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Evaluation of soil microorganisms with inhibitory activity against *Rhizoctonia solani* causal agent of the damping-off of canola*

Evaluación de microorganismos del suelo que poseen actividad inhibitoria contra *Rhizoctonia solani.*

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Pre-and post-emergence damping-off of canola seedlings caused by *Rhizoctonia solani* is a serious disease in Western Canada. Other fungi such as *Fusarium* spp. and *Pythium* spp. are also related to seedling damping-off. To-day, the search of soil bacteria is becoming a tool to use microorganisms as potential biocontrol agents for several plant diseases. The purpose of this research was to detect bacteria to biologically control *R. solani, Pythium* spp., and *Fusarium* spp.

Soil samples were collected throughout Alberta during 1987 to isolate bacteria. Canola seedlings were also used to obtain bacteria from the same samples. Plant pathogenic fungi were tested to detect the antagonistic activity of the isolates. Tests were made with coated canola seeds, amendments and fresh of freeze-dried cells.

Three hundred forty-one bacterial cultures were isolated. Only 16 inhibited fungal growth: 7 showed the same effects against *R. solani* and 9 showed uneven effects. Some isolates showed a weak action to *Pythium* spp. and *Fusarium* spp. Three isolates showed inhibitory effect on *R. solani* and *Pythium* spp.

Isolate F1 improved by about 50% the germination of canola seeds in inoculated pots when compared with the inoculated control. Coated seeds had low germination and emergence was below the inoculated control. The emergence of canola seedlings was very much improved when isolate 147 was delivered as an amendment in inoculated pots. Identification showed that 3 bacterial belonged to *Bacillus* spp., 4 to green fluorescent *Pseudomonas* spp. and 2 were *Streptomyces* spp.

Pre-and post-emergence damping-off of canola seedlings is a serious disease in Western Canada, where about 2.25 million Ha were planted in 1983 to rapeseed (*Brassica napus L.* and *B. campestris L*)(10). Even though losses are evident and economically important an estimate has not been made, but it is known that the disease can destroy up to 100% of a canola crop or it could partially thin it out (28). *Rhizoctonia solani* Kuhn, has been cited (10,28) as an important soil pathogen associated not only with damping-off but also with the root diseases complex of rapeseed (10). However, other fungi such as *Fusarium* spp. and *Pythium* spp. are also related to canola seeding damping-off. It is known that the degree of virulence varies considerably among the isolates of these fungi, but pathogenicity tests with these pathogens performed under laboratory conditions satisfied Koch's postulates (28). Evidence shows that all these pathogens are not only responsible for seedling blight or damping-off of young canola plants but also for root rot, foot rot and late root rot of adult plants as well (1, 10, 28).

Several control measures have been investigated in order to protect canola seeds and plants from these soil fungi.

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Among them, plant breeding, seed protection with fungicides and crop rotation can be mentioned. However, there has been little success on controlling this disease complex. Many years of research may suggest that there will be little effect on avoiding losses caused by these soil fungi using these traditional control measures.

In the last years, the use of soil microorganisms as agents of control of plant pathogens is becoming a very active research field. Some investigations suggest that under some conditions soil bacteria produce siderophores that chelates iron and make it unavailable for plant pathogens (15). Several sources to isolate potential bacterial antagonists have been used, periderm of potatoes (8), stem, roots, and daughter tubers of potatoes (36); soil organic matter (24), wheat roots (25), mycelial mats (35); sclerotia of *Sclerotium cepivorum* (30); and germinating cucumber seeds (5). Also, bacteria have been obtained for this purpose from commercial seed lots and apple blossoms (36) and specific strains of *P. fluorescens* and *P. putida* were isolated from potato and pea roots (5).

The use of bacteria from different sources is becoming an interesting tool for the search of microorganisms as potential biocontrol agents for several plant diseases induced by fungi and bacteria (31). The purpose of this research was to develop isolations procedures and techniques in order to detect from Alberta soils the presence of bacteria or other microorganisms with inhibitory action against *R. solani, Pythium* spp., and *Fusarium* spp., useful as potential biocontrol agents.

MATERIALS AND METHODS

Isolation of antagonistic bacteria from soil and the interior of canola seeds. Soil samples were collected from several parts of the Province of Alberta during the month of October, 1987. Four samples were taken from fields plowed under unknown crops in locations located 60, 10, 18 and 38 km south and west of Edmonton. Two other samples were obtained from fields that had rapeseed in the previous season and located 11 km north of Moronville. About 5 kg of soil were taken from each place and transported to the laboratory in a plastic bag and kept in a cold room at 4°C. For isolation, dilutions of soil samples were made as follow: 1 g of soil was placed in a test tube with 8 ml of sterile distilled water; serial dilutions were made up to 10^{-5}; between each dilution, agitation was done with a Vortex. Two plates of potato-dextrose agar (PDA-Difco) were used to isolate bacteria from each sample by dropping 0.1 ml of the sample on the plate surface, and spreading with a rod glass. The plates were incubated at room temperature for 3 to 5 days. After growth was evident, colonies were observed with a 10x hand magnifying lens and subsequent cultures were stored as pure cultures. Canola seeds were used to indirectly isolate bacteria from the same soil samples. For each pot, 25 surface sterilized seeds cv. Tobin were planted. After the seedlings were 5 cm high, they were carefully removed and washed by stirring in distilled water for 5 min. The seedlings were surface sterilized as follow: 5 min in 5% sodium hypochlorite solution and washed twice with sterile water. This process was repeated two times. Finally, with a sterile surgical blade, the lower portion of each seedling was removed. Twelve plates of PDA, each containing three lower parts of canola seedlings were assayed for each kind of soil. As soon as growth was observed, agar plugs containing mycelium of *R. solani* were inoculated at the opposite side of the plate (Fig. 1). Pure cultures were made on PDA plates which were stored on PDA slants.

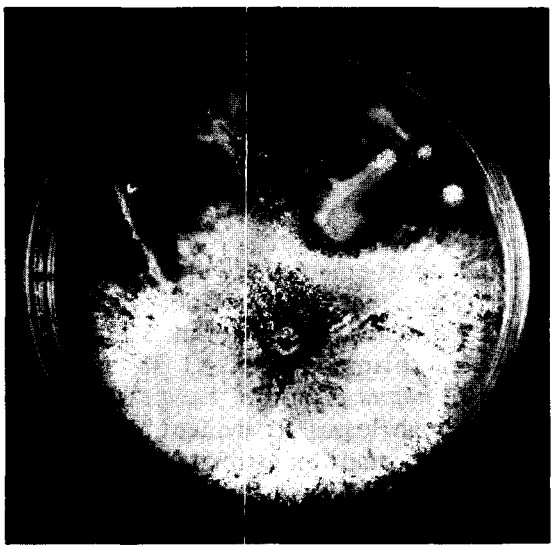

Fig. 1: The inhibitory action of bacterial growth products originating from cells growing around one of the roots (first from right) on the growth of *Rhizoctonia solani.*

Plant pathogenic fungi used to test antagonistic activity of bacteria isolated from soil. Several strains of plat pathogenic fungi were used in different tests to detect the antagonistic activity of the bacterial isolates obtained from the different soil samples and canola roots. Six strains of *R. solani* (N.70.03, N.28.0, C.51.25, N.69.32, P.04.

01, N.42.19); two of *Pythium* spp. (F.13.8, C.32.12) and two of *Fusarium* spp. (F.59.11, F.66.30) were used for this purpose. All the strains belonged to the Plant Pathogenic Fungi Collection of the Plant Science Department of the University of Alberta, Edmonton. Originally, they were isolated from several locations from the Province of Alberta, between May 15 and June 22, 1984.

Screening, selection and plate assays for the detection of potential antagonists. Pure cultures isolated from soil and seedlings were tested in 25 cm PDA Petri plates. The plates were inoculated with a pure bacterial culture isolate using a sterile cotton swab as stamp for plate replications. The plates were inoculated in such a way that surface spaces were left for fungal inoculation using agar plugs. When the development of the bacterial isolates was evident, *R. solani* agar plugs were inoculated in the empty spaces. This first selection of potential antagonists was performed with 5 different strains of *R. solani,* using all the isolates obtained from Alberta soils. Bacterial strains that inhibited the growth of any of the fungal strains were kept for re-testing their activity (Fig. 2).

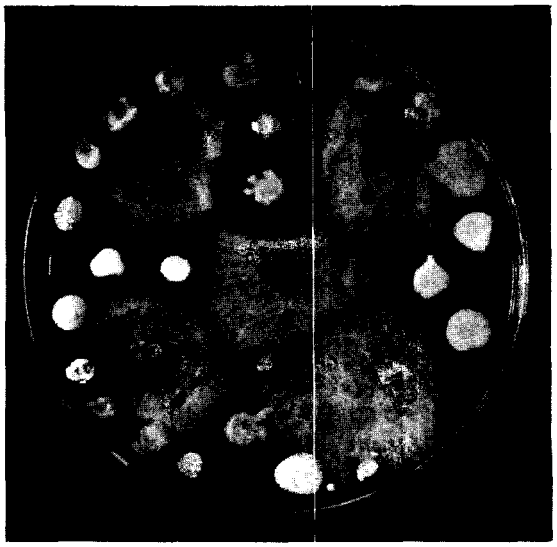

Fig. 2: Eight days old Petri Dish showing *Rhizoctonia solani* exposed to 26 different bacterial colonies. Two of the colonies produced diffusable substances that inhibited the growth of the fungus (clear zone around colonies on center-top of the dish). The plate was inoculated in 5 places with *R. solani* 3 days after bacterial growth.

The inhibitory effect of selected bacterial isolates was assayed again on PDA Petri dishes, inoculating the bacterial culture in 1/4 of the plate. The plates were incubated for 47 h, and then each plate was inoculated on the opposite side with a fungal strain. Two repetitions were made, one using only one bacterial strain for plate, and a second utilizing three bacterial strains per plate.

Preliminary identification of antagonistic bacterial isolates and effect of different media on the inhibitory activity. To study the effect of media on the antagonistic activity of several selected isolates (29, 39, 63, 101, 136, 149, 194, D1, F1, F2 in Table 2) different solid media were used: Czapex-Dox agar (CDA-Difco); Corn meal agar (CMA-Difco); Nutrient agar (NA-Difco); Potato dextrose agar (PDA-Difco). The following media were prepared as below: King's Medium B as described by Shaad (27); soybean yeast agar: soybean meal (Difco) 15 g, glucose 15 g, yeast extract 1.5 g, glycerol 5 cc, sodium chloride 2.5 g, calcium carbonate 1 g, agar 15 g, destilled water 1,000 cc; potato-dextrose yeast agar: PDA-Difco plus 2.5% yeast extract; casamino acids peptone glucose agar (CPG): proteose-peptone 15 g, glucose 10 g, casamino acids 2.5 g, agar 15 g, destilled water 1,000 cc.

A preliminary identification of the selected bacterial antagonistic isolates was conducted using the material and methods described by Shaas (27).

Preparation of bacterial cells for coating canola seeds. Selected isolates were used for either coating seeds or to be delivered in the amendment preparations. Four flasks for each culture containing 150 ml CPG broth, were inoculated with a loopful of bacteria from a 48 h old plate. The flasks were incubated on a shaker at 1,500 rpm for 48 h. The cells were concentrated by centrifugation at 10,000 rpm for 15 min. The pellet was resuspended in 200 ml of a 1:2 dilution of CPG broth, and the bacterial suspension was added to the autoclaved soil.

To coat seeds with bacteria 10 g of surface sterilized canola seeds cv. Tobin were placed in a 1:1 bacterial suspension with 1 or 2.5% dissolved methyl cellulose as sticking agent. The bacterial suspensions were prepared as described in the previous section. The seeds were: a) air-dried at room temperature and coated with calcium carbonate, and b) air-dried inside a laminar flow cabinet with the methyl cellulose mixed with calcium carbonate. The coated seeds were inmediately used for pot experiments.

Preparation of amendment with freeze-dried bacterial cells. Selected isolates were freeze-dried as follows: bacteria were grown on potato-dextrose broth (PDB-DIFCO) for 4 days in case of *Bacillus* sp. and 1 week for *Actinomyces* sp. strains. The cells were concentrated by centrifugation at 10,000 rpm for 15 min. The pellet was resuspended in 100 ml of a solution containing 1% L-glutamic acid, 5% inositol and 0.5% ascorbic acid. This suspention was frozen by immersing 500 ml Virtis bottles in acetone and dry ice. When the suspention was completely frozen, the bottles were taken to a Virtis freeze-drier and kept at -60 Torr for 48 h. The freeze-dried product was dispensed in sterile plastic dishes and stored at room temperature to be delivered as an amendment to soil.

The amendment was prepared as follows: a rich organic soil was air-dried and sieved through a 2 mm mesh sieve. The soil was dispensed in 500 g amounts in double paper bags and sterilized in an autoclave at 121°C for 30 min. The bags

were stored at room temperature and inoculated with 1 g of freeze-dried cells every 200 g of dry soil. Also, 10 gr of $CaCO_3$ were added to each 200 gr of soil bring the original pH of 4.5 to 5.7. The amendment was stored in a cold room at 4°C for 18 h and used for pot experiments.

Testing the antagonistic activity against ***Rhizoctonia solani*** *under controlled conditions and data analyses.* Both, fresh bacterial preparations and freeze-dried cells were used in pot experiments to study the inhibition of *R. solani.* Jiffy pots were 3/4-filled with a mixture of 3 parts of sterile soil and 1 part of Jiffi Mix. Twenty-five surface sterilized canola seeds were placed on top of the mixture and covered with 1 cm layer of soil infested with *R. solani* (C.51.25), containing 3.5 g of dry inoculum/10 kg of sterile soil. Ten pots were used for each treatment, each pot with 25 seeds. Every experiment included the following treatments: inoculated control with *R. solani* seeds alone, seeds with the antagonistic bacteria (coated or in the amendment), and seeds with the antagonist alone. The pots were placed inside growth cabinets in trays and watered daily with distilled water. The cabinet was set for 16 h light and day/night temperatures of 18/7°C to simulate western Canadian springtime conditions (1). Observations on seedlings emergence and symptoms caused by *R. solani* were recorded 15 days after planting.

The data on seedling emergence were statistically analyzed and is presented in Tables 3, 4 and 5. Analysis of variance of data from these Tables is presented in Table 6. The analyses were conduced with no data transformation using SAS (System Analysis Statistics, 1988) through the main computer of Computer Services of the University of Alberta, Edmonton, Alberta.

RESULTS

Three hundred forty-one pure bacterial cultures were isolated from six soil samples. Out of these 47 were selected from dilution 10^{-1}, 91 from 10^{-2}, 93 from 10^{-3}, and 110 from 10^{-4}. A few plates were contaminated with fast growing soil fungi but this did not interfere with the isolation process. Initially six strains of *R. solani* were challenged with these 341 bacterial cultures to detect the potential antagonists. The results of this experiment are included in Table 1, where it is evident that only 16 isolates out of 341 (4,6%) showed some kind of effect by slowing or stopping fungal growth. Out of these 16 isolates, only 7 (39, 61, 63, 136, 137, 149 and 194) consistently showed the same effects against the six strains of *R. solani.* On the other hand, nine isolates (16, 29, 40, 40B, 94, 101, 111, 150 and 169) were erratic and showed uneven effects on the *Rhizoctonia* isolates. Two of the isolates belonged to *Actinomyces* spp. (isolates 61 and 137).

Data presented in Table I also shows the antagonistic activity and the inhibition induced by the 16 isolates on different strains of *Pythium* spp., and *Fusarium* spp. Some of the isolates showed, in several tests, a weak or non-inhibitory action to the two *Pythium* spp. isolates (16, 39, 40B, 61, 63, 94, 101, 111, 150, 169, and 194). Three isolates (136, 137, and 149) showed inhibitory effect, both on *R. solani* and to *Pythium* spp. isolates.

The effect of bacterial isolates on the two *Fusarium* spp. strains was different from that observed on *Pythium* spp., and *R. solani* (Table I). Five isolates (61, 63, 136, 137, and 149) showed a consistent inhibition. One isolate (39) showed different effects on the two *Fusarium* strains, and ten isolates showed no effect (16, 29, 40, 40B, 94, 101, 111, 150, 169, and 194).

Few isolates showed a specific action such as isolate 29, active only against *Pythium* spp. and inconsistent action against *R. solani* and *Fusarium* spp. isolates. Isolate 194 affected only *R. solani* and isolates 29 and 40 were consistently active only agains *Fusarium* spp. Table I also shows that *R. solani* was inhibited by seven strains, *Pythium* spp. by five strains and *Fusarium* spp. also by five strains.

Isolations performed from 72 plates containing canola seedling roots yielded nine plates with bacteria with evident antagonistic action against *R. solani.* From these plates 13 pure bacterial cultures were made, and only five showed activity to plant pathogenic fungi in later tests. Isolates designed as D1 and D2 induced plate inhibition of *Fusarium* spp. (strain F.59.11) and *Pythium* (F.13.8); isolates F1, F2, and F3 induced plate inhibition of *Fusarium* (Strain 59.11), *Pythium* spp. (F.13.8) and *R. solani* (Strain N.51.25).

The isolates that consistently gave variable results in several tests were eliminated. While a selection of most promis-

ing isolates originating from soil dilutions and roots of canola seedlings were tested further. Table II shows the results of another test were several isolates of fungi were challenged with 10 selected bacterial isolates using different agar media. It was possible to observe that 4 isolates (39, 63, 136, 149) were consistently inhibitory to *R. solani, Pythium* sp., and *Fusarium* sp. The other isolates (29, 101, 194, D1, F1 and F2) caused different responses in the various fungi.

Preliminary identification of some selected isolates showed that bacterial cultures belonged to the endospore forming genus *Bacillus* sp. (39, 63 and 149), green fluorescent *Pseudomonas* spp. (136, D1, F1 and F2) and *Actinomyces* spp. (61 and 137).

Canola seeds were coated with isolated F1, 39, 63 and 149 in order to test their effects on seed germination and inhibition of *R. solani* (C.51.25). Results are presented in Table III which show that one of the bacterial strains (F1R treatment) improved, by about 50%, the germination of canola seeds in inoculated pots when compared with the inoculated control (RC). Also, there were no statistically significant differences among the non-coated control (HC), the bacteria coated controls (F1, 39, 63 and 149) and strain F1 in infested soil (F1R). The other strains improved germination of seeds as well, such as the isolate 63 (63R treatment). The isolates 39 and 149 did not protect canola seeds from infection as compared with the other strains.

Canola seeds were also coated with isolates 61, 137, F1 and D1 and tested for inhibition of *R. solani* in infested pots. Isolates 61 and 137 were also delivered in an amendment. Results of this experiment are presented in Table IV which show that the coated seeds with 2.5% xanthan gum mixed calcium carbonate had low germination and that emergence was below the inoculated control (RC treatment). However, the emergence of canola seedlings was very much improved when isolate 137 was delivered as an amendment in

TABLE I

Inhibition of strains of *Rhizoctonia solani, Pythium* spp. and *Fusarium* spp., by 14 bacterial and 2 *Actinomyces* spp. cultures, isolated from Alberta soils

Isolate	Inhibition of strains of fungi**									
	R. solani						*Pythium*		*Fusarium*	
	R1	R2	R3	R4	R5	R6	P1	P2	F1	F2
16	–	–	W	W	W	–	–	–	–	–
29	V	W	W	W	W	–	+	+	–	–
39	+	+	+	+	+	+	+	–	+	W
40	W	W	+	+	+	+	+	+	–	–
40B	W	+	V	W	–	–	–	–	–	–
61*	+	+	+	+	+	+	W	+	+	+
63	+	+	+	+	+	+	+	W	+	+
94	–	W	–	–	–	–	–	–	–	–
101	V	V	V	W	W	W	–	–	–	–
111	V	V	V	V	V	V	–	–	–	–
136	+	+	+	+	+	+	+	+	+	+
137*	+	+	+	+	+	+	+	+	+	+
149	+	+	+	+	+	+	+	+	+	+
150	–	W	W	–	–	–	–	–	–	–
169	W	–	W	–	–	–	–	–	–	–
194	+	+	+	+	+	+	–	–	–	–

\+ = Inhibition observed.
– = No inhibition observed.
W = Weak inhibition observed.
V = Variable response of isolate after several tests.
* = *Actinomyces* spp.
** = R1 N.70.03; R2 = N.28.0; R3 = C.51.25; R4 = N.69.32; R5 = P.04.01; R6 = N.42.19 (*R. solani*). P1 = F.13.8; P2 = C.32.12 (*Pythium* spp.); F1 = F.59.11; F2 = F.66.30 (*Fusarium* spp.).

TABLE II

Inhibition of growth of *Rhizoctonia solani, Pythium* spp., and *Fusarium* spp. on several agar media by nine selected bacterial isolates obtained Alberta soils and canola seedling roots

Fungus spp. and Agar Base Medium	Antagonistic action of bacterial strains (1)									
	29	39	63	101	136	149	194	D1	F1	F2
R. solani N.70.03 on King's Medium B	–	+	+	–	+	+	–	–	–	–
R. solani N. 51.25 on Czapex Dox	W	+	+	–	+	+	–	+	+	+
R. solani R.04.01 on Corn meal	W	+	+	W	+	+	W	–	–	–
R. solani N.42.19 on Soybean yeast	–	+	+	W	+	+	–	+	+	+
Pythium F.13.8 on Casamino acids glucose	–	+	+	–	+	+	–	+	+	+
Fusarium F.66.30 on Nutrient Agar	W	+	+	–	+	+	–	+	+	+

\+ = Inhibition observed.
– = No inhibition observed.
W = Weak inhibition observed.
(1) = 29 to 194 isolated from soil; D1, F1, and F2 from roots of seedlings.

TABLE III

Emergence of canola seedlings in pots infested with *Rhizoctonia solani* (C.51.25). Non-inoculated controls were also run. Coating of seed with bacteria was by bacterial cell suspensions (1 : 1 volume) mixed with 1% methyl cellulose, air-dried at room temperature and coated with calcium carbonate

Pot	Non-inoculated controls (1)					*R. solani* inoculated				
	F1	39	63	149	HC	RC	F1R	39R	63R	149R
1	17	12	15	18	12	9	21	9	15	6
2	22	19	16	12	21	9	10	4	13	10
3	16	16	16	20	23	4	14	14	12	13
4	21	20	16	14	12	10	17	10	10	9
5	15	17	19	24	25	10	21	13	18	14
6	23	22	19	20	12	9	21	7	15	16
7	15	14	20	23	21	7	10	7	15	13
8	20	21	17	29	23	5	14	14	14	12
9	20	21	18	25	25	5	17	10	14	12
10	20	16	18	23	12	9	19	10	15	11
Av*	19.9 a b	17.9 a b	17.5 a b	20.2 a	18.6 a b	7.7 e	16.4 a b c	9.8 d e	14.1 c d	11.6 c d e

* Tukey's Studentized Range Test (HSD).
Means with the same letter are not significantly different.
Critical value of studentized range = 4.588.
Minimum significant difference = 5.053.

(1) Isolate F1 (*Pseudomonas* spp.) obtained from seedlings from seedlings 39, 63 and 149 (*Bacillus* spp.) from soil.
HC = Healthy control. No bacterial seed coating.
RC = *Rhizoctonia solani* infested control. No bacterial seed coating.
R = *Rhizoctonia solani* infested.

TABLE IV

Emergence of canola seedlings from seeds coated with four strains of antagonistic bacteria and sown in *Rhizoctonia solani* (C.51.25) inoculated and non-inoculated pots. Coating of seed with bacteria was by bacterial cell suspensions (1 : 1 by volume) with 2.5% of xanthan gum, them mixed with calcium carbonate and air-dried in a laminar flow cabinet

Pot	Non-inoculated controls (1)				Controls		Coated treatments				Amendments	
	61	137	F1	D1	HC	RC	61R	137R	F1R	D1R	61AR	137AR
1	17	20	12	16	22	7	0	2	2	0	20	19
2	16	16	12	20	24	5	0	8	1	0	20	22
3	17	16	15	16	24	6	3	8	0	0	21	21
4	16	14	12	14	18	7	2	0	0	1	15	20
5	17	17	10	13	18	7	4	0	0	1	20	24
6	15	18	6	21	25	8	1	1	1	1	16	22
7	17	18	11	16	23	11	2	5	0	1	15	24
8	18	19	6	14	21	11	4	9	1	3	15	22
9	15	16	14	15	24	7	2	6	1	3	18	22
10	13	15	16	14	24	5	3	4	1	2	16	18
Av	16.1	16.9	11.4	15.9	22.3	7.4	2.1	4.3	0.7	1.2	17.6	21.4
	b	b	c	b	a	d	e f	e	d f	e f	b	a

* Tukey's Sudentized Range Test (HSD).
Means with the same letter are not significantly different.
Critical value of studentized range = 4.725.
Minimum significant difference = 4.3505.

(1) Isolates F1 and D1 (*Pseudomonas* spp.) obtained from seedlings; 61 and 137 (*Actinomyces* spp.) from soil.
HC = Healthy control. No bacterial seed coating.
RC = *Rhizoctonia solani* infested control. No bacterial seed coating.
R = *Rhizoctonia solani* infested.
AR = Bacteria in the amendment established in *R. solani* infested soil.

inoculated pots (treatment 137AR). The number of emerged plants in inoculated pots with *R. solani* and protected with isolate 137 ((treatment 137AR in Table IV) are statistically similar to the healthy control (HC)). However, non-inoculated controls (61, 137, F1, D1) showed lower rates of seed germination than the non-coated treatment (HC).

In another experiment isolates 61, 137, F1 and D1 were used as freeze-dried cells and delivered in an amendment. Also, isolates F1 and D1 were used as fresh preparations and also delivered in an amendment (61R, 137R, F1R, D1R, FiFR and D1FR) showed higher germination of canola seeds than the inoculated control (RC). However, these figures are statistically similar among themselves and lower than the non-inoculated control (HC). Also, there are no differences between using freeze-dried or fresh bacterial cells when they are independently delivered in an amendment.

The analysis of variance with the results are presented in Table IV. Statistical data analyses and F values show that there are significant differences among the treatments used in each set of experiments which are presented in Tables III, IV and V.

DISCUSSION

A number of bacteria with inhibitory activities *in vitro* to several species and strains of plant pathogenic agents of damping-off of canola were isolated from Alberta soils. Some prokaryotic filamentous organisms included in the *Actinomycetes*, causing the same effect the other bacterial isolates were also isolated.

TABLE V

Emergence of canola seedlings sown in *Rhizoctonia solani* (C.51.25) inoculated and non-inoculated pots. The antagonistic bacteria were delivered in an amendment either as a fresh cell preparation or a freeze-dried product

Pot	Freeze-dried controls				Fresh cells (1)		Controls		Freeze-dried cells			Fresh cells		
	61	137	F1	D1	F1F	D1F	HC	RC	61R	137R	F1R	D1R	F1FR	D1FR
1	22	25	23	24	25	23	25	8	15	18	13	14	17	14
2	23	23	23	18	22	22	18	5	9	14	14	15	20	17
3	22	24	22	24	23	23	22	10	8	15	13	14	13	17
4	22	24	25	23	24	21	23	10	14	15	10	14	14	16
5	23	24	24	21	22	24	25	8	13	11	14	14	14	9
6	23	23	22	20	20	19	24	4	16	17	11	16	12	14
7	19	22	23	22	24	18	20	7	11	17	9	14	15	22
8	23	19	22	15	22	24	23	8	15	20	12	17	17	15
9	19	19	22	19	18	24	19	10	9	15	14	18	16	10
10	19	20	20	24	21	20	22	10	8	11	10	13	15	15
Av*	21.5 a	22.3 a	22.6 a	21.0 a	22.1 a	21.8 a	22.1 a	7.9 c	11.8 b	15.3 b	12.0 b	14.9 b	15.3 b	14.9 b

* Tukey's Studentized Range Test (HSD).
Means with the same letter are not significantly different.
Critical value of studentized range = 4.837.
Minimum significant difference = 3.6969.

(1) Isolates F1 and D1 (*Pseudomonas* spp.) obtained from seedlings; 61 and 137 (*Actinomyces* spp.) from soil.
HC = Healthy control. No bacterial amendment.
RC = *Rhizoctonia solani* infested control. No bacterial amendment.
R = *Rhizoctonia solani* infested.

TABLE VI

Analysis of variance of data presented in Tables III, IV and V

Data from Table III: Canola seeds coated with bacteria and tested in inoculated (F1R, 39R, 63R, 149R), non-inoculated (F1, 39, 63, 149) and controls (HC, RC) with *R. solani.*

Source of Variation	DF	Sum of Squares	Mean square	F Value
Model	9	1638.01000	182.00111	14.99
Error	90	1091.70000	12.13000	
Corrected Total	99	2729.71000		

Data from Table IV: Canola seeds coated with bacteria (61R, 137R, F1R, D1R), delivered in amendments (61AR, 137AR), non-inoculated (61, 147, F1, D1) and controls (HC, RC) with *R. solani.*

Source of Variation	DF	Sum of Squares	Mean square	F Value
Model	11	7012.49166	637.49924	126.77
Error	108	543.10000	5.02870	
Corrected Total	119	7555.59166		

Data from Table V: Bacteria delivered as freeze-dried cells (61R, 137R, F1R, D1R), fresh cells (F1FR, D1FR), non-inoculated (61, 137, F1, D1, F1F, D1F) and controls (HC, RC) with *R. solani.*

Source of Variation	DF	Sum of Squares	Mean square	F Value
Model	13	3142.74285	241.74945	41.39
Error	126	736.00000	5.84126	
Corrected Total	139	3878.74285		

Some authors (34) recommend that the potentially useful bacteria to be used to control plant pathogenic fungi, should be isolated from the environment in which they will be expected to function and also that these bacteria should be isolated from the rhizosphere or rhizoplane of the target host. Our results clearly showed that not all the isolates found initially and that caused fungal inhibition mantained this effect on plates for several weeks. From Table II it is possible to conclude that only 4 isolates out of 341 obtained from soil and that 3 out of 13 isolated from roots of canola seedlings showed a potential use for further studies.

Even thought the results of *in vitro* experiments may not necessarily correlate with what may happen in the field, extensive experiments must be carried out in the laboratory before taking an isolate to outdoors tests (11). We believe and support the idea that *in vitro* antibiosis is the first desirable quality of a long process which should include testing of the isolates several times in plates and growth chambers to prove the persistency of the strains to cause antibiosis. Field trials should be the conclusive and the ultimate tests for any particular strain (9).

Ideally, a potential isolate to be used in biocontrol of damping-off of canola should show antagonistic activity to the different species of plant pathogenic fungi that are causal agents of this complex disease. We were able to select a few isolates which showed a consistent effect *in vitro* toward *R. solani, Fusarium* spp., and *Pythium* spp. This selection process was conducted gradually, starting with the most important pathogen to be controlled, in this case several strains of *R. solani.* After this initial screening, the number of bacterial isolates under study decreased dramatically (about 95%). At this point it was possible to re-test the cultures against more strains of *R. solani* and at the same time to extend the experiments to other plant pathogenic fungi, such as strains of *Fusarium* and *Pythium.* This process had to be conducted in this way, otherwise the amount of material being used would have reached unmanageable proportions.

Isolations performed from roots of canola seedlings growing in pots with Alberta soil samples, proved to be less laborious than isolations performed from soil dilutions. This sample test yielded bacteria which were closely related to rhizoplane and rhizosphere of canola, as well as bacteria that may be able to colonize the superficial layers of canola roots. Bacterial isolates obtained through this are likely to be more specific to canola than the ones isolated from soil dilutions. The source of specific root organisms for protection against fungi inducing damping-off is very important, since inhibition of the pathogen must occur on the root surface. In studies with bacterial colonization and antagonism on plant roots, it was reported that *R. solani* failed to grow on roots of protected seedlings of cucumber, and that the absence of the fungus was either due to competition or to a fungistatic substance produced by antagonistic microorganisms (21).

The isolations performed from seedlings of canola yielded only strains of *Pseudomonas.* These after final selection were antagonistic to the damping-off pathogens. Closed related species of this genera such as *P. putida* (strain R20) reduced the incidence of *P. ultimum* damping-off in the greenhouse by up to 79% (19). Members of *Pseudomonas* group are natural inhabitants of the soil, but more specifically of the particulate organic matter component and rhizosphere of plants (11, 24).

The relation of our isolates to either the rhizosphere of plants or strictly the soil phase is possible to understand after preliminary identification of the soil and seedling isolate inhibitors of *R. solani, Pythium* spp. and *Fusarium* spp. The isolates obtained from Alberta soil samples were mostly *Bacillus* spp. and those isolated from seedlings grown in the same samples were mainly green fluorescent *Pseudomonas* spp. Normally, *Bacillus* is considered to be a genus typical of the soil environment (3), and many green fluorescent pseudomonads are primary inhabitants of the rhizosphere and rhizoplane of plants

(34). These soil and rhizosphere genera contain specific strains that when incorporated into growth medium can reduce the development of plant pathogenic fungi (7, 11, 13). Fluorescent pseudomonads included in the *P. fluorescens* group, appear frequently among isolates from plant rhizospheres, and some strains that improve plant growth may also decrease root damage caused by root rot pathogens (32), and protect seeds from colonization by pre-emergence demping-off caused by *P. ultimum* (11). It is known that pyrrolnitrin, an effective inhibitor of *R. solani* is an antibiotic substance produced by several isolates of *P. fluorescens.* This antibiotic is less effective against *Fusarium* spp. and ineffective against *P. ultimum.* Also, evidence shows that *P. fluorescens* may be useful as an antagonist against *R. solani.* The antibiotic may also facilitate the establishment of stands of healthy seedlings by protecting the seeds through production of the antibiotic and therefore, act as an effective protectant against damping-off (26). Several authors have demonstrated that some *Pseudomonas* strains have the ability to improve the growth of crops such as sugar beet and potato (11, 32). Recently, strains of this genus were found to have a mechanism of antagonism related to iron deprivation by $iron^{+3}$ complexing siderophores produced during growth (14).

Bacillus species similar to those isolated in this research, belong to the aerobic endospore forming group. For biological control, a strain of *B. subtilis* was succesfully used to control *Fusarium* stem rot in carnation (18). Also there, is circumstantial evidence that *B. subtilis* is antagonistic to *F. roseum* and *R. solani* by the production of antibiotic substances (2, 4). Strains of *B. mycoides* and *B. pumilus* were used to achieve good control of *Gaeumannomyces graminis.* However, the mode of action of this antagonistic effect has not been established (16). Some implications for possible side effects on some crops should also be considered as members of this genus, particularly *B. pumilus,* an opportunistic pathogen, has been implicated in potato soft rot and is a very active pectinase producer (6).

There is some evidence that *Bacillus* spp. and *Streptomyces* spp. strains selected for biological control of *R. solani* may also promote plant growth through mechanisms not well understood yet, but not appearing to be specific to soil types or plants groups (17, 31). Some strains of *Streptomyces* isolated from soil have effective antagonism toward *R. solani* in culture, and have been selected for evaluating their roles in the biological control of the fungus causing serious problems in cauliflower seedlings. The inhibition was maintained in pot experiments and was related to high concentrations of the selected strains of *Streptomyces* (12). Also, the action of *Streptomyces* against soil-borne plant pathogens was observed and studied by Thuran (29), using isolate C-2-9, a new race of *S. achraceiscleroticus.* This was done by immersing seeds of different plants or by dipping the roots or lower parts of the stems of seedlings into the homogenate of isolate C2-9 just before planting. Another filamentous bacterium, *S. hygroscopicus* var. *geldanus* controls rhizoctonia root of pea only if the antagonist is added to previously sterilized soil, 2 or more days prior to the addition of *R. solani.* The control mechanism is related to production of the antibiotic geldanamycin in soil (23).

The indirect isolation of strains from roots of canola seedlings as described in the Material and Methods is useful, reliable and simple to conduct. Since surface sterilization of the canola seeds was used, the only remaining source of bacteria was from the soil sample where the seedlings were growing. This may be explained through growth of fluorescent pseudomonads in large amounts on the roots of plants where a suitable environment for their growth and survival may exist (34).

Selection of bacteria antagonistic to take-all of wheat was achieved from roots of wheat growing in pots (33). The production of antibiotic-like substances and siderophores appear as an important characteristic of some pseudomonads that suppress disease (11, 34). Furthermore, because there are probably other factors involved in the suppression of disease, *in vitro* antibiosis cannot be used as a sole determinant

in the selection of bacterial strains in the search of good and probably valuable antagonists. However, our findings, suggest that Alberta soils contain potentially useful bacteria, that may lead to development of novel biocontrol agents.

The lack of consistency of behaviour of the isolates is reflected throughout the several sets of experiments conducted in this research. The are many factors involved in this variable response (11, 31) which are related to soil and environmental factors instead of the bacterial strain under test.

Emergence of canola seedlings grown in *R. solani* infested soil was highly improved by delivering the two different *Actinomyces* spp. strains in an amendment or as freeze-dried cells as well as with some bacterial isolates as fresh cell preparations. Successful field trials of bacteria that decreased pre- and post-emergence damping-off caused by *Pythium* spp. were conducted by applying bacteria to sugar beets seeds in a mixture of 0.1 M $MgSO_4$ and 2% methyl cellulose, and coating with talc (20). However, recent evidence suggests that treating seeds or plants parts with methyl cellulose or xanthan gum as sticking agents of bacterial cells, may reduce their population by up to 90%. This detrimental effect was observed and detected after air-drying coated seeds with root-colonizing *Pseudomonas* (22). With other strains of actinomycete-like bacteria (17), similar effects were achieved when delivered in an amendment. It seemed that the filamentous slow growing prokaryotic microorganisms (*Streptomyces* spp.) gave good protection to canola seedlings if delivered in an amendment. On the other hand, typical prokaryotic cells such as green fluorescent *Pseudomonas* spp. and *Bacillus* spp., worked much better if coated around the canola seeds.

Freezing and drying of bacterial cells sometimes represents a laborious and expensive process, but in our case had the great advantage of avoiding cell carrier and eliminated the complexity of preparations of amendments and other similar seed carriers. To enhance viability, high bacterial populations can be successfully maintained by lyophylizing the cells in a protective medium (22).

Biological control of plant pathogens using unaltered antagonists is very appealing as it is Nature own way of keeping a balance among populations of microorganisms. Such an approach also answers very favourably to the public concerns on agricultural chemicals. We believe that it is safe to predict, than in a few decades, antagonists of the kind reported in this paper, will be commonly used as plant protection agents. However, before this happens, a lot of basic research needs to be done. Besides selection of antagonists, proper scale-up formulation and delivery systems compatible with the farming practices and cost considerations will have to be worked out. Also, there is a need to study the ecology and interactions among microorganisms in soil, so that the fate if applied the biocontrol agent in the same and subsequent seasons, can be consistently predicted.

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