plants were grown in a growth room. In addition, three conventional corn (39T68) and soybean (OAC Bayfield) plants were grown as negative controls for PCR analysis. Plants were grown in 3 L (10.5 cm diameter, 38 cm height) transparent plastic pots with 2 cm diameter holes in their bottoms. The pots were covered with aluminum foil to exclude light. Two layers of fiberglass screen (0.5 mm) were placed at the bottom of each pot. A thin layer of autoclaved aquarium gravel was placed between the screen and 2.2 L of a silt loam soil that had been passed through a 3 mm sieve. This resulted in a porosity of ∼59%. The silt loam soil (sand, 26.1%; silt, 60.1%; clay, 13.8%; pH, 7.3; OM, 5%; CEC, 27.1 cmol + kg^{-1}) was obtained from the Elora Research Station, ON, Canada (43° 41′ N, 80° 26′ W) and tested for corn and soybean rDNA content prior to use with the methods described by Lerat et al. (*24*). In each pot, the soil surface was shaped into a deep concave to prevent water from preferentially flowing between the soil and the pot during leaching treatment.

Seeds were surface sterilized in bleach (5.25% sodium hypochlorite) for 3 min, triple rinsed with double-distilled (ddi) H_2O, and germinated on sterile wet filter paper in Petri dishes in darkness. After 48 h [= 2 days after planting (DAP)], single germinated seeds were planted in each pot. At this time, the wet soybean seedlings were coated with a commercial peat-based inoculum (Nitragin soybean S culture, Liphatech, Inc., Milwaukee, WI). The soil in each pot was irrigated immediately prior to planting and maintained above 50% of field capacity (FC = 440 mL L^{-1} soil) (determined gravimetrically), and the plants were maintained at 25/20 °C and 16/8 h day/night at a relative humidity of 75% throughout the study. Twenty-nine days after planting, glyphosate was applied at 1.8 kg of active ingredient (ai) ha^{-1} to four replicates of the RR plants of each species. Forty-three DAP, all shoots were removed, whereas the root systems were left in the pots for 16 more days before the study was terminated.

At weekly intervals beginning at 7 DAP, each pot was flushed with 1.5 L (1.1 times the pore volume) of rain water. Rain water was collected locally during rainfall events and stored at 4 °C until used. The leachate water from the pots was collected in 75 mL containers, and 1.3 mL subsamples for DNA recovery were taken from the first, third, and fifth 75 mL fractions. DNA extraction and purification were conducted immediately after sampling.

Prior to planting, four 3 cm × 3 cm squares with 1 cm grid gradations printed on transparent plastic sheets were fixed to the outside of each pot. Two were used to determine root length density (RLD) of the top half of the root system, and two were used to determine RLD of the bottom half of the root system. Coinciding with weekly leachate sampling, the aluminum foil was temporarily removed to expose only the grids, and the number of root−grid line intersections was counted in each square.

Three days after the last leaching treatment, the roots were removed from each pot and washed from the soil. At this time, two soil samples were taken from each pot for soil DNA analysis. Care was taken not to include root tissue in these samples. Target DNA was extracted and quantified using the method described by Lerat et al. (*24*). Shoot and root dry matter was determined for each plant after drying for 48 h at 60 °C.

DNA Persistence in Leachate Water. The effects of temperature on the persistence of RR corn and RR soybean DNA spiked into leachate water obtained at 14 DAP from the respective negative control plants of the leaching study were investigated. From each plant, 24 mL of leachate water was spiked with $\sim 10^5$ target sequence copies mL^{-1} of corn or soybean DNA. The plant DNA was extracted from leaf tissue of RR corn (39T67) and RR soybean (DKB06-52) using the Qiagen DNeasy kit (Misissauga, ON, Canada), quantified with a spectrophotometer (260 nm), and stored at −20 °C until used. The number of genome copies mL^{-1} was estimated using genome masses published by Arumuganathan and Earle (*25*), and these were adjusted for target sequence copy numbers, assuming that each corn genome contained two copies of the *CTP2/EPSPS* rDNA target sequence (heterozygous) and two copies of the *10kDa*-zein indigenous DNA target sequence (homozoygous), whereas homozygous soybean contained four copies of the *CTP4/EPSPS* rDNA target sequence. After thorough mixing, 21 aliquots of 1 mL each were prepared in sterile 1.5 mL microtubes, and seven tubes from each leachate sample were incubated at 5, 15, or 25 °C.

DNA Purification and Concentration. To enable real-time PCR analysis of the leachate water samples, a simple and rapid DNA purification method based on chelation using Instagene Matrix (IM) (Bio-Rad, Mississauga, ON, Canada) followed by DNA concentration through 2-propanol precipitation was developed. Instagene Matrix (200 μL) was added to each leachate water subsample. In addition, 1 μL (1 ng μL^{-1} or $\sim 10^9$ copies) of green fluorescent protein (*GFP*) gene PCR product (*24*) was added to one of the three subsamples of each plant as an internal standard to determine DNA recovery. The *GFP* gene originates from *Aequorea victoria* (jellyfish) and had been cloned into *Escherichia coli* JM109 (*24*). The samples were shaken by hand and incubated for 20 min at 56 °C. Following incubation, the samples were shaken by hand again and centrifuged in a microfuge at 14000*g* for 10 min to remove particulates, bacteria, and the IM. Following centrifugation, DNA was precipitated from 700 μL of supernatant using 700 μL of cold (−20 °C) 2-propanol, 70 μL of 5 M NaCl, and 1 μL of glycogen (20 mg mL^{-1}, Fermentas, Burlington, ON, Canada). Following DNA precipitation (2−4 h) and centrifugation (14000*g* for 40 min at 4 °C), the pellets were washed with 70% ethanol, centrifuged (14000*g* for 15 min at 4 °C), dried, and resuspended in 35 μL of sterile ultrapure H_2O.

Table 1. Sequences (5′ to 3′) of Forward and Reverse Primers and Probes Used during Real-Time PCR Analysis for the *10kDa*-Zein, RR Corn, RR Soybean, and *GFP* DNA Target Sequences[a]

target	name	sequence	size (bp)	source
		Forward Primer		
10kDa-zein	ZeinF2	gcacttgccaccagtcatgc	134	
RR corn	CTP2-7	ggctctgagcttcgtcctcttaag	124	*21*
RR soybean	CTP4-5	atcagtggctacagcctgcat	92	*21*
GFP	GFP-Rep1	agtggagagggtgaaggtgatg	143	*21*
		Reverse Primer		
10kDa-zein	ZeinR2	tgaagcggtaaggccaacagttg		
RR corn	CP4-12	gaatgcggacggttccggaaag		*21*
RR soybean	CP4-12	gaatgcggacggttccggaaag		*21*
GFP	GFP-Rep2	tgatctgggtatctcgcaaagc		*21*
		Probe		
10kDa-zein	Zeinmb	TexRd-cgcgatcggacggacacgccatcaagctggatcgcg-BHQ2		
RR corn	RRmb	FAM-cgcgatcatttgcgggcggttgcgggcgatcgcg-Dabcyl		*21*
RR soybean	RRmb	FAM-cgcgatcatttgcgggcggttgcgggcgatcgcg-Dabcyl		*21*

[a] The size of the amplicons is indicated.

The same sterile ultrapure water also served as a negative control in real-time PCR reactions. DNA samples were stored overnight at 4 °C before real-time PCR analysis.

Optimization of DNA Recovery. To optimize the detection limit and minimize the likelihood of false negative results during real-time PCR analysis, DNA recovery was optimized in a series of experiments. In these experiments, DNA recovery was measured as template recovery using real-time PCR. After rain water had been leached through pots containing soil but no plants, water samples were spiked with known quantities of genomic RR corn or RR soybean DNA, and the effects of various concentrations of IM (100, 200, or 300 μL sample^{-1}) and filtration through 0.45 μm membrane filters (Millipore) prior to treatment with IM were evaluated. The effect of the optimized recovery and concentration method on lysis of bacteria was also determined. *E. coli* (JM109 containing the GFP plasmid) was grown in liquid Luria−Bertani (LB) medium for 8−12 h at 37 °C and added to leachate water for a final concentration of $\sim 10^7$ *E. coli* (as determined by hemocytometer counts under 1000× phase contrast microscopy). To determine the background (extracellular) DNA content, an equal amount of supernatant of the LB medium was added to leachate water after centrifugation for 10 min at 14000*g*. In two of the three repetitions of this experiment, 10^6 target DNA sequence copies mL^{-1} of genomic corn and soybean also were added to the leachate water samples to determine relative recovery of plant DNA. All samples were subjected to the recovery and concentration method, and the influence of IM incubation temperatures of 100 °C, 56 °C, and room temperature (22−25 °C) on cell lysis was examined.

An experiment to determine the detection limit of the method and the combined effects of DNA recovery and PCR inhibition was conducted. The optimized DNA purification and concentration method was applied to a 10-fold dilution series of sterile ultrapure or soil leachate water spiked with transgenic corn or soybean DNA to obtain $\sim 10^1-10^6$ copies of target DNA sequence mL^{-1}. In addition, data generated from this experiment were used to prepare standard curves for DNA content determination in leachate water samples with unknown quantities of target DNA. Each treatment in the optimization and standard curve experiments contained three independent replicates, and experiments were repeated two or three times.

Total DNA Content and Humic Acid Removal. Total DNA extraction from leachate samples and the removal of humic acid, a potent inhibitor of the Polymerase Chain Reaction, by IM treatment was determined. The DNA content of purified and concentrated leachate water samples was determined from four randomly chosen corn and soybean samples at 14, 28, 42, and 56 DAP to which *GFP* was not added. Total DNA was quantified in 100 μL of 10:90 DNA/ultrapure water using a spectrophotometer (260/280 nm).

Humic acid removal by treatment with IM was determined by measuring the humic acid content before IM addition and after centrifugation following IM treatment in 16 samples using a spectrophotometer (340 nm) (*26*). Humic acid standards [0.1, 1.0, 10, or 100 ng μL^{-1} humic acid (Sigma-Aldrich, Oakville, ON, Canada)] were prepared and used to generate a standard curve for humic acid determination in water samples.

Primers and Probes. Sequences and sources of primers and molecular beacons used in these experiments are shown in **Table 1**. The primers and the molecular beacon (RRmb) for corn (event NK603) and soybean (event 40-3-2) rDNA and the primers for the *GFP* reporter gene were developed in our laboratory during a previous study (*24*). In corn and in soybean, the junction of the CP4 EPSPS gene and the preceding chloroplast transit peptide gene was targeted for PCR analysis to avoid possible coamplification of EPSPS genes from bacteria present in environmental samples. For the indigenous *10kD*a-zein gene in corn, primers and a molecular beacon (Zeinmb) were developed on the basis of those reported by Einspanier et al. (*27*). Primers described by Einspanier et al. (*27*) were used to amplify a 275 bp region of the *10kDa*-zein gene in six corn genotypes (39T67, 39T68, DK26-75, DK27-12, DK27-11, and DK35-51). The PCR mixture was the same as described in the following section; however, the molecular beacon and T4 gene 32 protein (Roche, Laval, PQ, Canada) were omitted. PCR conditions were 3 min at 95 °C followed by 30 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. The PCR products were separated on a 1% (w/v) agarose gel, excised, cloned into the *Topo TA* vector, and transformed into OneShot *E. coli* as described by the manufacturer (Invitrogen, Mississauga, ON, Canada). After culture of the transformed *E. coli*, plasmids containing the target sequence were purified using the QIAprep kit (Qiagen, Inc., Mississauga, ON, Canada). The insert was excised from the plasmid using *Eco*RI and sequenced using the CEQ2000xl system (Beckman Coulter, Mississauga, ON, Canada) as directed by the manufacturer. From these sequences, a molecular beacon (Zeinmb) (**Table 1**) was designed for a homologous region using Beacon Designer software 3.0 (Bio-Rad, Hercules, CA). The molecular beacon was synthesized by Integrated DNA Technologies (Coralville, IA). The primers for Zeinmb (**Table 1**) were designed in-house.

Real-Time PCR Amplification. Real-time PCR amplifications were performed on a Bio-Rad iCycler in 96-well plate microtubes containing a final volume of 20 μL of the following reaction mixture: 1× iQ Supermix (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.8 mM dNTPs, 0.5 unit of *Taq* polymerase, and 3 mM $MgCl_2$) (Bio-Rad), 500 nM forward and reverse primers, 400 nM RRmb or 500 nM Zeinmb, and 20 ng μL^{-1} of T4 gene 32 protein, and 1 μL of template DNA from leachate water samples (30.6 ± 2.8 ng of DNA) or sterile ultrapure water. Conditions for real-time PCR amplifications were 3 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 20 s at 53 °C. Fluorescence was monitored during the 53 °C annealing step. The *GFP* reporter gene was quantified with real-time PCR as described by Lerat et al. (*24*). The PCR mixture contained 1× iQ Supermix (as above plus SYBR Green I dye and 10 nM fluorescein) (Bio-Rad), 500 nM forward and reverse primers, 20 ng μL^{-1} T4 gene 32 protein, and 1 μL of DNA template or sterile ultrapure H_2O. PCR conditions for *GFP* amplification were 3 min at 95 °C followed by 30 cycles of 10 s at 95 °C and 30 s at 60 °C. Fluorescence was monitored during the 60 °C annealing step.

Data Analysis. For each sample of the leachate and persistence studies, quantities of DNA in leachate water were calculated from the standard curves [log copies corn rDNA $= -0.257x + 9.454$, $R^2 = 0.999$; log copies corn *zein* DNA $= -0.265x + 9.893$, $R^2 = 0.999$; log copies soybean rDNA $= -0.255x + 8.622$; $R^2 = 0.999$, where x is the threshold cycle (Ct) in all cases]. To eliminate the effects of variation in DNA recovery and PCR inhibition among samples, target DNA copy numbers of individual samples were standardized for mean DNA recovery and PCR inhibition. Samples were standardized for mean *GFP* recovery as spiked leachate water samples used to develop standard curves already accounted for DNA losses during purification and PCR inhibition. From these data, mean rDNA content of leachate water for each RR plant was determined for each sampling date. *GFP* target gene copy numbers were determined using the standard curve equation: log copies *GFP* DNA $= -0.304x + 10.180$, $R^2 = 0.999$, where x is the Ct. From the three subsamples, the proportion of positive samples was determined for each RR plant at each sampling date. DNA content of soil samples taken at the end of the study were determined using the equations provided by Lerat et al. (*24*). In all cases, the DNA content of samples negative for template DNA after PCR was assumed to be zero. From the optimization experiments, relative DNA recoveries as affected by IM incubation temperature were determined by calculating the target sequence copy numbers using the above equations. These data were converted to relative percentage values of target DNA recovery compared to target DNA sequence recovery at 56 °C.

Root grid-intersection data were converted to root length density (RLD) values as described by Giovannetti and Mosse (*28*). For each sampling date, means for the top and bottom halves of the root system and grand means of each root system were calculated. The relative distribution of each root system through the soil profile was estimated by calculating the RLD ratio, which is the quotient of RLD of the top half of a root system divided by RLD of the bottom half of that root system. To determine the role of RLD on rDNA content of leachate water, weekly mean leachate water rDNA contents were regressed against weekly mean RLD in each species.

Due to large differences observed between corn and soybean, all statistical analyses were conducted within species and sampling dates. Two-tailed *t* tests were used to determine whether treatment with glyphosate influenced rDNA content of leachate water, proportion of positive samples, and mean RLD after and including the 35 DAP sampling date. Means for these response variables were summarized within sampling date as the *t*-test results dictated, and appropriate standard errors of the means were determined.

PCR efficiencies were calculated for the standard curves using the method described by Rasmussen (*29*). The equation $E = 10^{(-\text{slope})}$, where E is the efficiency and slope is the slope of the standard curve, was used to determine the real-time PCR efficiency for each DNA target sequence examined. Grand means were calculated after it was determined that the amplification efficiencies of the three target sequences (RR corn, RR soybean, and *10kDa*-zein) were within 5%, and efficiencies were converted to percentage values. Real-time PCR efficiency of the *GFP* reporter gene is reported separately.

Humic acid content in the selected samples was determined from spectrophotometer readings using the equation HA (ng μL^{-1}) $= 63.93x + 0.110$, where x is absorbance at 340 nm; $R^2 = 0.999$. Means for each treatment were summarized, and the appropriate standard errors of the means were calculated.

RESULTS

DNA Leaching Study. The quantity and temporal distribution of target DNA content in leachate water differed between corn and soybean (**Figure 1A**,**B**). In corn, maximum target DNA content in leachate water was detected at the onset of anthesis and declined rapidly after the shoots were removed (**Figure 1A**). In soybean, maximum rDNA content in leachate water occurred after shoot removal during early decomposition of the roots. The maximum levels of target DNA content in leachate water were 3 orders of magnitude lower in soybean than in corn. In both species, the number of samples positive for target DNA was closely associated with total target DNA content at each sampling date (**Figure 1A**,**B**). No false positives for target rDNA templates were found in the negative control plants (conventional genotypes).

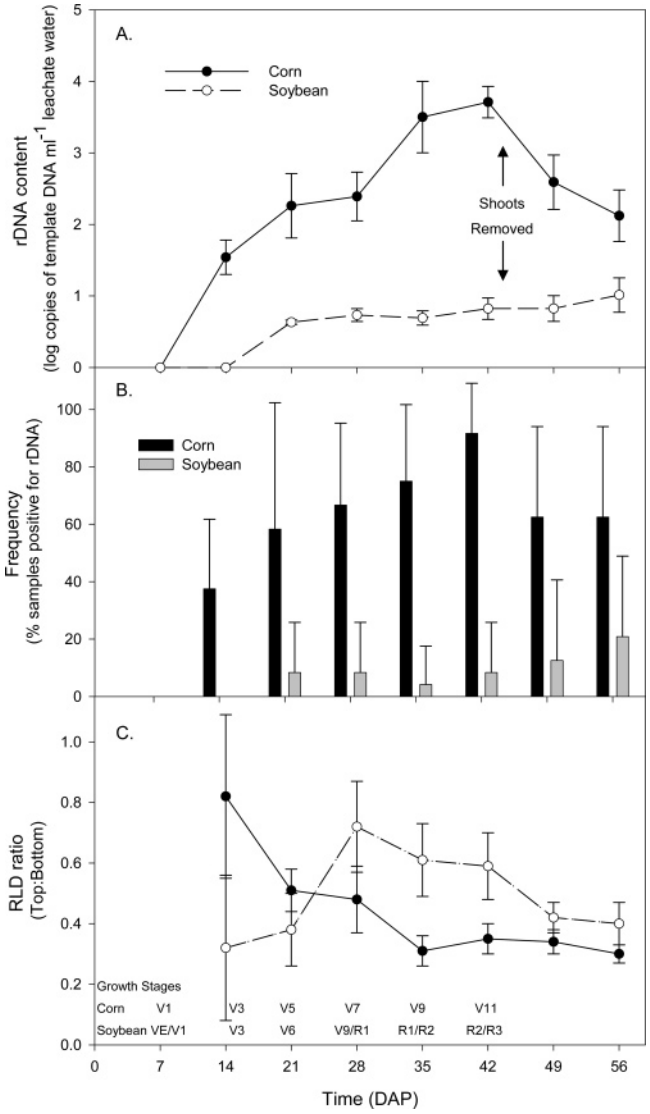

Figure 1. Recombinant DNA (rDNA) in leachate water (**A**), frequency of samples positive for rDNA (**B**), and root length density (RLD) ratios (**C**) in corn and soybean over time. Bars indicate 1 SEM for $n = 8$. Plant growth stages are indicated (**C**).

RLD ratios were different between corn and soybean during early plant growth (**Figure 1C**). In corn, the RLD ratio was greatest when roots were first visible (14 DAP) and declined curvilinearly during the remainder of the study to about one-third of the initial value. In contrast, soybean RLD ratios increased ~3-fold between 14 and 28 DAP, after which soybean RLD declined linearly to 50% of the maximum value. In both species, weekly mean RLD regressed well with weekly mean leachate water target DNA content and explained >80% of the variation between these variables (**Figure 2**). The residuals did not indicate a more complex relationship.

Treatment with glyphosate did not affect target DNA content of leachate water despite influencing soybean dry weights. In soybean, two-tailed *t* tests indicated that shoot ($P < 0.042$) and root ($P < 0.038$) weights of RR plants treated with glyphosate were lower than in RR plants not treated with glyphosate. Nevertheless, the treatments were combined as no differences in target DNA content in leachate water were observed (data not shown) and mean shoot and root dry weights were 11.2 (±

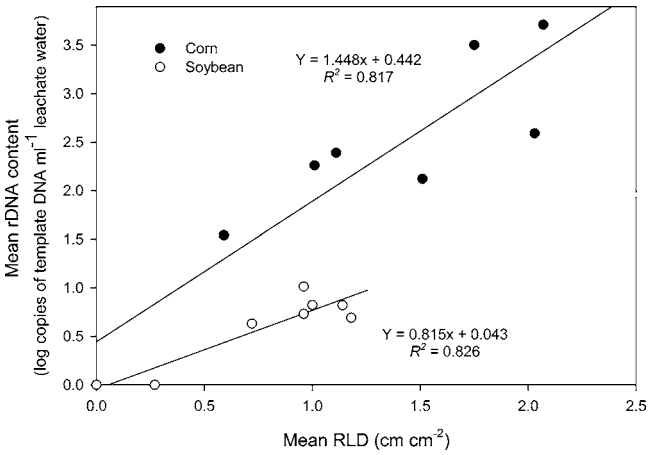

Figure 2. Relationship between mean RLD and mean rDNA content of leachate water in corn and soybean. Regression equations and coefficients of regression are indicated.

Table 2. Half-Life of Corn and Soybean DNA Incubated at Three Temperatures in Leachate Water from Soil Growing the Respective Plants ($n = 3$)

	DNA half-life (h)			
	corn		soybean	
incubation temp (°C)	*10kDa*-zein	CTP2/CP4 EPSPS	CTP4/CP4 EPSPS	$LSD_{0.05}$
5	8.9	9.1	26.7	7.8
15	5.9	4.5	12.9	4.2
25	1.2	1.8	4.2	1.6
$LSD_{0.05}$	3.4	1.3	8.2	

1.5) and 19.3 (± 3.4) g, respectively. In corn, glyphosate had no effect on target DNA content in leachate water and plant dry weights (27.5 ± 1.2 g $shoot^{-1}$, 31.6 ± 7.0 g $root^{-1}$).

At the end of the experiment, target DNA content of soil was greater in soybean than in corn. In corn only 1 of 16 samples was positive for rDNA (grand mean = 4.0 ± 4.0 copies g^{-1} of FW of soil), whereas in soybean 10 of 16 samples were positive for rDNA (grand mean = 30 ± 12 copies g^{-1} of FW of soil).

DNA Persistence Study. Plant species and temperature strongly influenced DNA persistence in leachate water (**Table 2**). In soybean leachate water, the half-life of DNA was 2.5−5.5 times greater than in corn leachate water at all incubation temperatures. No differences in DNA persistence in leachate water were observed between the indigenous and recombinant target DNA sequences in corn. Incubation temperature also strongly influenced DNA persistence in leachate water. In corn, the DNA half-life in leachate water decreased on average 2.6 times for each 10 °C increase in incubation temperature (Q_{10}), whereas in soybean a Q_{10} of 1.9 was observed. The data generated in the DNA persistence study fit well to the negative exponential function used to determine DNA half-life, and the residuals did not indicate a more complex relationship.

Leaching of rain water through soil increased the humic acid content, pH, and aerobic bacteria content (**Table 3**). No significant differences in humic acid content and pH were found between corn and soybean leachate water; however, culturable aerobic bacteria densities were ∼10 times greater in corn leachate water than in soybean leachate water.

DNA Purification and Detection. DNA purification and concentration from leachate water samples improved the detection limit by 2 orders of magnitude and the PCR efficiency by 13% (**Table 4**). DNA purification and concentration resulted in the same detection limit in leachate and ultrapure water (10^2 copies mL^{-1}). However, the percentage of samples that were positive for the target DNA was 3 times greater in ultrapure water samples than in leachate water samples (**Table 4**). The effects of the optimized DNA purification and concentration procedure on the detection limits of the target sequences (RR corn, *10kDa*-zein, and RR soybean) were determined for mean values of all three sequences because the detection limits of the three target templates were the same. The 95% confidence intervals of the standard curves (log) used to determine target DNA content in unknown samples ranged from 0.234 for *10kDa*-zein DNA to 0.417 for corn rDNA (data not shown). PCR efficiencies for the three plant target sequences were within 3% in leachate water after DNA purification and concentration (data not shown), and therefore grand means for PCR efficiency were determined (**Table** 4). Real-time PCR efficiency for the *GFP* reporter gene was 92% (data not shown). The molecular beacon (Zeinmb) designed for indigenous corn DNA (*10kDa*-zein) was highly specific and resulted in accurate detection of the target DNA sequence (**Figure 3**). Real-time PCR samples were positive only when the target sequence was present and amplified. All other amplicons resulted in negative results during real-time PCR. The origin of the other amplicon observed in lanes 1−3 is not known. The absence of this amplicon in the positive control (genomic corn DNA only) suggests that the template DNA is most likely from a microbial source present in environmental samples. No attempt was made to lessen the presence of this amplicon in the PCR reaction.

The concentration and incubation temperature of IM influenced target DNA recovery. Increasing the IM concentration to 300 μL $sample^{-1}$ for sample extraction did not further improve DNA recoveries, whereas a substantial reduction in DNA recovery was observed when the IM concentration was lowered to 100 μL $sample^{-1}$ (data not shown). DNA recovery of the reporter gene and all plant target genes was greatest when samples were exposed to 56 °C during IM treatment (**Table 5**). Almost half of all target DNA was lost when samples were incubated at 100 °C during IM treatment, which is recommended by the manufacturer for release of cellular DNA (Bio-Rad), whereas even lower DNA recovery was observed in samples that were incubated at room temperature.

A broad range in recovery of the GFP reporter gene and humic acid removal was observed among samples. Mean recovery of the *GFP* reporter gene was 36.5% (± 2.2, $n = 112$) among all samples from the leachate study, but ranged from 1.6 to 83%. The optimized protocol for DNA purification and concentration resulted in removal of 26.5% (± 1.03, $n = 32$) of the humic acids (**Table 3**) before DNA concentration, but ranged from 7.5 to 44.8%. No differences in humic acid removal by this purification and concentration method were observed between corn and soybean leachate water samples.

DISCUSSION

DNA Leaching and Persistence. The leaching study showed that detectable quantities of plant target DNA were released into the soil environment and were moved by leachate water during growth and early decomposition of roots with distinct differences in quantity and temporal distribution between corn and soybean plants. Greater final root weights, greater RLDs, and a greater proportion of total root system in the bottom half of the pots in corn versus soybean may explain the greater target DNA content of corn leachate water; however, differences in these parameters were ≤2-fold, whereas maximum target DNA content of leachate water differed by up to 3 orders of magnitude between these species. Therefore, it is likely that inherent differences in root growth rates and root cell turnover between corn and soybean also played a role. Soybean roots form a complex

Table 3. Aerobic Colony-Forming Units (CFU) and pH in Rain Water and Leachate Water and Humic Acid Content before and after Instagene Matrix Treatment in Leachate Water from Soil Growing Corn or Soybean Only[a]

treatment	aerobic plate counts (CFU mL^{-1})	pH	humic acid content (ng μL^{-1})	
			before	after
rain water	3.0×10^3	6.58	1.71	ND[b]
corn leachate	4.8×10^5 (1.2×10^5)	7.77 (0.19)	16.3 (2.0)	11.0 (1.0)
soybean leachate	4.4×10^4 (1.4×10^4)	7.84 (0.08)	14.3 (1.9)	11.1 (1.3)
t-test *P* value	0.014	0.720	0.442	0.927

[a] The SEM is indicated in parentheses for $n = 16$. [b] Not determined.

Table 4. Mean Detection Limit of the Three Target Sequences (RR Corn, *10kDa*-Zein, and RR Soybean), Reported as the Percentage of Samples Positive for the Template DNA, before and after DNA Purification and Concentration (P&C), and Mean PCR Efficiency in Ultrapure and Leachate Water ($n = 36$)

	positive samples (%) at detection limit of					
	10^5 template copies mL^{-1}	10^4 template copies mL^{-1}	10^3 template copies mL^{-1}	10^2 template copies mL^{-1}	10^1 template copies mL^{-1}	PCR efficiency *E* (%)
before P&C						
ultrapure water	100	50	25	0	0	99
leachate water	100	25	0	0	0	71
after P&C						
ultrapure water	100	100	100	25	0	83
leachate water	100	100	75	8	0	80

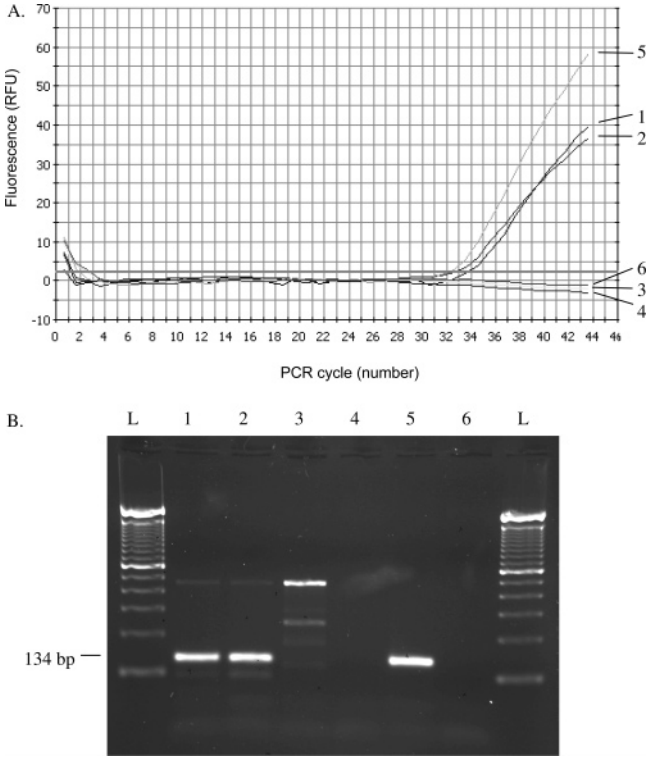

Figure 3. Real-time PCR results (**A**) and DNA separation on a 1% (w/v) agarose gel of 5 μL of PCR product (**B**) of the same PCR for the corn *10kDa*-zein target sequence: lane 1, leachate water sample positive for corn *10kDa*-zein DNA; lane 2, the same leachate water sample positive for corn *10kDa*-zein DNA; lane 3, negative control, leachate water; lane 4, negative control, leachate water (different sample from lane 3); lane 5, positive control, genomic corn DNA; lane 6, negative control, ultrapure water; L indicates 100 bp ladder.

Table 5. Effect of Instagene Matrix Incubation on Target Sequence (*GFP*, RR Corn, *10kDa*-Zein, and RR Soybean) Recovery at Room Temperature (20–22 °C) and 100 °C Relative to Target Sequence Recovery at 56 °C[a]

incubation temp (°C)	recovery (%)			
	GFP	RR corn	*10kDa*-zein	RR soybean
20–22	36.2 (13.7)	52.8 (12.5)	35.5 (18.2)	46.1 (31.8)
56	100	100	100	100
100	55.8 (10.0)	57.4 (37.4)	57.2 (32.8)	63.9 (21.3)
supernatant	23.2 (3.4)			

[a] The SEM is indicated in parentheses for $n = 3$.

symbiotic relationship with *Bradyrhizobium japonicum*, resulting in nitrogen fixation. This association does not occur in corn roots. The soybean roots were inoculated and some nodules developed; however, it is not known to what extent the symbiosis influenced our observations. Greater target DNA content in soybean leachate water after shoot removal also indicated fundamental differences in root dynamics between corn and soybean. These observations may be related to more rapid breakdown of soybean roots compared to corn. Browning of soybean roots, which suggests decomposition, was observed shortly after shoot removal, although the same was not observed in corn. Our observations on DNA mobility agree well with those of Poté et al. (*19*), who observed high potential for movement of biologically active bacterial DNA in soil water. Recombinant DNA content in leachate water was directly linked to RLD in both plant species. However, the RLD determined from the outside of each pot may be an overestimate of the true RLD because it was observed at the time of root washing that, in both species, root biomass was more concentrated at the pot periphery.

Persistence of plant target DNA in leachate water was temporary in both species, despite differences between corn and soybean. The values measured in this study are similar to those reported for *Legionella* and *Salmonella* DNA persistence in sea water (*23*, *30*) and are similar to half-lives of transformable plasmid DNA in soil (*31*), but were greater than DNA decline rates in decomposing plant leaf tissue buried soil (*5*). We did not observe differences in persistence between indigenous and recombinant corn DNA in leachate water, which shows that there was no discrimination in degradation between these two types of DNA in corn. Slower degradation of soybean DNA in soybean leachate water was linked to lower bacterial densities,

presumably resulting in fewer extracellular DNases and nucleases in soybean leachate water. This is supported by the mean temperature quotients ($Q_{10} \geq 2$) of DNA half-lives which indicated that DNA degradation in leachate water was enzymatic. Moreover, the short residence life of DNA in leachate water suggested that the plant-derived DNA in leachate water detected at each sampling time in the leachate study was recently released from the plant and did not reflect free plant DNA accumulation in soil water over time. DNA persistence determined in this study may be an overestimation of persistence of intact sequences of the entire genes investigated here. The short target sequences (<150 bp) required for real-time PCR analysis represent only a small size range of the respective genes, and DNA persistence of short gene fragments is generally longer than persistence of the DNA of entire genes (*32*).

The target sequence DNA content of soil near the rhizosphere was low for both species at the end of the experiment, but was greater in soybean than in corn (see Results). In soybean, small root fragments are more prevalent and root degradation appeared to be more rapid, which may have contributed to these observations.

DNA Purification and Detection. The DNA purification and concentration method described here is simple, safe, rapid, and inexpensive. The optimized purification and concentration method significantly improved the detection limit of target DNA in environmental water samples and increased the efficiency (*E*) of real-time PCR. The average recovery of target DNA using our method was equivalent to or slightly lower than total DNA recovery using more complex and time-consuming methods (*33*, *35*). Removal of humic acids using our method was lower than that reported for spin column methods (*38*); however, this appeared to have no appreciable negative effect on target DNA detection in purified and concentrated DNA from environmental water samples. The addition of T4 gene 32 protein in the PCR mixture alleviated the inhibitory effects that coprecipitated humic acids may have had during real-time PCR analysis. T4 gene 32 protein was added to the PCR mixtures at a concentration reported to counteract the inhibitory effects of humic acids ~2.5 times (Roche).

Samples positive for plant-derived DNA contained >100 copies of the target gene mL^{-1} of leachate water. This corresponds to two target sequence copies in 1 μL of 20 times concentrated template DNA added to the reaction mixture for real-time PCR analysis. Conservatively, the confidence intervals reported in the results indicate that accurate quantitation of target DNA in individual samples was not possible below ~700 copies of target DNA mL^{-1}. Our detection limit compares favorably to those reported in the literature for bacterial and viral DNA in environmental water samples (*33*−*35*).

Although our method of DNA recovery was gentle, recovery of DNA from bacterial cells cannot be excluded. The incubation temperature for IM (56 °C) was chosen because it maximized target template recovery and inactivates nucleases while limiting bacterial cell lysis according to the manufacturer (Bio-Rad). Some rupture of bacterial cells was evident, and therefore it is possible that some recovered target DNA was from lysed bacterial cells that had taken up target plant DNA. Filtration (0.45 μm) of water samples prior to DNA purification, as recommended by Dupray et al. (*23*) and Guy et al. (*39*), did not improve detection of the plant target DNA (data not shown).

The total quantity of plant-derived DNA released into the rhizosphere in the leaching study is not known as some DNA was likely retained by the soil matrix (*9*) and rapidly degraded. Pietramellara et al. (*9*), however, showed that flushing soil with water results in some desorption of bound DNA, and chelation of divalent cations by IM likely also resulted in release of DNA adsorbed to clay particles that were present in leachate water samples. Chelation of cations would break the cation bridges presumed to be responsible for DNA adsorption to soil (*14*, *15*). All target DNA found in corn leachate water originated from roots as shoots were removed before tasseling. In soybean, anthesis had begun before shoot removal, and therefore it is possible that pollen may have contributed to the target DNA content of leachate water; however, soybean is a primarily self-pollinating species (*36*), and therefore pollen release into the environment was likely minimal.

Similar to previous studies (*37*), we observed a large range of reporter gene (*GFP*) DNA recoveries among leachate water samples. These observations justified the use of the reporter gene to standardize target DNA recoveries. For target DNA standardization, we assumed that recovery of the reporter *GFP* gene was similar to the recovery of our target genes. Using the SYBR Green I method for detecting the reporter gene compared to detection with molecular beacons for all other target sequences may have contributed to the difference in PCR efficiencies observed. Our attempts to use a molecular beacon for detecting *GFP* recovery in a different study failed (see ref *24*).

Conclusions. To our knowledge, this is the first study showing in situ release of plant DNA by roots and movement of this DNA in leachate water through the soil environment during plant growth and early plant decomposition. During early decomposition, DNA was present in leachate water despite reports indicating that the majority of plant DNA is rapidly degraded within the tissue (*5*). Although persistence and therefore the period of biological activity of plant DNA in leachate water appear to be short, contact between soil bacteria and plant DNA outside the rhizosphere is likely. Persistence of two corn genes in leachate water was evaluated, and no difference was observed between the indigenous and recombinant corn DNA. In agricultural fields, soybean plant densities are higher than those of corn, and therefore some of the effects observed between individual plants of these species may not exist in these communities. We are currently conducting similar measurements in the field.

ABBREVIATIONS USED

CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; CTP, chloroplast transit peptide; FW, fresh weight; GFP, green fluorescent protein; GM, genetically modified; IM, Instagene Matrix; P&C, purification and concentration; PCR, Polymerase Chain Reaction; RLD, root length density; RR, Roundup Ready; RRmb, Roundup Ready molecular beacon; Zeinmb, *10kDa*-zein molecular beacon.

ACKNOWLEDGMENT

We thank L. England and M. Vincent for assistance and the Canadian Foundation for Innovation and the Ontario Challenge Fund for infrastructure support.

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Received for review March 1, 2005. Revised manuscript received May 13, 2005. Accepted May 20, 2005. We thank the NSERC Strategic Grant Program (STPGP 258065-02) (Canada) for supporting this project.

JF0504667