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(54) Title: BIOLOGICAL CONTROL OF PYTHIUM DISEASE IN CROPS

(57) Abrégé/Abstract:
Strains of Rhizobium leguminosarum biovar viceae have antifungal activity against the pathogen Pythium ultimum. Compositions and methods for treating or protecting plants susceptible to Pythium ultimum damage, and Pythium sp. "group G" damage in particular, are provided. Such strains include, for example, the strains deposited in the International Depository Authority of Canada under accession numbers IDAC 200704-01, IDAC 200704-02, IDAC 200704-03, and IDAC 200704-04.

Abstract

Strains of *Rhizobium leguminosarum* biovar *viceae* have antifungal activity against the pathogen *Pythium ultimum*. Compositions and methods for treating or protecting plants susceptible to *Pythium ultimum* damage, and *Pythium* sp. "group G" damage in particular, are provided. Such strains include, for example, the strains deposited in the International Depository Authority of Canada under accession numbers IDAC 200704-01, IDAC 200704-02, IDAC 200704-03, and IDAC 200704-04.

BIOLOGICAL CONTROL OF *PYTHIUM* DISEASE IN CROPS

Field of the Invention

[001] The invention relates to control of crop disease caused by the fungus *Pythium*, and compositions and methods therefore. In particular, the invention relates to the preparation and application of biocontrol agents from novel strains of *Rhizobium leguminosarum* biovar *viceae* affective in controlling *Pythium* disease.

Background of the Invention

[002] "Damping-off" is the sudden plant death in the seedling stage due to the attack of fungal pathogens such as *Pythium* spp. and *Rhizoctonia solani*. The pathogens are soilborne and are stimulated to grow and infect the seed or seedling of host crops by nutrients released from a germinating seed. Damping-off disease of seedlings occurs in most soils, temperate and tropical climates, and in greenhouses. The disease affects seeds and seedlings of various crops grown under greenhouse and/or field conditions. The amount of damage the disease causes to seedlings depends on the fungus, host tolerance/susceptibility soil moisture, and temperature. Normally, however, cool wet soils favor development of the disease caused by *Pythium* spp.. Roots may rot, or the hypocotyls (lower stem) may either collapse or become wiry. Seedlings may die before or after they emerge from the soil (pre-emergence and post-emergence damping-off, respectively). Seedlings in seedbeds often are completely destroyed by damping-off, or they die after transplanting. Severe loses of plants due to pre- and post-emergence damping-off often results in poor stands of many crops.

[003] *Pythium* spp. are the causal agents of seed, root, and crown rot diseases of economically important crops worldwide. *Pythium* sp. "group G", a sterile form of *Pythium ultimum* Trow, is a major plant pathogen of numerous crops grown in southern Alberta, including sugar beet (*Beta vulgaris* L.) and field pea (*Pisum sativum* L.). Indoor experiments, using soil artificially inoculated with *Pythium* sp. "group G", showed that safflower (*Carthamus tinctorius* L.), canola (*Brassica rapa* L.), field pea and sugar beet are highly susceptible to the pathogen (Huang et al 1992). Field surveys showed that *Pythium* spp. were the main cause of poor stands of sugar beet in southern Alberta (Bardin and Huang 2001). *Pythium ultimum* Trow and *Pythium irregulare* Buisman were the principal pathogens causing seed rot and damping-off of field pea and reduced seedling establishment in the

northern Canadian prairies (Hwang and Chang 1989). *Pythium* diseases in field crops are usually controlled by seed treatment with fungicides such as Thiram™ 75 WP and (or) Apron™.

[004] Increased health and environmental concerns with the use of chemical fungicides have stimulated the search for alternative ways to control the disease using antagonistic microorganisms as biological control agents. Considerable research has been conducted on biological control of *Pythium* species using antagonistic bacteria and fungi (Martin and Loper 1999). Satisfactory biocontrol of *Pythium* damping-off has been achieved using seed treatment with rhizobacteria that are antagonistic to the pathogen (Bardin et al. 2003).

[005] *Rhizobium* spp. are soilborne bacteria that can establish a symbiotic relationship with legume plants. The symbiosis takes place in plant root nodules, in which the differentiated rhizobia known as bacteroids convert atmospheric nitrogen to a nitrogenous compound that can be used by the plant. *Rhizobium* species are host specific. For instance, *Rhizobium leguminosarum* bv. *viceae* Frank nodulates only plants from the genera *Pisum*, *Lens*, *Vicia*, and *Lathyrus*. Inoculation of legume seeds with *Rhizobium* prior to planting is commonly used to improve legume crop production by increasing nodulation, thereby reducing the need for application of nitrogen fertilizer (Brockwell et al. 1995).

[006] Several reports have indicated that *Rhizobium* and *Bradyrhizobium* have potential as biocontrol agents of plant pathogens. Rhizobia inhibited mycelial growth of plant pathogens such as *Aphanomyces euteiches*, *Phoma medicaginis* (Dileep Kumar et al. 2001), *Macrophomina phaseolina*, *Rhizoctonia solani* (Omar and Abd-Alla 1998), *Phytophthora cactorum* (Drapeau et al. 1973), *Fusarium* spp. (Drapeau et al. 1973; Omar and Abd-Alla 1998; Dileep Kumar et al. 2001), and *P. ultimum* (Ozkoc and Deliveli 2001). In addition to in vitro inhibition, some *Rhizobium* strains reduced disease severity caused by *Phytophthora clandestina* (Simpfendorfer et al. 1999), as well as *Fusarium solani*, *M. phaseolina*, and *Rhizoctonia solani* (Siddiqui et al. 2000), in greenhouse experiments in which soil was artificially infested with the pathogen. In other studies, *Rhizobium* inoculation effectively suppressed diseases caused by *F. solani* (Estevez de Jensen et al.

2002), *Fusarium oxysporum*, *Rhizoctonia bataticola*, and *Pythium* sp. (Nautiyal 1997) in soil naturally infested with these pathogens.

[007] What is needed are biocontrol agents for *Pythium* spp., particularly biocontrol agents that will protect the crops from disease caused by *Pythium ultimum*.

Summary of the Invention

[008] According to the present invention, strains of the nitrogen-fixing bacteria *Rhizobium leguminosarum* biovar *viceae* are utilized to control *Pythium* infection on crops. The invention relates in particular to microbial pure cultures of four such strains, identified herein as R5, R12, R20 and R21, which were deposited on July 20, 2004 with the International Depository Authority of Canada (IDAC), 1015 Arlington Street, Winnipeg, Manitoba, R3E 3R2, Canada, under the auspices of the Budapest Treaty, under the following IDAC Deposit Accession numbers:

R5: IDAC 200704-04;

R12: IDAC 200704-03;

R20: IDAC 200704-02;

R21: IDAC 200704-01.

[009] According to one embodiment, the invention provides an antifungal composition comprising bacteria of at least one isolated *Rhizobium leguminosarum* biovar *viceae* strain effective in inhibiting growth of *Pythium ultimum*.

[010] According to another embodiment, the invention is directed to a method for treating or protecting a susceptible plant from *Pythium ultimum*. The plant or part thereof, or soil surrounding the plant, is contacted with an effective amount of at least one *Rhizobium leguminosarum* biovar *viceae* strain which has suppressive activity against *Pythium ultimum*.

[011] The bacterial strains may be selected on the basis of their ability to inhibit the colonization of *Pythium ultimum* on an agar plate. When a bacterial strain is said to "inhibit the colonization of *Pythium ultimum* on an agar plate" means that no mycelial growth of the fungus occurs on a streak of the bacterial strain laid down four centimeters distant from a *Pythium ultimum*-colonized potato dextrose agar plug on the plate, following incubation of the plate at room temperature for five days. The assay technique is described in more detail below.

[012] According to preferred embodiments of the invention, the *Rhizobium leguminosarum* biovar *viceae* strain is effective in inhibiting growth of *Pythium* sp. "group G", a sterile form of *Pythium ultimum*, and the strains are selected on the basis of their ability to inhibit the colonization of *Pythium* sp. "group G" on an agar plate. Plants susceptible to *Pythium* sp. "group G" are treated or protected.

[013] As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

[014] "Antifungal" means the ability to inhibit the growth of or kill fungi. It should be noted that a biological control agent can act in an antifungal manner by not only exerting a direct effect on a fungal pathogen, but also in an indirect manner, such as by competing with the pathogen for nutrient. Both such direct and indirect actions are understood to be "antifungal".

[015] As used herein, "biovar" or "biological variant" (or the abbreviation "bv.") means a strain of a bacterium that is differentiated by biochemical or other non-serological means from another strain. A "strain" is a subset of bacterial species differing from other bacteria of the same species by some minor but identifiable difference.

[016] As used herein, "biological control" is defined as control of a pathogen or insect by the use of a second organism.

[017] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others.

[018] The term "culturing" refers to the propagation of organisms on or in media of various kinds.

[019] A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

[020] An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be applied in one or more applications. In terms of

treatment and protection, an "effective amount" is an amount sufficient to ameliorate, stabilize, reverse, slow or delay progression of a fungal infection.

[021] An "isolate" is a pure culture derived from a heterogeneous, wild population of microorganisms.

[022] The term "isolated" is used interchangeably with "biologically pure" and means separated from constituents, cellular and otherwise, in which the strain or metabolite is normally associated with in nature.

[023] As used herein, "*Pythium ultimum*" is meant to include all forms of the species of this name, including but not limited to *Pythium* sp. "group G".

[024] By "suppressive activity" of a biological control agent against a fungal pathogen is meant the ability of the agent to ameliorate, stabilize, reverse, slow or delay progression of an infection by the fungal pathogen.

[025] "Whole broth culture" refers to a liquid culture containing both cells and media.

Detailed Description of the Invention

[026] We have found that *Pythium* diseases may be controlled by strains of the nitrogen-fixing bacteria *Rhizobium leguminosarum* bv. *viceae*. In one study, fifty-six percent of strains of *R. leguminosarum* bv. *viceae* obtained from field pea and lentil nodules were found to improve emergence of sugar beet seedlings in soil artificially infested with the pathogen *Pythium* sp. "group G" in indoor experiments. Strains tested as seed treatments in diseased fields effectively controlled damping-off of sugar beet and field pea caused by *Pythium* spp. The effectiveness of the *Rhizobium* strains is similar to that of *Pseudomonas fluorescens* Migula LRC 708, a biological control agent against *Pythium* damping-off of sugar beet, field pea, canola, and safflower (Bardin et al. 2003). The biological control activity of the *Rhizobium* strains is not host dependent, as they are effective agents for both legume (pea) and nonlegume (sugar beet) plants.

[027] According to the present invention, *R. leguminosarum* bv. *viceae* strains are utilized to control or alleviate *Pythium* infection on crops. *Rhizobium leguminosarum* bv. *viceae* strains are useful for promoting plant health by protecting inoculated seeds from attack

by *Pythium ultimum*, and *Pythium* sp. "group G" in particular, thereby reducing incidence of damping-off diseases.

[028] The bacteria *Rhizobium leguminosarum* bv. *viceae* comprising the active agent of the invention may be isolated from root nodules of host legumes such as pea and lentil. The bacteria are collected from root nodules which are removed from the plants and crushed. The nodule contents are plated on an appropriate medium to support the growth of the bacteria. The bacteria are cultured under conditions favoring the growth thereof. Conditions for culturing *Rhizobium leguminosarum* strains are known to those skilled in the art, and are exemplified in the Examples which follow. Colonies are isolated from the culture.

[029] The antagonistic activity of isolates against *Pythium ultimum* may be determined using the dual-culture technique. Each bacterial strain is streaked on a medium which will support the growth of *Pythium ultimum*, e.g., Tryptone Yeast Extract agar near the edge of a Petri dish (9 cm diameter). After incubation for one day at room temperature, a mycelial plug (0.6 mm diameter) of *Pythium ultimum* from a 48-hour culture grown on potato dextrose agar is placed in the center of each dish containing the bacterial streaks. The plates are incubated for five days at room temperature (20 ± 2 °C) and the inhibitory effect of each bacterial strain is determined by measuring the inhibition zone of mycelial growth. The inhibitory effect is scored as positive where the *Pythium* growth stops on or before the bacterial streak line.

[030] The bacteria may be utilized in the form of cultures of bacteria, such as a suspension in a whole broth culture, to prepare appropriate compositions for ground treatment, plant treatment, soil and/ or growing media treatment, or seed treatment.

[031] The compositions containing the bacteria as the sole active ingredient, or as a combination with one or more other active ingredients, are prepared in known manner, such as by using standard fermentation methods, processes and equipment, followed by homogeneously mixing and/or grinding the active ingredients with extenders, growth media ingredients (for example such as nutrients, stabilizers, buffering systems, plant growth hormones, and pH adjustment ingredients) and liquid or dry organic or inorganic carriers. Suitable carriers include sterilized and sanitized liquid carriers; pre-sterilized (irradiated or steam sterilized) and non-sterilized peat powders; granulated, spheronized or pelletized peat,

clay; and other extenders, filler pigments or minerals. Other carriers for granular formation include talc, gypsum, kaolin, attapulgite, montmorillonite, bentonite, wood flour, ground corn cob grits, starch, cellulose, and bran. The formulations can also contain additives such as adhesives, stickers, binders, polymers and other adjuvants applicable to agricultural or horticultural applications. Stickers or binders may comprise, for example, ethylene glycol, mineral oil, polypropylene glycol, polyvinylacetate, lignosulfonate, polyvinyl alcohol, polyvinylpyrrolidone, graphite, gum Arabic, methyl cellulose, and sucrose.

[032] The compositions of this invention can be formulated in powder or granular form by mixing together all the components, including any carrier and/or other additive(s) which may be utilized until a homogeneous mixture is formed. A sticker, if employed, may then be added and the entire mass mixed again until it has become essentially uniform in composition. The composition may or may not be formulated in a pre-sterilized carrier system.

[033] The optimum concentration of *Rhizobium leguminosarum* bv. *viceae* employed in the compositions of the invention for a particular application can be readily determined by those skilled in the art. In general, the concentration of bacteria can range from about 0.001 to about 1%, preferably from about 0.01 to about 0.5%, more preferably from about 0.05 to about 0.1%, by weight.

[034] In the case of a liquid formulation, an aqueous liquid nutrient medium may be utilized, optionally comprising adjuvants such as stickers, stabilizers and colorants.

[035] The composition of the invention may contain, as additional active agents, cells, spores or propagules of other biological control agents, one or more chemical fungicides, or one or more other pesticidal materials, such as insecticides. The chemical fungicide may be selected on the basis of its activity against *Pythium* spp., or may be selected on the basis of activity against other fungal pathogens.

[036] The method of the invention comprises applying to plants an antifungal effective amount of a composition containing *Rhizobium leguminosarum* bv. *viceae*. The composition is most advantageously applied to roots or seeds. The composition may also be utilized as ground treatments in fields or greenhouses. They may be applied to soil surrounding plants, or applied to soil into which seeds or seedlings are planted.

[037] The compositions are applied by methods which include, for example, seed treatments, spray applications, in-furrow applications, soil and growing media inoculation, application through irrigation system, and the like. The compositions can be applied as stand alone or with the standard chemical treatments to control *Pythium*.

[038] When employed as a seed dressing, the amount of composition is applied such that the seed is coated with a concentration of bacteria adequate to provide protection against *Pythium* spp. The actual amount to utilize depends on the nature and size of the seed, the amount of the protection desired, the local soil conditions, and other factors which may be taken into account in selecting the appropriate dosage of bacteria. Appropriate application rates of bacteria in terms of colony forming units (cfu) per seed are as follows: large seeded crops - from about 10^4 to about 10^8 for legumes, and from about 10^6 to about 10^7 for non-legumes; medium size-seeded crops - from about 10^3 to about 10^7 for legumes, and from about 10^5 to about 10^6 for non-legumes; small seeded crops - from about 10^2 to about 10^6 for legumes, and from about 10^4 to about 10^5 for non-legumes. Greater concentrations of bacteria may be applied.

[039] Seeds may be bacterized according to the present invention by steeping the seeds in a suspension of bacteria for an appropriate time, e.g. one hour. According to one such technique, bacterial slurries are prepared by adding 3 ml of 1% methyl cellulose to tryptone yeast medium plates on which the culture is grown, and gently scraping the culture off the plate. For seed treatment, seeds are soaked for, e.g., 20 minutes in the bacterial slurry. The bacterial concentration in the slurry may be determined by plating serial dilutions on tryptone yeast medium (Beringer, 1974).

[040] According to one embodiment, an inoculant composition may be prepared for application to seeds, using ground peat. Methods for the preparation of inoculant compositions of *Rhizobium* sp. for inoculation of crops, e.g., legumes, to increase nitrogen fixation are known. See, e.g., U.S. Pat. 5,484,464, the entire disclosure of which is incorporated herein by reference. One such inoculant composition is prepared from sterilized powdered peat with a moisture content of 6-20%, with or without a sticker. Using aseptic techniques, a suspension of *Rhizobium leguminosarum* bv. *viceae* is added to the peat at a rate of from about 10^5 to about 10^8 colony forming units of bacteria per gram of peat.

[041] The composition may be utilized to protect any crop which is susceptible to infection and damage by *Pythium ultimum,* and *Pythium* sp. "group G" in particular. Such crop species include, for example, sugar beet (*Beta vulgaris* L.), field pea (*Pisum sativum* L.), lentil (*Lens* spp.), safflower (*Carthamus tinctorius* L.), canola (*Brassica rapa* L. and *Brassica napus* L.), chickpea (*Cicer* spp.), sunflower (*Helianthus* spp.), alfalfa (*Medicago* spp.), soybean (*Glycine* spp.), and field bean (*Vicia faba*).

Examples

[042] In the following Examples, the viable counts of bacterial agents in slurries and on seeds were expressed as mean cfu ± SE. Shoot dry mass and emergence data of both indoor and field experiments were analyzed statistically using the Statistic Analysis Software package Version 6.0.9 (Examples 1-6) or Version 8.2 (Example 7) (SAS Institute Inc., Cary, N.C.). Analysis of variance was done using the general linear model procedure. Differences between treatments were analyzed using Fisher's least significant difference (LSD) test. All analyses were performed at the $P = 0.05$ level.

Example 1
Isolation of *Rhizobium* Strains

[043] Strains of *R. leguminosarum* bv. *viceae* were isolated from root nodules of field pea and lentil grown in southern Alberta, Canada, as follows. Roots from two plants per crop were washed in water to remove soil particles. The nodules were excised, surface sterilized in 2% sodium hypochlorite for 1 min, washed eight times in sterile distilled water, and crushed with a sterile spatula in 200 µL sterile water. The nodule contents were plated on tryptone – yeast extract medium (TY; Beringer 1974) containing 1.5% agar (Difco, Detroit, Mich.). Following incubation for 3–4 days at room temperature (20 ± 2 °C), a colony from each plate was purified by three successive single colony isolations. Eighteen strains of *R. leguminosarum* bv. *viceae* were isolated in this manner. Ten strains were isolated from field pea root nodules and eight from lentil root nodules. Of these strains, 8 showed no potential for control of *Pythium* damping-off of sugar beet in preliminary indoor experiments and were not tested further. The identities of the 10 remaining strains, 8 from pea and 2 from lentil (Table 1), were confirmed by performing plant nodulation experiments (see below) and by streaking the bacteria on Luria-Bertani (LB, Miller 1972) agar. The

strains did not grow on LB, which is consistent with the fact that *R. leguminosarum* is sensitive to the high salt concentration contained in this media.

Example 2:
Plant Nodulation by *Rhizobium* Strains

[044] The ability of the ten *Pythium*-antagonizing *Rhizobium* isolates to form nitrogen-fixing nodules on pea and lentil plants was determined in a nitrogen-free medium. Seeds were surface sterilized for 5 min in 50% aqueous sodium hypochlorite, washed 8–10 times with sterile distilled water, and germinated for 2 days in the dark on water agar (1.5%) in Petri dishes. Six seeds were planted in each sterile Leonard jar assembly (Leonard 1943), containing a mixture of quartz sand and vermiculite (1:1; *v/v*) saturated with nitrogen-free Jensen's nutrient solution (Vincent 1970). Two days after planting the seeds, each jar was inoculated with 10 mL of an aqueous bacterial suspension (10^7–10^8 cfu/10 mL) of *Rhizobium* or with 10 mL water for the uninoculated control. Each treatment was performed in duplicate. The experiment was repeated once. The plants were kept in a growth cabinet in a 16 h light (20 °C): 8 h dark (15 °C) cycle. They were watered with sterile distilled water as required. Lentil and pea plants were collected 26 and 27 days after inoculation, respectively. The shoots of the plants were excised, dried in a 60°C oven for 5 days, and weighed to determine nodulation efficacy.

[045] Plants inoculated with each of the ten *Pythium*-antagonizing *Rhizobium* strains were green and healthy compared with the brown and stunted plants of the uninoculated control. There were pinkish nodules formed on the roots of plants inoculated with the strains, while no nodules developed on the roots of uninoculated plants. In addition, the dry shoot masses of the inoculated plants were significantly ($P < 0.05$) greater than those of uninoculated plants (Table 1). Strain R12 was effective in establishing a beneficial symbiotic interaction with both lentil and pea plants (Table 1).

Table 1: Source of *Rhizobium leguminosarum* bv. *viceae* strains and their nodulation efficacy on field pea and lentil.

Rhizobium leguminosarum bv. *viceae**	Plant source†	Shoot dry mass (% control) Pea	Lentil
Strain			
R3	*Pisum sativum*	245a‡	nd
R4	*Pisum sativum*	243a§	nd
R5	*Pisum sativum*	295a‡	nd
R7	*Pisum sativum*	288a‡	nd
R8	*Pisum sativum*	263a‡	nd
R9	*Pisum sativum*	294a‡	nd
R12	*Lens culinaris*	273a‡	306a¶
R19	*Lens culinaris*	nd	327a¶
R20	*Pisum sativum*	235a§	nd
R21	*Pisum sativum*	224a§	nd
Uninoculated control		100b	100b

Note: Nodulation efficacy is expressed as percent increase in shoot dry mass of a pea or lentil plant inoculated with a *R. leguminosarum* bv. *viceae* strain compared with the uninoculated control (100%). The values represent the means of 12 plants (two pots of 6 plants each) from two independent experiments. Means within the same column followed by the same letter are not significantly different at $P = 0.05$ level (Fisher's LSD test). nd, not determined.

*Strains of *R. leguminosarum* bv. *viceae* isolated from the root nodules of pea or lentil plants collected in southern Alberta.

†Plant nodules where the bacteria were isolated.

‡Dry shoot mass of the uninoculated control was 202.3 mg/plant.

§Dry shoot mass of the uninoculated control was 271.0 mg/plant.

¶Dry shoot mass of the uninoculated control was 83.0 mg/plant.

Example 3
Control of *Pythium* Damping-off of Sugar Beet by *Rhizobium* Strains (dual culture experiments)

[046] The antagonistic activity of the ten remaining *R. leguminosarum* bv. *viceae* strains against *Pythium* sp. "group G" strain LRC 2105 (Huang et al. 1992) was determined by streaking a *Rhizobium* strain 4 cm away from a potato dextrose agar (PDA) plug colonized by *Pythium* on TY agar plates (dual culture technique). After incubation at room temperature for 5 days, the inhibitory activity of the *Rhizobium* strain was determined by measuring the zone of mycelial growth inhibition around the bacterial streak. Three ratings were used: –, no inhibition zone and growth of *Pythium* over the bacterial streak; +, no inhibition zone, but no growth of *Pythium* on the bacteria streak; and ++, 1–5 mm inhibition zone. There were three

replicates for each treatment and the experiment was repeated once. Strain R5 was rated as ++. It was the only strain showing antagonistic effects to *Pythium* sp. "group G", with formation of a small zone of inhibition 2 mm in size. The other 9 strains did not exhibit zones of inhibition but were able to prevent colonization of the bacterial streak by the pathogen and were therefore rated as + (slight inhibition).

Example 4
Test for Protease Production by *Rhizobium* Strains

[047] Protease production was determined by incubating colonies of *R. leguminosarum* bv. *viceae* on skim milk agar plates (Dunne et al. 1997) for 5 days at room temperature (20 ± 2 °C). Protease activity was compared with the protease positive strain, *Pseudomonas fluorescens* Migula LRC 708, which degrades casein and causes clearing of the skim milk agar plate (Bardin et al. 2003). There were three replicates for each treatment and the experiment was repeated once. Unlike strain *P. fluorescens* 708, none of the *Rhizobium* strains tested showed protease activity, as they failed to produce clearing zones around the colonies when plated on the skim milk agar plates. Thus, production of extracellular proteases is not the mechanism of action of the *Rhizobium* strains.

Example 5
Seed Treatment by *Rhizobium* Strains (indoor experiments)

[048] The strains of *R. leguminosarum* bv. *viceae* were further tested as seed treatments for control of *Pythium* damping-off of sugar beet in nonsterile soil. Bacterial cultures were grown on TY agar in Petri dishes (5.5 cm in diameter) for 48 hours at room temperature. The bacterial culture was resuspended in 3 mL of 1% methyl cellulose (MC) (Aldrich Chemical, Milwaukee, Wis.) by scraping the agar surface gently with a spatula. This resulted in bacterial slurries with a concentration averaging $3.9 \times 10^9 \pm 0.5 \times 10^9$ (mean ± SE) cfu/mL. Sugar beet (*Beta vulgaris* 'HM Bergen') (Novartis Seeds – Hilleshög, Longmont, Colo.) seeds were soaked for 20 minutes in the MC–bacterial slurry and were seeded directly into soil artificially infested with *Pythium* sp. "group G" strain LRC 2105. The soil consisted of 3 parts topsoil (Bzdell Soil Service, Lethbridge, Alberta.), 1 part sand (Tollestrup Construction, Lethbridge, Alberta), and 1 part peat moss (Premier Horticulture, Red Hill, PA.). The *Pythium* inoculant was prepared in pans containing a sterile mixture of 150 g wheat bran (Ellison Milling, Lethbridge, Alberta), 150 g corn meal (McCormick,

London, Ontario), and 300 mL distilled water. Twenty plugs (8 mm in diameter) of a 48-hour-old PDA culture of *Pythium* sp. "group G" were placed in each pan. After incubation for 2 weeks at room temperature in the dark, the wheat bran – corn meal mix was completely colonized by the pathogen. The *Pythium* inoculum was air-dried at room temperature for 4 days and ground using a Thomas-Wiley model 4 laboratory mill (Thomas Scientific, Philadelphia, Pa.) equipped with a 1-mm mesh screen. The soil, artificially infested with *Pythium* sp. "group G" at a concentration of 2 g inoculum/kg soil, was used to fill root trainers (Spencer-Lemaire Industries, Edmonton, Alta.), each containing 17 books of six cells per book. One sugar beet seed was planted per root trainer cell at a depth of 1.5 cm. Uninoculated seeds were also planted in non-infested soil. The root trainers were soaked in a water-filled tray until the soil was saturated by capillary action, and were then placed in propagator trays (The Stewart Company, Croydon, Surrey, UK) to create a high-moisture environment. The propagator trays were kept in a growth chamber in a 16 hour light (20 °C): 8 hour dark (15 °C) cycle. In each experiment there were three replicates per treatment and 18 seeds per replicate. The treatments were arranged in a completely randomized design. Seedling emergence was recorded 14 days after planting, and data from bacterial seed treatments were compared with the uninoculated control. Each set of experiments was repeated twice. Non-germinated seeds were collected, washed with sterile water, surface sterilized in 70% ethanol for 2 min, and plated on PDA in Petri dishes. The fungi isolated from the seeds were purified on PDA, and the genus of each fungus isolated was determined based on morphological characteristics.

[049] Emergence of uncoated sugar beet seeds planted in the *Pythium*-infested soil used in the indoor experiment was reduced by 37% (21% emergence) compared with seeds planted in non-infested soil (58% emergence). *Pythium* was reisolated from 65% of the non-germinated seeds tested. Despite the lack of clear antagonism against *Pythium* sp. "group G" in the *in vitro* assays, seed treatment with the *Rhizobium* strains significantly ($P < 0.05$) increased emergence of sugar beet in soil artificially infested with *Pythium* sp. "group G" compared with the untreated control (Table 2). The most effective strains for biological control of damping-off of sugar beet were R3, R4, R5, R7, R12, R20, and R21 (Table 2).

Table 2: Control of *Pythium* damping-off of sugar beet (*Beta vulgaris*) by seed treatment with *R. leguminosarum* bv. *viceae* (indoor experiments).

Rhizobium leguminosarum bv. *viceae*	
Strain	Emergence (%)
R12	52a
R20	46ab
R21	44ab
R4	43abc
R3	42abc
R7	42abc
R5	41abc
R9	39bcd
R8	36bcd
R19	32cd
Untreated control	21e

Note: Emergence of sugar beet seedlings was determined 14 d after planting. Means are of three replicates from three independent experiments. All experiments gave similar results. Means followed by the same letter are not significantly different at $P = 0.05$ level (Fisher's LSD test).

Example 6:
Control of *Pythium* Damping-off of Sugar Beet and Field Pea by *Rhizobium Leguminosarum* bv. *Viceae* Strains (field experiments)

[050] The selected strains of *R. leguminosarum* bv. *viceae* (R12, R20, and R21) effective against *Pythium* damping-off of sugar beet in indoor experiments were tested for control of damping-off of sugar beet and field pea in fields naturally infested with *Pythium* spp. at the Lethbridge Research Centre, Alberta. The efficacy of the *Rhizobium* strains was compared with the biocontrol agent *P. fluorescens* 708, which was shown to improve emergence of sugar beet, field pea, canola, and safflower in soil naturally infested with *Pythium* spp. (Bardin et al. 2003). The seeds were coated with the bacterial slurry as described previously using 2.4 and 9.5 mL bacterial slurry/100 seeds of sugar beet and field pea, respectively. The seeds were dried overnight at room temperature on a metallic mesh, which was placed on a paper towel to absorb the excess slurry. The number of bacteria coated onto the seeds was similar for the four bacterial strains, ranging from $1.4 \times 10^6 \pm 0.2 \times 10^6$ to $2.3 \times 10^7 \pm 0.2 \times 10^7$ cfu/seed for sugar beets and $3.0 \times 10^7 \pm 0.3 \times 10^7$ to $1.2 \times 10^8 \pm 0.4 \times 10^8$ cfu/seed for field peas. The coated seeds were then stored at 4 °C until planting. Bacterial counts on the seeds were determined by vortexing five coated seeds in 5 mL of

distilled sterile water for 30 seconds, and by plating serial dilutions on TY agar medium in Petri dishes for *Rhizobium* strains and on PDA in Petri dishes for *P. fluorescens*. Each bacterial count was performed in duplicate, and bacterial determinations for each treatment were performed twice. The *Rhizobium*-treated and untreated seeds were machine seeded into 0.9 m wide × 5.0 m long plots made of 4 rows of 100 seeds/row in a field naturally infested with *Pythium* spp. The plots were trimmed to 3.5 m after all seedlings emerged. Treatments were arranged in a randomized complete block design, with six replicates per treatment. The field experiments were performed twice, once in May and again in August 2001 in Fairfield Farm, Lethbridge, Alberta. Seedling emergence was recorded 4 weeks after planting and was compared with the uninoculated and fungicide controls. The amount of Thiram™ for the fungicide-treated seeds was 90 g/25 kg sugar beet seeds and 30 g/25 kg field pea seeds.

[051] Treatment of pea seeds with *R. leguminosarum* bv. *viceae* strain R12 or R20 caused a significant ($P < 0.05$) increase in seedling emergence compared with the untreated control in the two field experiments (Table 3). The efficacy of the two *Rhizobium* strains was similar to that of seed treatments with the rhizobacterium *P. fluorescens* 708. *Rhizobium leguminosarum* bv. *viceae* R21 significantly increased pea seedling emergence compared with the untreated control in the second (August 2001) but not in the first (May 2001) field experiment. The level of seedling emergence in the second field experiment was lower but not significantly ($P > 0.05$) different from that of *R. leguminosarum* bv. *viceae* R20 and *P. fluorescens* 708. None of the bacterial treatments were as effective as the fungicide Thiram™ for control of damping-off of field peas.

[052] In the sugar beet experiments conducted in May and August of 2001, seed treatment with *R. leguminosarum* bv. *viceae* R12, R20, or R21 increased seedling emergence compared with the untreated control (Table 3). This increase was significant ($P < 0.05$) in the August experiment. In both the May and August field experiments, the percent emergence of the *Rhizobium*-treated seeds was not significantly different from that of seeds treated with *P. fluorescens* 708. *Rhizobium leguminosarum* bv. *viceae* R12 and R21 were as effective as the fungicide treatment for protection of sugar beet seedlings against *Pythium* damping-off in the August field experiment, while *P. fluorescens* 708 was as effective as the fungicide treatment in the May field experiment (Table 3).

Table 3: Effect of bacterial seed treatment on field pea and sugar beet emergence in a field naturally infested with *Pythium* spp.

	Emergence (%)			
	Pea (*Pisum sativum*)		Sugar beet (*Beta vulgaris*)	
Treatment	May 2001	August 2001	May 2001	August 2001
Untreated control	41.4d	12.8d	10.0c	6.5d
Fungicide (Thiram™)*	71.4a	48.3a	28.6a	27.0a
R12	54.3bc	34.4b	18.6bc	24.7abc
R20	51.4c	30.6bc	14.3bc	21.9c
R21	37.1d	23.7c	17.1bc	24.9ab
Pseudomonas fluorescens 708	60.0b	29.6bc	20.0ab	23.5bc

Note: Seedling emergence was determined 4 weeks after planting. Values are means of six replicates. Means within each column followed by the same letter are not significantly different at $P = 0.05$ level (Fisher's LSD test).

*The concentration of Thiram™ was 90 g/25 kg sugar beet seeds and 30 g/25 kg field peas.

Example 7:
Control of *Pythium* Damping-off of Pea and Lentil by *Rhizobium* Strains (field experiments)

[053] The strains of *Rhizobium leguminosarum* bv. *viceae* used for the study were 99A1, R12, R20, and R21. Strain 99A1 was originated from the commercial pea inoculant produced by Agrium, Inc. Calgary, Alberta. Bacterial cultures were grown on tryptone-yeast agar (TYA) (Beringer, 1974) in Petri dishes for 48 h at room temperature (20±2°C). The resulting colonies were suspended in 5 ml per dish of 1% methyl cellulose (Sigma-Aldrich, Milwaukee, WI) in sterile distilled water, and scraped gently with a spatula to obtain bacterial slurries. Seeds of field pea cv. Trapper and lentil cv. Laird were soaked for 20 minutes in the slurries, spread on a metallic mesh sheet with paper towel underneath to absorb the excess slurry, and air-dried overnight under a fume hood. Enumeration of bacteria coated onto seeds was done by placing 5 seeds in a test tube with 5 ml of sterile distilled water, vortexing for 30 sec, and plating serial dilutions on TYA, 0.1 ml per 9-cm dish. After incubation at room temperature for 3 days, bacterial colonies developed in each dish were counted. There were two replicates for each treatment.

[054] Field experiments were conducted at the Agriculture and Agri-Food Canada Research Centre near Lethbridge, Alberta, Canada, in a field naturally infested with *Pythium* spp. (predominantly *Pythium* sp. 'group G'). For the pea experiment, seeds were planted using a plot seeder on 28 May 2004, in 4-row plots with a row length of 5 m, a row spacing of 22.5 cm, and a plant spacing of 5 cm (i.e. 20 seeds/m). Untreated seeds and fungicide-treated seeds (Thiram™ at the rate of 30 g/25 kg seed) (Gustafson; Calgary, Alberta, Canada) were used as controls. Treatments were arranged in a randomized block design with 6 replicates. For the lentil experiment, seeds were planted on the same date and using the same parameters as for field pea.

[055] Seedling emergence for each plot was determined. The number of healthy seedlings and the number of wilted seedlings were counted in the middle 3 m of each row, and the percent loss due to pre-emergent and post-emergent damping-off were calculated, as well as the final stand establishment. The causal agent of seedling death was determined by collecting 10 non-emerged seedlings and all of the wilted seedlings from each plot, washing in running water, surface sterilizing in 70% ethanol for 90 sec, incubating on potato dextrose agar (PDA) in Petri dishes at room temperature for 7 days, and examining the organisms derived from each sample. Results of reisolation of diseased seedlings were used to calculate the incidence of damping-off due to *Pythium* spp. for each plot.

[056] Seedling height for each plot was determined (6-node stage for peas; 5-node stage for lentils). For each row, ten seedlings were randomly selected and the distance from the first node to the terminal branch of each seedling was measured.

[057] Differences between treatments for incidence of damping-off, seedling emergence and seedling height data were analyzed for statistical significance using analysis of variance (ANOVA) and means of treatments for each set of data were separated using Duncan's multiple range test at the P=0.05 level. All statistical analyses were done using SAS Statistical Analysis Software, Version 8.2 (SAS Institute Inc., Cary, North Carolina 2001). The results are set forth in Tables 4 -7.

Table 4: Effect of seed treatment with *Rhizobium leguminosarum* biovar *viceae* strains on damping-off of field pea.

Treatment	Damping-off (%)[1] Pre-emergent	Post-emergent	by *Pythium*	Final Stand (%)[1]
Control	62.0 a[2]	0.2	55.4 a[2]	37.8 a[2]
99A1	62.0 a	0.3	54.8 a	37.7 a
R12	55.2 ab	0.1	47.0 ab	44.7 ab
R20	51.6 b	0.7	43.4 b	47.9 b
R21	28.5 c	0.8	23.7 c	70.7 c
Fungicide (Thiram)	20.3 c	0.6	16.7 c	79.1 c

[1] Based on 60 seeds planted per 3-meter section of row, 4 rows per plot.
[2] Means within each column followed by the same letter are not significantly different (Duncan's multiple range test; $P>0.05$).

Table 5: Effect of seed treatment with *Rhizobium leguminosarum* biovar *viceae* strains on seedling height of field pea.

Treatment	Plant Height (cm)[1]
Control	11.1 a[2]
R20	11.1 a
99A1	12.2 ab
R12	12.8 b
R21	12.8 b
Fungicide (Thiram)	14.6 c

[1] Distance from the first node to the terminal branch; measured at the 6-node stage (4 weeks after planting). Based on random selection of 10 seedlings per row, 4 rows per plot.
[2] Means within each column followed by the same letter are not significantly different (Duncan's multiple range test; $P>0.05$).

Table 6: Effect of seed treatment with *Rhizobium leguminosarum* biovar *viceae* strains on damping-off of lentil.

Treatment	Damping-off (%)[1] Pre-emergent	Post-emergent	by *Pythium*	Final Stand (%)[1]
99A1	50.6 a[2]	2.3	38.1 a[2]	47.1 a[2]
Control	46.3 ab	2.7	35.3 ab	51.0 ab
R21	44.1 ab	1.5	31.5 bc	54.4 bc
R20	43.1 b	3.3	30.2 bc	53.6 bc
R12	39.9 bc	2.2	27.8 cd	57.9 cd
Fungicide (Thiram)	34.2 c	2.6	22.8 d	63.2 d

[1] Based on 60 seeds planted per 3-meter section of row, 4 rows per plot.
[2] Means within each column followed by the same letter are not significantly different (Duncan's multiple range test; P>0.05).

Table 7: Effect of seed treatment with *Rhizobium leguminosarum* biovar *viceae* strains on seedling height of lentil.

Treatment	Plant Height (cm)[1]
Control	11.2 a[2]
R20	11.2 a
99A1	11.2 a
R12	11.3 a
R21	11.3 a
Fungicide (Thiram)	11.4 a

[1] Distance from the first node to the terminal branch; measured at the 5-node stage (4 weeks after planting). Based on random selection of 10 seedlings per row, 4 rows per plot.
[2] Means within each column followed by the same letter are not significantly different (Duncan's multiple range test; P>0.05).

[058] Enumeration of bacteria coated onto seeds revealed similar numbers of bacteria per seed for all four strains of *R. leguminosarum* bv. *viceae*, for both field pea and lentil. The number of colony-forming units (cfu) per seed ranged from 2.3×10^5 to 2.9×10^5 for pea, and from 2.2×10^5 to 5.1×10^5 for lentil.

[059] Reisolation of diseased pea seedlings revealed that 84% of the seedlings killed by pre- and post-emergent damping-off were infected with *Pythium* spp., whereas the remaining seedlings were colonized by *Fusarium* spp. Treatment of pea seeds with R20, R21 or Thiram™ significantly ($P<0.05$) reduced pre-emergent damping-off compared to the untreated control (Table 4). The incidences of pre-emergent damping-off for the treatments of R20, R21 and Thiram™ were 51.6%, 28.5% and 20.3%, respectively, compared to 62.0% for the untreated control. There was no significant difference in incidence of pre-emergent damping-off between the treatments of R21 and Thiram™. Damping-off losses of pea due to *Pythium* spp. alone followed a similar trend, ranging from 16.7% in the fungicide treatment and 23.7% in the treatment of R21, to 55.4% in the untreated control (Table 4). The height of pea plants arising from seed treated with R12, R21 or Thiram™ was significantly ($P<0.05$) greater than for plants arising from untreated seed (Table 5). Seedling height for the treatments of R12 and R21 was 12.8 cm for both *Rhizobium* strains, compared to 14.6 cm for the treatment of Thiram™, and 11.1 cm for the untreated control.

[060] For the lentil experiment, results of plating of diseased seedlings showed that 68% were infected with *Pythium* spp., 22% were infected with *Botrytis cinerea*, and the remainder was colonized by *Fusarium* spp. Treatment of lentil seeds with R20, R12 or

Thiram™ significantly (P<0.05) reduced incidence of pre-emergent damping-off compared to the untreated control (Table 6). The disease incidences for the treatments of R20, R12 and Thiram™ were 43.1%, 39.9% and 34.2%, respectively, compared to 50.6% for the untreated control. Incidence of damping-off of lentil due to *Pythium* spp. alone followed the same trend, ranging from 22.8% in the fungicide treatment and 27.8% in the treatment of R12, to 38.1% in the untreated control (Table 6). No significant differences in seedling height of lentil were detected among the treatments (Table 7).

[061] Among the four strains of *R. leguminosarum* bv. *viceae* tested, strains R20 and R21 from pea were most effective for control of damping-off of pea (Table 4), whereas the strain R12 from lentil was most effective for control of damping-off of lentil (Table 6). The study on pea also suggests that the strains may have a growth promoting effect on seedlings, as seen in the case of increased height of pea seedlings for the treatments of R12 and R21 (Table 5).

[062] All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

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We claim:

1. A method for treating or protecting a plant susceptible to *Pythium ultimum* comprising contacting the plant or part thereof, or the soil surrounding the plant, with an effective amount of at least one *Rhizobium leguminosarum* biovar *viceae* strain which has suppressive activity against *Pythium ultimum*.

2. The method according to claim 1 wherein the at least one *Rhizobium leguminosarum* biovar *viceae* strain which has suppressive activity against *Pythium* sp. "group G".

3. The method according to claim 1 wherein the plant is in the form of a seed.

4. The method according to claim 1 wherein the plant part contacted comprises root.

5. The method according to claim 1 wherein the plant is selected from the group consisting of sugar beet, field pea, lentil, safflower, canola, chickpea, sunflower, alfalfa, soybean and field bean.

6. The method according to claim 1 wherein the plant is treated or protected from seed rot or damping-off.

7. The composition according to claim 2 wherein the strain inhibits the colonization of *Pythium* sp. "group G" on an agar plate.

8. The method according to claim 1 wherein the bacteria comprises the bacterium deposited under International Depository Authority of Canada Accession number IDAC 200704-01.

9. The method according to claim 1 wherein the bacteria comprises the bacterium deposited under International Depository Authority of Canada Accession number IDAC 200704-02.

10. The method according to claim 1 wherein the bacteria comprises the bacterium deposited under International Depository Authority of Canada Accession number IDAC 200704-03.

11. The method according to claim 1 wherein the bacteria comprises the bacterium deposited under International Depository Authority of Canada Accession number IDAC 200704-04.

12. The isolated *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-01.

13. The isolated *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-02.

14. The isolated *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-03.

15. The isolated *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-04.

16. An antifungal composition comprising bacteria of at least one isolated *Rhizobium leguminosarum* biovar *viceae* strain which has suppressive activity against *Pythium ultimum*.

17. The composition according to claim 16 wherein the at least one *Rhizobium leguminosarum* biovar *viceae* strain has suppressive activity against *Pythium* sp. "group G".

18. The composition according to claim 17 wherein the strain inhibits the colonization of *Pythium* sp. "group G" on an agar plate.

19. The composition according to claim 16 comprising one or more biologically inert components.

20. The composition according to claim 19 wherein the one or more inert component is selected from the group consisting of carrier materials, stickers, binders, adhesives, extenders, and mixtures thereof.

21. The composition according to claim 19 comprising cells or spores of other biological control agents, one or more chemical fungicides, or one or more pesticides.

22. The composition according to claim 16 wherein the bacteria strain is the *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-01.

23. The composition according to claim 16 wherein the bacteria strain is the *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-02.

24. The composition according to claim 16 wherein the bacteria strain is the *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-03.

25. The composition according to claim 16 wherein the bacteria strain is the *Rhizobium leguminosarum* biovar *viceae* strain deposited under International Depository Authority of Canada Accession number IDAC 200704-04.