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Full Length Research Paper

Monitoring the persistence of a genetically modified strain of the diazotroph *Azorhizobium caulinodans* in the rhizosphere of wheat (*Triticum aestivum L.*)

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A protocol was developed to monitor persistence and spread of a genetically modified strain of the growth promoting diazotroph *Azorhizobium caulinodans* used to inoculate field grown wheat. The protocol was used to identify an endogluconase(*egl*) overproducing strain of *A. caulinodans*re-isolated from inoculated soils. A detection limit of 2500 cfu/g and 10^8–10^{10}CFU/g of soil was determined using the BIOLOG® fingerprinting method and PCR technique, respectively. Since none of the tested samples were positive for the bacteria or the *Egl* 1 gene construct, it was concluded that *A. caulinodans* ORS 571 pGV910-C1 did not persist or spread in any of the tested field locations. This result may be due to low soil temperatures and competition of indigenous microorganisms: environmental factors that were not favorable for the diazotroph to thrive in the test locations. The application of different inoculation methodologies, as well as the study of other rhizobial genera for the inoculation of wheat in further experiments, is strongly recommended.

Keywords: ORS 571, detection, diazotroph, endogluconase, wheat.

INTRODUCTION

Azorhizobium caulinodans is a stem and root nodulating nitrogen-fixing bacterium, which was isolated from the stem and root nodules of the semi-aquatic tropical legume *Sesbania rostrata* that grows in the Sahel region of West Africa (Dreyfus *et al*., 1988). Several studies have shown that this bacterium is able to colonize intercellular spaces of cortex, xylem and root meristems of several non-legumes through crack entry of emerging lateral roots (Sabry *et al*., 1997; Webster *et al*., 1997; Cocking, 2005). In the studies of Sabry *et al.* (1997), inoculation of aseptically grown wheat with *A. caulinodans* stimulated formation of large number of short (up to 3 mm) lateral roots accompanied by increased grain yield and nitrogen content. On the other hand, *A. caulinodans* were able to enter the roots of rice at emerging lateral roots (lateral root cracks) by crack entry and bacteria moved into intercellular space within the cortical cell layer of roots (Mia and Shamsuddin, 2010).

In greenhouse experiments, increased biomass, grain yield, leaf area, leaf area index and other agronomical variables of wheat cv. CDC Teal inoculated with the genetically modified strain of *A. caulinodans,* ORS571 pGV910-C1, was reported by Anyia *et al.* (2004), as well as by Bécquer *et al.* (2007) and Anya *et al*. (2009). This strain overproduces endoglucanase and shows a normal nodulation behavior on the legume host plant (Geelen *et al*., 1995). On the basis of these arguments, the objective of the present work was monitoring of persistence and spread of a genetically modified strain of the growth promoting diazotroph *Azorhizobium caulinodans* used to

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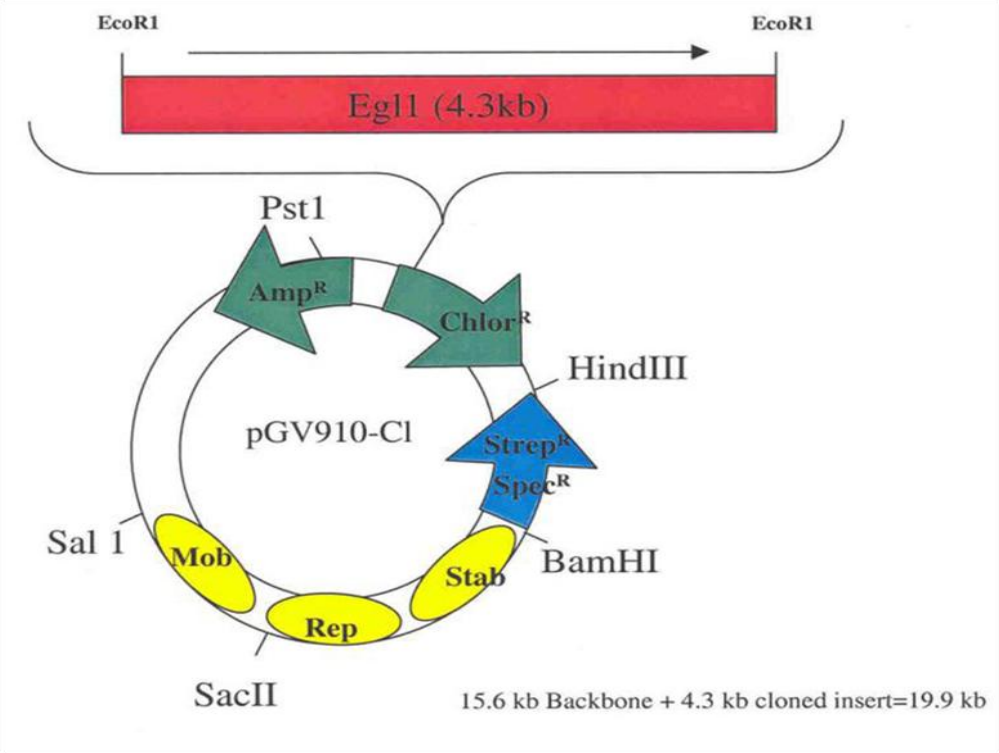

Figure 1. Physical map of the pGV910-C1plasmid. The pGV910-Cl was derived from the pGV910 plasmid vector by insertion of a 4.3 kb EcoR1 fragment of the Egl 1 gene (total=19.9 kb). The 4.3 kb fragment of the endoglucanase gene Egl1 is from *A. caulinodans*. The plasmid contains the ColE1 and pVS1 origins of replication and is stably maintained in *A. caulinodans*ORS571. The construct has a 5.3 kb pBR325 backbone (green) + 2.3 kb from pR702 (blue) + 8 kb from pVS1 (yellow).

inoculate field grown wheat.

MATERIALS AND METHODS

Strain used

One genetically modified rhizobial strain (ORS571 pGV910-C1), pertaining to *Azorhizobium caulinodans* was used. Strains purity was verified on yeast extract-mannitol agar (YMA) medium (Vincent, 1970) containing Congo red (0.00125%). Stocks were prepared on tryptone-glucose-yeast-extract (TGYE) (Dreyfus *et al*., 1988) and kept at -70 ^{o}C (under 30% of glycerol) for long-term storage and at 4 ^{o}C as source cultures.

Molecular procedures

The pGV910-C1 plasmid was derived from the pGV910 plasmid vector by insertion of a 4.3 kb EcoR1 fragment of the Egl 1 gene (total = 19.9 kb) (Figure 1). The 4.3 kb fragment of the endoglucanase gene Egl1 is from *A. caulinodans* (Geelen *et al*., 1995). Expression of the endoglucanase gene is driven from the chloramphenicol resistance gene promoter. The gene can restore endoglucanase activity in Egl^{-} strains of *A. caulinodans*. The host plasmid pGV910 was originally constructed by Van den Eede *et al.* (1992). It contains the ColE1 and pVS1 origins of replication and is stably maintained in *A. caulinodans* ORS571. Selection of the ORS571 pGV910-C1 strain is enhanced by antibiotic resistance genes including streptomycin/spectinomycin, ampicillin and chloramphenicol (Geelen *et al*., 1995).

Conditions of field experiments

A field study approved by the Canadian Food Inspection Agency was conducted at four sites located in different soil zones of Alberta. The sites included the following soils: Malmo series of an Eluviated Black Chernozem (at Vegreville), Lacorey soil series of an Orthic Gray Luvisol (St. Vincent), Black Loam Soil Zone, Niobe Silt Loam (80%), Penhold Meadow (20%) (atInnisfail) and Clay Loam within the Brown Soil Zone (at Taber, irrigated). On each of four trial sites, the experimental plots were secluded from other field operations with a 12 m fallowed buffer zone that isolated the treated crop and minimized treatment drift to non-target plants.

Experimental design and statistics for the field experiment

A factorial scheme in a randomized complete block design (RCBD) with 4 replicates per treatment was used. Three inoculation levels including inoculated (live inoculum), filtered (sterile filtered inoculum containing metabolites/compounds generated by *A. caulinodans*) and control (no inoculation) and four nitrogen levels (0%N, 25%N, 50%N and 100%N of recommended rates) were applied.

Seed inoculation procedures

Pure culture of *A. caulinodans* (strain ORS 571 pGV910-C1)was applied to the seeds as a liquid inoculant in a tryptone, glucose yeast extract (TGYE) fermentation broth. The concentration of cells in the inoculant was 9.4 x 10^8 CFU/mL. The treatment (live inoculum and filtered inoculum) was applied at a rate of 75 mL/ 27 kg of wheat seed. In order to maximize the number of cells delivered to soil, a relatively high seeding rate of 265 kg/ha was used. As a result, about 230 cells/g soil was introduced to the top 30 cm of soil.

Monitoring techniques and estimation of detection limits

Two independent techniques were used to monitor persistence of *A. caulinodans* in the fields used for experiments, including BIOLOG® (Hayward, CA) fingerprinting employing morphology and substrate utilization patterns of *A. caulinodans*, and a PCR technique with specific primers designed to target highly conserved regions of the plasmid including the Egl1 gene. To establish the detection limit using BIOLOG®, sterilized and unsterilized field soil was spiked with a serial dilution of *A. caulinodans* containing 0, 7.2 x 10^3, 3.6 x 10^4, 7.2 x 10^4, 3.6 x 10^5, 7.2 x 10^5, and 3.6 x 10^6CFU/mL. Five g of the spiked soil was extracted with 50 mL of 0.05 M sodium phosphate buffer (Slaski *et al*., 2002). The soil extract (100 µL) was plated on TGYE plates containing ampicilin and streptomycin antibiotic markers to select for *A.caulinodans* ORS 571 pGV910-C1. Plates were incubated for 24 to 48 hours at 30°C±2. Some plates contained Congo Red (0.00125%) Circular and creamy single colonies typical of *A.caulinodans* on agar (Dreyfus *et al*., 1988) were counted. Isolated colonies, usually 2-10, were re-streaked on selective media and incubated for a further 48 hours to obtain pure cultures. The colonies from individual plates were then collected with sterile swabs and dissolved in BIOLOG® inoculating fluid. One hundred µL of the suspension was pipetted into 96 wells on the BIOLOG®GN2 microplates. Plates were incubated for 24 to 48 h at 30°C±2 and read on an Emax BIOLOG® plate reader. The identity of the colonies was confirmed by BIOLOG® analysis of substrate utilization pattern. Since *A. caulinodans* was not present in the BIOLOG® database, a visual comparison was made with the substrate utilization pattern produced by the pure culture of *A. caulinodans* ORS 571 pGV910-C1 obtained from Dr. E. Cocking (Not tingham, UK). Isolates showing patterns similar to the original stock culture of *A.caulinodans* ORS 571 pGV910-C1 and wild strains of the bacterium were used to generate a database containing 36 entries, which served for identification of *A. caulinodans* in the monitoring process.

The lowest soil preparation contained 7,200 cells/g of soil, which means that it was theoretically possible to extract 720 cells/mL assuming 100% extraction efficiency (EF), (1g/10 mL extraction buffer). We successfully extracted 30 (sterile soil) and 20 (non-sterile soil) cfu/mL with an EF of 4.2 and 2.8%, respectively. Using the relationship CFU = cells/g soil / EF, it was possible to detect *A. caulinodans* ORS 571 pGV910-C1 using our BIOLOG® assay in non-sterile soils at about 2500 CFU/g of soil.

For the PCR analysis, 5g of soil was initially extracted as described by Yeates *et al*., (1998). To improve DNA yield and extraction efficiency, the freshly prepared extracts were processed with MoBioUltraClean™ Soil DNA Isolation kits (Mo Bio Lab. Inc., Carlsbad, CA). This protocol yielded good quality amplifiable DNA (using 16S universal primers) comparable to, or better than, the Yeates protocol in a side-by-side comparison. The average total DNA yield from freshly extracted soil measured fluorometrically was 40.97µg/mL, with a reliable range of 25–45 µg/mL. Poor DNA recovery from stored soil extracts was experienced, most likely due to co-extraction of DNAses and nucleases that are known to degrade free DNA and humic and fulvic acids known to be PCR inhibitors (Gulden *et al*., 2005). To enhance DNA recovery, glass beads were used to facilitate cell rupture, while $AlNH_4(SO_4)_2$ and aurintricarboxylic acid were added to the extraction mixture to precipitate humic acids and to inhibit DNA degradation, respectively. This method, when applied to freshly extracted soil samples, resulted in the generation of high quality DNA in a consistent fashion.

The PCR detection limit of *A. caulinodans* ORS 571 pGV910-C1 in soil was established from soil spiked with different dilutions of the bacterium culture ranging from 10 to 1.0 x 10^6cfuper gram of soil. The spiked soil was extracted using the procedure described above. Once DNA amplifiability using the universal 16s primer was determined, specific oligonucleotides designed to target the endoglucanase-chloramphenicol construct were used to detect the Egl1 gene in the soil DNA extract. The primers used were Chlor F1 (5'-acctataaccagaccgttcagc-3' forward primer, which hybridized to the chloramphenicol sequence-Genbank accession # LO8855) and Egl R24 (5'-agtgtgagagagagggtctcg-3' reverse primer, which hybridized to the endoglucanase sequence-Genbank accession # Z48958). This primer set

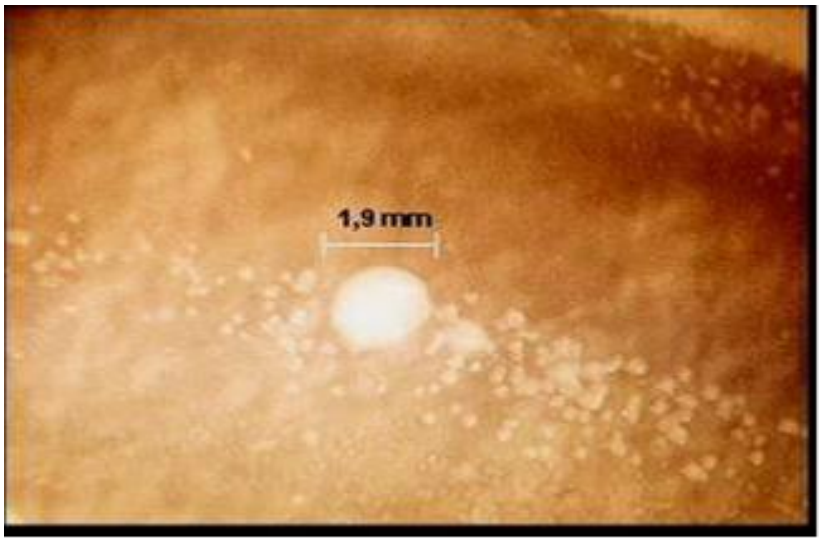

Fig 2. Colonies on a TGYE media, containing antibiotics (ampicilin and streptomycin antibiotic markers), exhibiting similar morphological characteristics to *Azorhizobiumcaulinodans* ORS 571 pGV910-C1.

had high specificity and sensitivity resulting in the generation of a single 900 bp amplification product at annealing temperatures from 50-60°C. Amplification conditions were established on an Eppendorf Mastercycler EP thermocycler using the following PCR program:1: 95 ^{o}C for 9 min. 2: 95 ^{o}C for 1 min. 3: 60 ^{o}C for 1 min. 4: 72 ^{o}C for 1 min. 5: Cycle back to step 2; 39 cycles. 6: 72 ^{o}C for 4 min. 7: 4 ^{o}C hold. These PCR parameters resulted in the production of 900 bp amplificon at the lowest dilution tested (10^{10}CFU/g soil). Because of the specificity and sensitivity of the primers, detection of *A. caulinodans* at lower dilution is possible, with the only limitation being the DNA extraction efficiency. Using the Chlor F1 and Egl R24 primers, direct PCR of serial dilution of pure culture of *A. caulinodans* ORS 571 pGV910-C1 yielded the expected 900 bp product at a minimum cell concentration of10^{8}CFU/mL.

Protocol procedures

Once detection limits of the BIOLOG® and PCR methods were established, the protocols were used to analyze soil samples from plots of each trial site seeded with wheat cv. CDC Teal inoculated with *A. caulinodans* ORS 571 pGV910-C1. Time zero soil samples were taken from inoculated and uninoculated plots as a reference for comparison. The same plots were sampled every 30 days throughout the growing season until the ground froze into the following seasons until the organism could no longer be detected. Additionally, soil was sampled at 1 mintervals in a perimeter approximately 5m into the buffer around the experimental site. These samples were used primarily to monitor for possible dispersion from the treated plots. In addition to the extensive soil sampling program, three wheat plants from each inoculated plot were randomly collected. DNA was extracted from root, leaf and grains and tested for the presence of *A. caulinodans* or the Egl1 gene using the Chlor F1 and Egl R24 primers combination. For plant tissue DNA extraction, the Qiagen DNeasy® Plant Mini kit (Mississauga, ON) was used.

RESULTS AND DISCUSSION

Several antibiotic resistant bacteria were found in soil extracts. From these, some produced colonies exhibiting similar morphological characteristics to *A. caulinodans* ORS 571 pGV910-C1: circular and creamy single colonies; in a media that included Congo Red in the formulation, no absorption of this dye was observed (Dreyfus *et al*., 1988) (fig. 2, fig. 3).

None of these antibiotic resistant bacteria were confirmed to be *A. caulinodans* using the BIOLOG® substrate utilization pattern protocol or the PCR analysis. A search of the BIOLOG® GN database suggests that most of the isolates were *Pseudomonas, Burkholderia* and *Xanthomonas* (99-100% statistical probability) (table1).

The origin of the multi-antibiotic resistance in those isolates is unknown. However, the fact that these bacteria were present in time zero samples suggests that resistance was not acquired from *A. caulinodans* ORS 571 pGV910-C1. The pGV910 plasmid is not self-transmissible and can only be mobilized with the helper plasmid pRK2013 in tri-parental mating (Van den Eede *et al.,* 1992). The fact that the indigenous antibiotic resistant bacteria were highly prevalent in the soil samples suggests that they might have outcompeted the

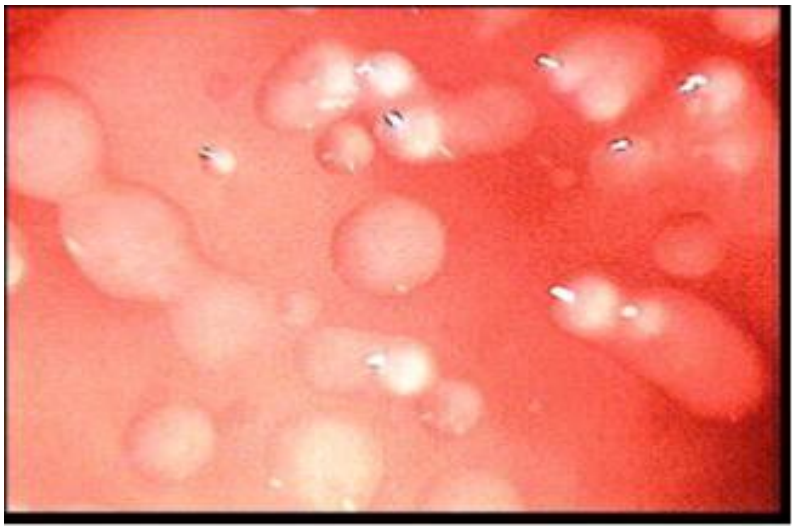

Fig 3. Colonies on a TGYE media, containing antibiotics (ampicilin and streptomycin antibiotic markers) and congo red (0.00125%), exhibiting similar morphological characteristics to *Azorhizobium caulinodans* ORS 571 pGV910-C1.

Table 1. Example of bacterial species more commonly found on the basis of the substrate utilization pattern in the BIOLOG® protocol.

Species included in the BIOLOG® data base	% probability	Similarity coefficient	Distribution	Identified specie on the basis of its substrate utilization pattern	Species type
Pseudomonas fluorescens	99-100	0,508-0,602	6,10-7,82	*P. fluorescens*	G (-)
Burkholderiacocovenenans	99-100	0,563-0,590	6,31-6,85	*B. cocovenenans*	G (-)
Xanthomonascampestris	100	0,771	6,29	*X. campestris*	G (-)
Pseudomonas tolaasi	NS	NS	NS	-	-
Pseudomonas synxantha	NS	NS	NS	-	-
Pseudomonas mucidolens	NS	NS	NS	-	-
Burkholderiamultivorans	NS	NS	NS	-	-
Alcaligenesfaecalis	NS	NS	NS	-	-
Sphingomonassanguis	NS	NS	NS	-	-
Sphingomonasparapaucimobilis	NS	NS	NS	-	-
Stenotrophomonasmaltofilia	NS	NS	NS	-	-

NS- Non significant.

introduced*A. caulinodans* ORS 571 pGV910-C1, which appears not to have thrived under the field conditions. Perhaps linked to this fact, Bécquer *et al*. (2012) obtained experimental results that suggested a marked influence of the indigenous rhizosphere microorganisms as compared with inoculated rhizobial strains in different variables assessed in *Triticumaestivum* and *T. secale* under field conditions.

The monitoring protocol described in this communication was used to test over 1200 soil and 400 plant samples collected from the 4 field locations used for experiments with *A. caulinodans* ORS 571 pGV910-C1. Since none of the tested samples were positive for the bacteria or the Egl 1 gene construct, it is obvious that *A. caulinodans* ORS 571 pGV910-C1 did not persist or spread in any of the tested field locations. It is possible that the organism was outcompeted by indigenous microbes and moreover, since it was isolated from tropical soils, it can be assumed that environmental conditions were not favorable for it to thrive in the test locations. In a non-sterile soil, native microorganisms may interfere with the root colonization of introduced bacteria (Okon, 1982). According to Van Berkum and Bohlool (1980), since it is impossible to simulate the plethora of interactions that operate in nature, superior strains and improved mutants manufactured in laboratories may have little ecological relevance and even less agronomic value.

Low soil temperature was suggested as an important environmental factor drastically affecting survival of *A. caulinodans* in temperate climatic conditions of UK soils

(Bullard, 1999). The author reported that moderate temperatures 18/8 ^{o}C reduced introduced populations of *A. caulinodans* by an order of magnitude in 35 days. There are arguments on how low temperatures may influence uptake of organic and inorganic substrates in different bacterial communities (Nedwell, 1999). According to Wiebe *et al.* (1993), even mesophilic marine bacteria, isolated from the southeastern subtropical shelf waters of the USA, were inhibited by lack of substrate at temperatures near their minimum for growth.

Therefore, it appears that low spring temperatures in Alberta with frozen soils at depths below 40 cm observed at times in April may have resulted in the cell rupture and death of*A. caulinodans*. Without growth and multiplication, the introduced cells were probably present in numbers below the detection limit of our protocols, yet were apparently in sufficient numbers to have some effects on plant growth. Similar results, on the identification of cyanobacteria in a rice paddy rhizosphere, were found by Wartiainen *et al.* (2008). These authors suggested that failure in the identification of the microorganism might be due to template discrimination in the PCR reactions, or low abundance of cyanobacteria compared to heterotrophic nitrogen-fixing bacteria. There also isa possibility that the method of inoculation used in this experiment, might have notensured a satisfactory amount of rhizobial cells per seed to succeed competition with the indigenous microbial population in the rhizosphere of wheat.

It is concluded that*A. caulinodans* ORS 571 pGV910-C1 did not persist or spread in any of the tested field locations probably due whether to the negative influence of several stress environmental conditions in the test locations, or the disadvantageous competition with the indigenous microbial population, or even the combination of both factors.

It is strongly recommended to apply different methodologies of seeds inoculation in further studies, as well as to study other rhizobial genera for the inoculation of wheat.

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