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The effect of crop species on DNase-producing bacteria in two soils

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Abstract

Purpose: Extracellular deoxyribonucleases (exDNases) from microbial origin contribute substantially to the restriction of extracellular DNA (exDNA) in the soil. Hence, it is imperative to understand the diversity of bacterial species capable of performing this important soil function and how crop species influence their dynamics in the soil. The present study investigates the occurrence of DNase-producing bacteria (DPB) in leachate samples obtained from soils in which the crop species of alfalfa (*Medicago sativa* L.), canola (*Brassica napus* L.), soybean (*Glycine max* [L.] Merr.) and wheat (*Triticum aestivum* L.) were raised in a growth room.

Methods: Selective media containing methyl green indicator was used to screen for DPB from leachate samples, whereas the 16S rRNA sequence analysis was employed to identify the isolates.

Results: The proportion of culturable DPB ranged between 5.72 and 40.01%; however, we did observe specific crop effects that shifted throughout the growing period. In general, higher proportions of exDNase producers were observed when the soils had lower nutrient levels. On using the 16S rRNA to classify the DPB isolates, most isolates were found to be members of the *Bacillus* genera, while other groups included *Chryseobacterium*, *Fictibacillus*, *Flavobacterium*, *Microbacterium*, *Nubsella*, *Pseudomonas*, *Psychrobacillus*, *Rheinheimera*, *Serratia* and *Stenotrophomonas*. Five candidate exDNase/nuclease-encoding proteins were also identified from *Bacillus mycoides* genomes using online databases.

Conclusion: Results from this study showed that crop species, growth stage and soil properties were important factors shaping the populations of DPB in leachate samples; however, soil properties seemed to have a greater influence on the trends observed on these bacterial populations. It may be possible to target soil indigenous bacteria that produce exDNases through management to decrease potential unintended effects of transgenes originating from genetically modified organisms (GMOs) or other introduced nucleic acid sequences in the environment.

Keywords: DNase-producing bacteria, exDNA, exDNAse(s), GMOs, Wheat, Canola, Soybean, Alfalfa

Introduction

Plants and microbes are known to form complex and dynamic interactions within the soil matrix, and these can have significant implications to the functioning of the ecosystem (Hartmann et al. 2009). These series of complex interactions between the plant, microbes and the soil environment result to increased microbial activity in the rhizosphere resulting to the selection of unique plant species-specific microbial consortia (Berg and Smalla 2009; Hartmann et al. 2009). The structure and functions of microbial communities in the soil are strongly influenced by the residing plant species (Burns et al. 2015). For instance, the microbial communities in the soil associated with wheat and canola differ substantially (Hansen et al. 2019 Hansen et al. 2018; Lay et al. 2018).

Because of the intimate association between plants and microbes, the release of genetically engineered (GE) plants brought into question the ecological sustainability

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of the technology due to the perceived non-targeted effects of transgenes to soil organisms such as natural transformation of microbes through horizontal gene transfer mechanisms (HGT) (Tsatsakis et al. 2017). The increased rhizosphere activity has also been documented for nuclease-producing microorganisms which could be exploited for transgenes mitigation in the environment. For instance, Greaves and Webley (1965) observed that the total number of nuclease-producing microorganisms were higher on the root surface and in the rhizosphere soil of pasture grasses than in non-rhizosphere soil. The plethora of compounds secreted through the root cap mucilage including genetic material (Wen et al. 2009; Knox et al. 2020; Ropitaux et al. 2020; Chambard et al. 2021) provide a source of nutrients and energy that may result in differential growth of microbial communities (Berg and Smalla 2009; Haichar et al. 2014). Plant DNA enters the soil environment mainly through root exudates, root cap sloughing, pollen dispersal and degradation of plant materials (Levy-Booth et al. 2007; Monticolo et al. 2020).

One major fate of extracellular DNA (exDNA) in the soil environment is degradation by indigenous soil microbial extracellular deoxyribonucleases (exDNases) into smaller fragments resulting to the loss of genetic information (Blum et al. 1997; Levy-Booth et al. 2007; Nielsen et al. 2007; Ibáñez de Aldecoa et al. 2017). The presence of these restriction enzymes therefore serves as barriers to exDNA introgression into native soil bacteria through HGT (Dodd and Pemberton 1999; Wu et al. 2001) and its subsequent long-term persistence in the soil environment (Kunadiya et al. 2021). For instance, Stewart and Sinigalliano (1990) reported a decrease in natural transformation frequency in bacteria after incubating marine and artificial sediments with DNase 1. Microbial exDNases contribute to soil functions and have largely been associated with nutrient scavenging activities (Benedik and Strych 1998; Desai and Shankar 2003; Levy-Booth et al. 2007; Ibáñez de Aldecoa et al. 2017) and virulence of pathogens (Park et al. 2019; Monticolo et al. 2020). For example, an increase of up to 35-fold in viable bacterial counts was observed 3 days after spiking soils with nucleic acids, thereby implying that microbes use nucleic acids as substrates for growth (Greaves and Wilson 1970). Blum et al. (1997) also observed an increase in the number of soil microbes 12 h after injecting DNA into soils, with rapid degradation occurring to the unbound DNA spike.

Some bacteria and fungi closely associated with plant roots have been reported to exhibit exDNase activities (Greaves and Webley 1965; Bertagnolli et al. 1996; Tavares and Sellstedt 2001; Klosterman et al. 2001; Balestrazzi et al. 2007). It was thought that only a limited number of bacterial species produce nucleases extracellularly (Benedik and Strych 1998); however, studies have found that many bacterial species exude nucleases that are either anchored to the cell wall or exuded into the growth medium (Eaves and Jeffries 1963; Nakai et al. 1965; Rothberg and Swartz 1965; Greaves and Webley 1965; Jakubovics et al. 2013; Sheikh and Hosseini 2013). This makes it possible to detect exDNase activities on agar plates containing indicators such as methyl green that are intercalated into the double helix matrix, but become clear halos of enzyme activity around the growing colonies as the double helix is being degraded.

The majority of studies on soil bacteria report single isolates that produce exDNases, whereas only a few studies have identified multiple isolates that produce exDNases. Pioneer studies on nuclease-producing bacteria (Greaves et al. 1970) reported nuclease production by *Cytophaga johnsonii* isolated from the soil which utilized nucleic acids as a C and P source more efficiently than a N source. On the other hand, some bacterial species such as *Escherichia coli*, *Serratia marcescens*, *Myxococcus virescens*, *Myxococcus fulvus* and *Chondrococcus coralloides* can thrive solely on nucleic acids as a C source for their growth (Norén 1955; Redfield 1993; Benedik and Strych 1998). For instance, the proliferation of a marine thriving *Vibrio* sp. increased up to 4 orders of magnitude in the presence of DNA, with rapid microbial assimilation of a significant proportion of the degradation products (Maeda and Taga 1974). Ten et al. (2006) isolated and characterized a novel DNase-producing isolate *Pedobacter ginsengisoli* sp. nov. belonging to the *Bacteroidetes* phyla from field soils growing ginseng (*Panax ginseng*) in South Korea. Strong exDNase activity was also observed in *Frankia* strains which are known to form symbiotic relationships with many dicot plant species (Tavares and Sellstedt 1997).

Extracellular DNase production has been reported in several marine bacterial isolates with the majority belonging to the genera *Bacillus* (Al-Wahaibi et al. 2019; Asha and Krishnaveni 2020). Moreover, several soil bacterial isolates belonging to different phyla groups were observed to express exDNases whose activities were mostly optimal at neutral pH and at temperatures between 30 and 40 °C (Kamble et al. 2011). Greaves and Wilson (1970) also recorded large numbers of DPB from different soil types growing grassy vegetation with varying proportions of DPBs at 17% in kaolinite, 86% in montmorillonite, 58% in peat and 47% in sandy soils. In an indoor study carried out by Balestrazzi et al. (2007) using transgenic white poplars with the bar gene insert, 62.5 to 100% of the total culturable bacterial populations were observed to express exDNase activities. Moreover, bacterial isolates cultured and identified as DNase producers in their study belonged to five genera: *Bacillus*,

Brevibacillus, *Microbacterium*, *Pseudomonas* and *Stenotrophomonas*.

To date, no study has described DPB inhabiting agricultural soils nor has the effect of crop species on DPB been investigated. Thus, the current study was carried out to quantify and identify bacterial species that express DNase activity extracellularly in soils collected from an agricultural field planted to different crops. Since microbial exDNase activity is an important soil function and can contribute significantly to ecosystem services such as hindering the dissemination of transgenes in the environment, understanding the diversity of exDNase-releasing bacteria in the soil and how different crop species affect this diversity is important. To add on that, exDNase activity was used as a model function in the current study to understand how crop species and soil type influence soil functions which are mediated by soil microbes and may be indirectly or directly affected by management practices employed and/or new technological advances in agriculture. We hypothesized that a number of key DNase-producing bacteria inhabiting agricultural soils would be identified.

Materials and methods

Leaching study

Prior to the study, soils were collected at two different locations (A and B) within the University of Manitoba's Ian N. Morrison Research Farm in Carman, MB, Canada (49° 29′ 48″ N, 98° 2′ 26″ W, 267 m above sea level). Chemical and physical properties of these soils were determined at a commercial laboratory (Agvise Laboratories Inc., Northwood, ND, USA). The soil parameters are shown in Table 1, and the major difference between the soils was macronutrients (N, P, K), micronutrients (Zn, Fe, Mn) and pH. The experiments conducted in this study included a preliminary experimental run with soil A to develop the methods and a full experiment with soil A and one with soil B. All experiments were conducted in the growth room in a randomized complete block design. Six replicates each of four crop species, including, wheat (*Triticum aestivum* L.), canola (*Brassica napus* L.), alfalfa (*Medicago sativa* L.) and soybean (*Glycine max* [L.] Merr.) were used in the study. Unplanted pots were included as controls. Plants were grown in 1.5-L (10.5 cm diameter, 38 cm height) transparent, inverted plastic bottles each covered with aluminium foil to exclude light and having a 2-cm diameter hole in their bottoms. Two layers of fiberglass mesh (0.2 mm) were placed at the bottom of each pot followed by 150 mL of industrial quartz. One litre mixture of industrial quartz and soil (1:1, v/v) fertilized with 40 kg N ha^{-1} in the form of urea was added to the pots, and before planting, the canola seeds were treated with fungicide (trifloxystrobin, metalaxyl) and insecticide (clothianidin, carbathiin) while the alfalfa seeds were scarified briefly using an electric seed scarifier (Westinghouse Electric Corp AC Motor 317P044, USA).

Table 1 Parameters of the two soils used in this study

	Soil ID	
Parameters	**Soil A**	**Soil B**
N, kg ha^{-1}	77.6	172.4
S, kg ha^{-1}	19.1	23.6
P, kg ha^{-1}	31.4	56.0
K, kg ha^{-1}	412.0	555.5
Mg, kg ha^{-1}	788.0	822.1
Ca, kg ha^{-1}	4664.0	4715.2
Na, kg ha^{-1}	51.5	71.7
Zn, kg ha^{-1}	4.9	11.3
Fe, kg ha^{-1}	126.8	297.9
Mn, kg ha^{-1}	12.1	88.7
B, kg ha^{-1}	1.1	1.3
pH	6.3	5.1
Cation exchange capacity (CEC), meq	13.9	19.9
Organic matter (OM), %	3.3	4.3
Sand, %	76.0	72.0
Silt, %	11.0	15.0
Clay, %	13.0	13.0

Shortly after emergence, the pots were thinned to three seedlings per pot which were watered as required from here on. Three weeks post emergence, pots were fertilized with nitrogen-phosphorus-potassium (20:20:20) at the rate of 5 g L^{-1} of water. The plants were maintained at 25/20 °C and 16/8 h day/night at a relative humidity of 75% throughout the study. To prevent water from preferentially flowing between the soil and the pot during treatment leaching, at the time of seeding, the soil surface was shaped into a deep concave in each pot to facilitate leaching through the soil profile and leaching was done at least monthly with 200 mL of distilled H_2O. The first 30 mL of the leachate from each pot were collected in 50 mL falcon tubes placed on ice during leaching to minimize enzymatic degradation of DNA by enzymes with further analyses, and DNA purification performed immediately after sampling. The crops' developmental stages at the time of leaching are shown in Table 2. Coinciding with monthly leaching, the aluminium foil was removed temporarily and a 5 × 5 cm square grid with 1-cm grid gradations printed on transparent plastic sheets was placed on the exterior of each pot. The number of root-grid line intersections was counted in each square to determine root length density (RLD). At the end of the experiment, shoot and root dry matter were determined for each plant after drying to equilibrium at 55 °C.

Table 2 Developmental stages of the four crops at different times of leaching during experimental runs

	Experiment 1 (soil A)				Experiment 2 (soil B)		
	Sampling date				Sampling date		
Crop	1 (49)*	2 (63)*	3 (92)*	4 (126)*	1 (32)*	2 (59)*	3 (89)*
Alfalfa	early veg.	Mid veg.	Early budding	Flowering	7th trifoliate	Mid veg.	Early budding
Canola	4 leaf stage	5 leaf stage	Flowering	Phys. maturity	3 leaf stage	5 leaf stage	Flowering
Soybean	Flowering	Flowering	Phys. maturity	–	5th trifoliate	Flowering	Phys. maturity
Wheat	Bolting	Heading	Phys. maturity	–	Tillering	Heading	Phys. maturity

Abbreviations: *veg* vegetative, *phys* physiological
*Days after planting

Screening for DNase activity

One milliliter of aliquot of each leachate sample was transferred into a 2-mL centrifuge tube and each leachate was 10-fold serially diluted. A 100 μL aliquot of the 10^{-3} dilution was plated onto two replicate plates containing the Difco™ DNase Test Agar with methyl green as a substrate for DNase enzyme activity (DGM medium, Becton, Dickinson and Company Sparks, USA) prepared according to the manufacturer's instructions. Plates were incubated at 25 °C, and 48 h after plating, total culturable DNase-producing bacteria (TCDPB) and all the total colony-forming units (TCFU) with and without DNA degradation halos were enumerated and used to estimate the proportion of culturable DNase-producing bacteria (%CDPB). Finally, single DNase-forming colonies were picked randomly from the treatments and sub-cultured on DGM medium to obtain single isolated colonies for identification purposes.

Bacterial DNA extraction

Genomic DNA of bacterial single colonies were extracted using the InstaGene Matrix (IM) (Bio-Rad, Mississauga, ON, Canada), according to the manufacturer's protocol. In brief, picked isolated single colonies were suspended in 1 mL of distilled autoclaved water and vortexed briefly before centrifuging at 15,294×*g* for 1 min. The resulting pellet was suspended in 200 μL of InstaGene Matrix and incubated at 37 °C for 15 min using a water bath. Samples were vortexed at high speed for 10 s and placed in a 100 °C water bath for 8 min after which samples were vortexed for 10 s at high speed and centrifuged for 3 min at 15,294×*g*. DNA quantity and quality were assessed using a microplate spectrophotometer (Epoch Biotek, Winooski, VT, USA) and samples were stored in the freezer at − 20 °C until further analyses.

Target amplification for sequencing

Amplification of the partial 16S rRNA gene in DNA from bacterial single colonies was done in two replicate reactions in a total volume of 25 μL containing 12.5 μL of the 2x Phusion High Fidelity PCR Master Mix (Fisher Scientific, Ottawa, ON, Canada), 1 μL (10 pmol) of each of the universal primers 27F/1492R (Suzuki and Giovannoni 1996), 0.75 μL 100% dimethyl sulphoxide (DMSO), 2.5 μL of DNA and 7.25 μL distilled autoclaved water. The qPCR conditions were initial denaturation for 30 s at 98 °C, followed by 30 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 55 °C, extension at 72 °C for 30 s and a final extension step at 72 °C for 10 min. The two replicate samples of the qPCR products were pooled and verified on 1% agarose gel, and thereafter, the qPCR products were sent to Macrogen (Rockville, Maryland, USA) for Sanger sequencing using the universal primers used in this study.

Genomic DNA extraction from leachates

Total DNA was extracted from each leachate sample using the PowerSoil® total DNA isolation kit (MoBio, Laboratories, Solana Beach, CA, USA) with adjustments made to adapt the manufacturer's protocol to our experiment. Briefly, 10 mL aliquot of each leachate was pipetted into a 50-mL centrifuge tube and centrifuged at 5000*g* for 30 min at 4 °C to recover DNA and bacterial cells from the leachates. The pellets were washed with cold 70% ethanol and centrifuged at 5000*g* for 10 min at 4 °C followed by drying and resuspension in 1.5 mL of sterile Milli Q H_2O. The rest of the protocol followed the manufacturer's instructions except for the solution volumes which were optimized for leachate samples (500 μL bead solution buffer, 15 μL C1 solution, 100 μL C2 solution and 50 μL C3 solution per extraction). DNA was eluted from the column with 100 μL of Milli Q H_2O. The quantity and purity of DNA was assessed on a microplate spectrophotometer (Epoch Biotek, Winooski, VT, USA) at 260 and 280 nm.

Quantitative real-time PCR amplifications (qPCR)

Quantitative PCR for total bacterial load (TBL) was performed on DNA from leachate samples using a Bio-Rad CFX Real-time system (Bio-Rad, Hercules, CA, USA). The amplifications were performed in two replicates on 96-well reaction plates (Bio-Rad, Hercules, CA, USA) with a final volume of 20 μL reaction mixture containing 10 μL iTaq SYBR Green Supermix (Bio-Rad, Hercules,

CA, USA), 0.5 μL (10 pmol) of each of the eubacterial primers 338F/518R (Fierer et al. 2005), 1 μL of genomic DNA and 8 μL of distilled autoclaved water. The qPCR amplification conditions were 3 min at 95 °C followed by 35 cycles of 20 s at 95 °C, 20 s at 53 °C and 20 s at 72 °C. Following qPCR, a melt curve analysis was conducted with a temperature gradient from 65 to 95 °C in 0.5 °C increments for 5 s per cycle with continuous fluorescence monitoring performed after amplification to confirm amplification specificity to the target product. Purified DNA products amplified from soil DNA using similar primers were pooled and 10-fold serially diluted seven times to construct a standard curve to quantify the target DNA and evaluate primer efficiency.

Phylogenetic analyses

Nucleotide sequences were examined and edited using the Chromas software package (www.technelysium.com.au) after which the forward and reverse strands of the partial 16S rRNA sequences were aligned using the ClustalW multiple alignment option in the BioEdit program (Hall 1999). The GenBank databases were used to determine close phylogenetic associations using the Basic Local Alignment Tool (BLAST) at the National Center for Biotechnology Information (http://www-ncbi-nlm-nih-gov.uml.idm.oclc.org). The BioEdit program was used to assemble and align all sequences while maximum likelihood phylogenetic analyses were conducted using the Kimura 2-parameter (K2P) model (Kimura 1980). The final tree was constructed from 1000 bootstrap replicates in MEGA v7.0.18 (Kumar et al. 2016) after which edits were made using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The partial 16S rRNA nucleotide sequences of the isolates were deposited in the GeneBank database under the accession numbers (MN294613 to MN294681) (Table 9).

Identification of putative exDNase/nuclease-encoding genes

Bacterial genomes from the IMG/M database (Markowitz et al. 2012) were searched for genes encoding possible secreted DNases/nucleases. The protein sequences encoding the identified candidate exDNases/nucleases genes in *Bacillus mycoides* were retrieved from UNIPROT protein database (Apweiler et al. 2004). Finally, to identify candidate secreted DNases/nucleases, the candidate proteins from *Bacillus mycoides* were screened for secretion signals using SignalP (Petersen et al. 2011) and SecretomeP (Bendtsen et al. 2004) which generate non-classical neural network (NN) secretion scores for non-classical secreted proteins, whereas PSORTb 3.0.2 (http://www.psort.org/psortb/index.html) (Yu et al. 2010) was used to predict the subcellular location.

Statistical analyses

The SAS package 9.4 (SAS Institute, Inc., Cary, NC, USA) was used to conduct analysis of variance (ANOVA) using the mixed procedure. The preliminary experimental run with soil A which had fewer and incompatible harvest dates was not included in this final analysis. Fixed factors in the ANOVA model were crop, sampling date, experiment and their interactions. The replication blocks nested within experimental run were considered random. To satisfy the normality assumptions, log or square root transformations were applied to the dataset and normality of residuals was examined by using the Shapiro-Wilk test in the univariate procedure. In addition, Lund's test (Lund 1975) was used to identify and inspect potentially influential outliers, while the Akaike's information criterion was used to examine and correct (when necessary) the homogeneity of variance prior to final data analysis. Following this, the REPEAT ED statement was used to correct for heterogeneity of variance among treatments when necessary. Fisher's Protected LSD (α = 0.05) difference was used to compare treatment means using the pdmix800 macro (Saxton 1998). The method=type3 option was used to determine the partitioning of variance based on estimated type 3 sums squares To determine co-linearity among the response variables, correlation analysis was used. This analysis was conducted within experiment and sampling dates to minimize confounding effects with date and soil-specific effects.

Results for total culturable DNase-producing bacteria (TCDPB), proportion of culturable DNase-producing bacteria (%CDPB), total colony-forming units (TCFU) and total bacterial load (TBL)

Experiment was the most important factor influencing the interpretation of the results among the bacterial response variables in these experiments. The behaviour of the response variables between the two experiments was often quite different. As these response variables were determined from leachate samples, it was not unexpected that experiment played a major role in the partitioning of variance components as soil physical and chemical properties can affect leachate composition and some of these parameters were different between the two soils used in this study (Table 1). Leachate samples were used in these experiments for several reasons. First, they are non-destructive and allow for repeat sampling of the same experimental unit; second, they are integrative over the entire volume of soil for each experimental unit, and third, an exDNA disappearance assay (manuscript in preparation) was developed which was more reliable on leachate samples than soil samples, and these experiments contributed to understanding the exDNA dynamics in leachate water and the effects of crop

species, sampling date and soil type on this process. In the combined analyses, a relatively small, but significant portion of the variance was partitioned to the plant species and sampling date effects.

All main effects except one and all but two interactions were statistically significant among all bacterial response variables (Table 3). The relative contribution of sampling date and its interactions to total variation relative to the crop species effect was not the same among the response variables. In specific, effects including sampling date consumed less variation than all crop species effects in TCDPB and TCFU, whereas the opposite was observed for %CDPB and 16S rRNA-based TBL. Overall, the experimental factors and their interactions explained 53–71% of the total variation in these experiments indicating a significant amount of unexplained variation in TCDPB (43%), %CDPB (48%) and TBL (36%) suggesting high variability in these measurements or that critical factors that affect these bacterial parameters were not included in the experiment. When analyzing TCDPB within each experiment, crop species was the main source of variation in both experiments consuming 28% and 36% of the total variation. Additionally, date was the main source of variation in both experiments for TBL explaining about 16% and 42% of total variation in experiments 1 and 2, respectively. The same degree and consistency of variance partitioning to crop species and sampling date within experiments was not observed in the other bacterial response variables; however, either was significant depending on the experiment (data not shown). This shift in variance partitioning when analyzed within experiments clearly showed the importance of crop species and sampling date on bacterial response variables.

In experiment 1 with soil A, the number of TCDPB in leachates from alfalfa and soybean were equal to or greater than TCDPB in leachates from the unplanted control (UC) (Table 4). The number of TCDPB in wheat leachates was mostly intermediate, while generally the lowest TCDPB concentrations were observed in the canola leachates. Similar trends were also observed in the preliminary experiment (data not shown), and we are therefore relatively confident the observed differences between the experiments were influenced strongly by soil type. In contrast, a different response was observed in experiment 2 with soil B where canola leachates contained greater densities of TCDPB compared with the UC at all sampling dates, while TCDPB in alfalfa, soybean and wheat leachates were similar at most sampling dates except at 89 dap where the number of TCDPB were lower in soybean leachates than in alfalfa leachates. At 89 dap, soybean were at physiological maturity while alfalfa was still at anthesis; thus, the difference in plant maturity was a probable factor contributing to the difference observed.

The sampling date response (which signify plant development) in TCDPB was also unique to experiment, and interestingly, this effect was most prominent in experiment 1 and particularly in the alfalfa, canola and UC leachates. In canola leachates from experiment 1, a progressive increase in the population density of TCDPB was observed as this species developed and matured. On the other hand, a decrease in TCDPB occurred at the mid-vegetative developmental stages in alfalfa leachates, while in the UC leachates, lower TCDPB were observed at the first sampling date. Sampling date or its interaction with crop species had no effect on TCDPB in experiment 2 with soil B. While similar in texture, soil B was more nutrient rich, particularly in N and some of the micronutrients, than soil A (Table 1). Soil B also had a lower pH, and a higher CEC and OM content than soil A which likely contributed to the soil-specific observations. The nutrient profile of each soil was only assessed at the beginning of each experiment.

Culturable DNase-producing bacteria (%CDPB) expressed as a proportion of the TCFU ranged from 5.7 to 40.0% among treatments in these experiments (Table 5). While the maximum proportions of %CDPB were similar between the

Table 3 Percentage of total variance contributed by experimental factors and their interactions on measured bacterial parameters

Source	TCDPB[a]	*P* value	%CDPB[a]	*P* value	TCFU[a]	*P* value	TBL[a]	*P* value
Crop	7.66	< .0001	3.74	0.023	7.95	< .0001	2.86	0.022
Date	7.47	< .0001	10.78	< .0001	16.88	< .0001	22.68	< .0001
Crop x date	9.24	0.001	3.55	0.359	11.82	< .0001	5.03	0.030
Experiment	0.30	0.313	16.37	< .0001	10.99	< .0001	7.45	< .0001
Crop x experiment	21.24	< .0001	5.50	0.003	15.91	< .0001	8.22	< .0001
Date x experiment	0.22	0.693	2.91	0.012	2.33	0.003	10.21	< .0001
Crop x date x experiment	10.43	< .0001	9.65	0.001	5.43	0.001	8.09	0.0002
Error	43.44	–	47.5	–	28.69	–	35.46	–

[a]Column values are the proportions (%) of the variance component explained by the factor

Abbreviations: *TCDPB* total culturable DNase-producing bacteria, *%CDPB* proportion of culturable DNase-producing bacteria, *TCFU* total colony-forming units, *TBL* total bacteria load

Table 4 Effect of crop species on TCDPB expressed in $\log_{10}$ CFU per mL of leachate during experimental runs

	TCDPB in experiment 1 (soil A)				TCDPB in experiment 2 (soil B)		
	Sampling date				Sampling date		
Crop	1 (49)*	2 (63)*	3 (92)*	4 (126)*	1 (32)*	2 (59)*	3 (89)*
UC	$4.96^{c,B}$	$5.40^{a,A}$	$5.28^{a,AB}$	$5.65^{a,A}$	4.90^{c}	4.84^{b}	4.98^{c}
Alfalfa	$5.70^{a,A}$	$5.20^{ab,B}$	$5.35^{a,B}$	$5.54^{ab,A}$	5.13^{b}	5.36^{a}	5.46^{ab}
Canola	$4.42^{d,C}$	$4.79^{c,BC}$	$5.12^{ab,AB}$	$5.33^{b,A}$	5.49^{a}	5.42^{a}	5.53^{a}
Soybean	5.41^{ab}	5.35^{ab}	5.53^{a}	–	5.36^{ab}	5.38^{a}	4.99^{c}
Wheat	5.22^{bc}	5.19^{b}	4.73^{b}	–	5.36^{ab}	5.13^{ab}	5.16^{bc}
SEM	0.104	0.073	0.167	0.081	0.084	0.137	0.106
P value	**< .0001**	**< .0001**	**0.020**	**0.042**	**0.001**	**0.029**	**0.005**

*Days after planting
Abbreviations: *UC* unplanted control, *TCDPB* total culturable DNase-producing bacteria
Mean separation done for each sampling date within each experiment
^{a,b,c}Means with different letters within columns indicate statistical differences between treatments at $P < 0.05$.
^{A,B,C}Means with different letters between columns indicate statistical differences between sampling dates at $P < 0.05$
P values in bold indicate statistical significance at $P < 0.05$

experiments (40% and 36% in experiment 1 and 2, respectively), the lowest %CDPB was 10% lower in experiment 2 than experiment 1 which again, is indicative of soil-specific bacterial population dynamics. Generally, the effect of crop species was less consistent in this response variable as differences among treatments were observed at all sampling dates only in experiment 2. In experiment 1, alfalfa and wheat leachates had the greatest %CDPB at the earlier sampling times. Also, differences among treatments in %CDPB were only observed at 63 dap and in particular, %CDPB in alfalfa, were about half of those observed in the UC. This was caused principally by lower TCDPB in the alfalfa treatment (Table 4). In experiment 2, on the other hand, planted treatments resulted in %CDPB that were quadruple those of the UC at 59 dap (Table 5), which was probably caused by lower TCDPB and TCFU in the UC treatment (Tables 4 and 6). Moreover, canola leachates consistently had greater %CDPB than the UC at all sampling dates in this experiment. The %CDPB of all other crop species were similar to UC in experiment 2 except at 59 dap where alfalfa and soybean leachates had greater %CDPB.

Despite the observed differences in TCDPB and %CDPB among the experimental treatments, TCDPB and TCFU were correlated at each sampling date in both experiments. The Pearson *R* values ranged from 0.57 to 0.94 (p value < 0.001). The relationship between TCFU, the culturable portion, and TBL based on 16S rRNA copy number was less clear. In experiment 2 and most sampling dates of experiment 1, no correlations were found between these bacterial measures. However, positive correlations between TCFU and TBL were observed at 63 dap (Pearson $R = 0.40$, p value = 0.0001) and 92

Table 5 Effect of crop species on %CDPB during experimental runs

	% CDPB in experiment 1 (soil A)				% CDPB in experiment 2 (soil B)		
	Sampling date				Sampling date		
Crop	**1 (49)***	**2 (63)***	**3 (92)***	**4 (126)***	**1 (32)***	**2 (59)***	**3 (89)***
UC	30.16^{ab}	29.12^{ab}	30.81^{a}	31.87^{a}	$14.12^{b,AB}$	$5.72^{c,B}$	$17.33^{bc,A}$
Alfalfa	$40.01^{a,A}$	$15.47^{c,C}$	$29.05^{a,AB}$	$22.17^{a,BC}$	15.27^{b}	20.67^{a}	25.19^{abc}
Canola	29.37^{b}	34.21^{a}	30.55^{a}	23.72^{a}	$29.70^{a,A}$	$15.63^{a,B}$	$35.97^{a,A}$
Soybean	36.29^{ab}	28.31^{ab}	31.42^{a}	–	21.09^{ab}	14.40^{ab}	14.14^{c}
Wheat	$38.29^{ab,A}$	$18.09^{bc,B}$	$21.53^{a,B}$	–	$20.26^{ab,A}$	$9.88^{bc,B}$	$29.00^{ab,A}$
SEM	0.308	0.393	0.505	0.372	0.372	0.261	0.477
P value	0.145	**0.009**	0.644	0.129	**0.020**	**0.0001**	**0.022**

*Days after planting
Abbreviations: *UC* unplanted control, *%CDPB* proportion of culturable DNase-producing bacteria
Mean separation done for each sampling date within each experimental run
^{a,b,c}Means with different letters within columns indicate statistical differences between treatments at $P < 0.05$
^{A,B,C}Means with different letters between columns indicate statistical differences between sampling dates at $P < 0.05$
P values in bold indicate statistical significance at $P < 0.05$

Table 6 Effect of crop species on TCFU expressed in $\log_{10}$ per milliliter of leachate during experimental runs

	TCFU in experiment 1 (soil A)				TCFU in experiment 2 (soil B)		
	Sampling date				Sampling date		
Crop	1 (49)*	2 (63)*	3 (92)*	4 (126)*	1 (32)*	2 (59)*	3 (89)*
UC	$5.49^{c,C}$	$5.97^{a,AB}$	$5.80^{ab,B}$	$6.15^{a,A}$	$5.78^{b,B}$	$6.10^{a,A}$	$5.77^{bc,B}$
Alfalfa	$6.10^{a,AB}$	$6.02^{a,BC}$	$5.89^{ab,C}$	$6.21^{a,A}$	5.96^{ab}	6.05^{a}	6.09^{a}
Canola	$4.96^{d,D}$	$5.26^{b,C}$	$5.68^{bc,B}$	$5.97^{b,A}$	$6.02^{a,B}$	$6.24^{a,A}$	$5.98^{ab,B}$
Soybean	5.86^{ab}	5.91^{a}	6.04^{a}	–	6.04^{a}	6.23^{a}	5.85^{bc}
Wheat	$5.64^{bc,B}$	$5.95^{a,A}$	$5.43^{c,B}$	–	$6.07^{a,A}$	$6.15^{a,A}$	$5.71^{c,B}$
SEM	0.085	0.053	0.107	0.052	0.068	0.141	0.079
P value	**< .0001**	**< .0001**	**< .0001**	**0.015**	0.055	0.825	**0.011**

*Days after planting
Abbreviations: *UC* unplanted control, *TCFU* total colony-forming units
Mean separation done for each sampling date within each experimental run
^{a,b,c}Means with different letters within columns indicate statistical differences between treatments at $P < 0.05$
^{A,B,C}Means with different letters between columns indicate statistical differences between sampling dates at $P < 0.05$
P values in bold indicate statistical significance at $P < 0.05$

dap (Pearson $R = 0.56$, p value < 0.0001) in experiment 1 (data not shown). These results not only indicate soil-specific microbial dynamics, but as expected, also an important temporal component to soil microbial community dynamics. Furthermore, these findings also highlight the differences between culturable and unculturable techniques for bacterial studies.

In experiment 1, the range in TCDPB and TCFU (Tables 4 and 6) among the treatments diminished over the duration of the experiments showing a clear trend in the temporal dynamics in these response variables. More specific, the largest differences (greater than 10-fold among treatments) were observed at 49 dap in experiment 1, and by the end of the experiments, this range in differences diminished to about 0.32 and 0.24 $\log_{10}$ units for TCDPB and TCFU, respectively. The same was not observed in experiment 2 where the range in the difference among crop species was more consistent throughout the development of the plants (0.55–0.59 $\log_{10}$ units in TCDPB and 0.19–0.39 $\log_{10}$ units in TCFU). Differences among treatments were less common for TCFU in experiment 2 and TBL in experiment 1 (Tables 6 and 7). Nevertheless, a few plant species-specific trends were observed in these response variables, particularly in experiment 1. For example, canola consistently had the lowest TCFU densities at each sampling date in experiment 1 and the concentration increased at each subsequent sampling date in this species. In experiment 2, TCFU in canola leachates were not different from those in the other crop species and the greatest bacterial densities were observed at 59 dap. In experiment 1, alfalfa had among the highest TCFU densities, but these were lower during the mid-vegetative stages and increased again at the final sampling date. TBL, on the other hand, decreased in alfalfa at the last sampling date in both experiments. The same trend was observed for most other plant species as well, where TBL was lowest at the last sampling date in experiment 2, and for canola in experiment 1. Differences among treatments within sampling date were sporadic and inconsistent. In many cases, the observed differences were less than 10-fold and therefore likely of limited biological significance.

Shoot and root growth

Root length density measurements are a non-destructive two-dimensional method for estimating the density of

Table 7 Effect of crop species on TBL using 16S rRNA gene copies expressed in $\log_{10}$ per mL of leachate during experimental runs

	TBL in experiment 1 (soil A)				TBL in experiment 2 (soil B)		
	Sampling date				Sampling date		
Crop	1 (49)*	2 (63)*	3 (92)*	4 (126)*	1 (32)*	2 (59)*	3 (89)*
UC	$6.93^{a,AB}$	$7.19^{a,A}$	$6.66^{b,C}$	$6.86^{a,BC}$	7.27^{a}	6.76^{b}	6.68^{a}
Alfalfa	$6.84^{a,A}$	$6.83^{ab,A}$	$6.64^{b,AB}$	$6.43^{ab,B}$	$7.61^{a,A}$	$7.59^{a,A}$	$6.51^{a,B}$
Canola	$6.86^{a,A}$	$6.54^{b,A}$	$6.70^{ab,A}$	$5.96^{b,B}$	$7.52^{a,A}$	$7.68^{a,A}$	$6.80^{a,B}$
Soybean	6.91^{a}	6.96^{ab}	6.94^{a}	–	$7.65^{a,A}$	$7.13^{b,A}$	$5.65^{b,B}$
Wheat	$6.73^{a,B}$	$7.09^{a,A}$	$6.70^{ab,B}$	–	$7.75^{a,A}$	$7.71^{a,A}$	$7.04^{a,B}$
SEM	0.076	0.160	0.089	0.205	0.172	0.153	0.254
P value	0.333	**0.049**	0.157	**0.047**	0.372	**0.002**	**0.013**

*Days after planting
Abbreviations: *UC* unplanted control, *TBL* total bacteria load
Mean separation done for each sampling date within each experimental run
^{a,b,c}Means with different letters within columns indicate statistical differences between treatments at $P < 0.05$
^{A,B,C}Means with different letters between columns indicate statistical differences between sampling dates at $P < 0.05$
P values in bold indicate statistical significance at $P < 0.05$

root proliferation in a volume of soil. In experiment 1, no relationship between RLD and soil bacterial response variables were found, whereas in experiment 2, root length density correlated with TCFU at all sampling dates (Pearson R = 0.39 to 0.42, p values 0.0008 to 0.0027). At 32 dap in experiment 2, RLD also was correlated with %CDPB (Pearson R = 0.49, p value < 0.0001) and TBL (Pearson R = 0.38, p value = 0.003), and at 59 dap, RLD was correlated positively with TCDPB (Pearson R = 0.39, p value = 0.0019) and negatively with TBL (Pearson R = − 0.40, p value = 0.0014). Moreover, at the later sampling dates in experiment 1, RLD correlated well with per plant shoot and per plant root biomass (Pearson R = 0.46–0.72, p value < 0.0001). These results indicate that root length density alone may not be as important as other crop species-specific effects for the observed treatment differences among the crop species and soil type clearly modified these effects.

As expected, differences in shoot and root biomass were observed among the crop species (Table 8) and these also were influenced by experiment. At the end of the experiment, alfalfa had produced the greatest amount of root biomass in both experiments. The greatest shoot biomass was observed in alfalfa in experiment 1and in canola in experiment 2. Among the crop species, shoot dry weight was related to root dry weight only experiment 1 with Pearson R ranging from 0.52 to 0.74 (p value = < 0.001 to 0.0001). At the last sampling date, dry weights were related to TCDPB (Pearson R = 0.51, p value = 0.0016 for shoot; Pearson R = 0.67, p value < 0.0001 for root) and TCFU (Pearson R = 0.70, p value < 0.0001 for shoot; Pearson R = 0.80, p value < 0.0001 for root) in experiment 1. In experiment 2, neither shoot nor root biomass was related to any of the soil bacterial response variables further confirming observations above that crop species-specific factors other than plant biomass are more important at influencing soil microbial function.

DNase-producing bacterial isolates

Some of the DPB were isolated and picked for identification using Sanger sequencing. Bacterial isolates identified in the current study were classified into four phyla groups including the *Firmicutes* (37 isolates), *Actinobacteria* (14 isolates), *Proteobacteria* (10 isolates) and *Bacteroides* (8 isolates) with a total of 11 genera groups (Table 9). The genera groups of DPB isolates identified in this study included *Bacillus*, *Chryseobacterium*, *Fictibacillus*, *Flavobacterium*, *Microbacterium*, *Nubsella*, *Pseudomonas*, *Psychrobacillus*, *Rheinheimera*, *Serratia* and *Stenotrophomonas*. The largest proportion of culturable DPB (54%) was identified as *Firmicutes* with 6 different *Bacillus* species. The identity of the DPB isolates to sequences in the NCBI gene bank database ranged between 94 and 100% with the exception of isolates identified as *Microbacterium paraoxydans* (57-15C) and *Pseudomonas baetica* (24-14A) whose identities were 85% (Table 9).

Of the total 69 DPB isolates identified, about one-third (23 isolates) were isolated from the canola leachates (Fig. 1) and were mostly from the phyla *Firmicutes*. No isolates in the *Bacteroides* phyla were identified from alfalfa leachates, while few members in the *Bacteroides* phyla were isolated from wheat leachate. The number of *Proteobacteria* isolates was the same among all the treatments, whereas the lowest number of *Actinobacteria* was isolated from the UC and soybean leachates. Leachates from experiment 1 using soil A contained mostly *Firmicutes* and *proteobacteria*, with the canola treatment culturing only *Firmicutes*, while alfalfa leachates contained the lowest number of *Proteobacteria*. The identity structure of the isolates was different in experiment 2 using soil B, where *Firmicutes* were only cultured from canola and UC leachates with the

Table 8 RLD at different sampling dates and biomass components at the end of experimental runs expressed on per plant basis

	Experiment 1 (soil A)					Experiment 2 (soil B)				
	Sampling date					Sampling date				
	1 (49)*	2 (63)*	3 (92)*			1 (32)*	2 (59)*	3 (89)*		
Crop	RLD			SBPP	RBPP	RLD			SBPP	RBPP
Alfalfa	0.83^{b}	1.05^{a}	1.12^{a}	3.96^{a}	4.40^{a}	0.36^{b}	0.52^{b}	2.03^{a}	2.06^{c}	1.75^{a}
Canola	0.78^{b}	0.70^{b}	1.03^{a}	1.96^{bc}	0.80^{b}	0.87^{a}	1.30^{a}	1.24^{b}	2.72^{ab}	1.93^{a}
Soybean	1.16^{a}	0.71^{b}	0.89^{a}	2.37^{b}	0.88^{b}	0.94^{a}	1.22^{a}	1.16^{b}	2.34^{bc}	0.93^{b}
Wheat	0.73^{b}	0.33^{c}	0.54^{b}	1.64^{c}	1.02^{b}	0.67^{a}	0.51^{b}	0.43^{c}	3.11^{a}	1.00^{b}
SEM	0.097	0.089	0.097	0.181	0.278	0.116	0.113	0.147	0.197	0.211
P value	**0.027**	**0.0004**	**0.001**	**< .0001**	**0.0002**	**0.003**	**< .0001**	**< .0001**	**0.011**	**0.012**

*Days after planting

Abbreviations: *RLD* root length density, *SBPP* shoot biomass per plant, *RBPP* root biomass per plant

Mean separation done for each sampling date within each experimental run

[a,b,c]Means with different letters within columns indicate statistical differences between treatments at $P < 0.05$.

P values in bold indicate statistical significance at $P < 0.05$

Table 9 Identified DPB isolates from leachates grown with different crops using partial 16S rRNA sequences

Phylum	DPB[b] Isolate ID	Genus	Species	Identity (%)[c]	GenBank Closest NCBI database match (Accession no.)	GenBank Accession no.	Treatment	Exp[d]
Actinobacteria (20%)[a]	49-15SL	*Microbacterium*	*M. azadirachtae*	97	LC177121.1	MN294656	Soil	2
	50-15SL		*M. azadirachtae*	100	MH489019.1	MN294657	Soil	2
	52-15A		*M. azadirachtae*	98	MH489019.1	MN294659	Alfalfa	2
	53-15A		*M. azadirachtae*	98	MH489019.1	MN294660	Alfalfa	2
	66-15SY		*M. azadirachtae*	99	MH489019.1	MN294673	Soybean	2
	70-15SY		*M. azadirachtae*	97	MH489019.1	MN294677	Soybean	2
	71-15W		*M. azadirachtae*	97	MH489019.2	MN294678	Wheat	2
	58-15C		*M. foliorum*	99	CP041040.1	MN294665	Canola	2
	73-15W		*M. foliorum*	97	KF803585.1	MN294680	Wheat	2
	74-15W		*M. foliorum*	99	MG195155.1	MN294681	Wheat	2
	51-15A		*M. oxydans*	97	MF767919.1	MN294658	Alfalfa	2
	55-15A		*M. oxydans*	99	MF767919.1	MN294662	Alfalfa	2
	56-15C		*M. oxydans*	99	MF767919.1	MN294663	Canola	2
	57-15C		*M. paraoxydans*	85	KX280770.1	MN294664	Canola	2
Bacteroidetes (12%)[a]	62-15C	*Chryseobacterium*	*C. lathyri*	99	KU924001.1	MN294669	Canola	2
	67-15SY		*C. oranimense*	99	NR_044168.1	MN294674	Soybean	2
	68-15SY		*C. oranimense*	96	NR_044168.1	MN294675	Soybean	2
	72-15W		*C. oranimense*	98	NR_044168.1	MN294679	Wheat	2
	48-15SL		*C. taihuense*	95	KT719933.1	MN294655	Soil	2
	69-15SY	*Flavobacterium*	*F. ginsengiterrae*	96	NR_132661.1	MN294676	Soybean	2
	65-15C	*Nubsella*	*N. zeaxanthinifaciens*	98	NR_114146.1	MN294672	Canola	2
	46-15SL		*N. zeaxanthinifaciens*	96	NR_114146.1	MN294653	Soil	2
Firmicutes (54%)[a]	5-14SY	*Bacillus*	*B. cereus*	97	MG205787.1	MN294616	Soybean	1
	12-14W		*B. cereus*	97	KU721999.1	MN294622	Wheat	1
	14-14W		*B. cereus*	99	MG205902.1	MN294623	Wheat	1
	21-14A		*B. cereus*	100	MN232174.1	MN294630	Alfalfa	1
	22-14A		*B. cereus*	98	KF725719.1	MN294631	Alfalfa	1
	41-14C		*B. cereus*	96	KX350001.1	MN294648	Canola	1
	42-14C		*B. cereus*	94	KF500919.1	MN294649	Canola	1
	43-14C		*B. cereus*	98	KJ473716.1	MN294650	Canola	1
	44-14C		*B. cereus*	99	MF988724.1	MN294651	Canola	1
	59-15C		*B. muralis*	99	EU977778.1	MN294666	Canola	3
	1-14SY		*B. mycoides*	97	KU160370.1	MN294613	Soybean	1
	4-14SY		*B. mycoides*	99	KU160370.1	MN294615	Soybean	1
	9-14SY		*B. mycoides*	97	KU160370.1	MN294619	Soybean	1
	11-14W		*B. mycoides*	97	MK217082.1	MN294621	Wheat	1
	17-14W		*B. mycoides*	94	CP020743.1	MN294626	Wheat	1
	18-14W		*B. mycoides*	100	KU160370.1	MN294627	Wheat	1
	20-14A		*B. mycoides*	100	MK883205.1	MN294629	Alfalfa	1
	26-14A		*B. mycoides*	100	KU160370.1	MN294634	Alfalfa	1
	33-14C		*B. mycoides*	95	KU160370.1	MN294640	Canola	1
	34-14C		*B. mycoides*	100	KJ528876.1	MN294641	Canola	1
	36-14C		*B. mycoides*	100	KU160370.1	MN294643	Canola	1
	37-14C		*B. mycoides*	100	KU160370.1	MN294644	Canola	1

Table 9 Identified DPB isolates from leachates grown with different crops using partial 16S rRNA sequences *(Continued)*

Phylum	DPB[b] Isolate ID	Genus	Species	Identity (%)[c]	GenBank Closest NCBI database match (Accession no.)	GenBank Accession no.	Treatment	Exp[d]
	39-14C		*B. mycoides*	97	KU160370.1	MN294646	Canola	1
	40-14C		*B. mycoides*	99	KJ528876.1	MN294647	Canola	1
	45-14C		*B. pumilus*	96	MK491041.1	MN294652	Canola	1
	32-14SL		*B. simplex*	97	KX866680.1	MN294639	Soil	1
	47-15SL		*B. simplex*	98	FN435888.1	MN294654	Soil	2
	60-15C		*B. simplex*	96	FN435888.1	MN294667	Canola	2
	61-15C		*B. simplex*	97	KM817231.1	MN294668	Canola	2
	16-14W		*B. thuringiensis*	97	CP004870.1	MN294625	Wheat	1
	19-14A		*B. thuringiensis*	99	KX592862.1	MN294628	Alfalfa	1
	25-14A		*B. thuringiensis*	97	KU179338.1	MN294633	Alfalfa	1
	38-14C		*B. thuringiensis*	95	JF895480.1	MN294645	Canola	1
	28-14SL	*Fictibacillus*	*F. arsenicus*	98	CP016761.1	MN294636	Soil	1
	2-14SY	*Psychrobacillus*	*P. psychrodurans*	95	KC618486.1	MN294614	Soybean	1
	27-14SL		*P. psychrodurans*	95	KP334978.1	MN294635	Soil	1
	35-14C		*P. soli*	100	MH934924.1	MN294642	Canola	1
Proteobacteria (14%)[1]	24-14A	*Pseudomonas*	*P. baetica*	85	KY963434.1	MN294632	Alfalfa	1
	6-14SY		*P. fluorescens*	99	CP015225.1	MN294617	Soybean	1
	8-14SY		*P. moorei*	96	FM955889.1	MN294618	Soybean	1
	29-14SL		*P. mosselii*	98	CP024159.1	MN294637	Soil	1
	15-14W		*P. putida*	96	KJ819580.1	MN294624	Wheat	1
	10-14W	*Rheinheimera*	*R. soli strain*	99	KU597256.1	MN294620	Wheat	1
	31-14SL	*Serratia*	*S. fonticola*	96	CP013913.1	MN294638	Soil	1
	54-15A	*Stenotrophomonas*	*S. maltophilia*	96	MK641655.1	MN294661	Alfalfa	2
	63-15C		*S. maltophilia*	99	JN705917.1	MN294670	Canola	2
	64-15C		*S. maltophilia*	98	CP033829.1	MN294671	Canola	2

[a]Proportions of Phyla groups
[b]*DPB* DNase-producing bacteria
[c]Percent identity match of sequence based on the NCBI database
[d]Experiment

largest number cultured from canola leachates. DNase-producing *Proteobacteria* were found only in alfalfa and canola leachates in experiment 2. Furthermore, *Bacteroides* were cultured from leachates of all treatments except from alfalfa leachates with the most cultured from soybean leachates and the least cultured from wheat leachates. DNase-producing *Actinobacteria* were cultured from all leachates; however, their numbers were greatest in alfalfa leachates. These results further support the observations in this study that soil type is an important factor in shaping the soil bacterial community.

On clustering the DPB isolates using the maximum likelihood method, all isolates clustered close to their respective phyla groups (Fig. 2). The *Bacteroides* group clustered separately from all other bacterial groups, and this was strongly supported by the high bootstrap value of 100%. Among the *Bacteroides* cluster, *Flavobacterium ginsengiterrae* was strongly distinct as indicated by a bootstrap value of 71.1%. Moreover, the *Firmicutes* were separated from the *Actinobacteria* and *Proteobacteria* moderately with a 55.5% bootstrap value, while the bacterial species in the *Actinobacteria* phylum clustered tightly together with a strong bootstrap value of 99.9%. Within the *Proteobacteria* phylum, bacterial species seemed to have some variation from each other with the *Pseudomonas* genus separating more from the other species in this phylum than the different genera in the other phylum groups. At the same time, the *Strenotrophomonas* species clustered tightly together with a 100% bootstrap value.

Putative exDNase/nuclease-encoding genes

When the genomes for the sequenced bacterial isolates in the IMG/M database were queried, a total of 9 possible secreted exDNases/nucleases were identified

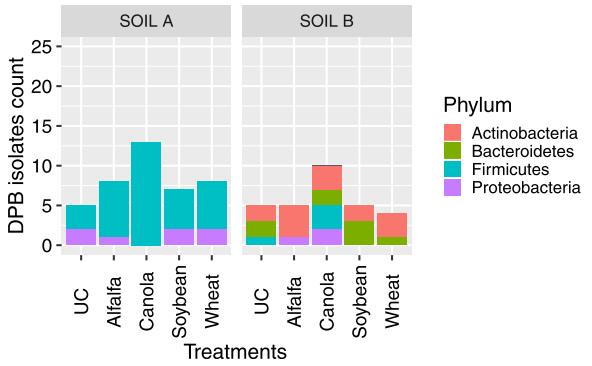

Fig. 1 The number of culturable DNase-producing bacteria isolates within each Phyla group identified from two experiments using different soils (**a** and **b**) growing four different crop species and unplanted control (UC) based on partial 16S rRNA gene sequences

(Additional file 1: Table S1). These enzymes included TatD-related DNase (COG0084/KOG3020/pfam01026), Deoxyribonuclease NucA/NucB (pfam14040), Staphylococcal nuclease homologue (pfam00565), Bacterial EndoU nuclease (pfam14436), DNase/tRNase domain of colicin-like bacteriocin (pfam12639), Endonuclease/Exonuclease/phosphatase family (pfam03372), endA-deoxyribonuclease I (K01150), EndA-DNA-entry nuclease (K15051) and a predicted extracellular nuclease (K15051 (COG2374). A total of five possibly secreted exDNases/nucleases were predicted in *Bacillus mycoides* (Table 10). Only the TatD-related DNase which was predicted to be localized in the cytoplasm did not possess a signal peptide nor predicted to be non-classically secreted whereas the colicin-like bacteriocin DNase did not map to a specific location.

Discussion

In the present study, both culture-dependent and molecular techniques were employed to quantify and identify DPB inhabiting soils planted to different agricultural plant species in a greenhouse study. In addition, the TBL was enumerated on selective culture medium coupled with commonly used universal bacterial primers. Soil bacteria that release DNases extracellularly are an important component in the chain for assessing avenues to mitigate the unintended effects of GMOs in the environment. Moreover, soil enzyme activities mediated by soil microbes are important as they perform beneficial ecosystem functions, and thus, understanding the dynamics of these microbes in the soil will help shed more light on the untargeted effects of evolving agronomic practices. To the best of our knowledge, no studies have reported DPB inhabiting soils cultivated to annual and perennial crop species and more specifically in the Canadian prairie region, which accounts for most of the arable agricultural land in Canada.

The crop species and developmental stage strongly influenced the TBL and DPB in leachates from the growth room study. The results observed in this study suggest that crop species exert specific selection pressures to the soil total bacterial population and DPB in the form of soil nutrient depletion and species-specific compounds released by plant roots which change the proportion of the selected bacterial groups during the crop's developmental stages. This observation is well documented in other studies (Miethling et al. 2000; Smalla et al. 2001; Dunfield and Germida 2003; Costa et al. 2006; Berg and Smalla 2009; Hartmann et al. 2009). In contrast to our results, an indoor study using transgenic poplars growing on loamy sand over a duration of 26 months did not observe shifts in the proportion of the culturable DPB (Balestrazzi et al. 2007). In addition, they did observe higher proportions of DPB (62.5 to 100%) in bulk soils associated with poplar, whereas the current study reports an overall lower proportion of DPB (5.72 to 40.01%) from leachates. It is important to bear in mind that poplars are woody tree species, while in the current study annual and herbaceous perennial crops species were used thus a probable factor contributing to the differences in the DPB populations observed.

The DPB constitute a large proportion (> 50%) of culturable organisms in soil and aquatic environments (Greaves and Wilson 1970; Maeda and Taga 1973, 1974). Furthermore, DPB have been reported in soils grown to pasture grasses where they constituted up to 42% of the total culturable isolates in the rhizosphere and bulk soils that were preferentially stimulated depending on the species and age of the grasses (Greaves and Webley 1965) which agrees with our findings. The sampling strategy of leaching experimental pots used in the current study may have contributed to the lower proportions of DPB observed from those of previous studies. Leaching of the soil may however present some advantages compared to using rhizosphere and bulk soils because it is less destructive, covers a larger volume of soil and integrates the effects from both the rhizosphere and bulk soils.

It is also interesting to note that experiment 2 had lower proportions of %CDPB than experiment 1, and the UC in experiment 2 consistently cultured lower TCDPB and %CDPB than experiment 1. The possible explanation to this is that under high nutrient levels as in the case of experiment 2 (soil B), the soil bacteria had less need to breakdown DNA as a nutrient source. Moreover, this effect was masked in the presence of plants as they release extra nutrients that can be utilized by the microbes. Under low nutrient levels in experiment 1 (soil A), bacteria use exDNase enzyme activity as an alternate nutrient acquisition mechanism which progressively decreases in the presence of plants. Degradation of exDNA

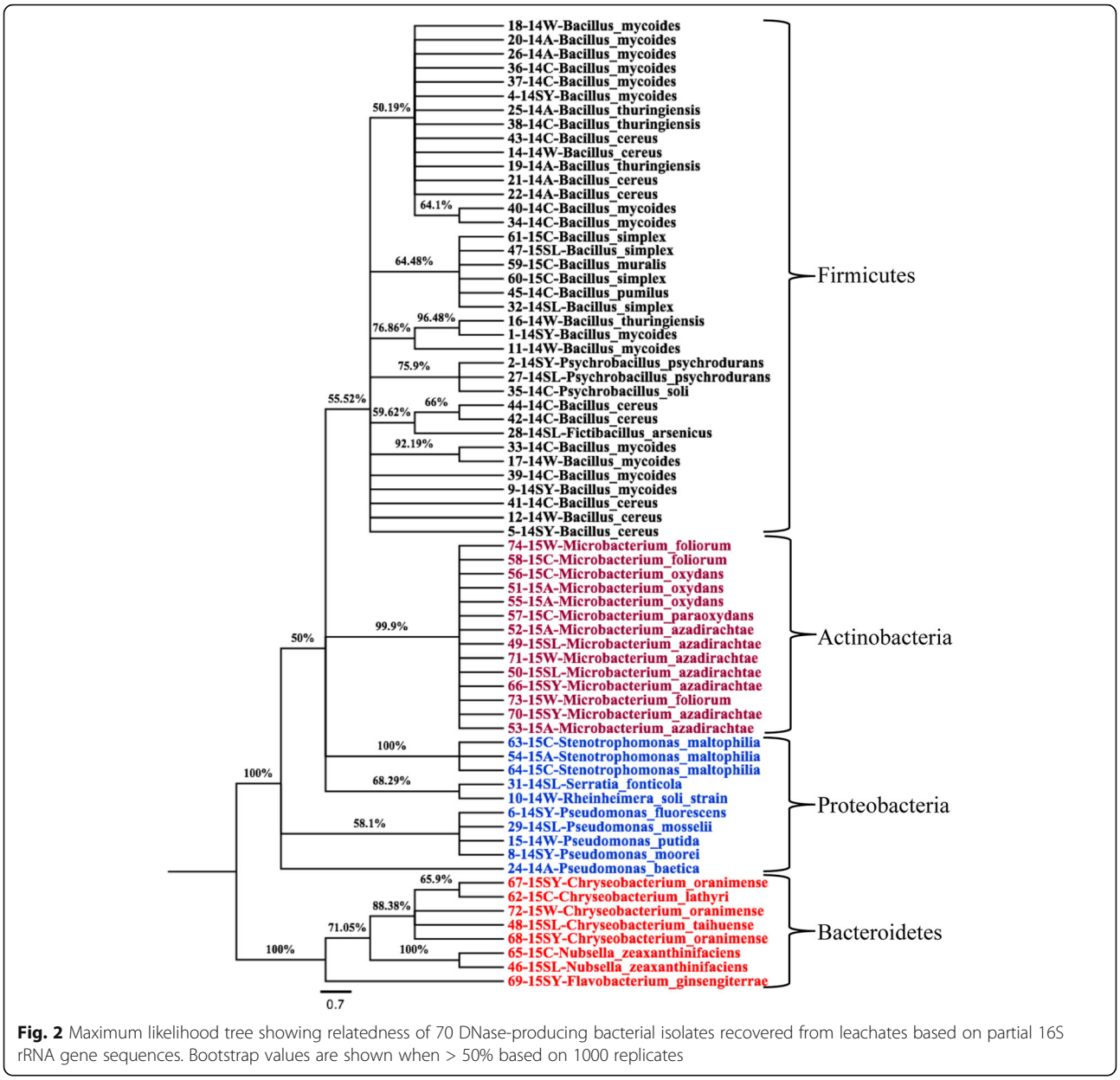

Fig. 2 Maximum likelihood tree showing relatedness of 70 DNase-producing bacterial isolates recovered from leachates based on partial 16S rRNA gene sequences. Bootstrap values are shown when > 50% based on 1000 replicates

Table 10 Candidate exDNase/nuclease-encoding proteins in *Bacillus mycoides*

IMG/M database annotation	Uniprot entry	Pfam[a]	SignalP	NN scores[b]	Localization (score)
TatD-related DNase	A0A084ITC0	Pfam01026	No	0.058	Cytoplasmic (9.97)
Endonuclease/Exonuclease/phosphatase family	A0A0A0WPY9	Pfam03372	Yes	0.931	Cytoplasmic membrane (4.60)
Deoxyribonuclease NucA/NucB	A0A090YLN6	Pfam14040	Yes	0.500	Cytoplasmic membrane (9.81)
Staphylococcal nuclease homologue (SNase)	C2Y446	Pfam00565	No	0.941	Extracellular (10)
DNase/tRNase domain of colicin-like bacteriocin	A0A0B5S5U9	pfam12639	No	0.698	All locations (2.5)

[a]Protein families
[b]Neural network prediction of signal peptides

by microbial nucleases contributes large proportions of the daily requirement of N and P for microbial growth in pelagic environments (Jørgensen and Jacobsen 1996). Other studies also have hypothesized that microbial extracellular nuclease activities are involved in scavenging for nutrients such as C, N and P from their environment (Blum et al. 1997; Benedik and Strych 1998; Dell'Anno 2005; Bais et al. 2006; Levy-Booth et al. 2007; Nielsen et al. 2007; Ibáñez de Aldecoa et al. 2017). Studies supporting this suggestion include those of Greaves et al. (1970) who reported that the production of nucleases in *Cytophaga johnsonii* was greatest in low-nutrient media, Salikhova et al. (2000) who observed an increase in the production of nuclease from *Proteus mirabilis* which exhibits both DNase and RNase activity when grown in low P conditions, Turk et al. (1992) who reported that the rate of DNA decomposition was 10-fold greater in P-limiting compared with N-limiting marine environments and Mulcahy et al. (2010) who observed that *Pseudomonas aeruginosa* highly expressed exDNase under P-limiting conditions to restrict DNA and use its constituents as a source of nutrients.

Sampling date-specific effects of crops on soil bacteria were observed on both the culturable and the 16S rRNA gene copies. We did not observe any particular trends consistently among the crop species over the growing period; however, alfalfa and soybean plants seemed to favour higher numbers of bacteria according to the culture technique while canola plants suppressed the proliferation of culturable bacteria in experiment 1 (the lower nutrient soil). Altogether these results imply that crops have dynamic and temporal effects on soil bacterial populations which are dependent on the growth stage, soil nutrient levels and the plant species. Both plant species and soil properties largely influence the structure and functions of soil microbial communities as previously reviewed (Berg and Smalla 2009). The growing season has previously been shown to influence the abundance of microbes associated with canola roots when fragments of the 16S rRNA were analyzed on denaturing gradient gel electrophoresis (Smalla et al. 2001). Studies by Dunfield and Germida (2003) revealed similar trends where they observed seasonal variability in the microbial community using the fatty acid methyl ester (FAME) profiles and community-level physiological profiles techniques on soils planted to genetically modified canola. Moreover, Germida et al. (1998) observed a plant-dependent effect on the diversity of rhizoplane bacteria associated with canola and wheat based on their FAME profiles. Differences in the microbial community associated with *Arabidopsis* shifted with the development stage and were highly correlated with the root exudates, and the seedling microbiome were observed to be distinct from the other stages (Chaparro et al. 2014). Changes in soil bacterial abundance over the growing season were also observed in fields planted with soybean (Sugiyama et al. 2014). Thus, it seems the soil bacterial population shifts more frequently under the influence of crop species in their surroundings and are highly transient over time which we also observed. Canola and other *Brassica* species are known to produce glucosinolates through their root exudates which when hydrolyzed to isothiocyanates act as biofumigants that actively suppress soil-borne pathogens consequently affecting the composition of rhizosphere microbial communities (Rumberger and Marschner 2003; Smith et al. 2004; Matthiessen and Kirkegaard 2006; Hansen et al. 2018). For example, soybean root colonization by arbuscular mycorrhizal fungi was reduced up to 30% when the preceding crop was canola in the rotation (Valetti et al. 2016). Similarly, in our study, we did observe a suppressing effect of canola on the total bacterial biomass and DPB in experiment 1 using the low nutrient soil (A).

Soil properties such as pH, nutrients, organic matter, texture and structure are known to act singly or in combination to influence the structure and functions of soil microbes (Garbeva et al. 2004). According to Reese et al. (2018), the soil factor having the most dominant effect on the soil microbes varies according to the environment. For instance, some studies have reported a reduction in microbial biomass as a result of N application (Treseder 2008; Janssens et al. 2010; Ramirez et al. 2012), while others reported an increase (Frey et al. 2004; Leff et al. 2015). Our present findings support the suggestion that soil factors influence microbes differently. Specifically, we observed that TCDPB and %CDPB were higher in leachates from UC of experiment 1 than in UC of experiment 2, which may be associated with the lower pH of the soil used in the latter experiment compared with the former. In addition, we also observed that compared with the UC, the TCDPB of crops planted in experiment 1 was either reduced or did not change, whereas TCDPB of all crops grown in experiment 2 were increased, suggesting that the differences in soil properties between the two soils imposed a strong selective pressure in favour of the growth of DNase-producing bacteria. Additionally, with less need to breakdown DNA in experiment using soil B, coupled with the absence of plants in the UC which eliminated microbial competition for nutrients, the DPB population was reduced significantly. However, due to competition in the presence of plants, DPB still needed to utilize DNA as a source of nutrient hence their increased population in the crop treatments compared to the UC.

Similarly, it is noteworthy that canola had reduced TCDPB and TCFU than the control treatment in experiment 1, whereas the opposite was true in experiment 2. The differences in pH between the two soils could have been a possible contributing factor to these observed

differences. As canola is known to acidify the rhizosphere for P acquisition (Hedley et al. 1982), the already strongly acidic environment in soil B could have presented an advantage for its uptake by this plant creating a P-limiting environment and hence the switch to the alternate mechanism of acquiring this nutrient by microbes. A more interesting observation was that although alfalfa did not do well in experiment 2 (as reflected by lower root and shoot biomass production in this experiment compared with experiment 1), a factor directly associated with the low pH of soil B, the TBL count from alfalfa leachates was higher in experiment 2 than in experiment 1 suggesting that the plant-soil-microbe interaction is complex and not only a function of plant biomass or species. The microbial populations were highly responsive to the presence of wheat and canola throughout the experiments as reflected by the shifts among sampling dates, while the least responses were observed in the presence of soybean and those in alfalfa were intermediate.

In the current study, we isolated DNase-producing soil bacteria belonging to *Bacillus*, *Chryseobacterium*, *Fictibacillus*, *Flavobacterium*, *Microbacterium*, *Nubsella*, *Pseudomonas*, *Psychrobacillus*, *Rheinheimera*, *Serratia* and *Stenotrophomonas* genera. This observation is in agreement with the findings of Farmer et al. (2014) who isolated soil DPB belonging to the *Bacillus*, *Pseudomonas*, *Serratia* and *Strenotrophomonas* genera; Balestrazzi et al. (2007) who isolated DPB belonging to the genera *Bacillus*, *Microbacterium*, *Pseudomonas* and *Stenotrophomonas* and Aparna and Sarada (2012) who isolated several DPB belonging to *Serratia* genera. Although we only identified six *Bacillus* species (*B. cereus*, *B. muralis*, *B. mycoides*, *B. pumilus*, *B. simplex* and *B. thuringiensis*) that produce exDNase, several other *Bacillus* species have been reported to exhibit extracellular nuclease activity including *B. subtilis* (Akrigg and Mandelstam 1978; Moreno et al. 2012); *B. licheniformis* (Nijland et al. 2010); *B. fusiformis*, *B. megaterium*, *B. sphericus*, *B. brevis* (Balestrazzi et al. 2007); and *B. seohaeanensis*, *B. stratosphericus*, *B. oceanisediminis*, *B. mojavensis* (Moreno et al. 2012). A study by Al-Wahaibi et al. (2019) reported the *Bacillus* genera group to constitute the largest proportion of culturable exDNase-producing bacterial isolates from different marine habitats. However, their findings that *Proteobacteria* (57%) and *Firmicutes* (34%) dominated culturable exDNase-producing bacterial isolates contrasts our results as the largest proportion belonged to *Firmicutes* (54%) while *Actinobacteria* were the second largest group (20%). The results of these studies, together with ours, indicate that a large proportion of culturable bacteria in the *Bacillus* group may be responsible for extracellular nuclease activities in the soil.

Out of the of 9 possible secreted exDNases/nucleases identified in the genomes of bacterial isolates in the present study, five of them were present in *Bacillus mycoides.* The prediction of signal peptides in endonuclease/exonuclease/phosphatase family and deoxyribonuclease NucA/NucB indicate that they may translocate across the bacterial membrane (Petersen et al. 2011). Moreover, tatD-related DNases, endonuclease/exonuclease/phosphatases and deoxyribonuclease NucA/NucB, have previously been shown to be important for virulence in some plant pathogens (Tran et al. 2016; Hawes et al. 2016; Park et al. 2019). The staphylococcal nuclease is a well-characterized nuclease from *Staphylococcus aureus*, in which this enzyme is secreted to degrade extracellular nucleic acids (Kiedrowski et al. 2014). On the other hand, colicin-DNases are secreted nucleases and have been observed to kill non-self-target cells and enhance survival under stress in *E. coli* (Yang 2011; Sharma et al. 2019).

To the best of our knowledge, this is the first study reporting *Bacillus mycoides* as an exDNase producer in the soil. This observation may be of interest in understanding the documented plant growth promotion activities by this bacterium which is abundant in the soil and rhizosphere and endosphere of some plants (Neher et al. 2009; Stefan et al. 2013; Bach et al. 2016; Ambrosini et al. 2016).

Conclusion

The results presented in this study show that plants have influence on total culturable soil bacteria communities, and this influence is variable depending on the crop species, soil abiotic properties and the stage of development of the plant. This observation is also true for culturable DNase-producing bacteria as we observed changes over time in the proportions cultured by individual crop species during the development of the plant and also among the species at the different sampling dates. In addition, our findings suggest that different soils exert variable selective pressure with potential to influence the composition, structure and possibly the functions of microbes inhabiting them. Furthermore, we also observed a complex interaction between the crop species and soil type suggesting that crop performance may not be a good indicator of microbial richness and diversity in the soil; hence, the focus should be directed onto the specific properties of the soil and crop with potential to exert selective pressure on to the resident microbial populations. Moreover, this study provided evidence suggesting that there seems to be large numbers of soil bacteria that produce exDNase into their surroundings. In this study, the DNase producers were identified as members of eleven different genera with a majority of the isolates belonging to the *Firmicutes*. Some isolates identified in this

work are well characterized and have been exploited for their growth-promoting properties; however, some are less studied and may require future studies to unveil important properties that may be used for the benefit of the agricultural industry. In the context of the continued debate on the use of genetically engineered plants and other nucleic acid-based technologies, these indigenous soil bacteria that produce exDNases can also be exploited to mitigate the potential effects of transgenes or emerging gene-editing technologies (e.g. CRISPR-Cas9) in the environment. To add on that, these soil bacteria may be beneficial for mitigating potential pleiotropic effects of emerging new technologies in crop protection such as RNA interference. DNases produced by these bacteria can be exploited for medical purposes to produce DNA vaccines and biological drugs for gene therapy targeting genetic diseases and dispersal of biofilm forming microbes. Moreover, these nucleases can be harvested for genetic engineering purposes and also for use in the food and pharmaceutical industries.

Supplementary Information

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Additional file 1: Table S1 Candidate exDNase/nuclease-encoding proteins in sequenced bacterial isolates putatively involved in DNA degradation. *Identified from Uniprot database

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Authors' contributions

LNK carried out the indoor experiments, laboratory studies, phylogenetic analysis and statistical analysis and drafted the manuscript. RHG participated in the design of the study, provided guidance in statistical analysis and proofread the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all data generated and analyzed are included in this article.

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Consent for publication

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Competing interests

The authors declare no competing interest.

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