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(54) Title: PSEUDOMONAS BIOCONTROL STRAINS

(57) Abstract

Mutant strains of *Pseudomonas* have been isolated which have enhanced biocontrol properties. The strains are particularly useful against *Rhizoctonia solani.*

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PSEUDOMONAS BIOCONTROL STRAINS

The present invention relates to the identification of mutant strains of *Pseudomonas* which have improved biocontrol properties. More specifically it relates to strains which are effective against plant pathogenic fungi.

It has been recognized that crops grown in some soils are naturally resistant to certain fungal pathogens. Furthermore, soils that are conducive to the development of these diseases can be rendered suppressive or resistant to the pathogen by the addition of small quantities of soil from a suppressive field (Scher and Baker (1980) Phytopathology 70: 412-417). Conversely, suppressive soils can be made conducive to fungal disease susceptibility by autoclaving, indicating that the factors responsible for disease control are biological. Subsequent research has demonstrated that root colonizing bacteria are responsible for this phenomenon which is known as biological disease control (Cook and Baker (1983), The Nature and Practice of Biological Control of Plant Pathogens; Amer. Phytopathol. Soc., St Paul, MN).

In many cases, the most efficient strains of biological disease controlling bacteria are fluorescent pseudomonads (Weller *et al.* (1983) Phytopathology, 73: 463-469). These bacteria have also been shown to promote plant growth in the absence of a specific fungal pathogen by the suppression of detrimental rhizosphere microflora present in most soils (Kloepper *et al.*(1981) Phytopathology 71: 1020-1024). Important plant pathogens that have been effectively controlled by seed inoculation with these bacteria include *Gaemannomyces graminis*, the causative agent of take-all in wheat (Cook *et al.*(1976) Soil Biol. Biochem 8: 269-273) and *Pythium* and *Rhizoctonia*, pathogens which cause damping off of cotton (Howell *et al.* (1979) Phytopathology 69: 480-482). *Rhizoctonia* is a particularly problematic plant pathogen for several reasons. Firstly, it is capable of infecting a wide range of crop plants and secondly, there are no commercially available chemical fungicides that are effective in controlling the fungus.

Many biological disease controlling *Pseudomonas* strains produce antibiotics that inhibit the growth of fungal pathogens (Howell *et al.* (1979) Phytopathology 69:480-482; Howell *et al.* (1980) Phytopathology 70: 712-715). These antibiotics have been implicated in the control of fungal pathogens in the rhizosphere. In particular, Howell *et al.* (Phytopathology 69: 480-482; 1979) disclose a strain of *Pseudomonas fluorescens* which was shown to produce an antibiotic substance that is antagonistic to *Rhizoctonia solani.* Indeed, several past studies have focused on the effects of mutations that result in the inability of the disease control bacterium to synthesize these antibiotics (Kloepper *et al.* (1981) Phytopathology 71: 1020-1024; Howell *et al.* (1983) Can. J. Microbiol. 29: 321-324). In these cases, the ability of the organism to control the pathogen is reduced, but not eliminated.

An important factor in biological control is the ability of an organism to compete in a given environment (Baker *et al.* (1982) Biological Control of Plant Pathogens, American Phytopathological Society, St. Paul, Minn., pages 61-106). Thus, it is desirable to obtain strains of biocontrol agents which are effective to control the growth of *Rhizoctonia solani* and other fungi and also able to aggressively compete with indigenous bacteria and microflora that exist in the rhizosphere of the plant.

The present invention is drawn to biocontrol strains of bacteria that are able to effectively control pathogenic attack on crop plants. The biocontrol strains of the invention produce at least one antifungal substance capable of inhibiting a broad spectrum of plant pathogens. Such strains have increased biocontrol properties and are able to aggressively compete in the plant rhizosphere. Methods of making the biocontrol strains as well as methods of using the strains for control of pathogenic attack on crops are described.

The present invention provides improved biocontrol strains which can be used to control pathogenic attack on crop plants. Such strains are able to aggressively compete in the plant rhizosphere as well as produce one or more antifungal substances that are effective against a broad spectrum of plant pathogenic fungi, particularly *Rhizoctonia*, more particularly *Rhizoctonia solani.*

The present invention further provides compositions comprising a pathogenically effective amount of an improved biocontrol strain according to the invention together with a suitable carrier, which can be used to control pathogenic attack on crop plants.

The invention further provides methods for for controlling or inhibiting the growth of a plant pathogenic fungus by applying an improved biocontrol strain according to the invention or a composition comprising same to (a) an environment in which the plant pathogenic fungus may grow, (b) a plant or plant part in order to protect said plant or plant part from a plant pathogenic fungus, or (c) seed in order to protect a plant which develops from said seed from a plant pathogenic fungus.

The biocontrol strains of the present invention are important for several reasons. Firstly, *Rhizoctonia solani* is a particularly pernicious plant pathogen. The affected plants include beans, wheat, tomato and potato, in addition to cotton. Secondly, there are almost no environmentally safe and effective fungicide treatments available for the protection of crops from *Rhizoctonia solani*. Therefore, the use of the disclosed biocontrol strains to control or prevent *Rhizoctonia solani* infections in crop plants provide the first environmentally really safe and effective method of control of this pathogen.

The biocontrol strains of the present invention are made by preparing a collection of insertion mutants as described in the Experimental section below. Such insertion mutant strains are then screened for *in vitro* fungal inhibition activity. Those strains which demonstrate the desired activity are then further characterized in greenhouse and field biocontrol assays.

In one embodiment of the invention, using molecular biological techniques, the plasmid pCIB116 was constructed. pCIB116 is suitable for use in transposon mutagenesis and was transferred to *Pseudomonas* strain CGA 267356 (a.k.a. 11c-1-38 - see EP 0 472 494 A2; ATCC # 55169) to generate a collection of insertion mutants. Suitable methods for transfer of pCIB116 and other such plasmids to *Pseudomonas* CGA 267356 are known in the art. Simon *et al.* (1983) Bio/Technology 1:784-791. Preferred methods for the transfer of DNA to *Pseudomonas* include conjugation from *E. coli* and electroporation. Maniatis *et al.*, (1989) Molecular Cloning, Ch. 1, Cold Spring Harbor Laboratory Press, New York.

In addition, the nature of the plasmid is not critical to the invention; it is sufficient that part of the transferred plasmid is able to transpose to various loci within the *Pseudomonas* genome and form a library of insertion transposon mutants which can subsequently be screened. Thus a library of approximately 10000 different insertion mutants of *Pseudomonas* were generated. These were tested for their ability to inhibit growth of the fungus *Neurospora in vitro.* Mutants were isolated which produced antifungal clearing zones which were distinctive from wild-type and these were tested for their ability to control infestation by the fungus *Rhizoctonia solani* of cotton in glasshouse tests. Two insertion mutants were found to provided better disease control against *Rhizoctonia* in tests on cotton. These two *Pseudomonas* strains have been deposited in connection with this application according to the regulations of the Budapest Treaty on January 21, 1994 with the Agricultural Research Service Culture Collection (NRRL), an International Deposit Authority recognized under the Budapest Treaty and given the accession numbers NRRL B-21172 and NRRL B-21173, respectively.

It is recognized that strains can be isolated which display the capacity to control a single or a range of fungal plant pathogens. Thus, in one embodiment of the invention strains are provided which have enhanced biocontrol properties against the fungus *Rhizoctonia solani.* In a further embodiment of the invention strains are provided which have enhanced biocontrol properties against a range of fungal plant pathogens.

In a further embodiment of the invention compositions are provided comprising a pathogenically effective amount of an improved *Pseudomonas* biocontrol strain according to the invention together with a suitable carrier, which can be used to control pathogenic attack on crop plants.

A preferred embodiment of the invention provides a composition, wherein the carrier are granules consisting of a finely divided carrier and a polymer layer comprising an improved *Pseudomonas* biocontrol strain, wherein the polymer is
a) a film-forming, water-soluble and essentially non-crosslinked polymer, and the granules comprise at least 0.5 % by weight of water, based on the granules, or
b) a film-forming, structurally crosslinked, water-swellable polysaccharide containing carboxyl or sulfate groups in the presence of sodium or potassium ions, and the granules comprise at least 0.5 % by weight of water, based on the granules.

A further embodiment of the invention provides a method for controlling or inhibiting the growth of a plant pathogenic fungus by applying the biocontrol strains of the instant invention or a composition comprising same to an environment in which the plant pathogenic fungus may grow. This could be to the plant/s or parts of the plant/s or seeds (prior to planting) of the plant/s to be protected, or alternatively to soil in which the plant/s to be protected are growing or will grow. The strains will be applied in an effective amount, preferably together with a suitable carrier. That is, in an amount sufficient to control or inihibit the pathogen. The rate of application may vary according to the crop to be protected, the efficacy of the biocontrol strain, the pathogen to be controlled, and the severity of the disease pressure. Generally, the rate of application will be about 1.3×10^5 cfu/cm to about 1.3×10^{10} cfu/cm, specifically about 1.3×10^6 cfu/cm to about 1.3×10^9 cfu/cm, more specifically about 1.3×10^7 cfu/cm to about 1.3×10^8 cfu/cm.

Another embodiment of the present invention provides methods of inhibiting the growth of the fungus *Rhizoctonia solani* by applying the biocontrol strains of the instant invention or a composition comprising same to an environment in which the plant pathogenic fungus may grow. This could be to the plant/s or parts of the plant/s or seeds (prior to planting) of the plant/s to be protected, or alternatively to soil in which the plant/s to be protected are growing or will grow. As noted above, the rate of application will vary depending on various factors. However, the general rate of application will be about 1.3×10^5 cfu/cm to about 5×10^9 cfu/cm, specifically about 1.3×10^6 cfu/cm to about 1.3×10^9 cfu/cm more specifically about 1.3×10^7 cfu/cm to about 1.3×10^8 cfu/cm.

If a composition is used wherein the carrier are granules consisting of a finely divided carrier and a polymer layer as indicated above, the charging level, measured by the cell concentration, can be particularly high. The microorganism is preferably present in a charging level of $1x10^5$ to $1x10^{11}$ cfu (colony-forming units) per g of granules.

A further embodiment of the present invention provides a method for the isolation of novel biocontrol strains comprising the steps of (1) creating a library of transposon insertion mutants in a *Pseudomonas* strain; (2) testing said mutants for their ability to inhibit the growth of a test fungus such as *Neurospora in vitro*; (3) comparing the zones of clearing in the test fungus produced by wild-type non-mutant and mutant strains; (3) selecting mutants

which produce zones of clearing which are distinctive from the zones of clearing of the wild-type non mutant strain; and (4) further selecting these isolated mutants for their biocontrol properties on plant pathogenic fungi using biocontrol tests which are well known in the art.

The biocontrol strains of the present invention and the compositions comprising same, respectively, may be used in any manner known in the art, including coating seeds with an effective amount of the biocontrol strain, or in furrow application of the biocontrol strain directly into the soil and foliar application. Such methods are well known in the art and are described, for example, in the published European Application EP 0 472 494 A2. Furthermore, the strains of this application can also be mixed in formulation with known pesticides in a manner described in WO 94/10845, which disclosure is herein incorporated by reference.

The biocontrol strains of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. These compounds can be both fertilizers or micronutrient donors or other preparations that influence plant growth. They can also be selective herbicides, insecticides, fungicides, bactericides, nematicides, molluscides or mixtures of several of these preparations, if desired, together with further agriculturally acceptable carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g. natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders or fertilizers

The compositions of the present invention are effective, for example, against the phytopathogenic fungi belonging to the following classes: Ascomycetes, e.g., Fusarium; Basidiomycetes, e.g., Rhizoctonia; Oomycetes belonging to the class of Phycomycetes, e.g., Phytophthora, Pythium and Plasmopara. As plant protective agents, the compositions of the present invention can be used against important noxious fungi of the Fungi imperfecti family, e.g., Cercospora and Botrytis. Botrytis and the gray mould on vines, strawberries, apples, onions and other varieties of fruit and vegetables are a source of considerable economic damage. Thus, the compositions of the present invention may be particularly useful because they demonstrate excellent microbicidal activity against a wide spectrum of

fungi. They control mold fungi such as Penicillium, Aspergillus, Rhizopus, Fusarium, Helminthosporium, Nigrospora and Alternaria, as well as bacteria such as butyric acid bacteria and yeast fungi such as Candida. Furthermore, the combinations of the present invention have excellent activity against fungi which occur in seeds or in the soil. As plant protective agents, the combinations are advantageous for practical application in agriculture for protecting cultivated plants, without damaging said plants by harmful side-effects.

Target crops to be protected within the scope of the present invention comprise e.g., the following species of plants: cereals (wheat, barley, maize, rye, oats, rice, sorghum and related crops), beet (sugar beet and fodder beet), pomes, drupes and soft fruit (apples, pears, plums, peaches, almonds, cherries, strawberries, raspberries and blackberries), leguminous plants (beans, lentils, peas, soybeans), oil plants (rape, mustard, poppy, olives, sunflowers, coconut, castor oil plants, cocoa beans, groundnuts), cucumber plants (cucumber, marrows, melons), fibre plants (cotton, flax, hemp, jute), citrus fruit (oranges, lemons, grapefruit, mandarins), vegetables (spinach, lettuce, asparagus, cabbages, carrots, onions, tomatoes, potatoes, paprika), lauraceae (avocados, cinnamon, camphor), or plants such as maize, tobacco, nuts, coffee, sugar cane, tea, vines, hops, bananas and natural rubber plants, as well as ornamentals (composites). The compositions may also be useful for storage protection of natural substances which are in freshly harvested or further processed form.

The biocontrol strains may be applied in any method known for treatment of seed or soil with bacterial strains. For example, see US Patent No.4,863,866. The strains are effective for biocontrol even if the bacterium is not living. Preferred is, however, the application of the living bacterium.

The biocontrol strains may be used in unmodified form or together with any suitable agriculturally acceptable carrier. Such carriers are adjuvants conventionally employed in the art of agricultural formulation, and are therefore formulated in known manner to emulsifiable concentrates, coatable pastes, directly sprayable or dilutable solutions, dilute emulsions, wettable powders, soluble powders, dusts, granulates, and also encapsulations, for example, in polymer substances. Like the nature of the compositions, the methods of application, such as spraying, atomizing, dusting, scattering or pouring, are chosen in

accordance with the intended objective and the prevailing circumstances. Advantageous rates of application are normally from about 50 g to about 5 kg of active ingredient (a.i.) per hectare ("ha", approximately 2.471 acres), preferably from about 100 g to about 2kg a.i./ha. Important rates of application are about 200 g to about 1kg a.i./ha and 200g to 500g a.i./ha. For seed dressing advantageous application rates are 0.5 g to 1000 g a.i.per 100 kg seed, preferably 3 g to 100 g a.i. per 100 kg seed or 10 g to 50 g a.i.per 100 kg seed.

Preferred methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention are leaf application, seed coating and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pathogen (type of fungus). The biocontrol strains according to the invention may also be applied to seeds (coating) by impregnating the seeds either with a liquid formulation containing biocontrol strains, or coating them with a solid formulation. In special cases, further types of application are also possible, for example, selective treatment of the plant stems or buds.

The formulations, compositions or preparations containing the biocontrol strains according to the invention and, where appropriate, a solid or liquid adjuvant, are prepared in known manner, for example by homogeneously mixing and/or grinding the biocontrol strains with extenders, for example solvents, solid carriers and, where appropriate, surface-active compounds (surfactants).

Suitable solvents for compositions include aromatic hydrocarbons, preferably the fractions having 8 to 12 carbon atoms, for example, xylene mixtures or substituted naphthalenes, phthalates such as dibutyl phthalate or dioctyl phthalate, aliphatic hydrocarbons such as cyclohexane or paraffins, alcohols and glycols and their ethers and esters, such as ethanol, ethylene glycol monomethyl or monethyl ether, ketones such as cyclohexanone, strongly polar solvents such as N-methyl-2-pyrrolidone, dimethyl sulfoxide or dimethyl formamide, as well as epoxidized vegetable oils such as epoxidized coconut oil or soybean oil; or water.

The solid carriers used e.g. for dusts and dispersible powders, are normally natural mineral fillers such as calcite, talcum, kaolin, montmorillonite or attapulgite. In order to

improve the physical properties it is also possible to add highly dispersed silicic acid or highly dispersed absorbent polymers. Suitable granulated adsorptive carriers are porous types, for example pumice, broken brick, sepiolite or bentonite; and suitable nonsorbent carriers are materials such as calcite or sand. In addition, a great number of pregranulated materials of inorganic or organic nature can be used, e.g. especially dolomite or pulverized plant residues.

Suitable surface-active compounds are nonionic, cationic and/or anionic surfactants having good emulsifying, dispersing and wetting properties. The term "surfactants" will also be understood as comprising mixtures of surfactants.

Suitable anionic surfactants can be both water-soluble soaps and water-soluble synthetic surface-active compounds.

Suitable soaps are the alkali metal salts, alkaline earth metal salts or unsubstituted or substituted ammonium salts of higher fatty acids (chains of 10 to 22 carbon atoms), for example the sodium or potassium salts of oleic or stearic acid, or of natural fatty acid mixtures which can be obtained for example from coconut oil or tallow oil. The fatty acid methyltaurin salts may be used.

More frequently, however, so-called synthetic surfactants are used, especially fatty sulfonates, fatty sulfates, sulfonated benzimidazole derivatives or alkylarylsulfonates.

The fatty sulfonates or sulfates are usually in the form of alkyli metal salts, alkaline earth metal salts or unsubstituted or substituted ammonium salts and have a 8 to 22 carbon alkyl radical which also includes the alkyl moiety of alkyl radicals, for example, the sodium or calcium salt of lignonsulfonic acid, of dodecylsulfate or of a mixture of fatty alcohol sulfates obtained from natural fatty acids. These compounds also comprise the salts of sulfuric acid esters and sulfonic acids of fatty alcohol/ethylene oxide adducts. The sulfonated benzimidazole derivatives preferably contain 2 sulfonic acid groups and one fatty acid radical containing 8 to 22 carbon atoms. Examples of alkylarylsulfonates are the sodium, calcium or triethanolamine salts of dodecylbenzenesulfonic acid, dibutyl-naphthalene-sulfonic acid, or of anaphthalenesulfonic acid/formaldehyde condensation

product. Also suitable are corresponding phosphates, e.g. salts are preferably in the form of halides, methylsulfates or ethylsulfates, e.g. stearyltrimethylammonium chloride or benzyldi(2-chloroethyl) ethylammonium bromide or salts of the phosphoric acid ester of an adduct of p-nonyl- phenol with 4 to 14 moles of ethylene oxide.

Non-ionic surfactants are preferably polyglycol ether derivatives of aliphatic or cycloaliphatic alcohols, or saturated or unsaturated fatty acids and alkylphenols, said derivatives containing 3 to 30 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenols.

Further suitable non-ionic surfactants are the water-soluble adducts of polyethylene oxide with polypropylene glycol, ethylenediamine propylene glycol and alkylpolypropylene glycol containing 1 to 10 carbon atoms in the alkyl chain, which adducts contain 20 to 250 ethylene glycol ether groups and 10 to 100 propylene glycol ether groups. These compounds usually contain 1 to 5 ethylene glycol units per propylene glycol unit.

Representative examples of non-ionic surfactants are nonylphenolpolyethoxyethanols, castor oil polyglycol ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethylene glycol and octylphenoxyethoxyethanol. Fatty acid esters of polyoxyethylene sorbitan and polyoxyethylene sorbitan trioleate are also suitable non-ionic surfactants.

Cationic surfactants are preferably quaternary ammonium salts which have, as N-substituent, at least one C_8-C_{22}alkyl radical and, as further substituents, lower unsubstituted or halogenated alkyl, benzyl or lower hydroxyalkyl radicals. The salts are preferably in the form of halides, methylsulfates or ethylsulfates, e.g. stearyltrimethyl-ammonium chloride or benzyldi(2-chloroethyl)ethylammonium bromide.

The surfactants customarily employed in the art of formulation are described, for example, in "McCutcheon's Detergents and Emulsifiers Annual, "MC Publishing Corp. Ringwood, New Jersey, 1979, and Sisely and Wood, "Encyclopedia of Surface Active Agents, "Chemical Publishing Co., Inc. New York, 1980.

The agrochemical compositions usually contain from about 0.1 to about 99%, preferably about 0.1 to about 95%, and most preferably from about 3 to about 90% of the active ingredient, from about 1 to about 99.9%, preferably from about 1 to about 99%, and most preferably from about 5 to about 95% of a solid or liquid adjuvant, and from about 0 to about 25%, preferably about 0.1 to about 25%, and most preferably from about 0.1 to about 20% of a surfactant.

Preferred within the present application are formulations comprising living microorganisms as active ingredient, which consist of polymer gels which are crosslinked with polyvalent cations and comprise these microorganisms. This is described, for example, by D.R. Fravel et al. in Phytopathology, Vol. 75, No. 7, 774-777, 1985 for alginate as the polymer material. It is also known from this publication that carrier materials can be co-used. These formulations are as a rule prepared by mixing solutions of naturally occurring or synthetic gel-forming polymers, for example alginates, and aqueous salt solutions of polyvalent metal ions such that individual droplets form, it being possible for the microorganisms to be suspended in one of the two or in both reaction solutions. Gel formation starts with the mixing in drop form. Subsequent drying of these gel particles is possible. This process is called ionotropic gelling. Depending on the degree of drying, compact and hard particles of polymers which are structurally crosslinked via polyvalent cations and comprise the microorganisms and a carrier present predominantly uniformly distributed are formed. The size of the particles can be up to 5 mm.

Compositions based on partly crosslinked polysaccharides which, in addition to a microorganism, for example, can also comprise finely divided silicic acid as the carrier material, crosslinking taking place, for example, via Ca^{++} ions, are described in EP-A1-0 097 571. The compositions have a water activity of not more than 0.3. W.J. Cornick et al. describe in a review article [New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases, pages 345-372, Alan R. Liss, Inc. (1990)] various formulation systems, granules with vermiculite as the carrier and compact alginate beads prepared by the ionotropic gelling process being mentioned. Such compositions are also disclosed by D.R.Fravel in Pesticide Formulations and Application Systems: 11th Volume, ASTM STP 1112 American Society for Testing and Materials, Philadelphia, 1992,

pages 173 to 179 and can be used to formulate the *Pseudomonas* biocontrol strains according to the invention.

A preferred embodiment of the invention provides a composition comprising granules consisting of a finely divided carrier and a polymer layer comprising an improved *Pseudomonas* biocontrol strain according to the invention, wherein the polymer is
a) a film-forming, water-soluble and essentially non-crosslinked polymer, and the granules comprise at least 0.5 % by weight of water, based on the granules, or
b) a film-forming, structurally crosslinked, water-swellable polysaccharide containing carboxyl or sulfate groups in the presence of sodium or potassium ions, and the granules comprise at least 0.5 % by weight of water, based on the granules.

Essentially non-crosslinked in the context of the invention means that no monomeric crosslinking agents which lead to covalent bonds or polyvalent cations which lead to ionotropic gel formation are added.

Structurally crosslinked here means the formation of a spatial network of an individual polymer or a mixture of 2 polymers via hydrogen bridges or via electrostatic interaction of sodium and potassium ions. A thermoreversible spatial structure (gel) which changes back into a solution on heating is thereby formed. Examples are the pronounced double helix structure of carrageenans in the presence of potassium ions or the structure formation of a carrageenan/carob bean flour mixture. A thermally irreversible structure formation by polyvalent ions does not fall under the above definition.

The polysaccharide can contain one or more carboxyl or sulfate groups per recurring structural unit.

Water-soluble in this connection means that preparation of an at least 0.5 % by weight aqueous polymer solution is possible in the temperature range between 5° and 95° C.

The granules preferably comprise the microorganisms in an amount of 0.1 to 10 % by weight, preferably 0.3 to 5 % by weight and particularly preferably 0.5 to 3 % by weight

of dry matter, based on 1 kg of granules. The sum of all the constituents of the granules is always 100 %.

The charging level, measured by the cell concentration, can be particularly high. The microorganism is preferably present in a charging level of $1x10^5$ to $1x10^{11}$ cfu (colony-forming units) per g of granules. In the formulation according to the invention, this concentration of live cells can be retained over a period of up to 10 months - under storage at room temperature - with only slight losses of microorganisms of less than one power of ten cfu.

The residual water content is preferably at least 1 % by weight, more preferably at least 3 % by weight and particularly preferably at least 5 % by weight. The upper limit of the water content is preferably not more than 40 % by weight, more preferably not more than 30 % by weight and particularly preferably not more than 20 % by weight. The upper limit of the water content is influenced by the carrier material, the water-solubility of the polymer and the preparation process. In the case of coating processes, for example coating in a fluidized bed, a water content of 0.5 to 20 % by weight can easily be achieved, while in the case of extrusion processes, the water content can be higher and can be, for example, 0.5 to 40 % by weight.

The finely divided carrier material can have an average particle diameter of 1 m to 0.8 cm, more preferably 10 m to 0.5 cm and particularly preferably 20 m to 0.2 cm. It can be an inorganic or organic material. Organic materials are preferably used for fungi and inorganic materials are preferably used for vegetative cells (bacteria). Examples of water-insoluble organic materials are comminuted bran, straw, wood flour and cellulose. Inorganic carriers are, in particular, water-insoluble metal oxides and metal salts (SiO_2, Al_2O_3, $BaSO_4$, $CaCO_3$) or silicates and aluminosilicates of the alkali metals and alkaline earth metals. Preferred silicates are the laminar silicates. Examples of silicates are clay minerals, attapulgite, kieselguhr, lime grit, diatomaceous earth, wollastonite, olivine and vermiculite. Vermiculite is particularly preferred.

The amount of carrier material can be, for example, 50 to 99 % by weight, preferably 65 to 95 % by weight and particularly preferably 75 to 90 % by weight.

The granules can have an average particle size of 0.01 mm to 8 mm, and the average particle size is preferably 0.2 to 4 and particularly preferably 0.5 to 2 mm.

The film-forming, water-soluble and essentially non-crosslinked polymer can be a synthetic or naturally occurring polymer. Examples of synthetic polymers are homo- and copolymers based on polyvinyl alcohol, polyethylene glycol or polyvinylpyrrolidone and polyacrylamides. The naturally occurring polymers are chiefly polysaccharides, which can be derivatized. A large number of preferred naturally occurring polymers are known, for example starch, alginates, carrageenans, in particular k-carrageenan, t-carrageenan and l-carrageenan, xanthan, carob bean flour or methylcelluloses. Mixtures thereof can also be used.

The polymers must be compatible with the microorganism, which can be determined by the expert in a simple manner by bringing the microorganism and polymer together. Alginates and carrageenans are particularly preferred. Vermiculite with k-carrageenan is a particularly preferred combination of carrier and water-soluble polymer.

The film-forming, structurally crosslinked, water-swellable polymer is a polysaccharide, preferably k-carrageenan, t-carrageenan, carob bean flour/xanthan or mixtures thereof, which is in the presence of sodium or potassium ions. These polymers form thermally reversible gels in which intermolecular hydrogen bridges or ionic bonds are predominant.

The amount of water-soluble or water-swellable polymer can be, for example, 0.1 to 20 % by weight, preferably 0.1 to 10 % by weight and particularly preferably 0.5 to 5 % by weight.

The sodium or potassium ions are preferably in a molar ratio to the carboxyl or sulfate groups of the polymers of 0.001:1 to 1:1.

The process for the preparation of granules consisting of a finely divided carrier and a polymer layer comprising an improved *Pseudomonas* biocontrol strain according to the invention, wherein the polymer is
a) a film-forming, water-soluble and essentially non-crosslinked polymer and the granules comprise at least 0.5 % by weight of water, based on the granules, or
b) a film-forming, structurally crosslinked, water-swellable polysaccharide containing carboxyl or sulfate groups in the presence of sodium or potassium ions and the granules comprise at least 0.5 % by weight of water, based on the granules, comprises
(A) for preparation of granules a), suspending, or dissolving at temperatures of not more than 95 C, a film-forming and water-soluble polymer and, after cooling to room temperature, suspending a microorganism in this suspension or solution, or
(B) for preparation of granules b), dissolving a polysaccharide containing carboxyl or sulfate groups in an aqueous buffer solution containing sodium or potassium ions and then suspending a microorganism in this solution,
(C) mixing the resulting suspensions directly with a finely divided carrier material or with an aqueous suspension of the finely divided carrier material, and
(D) removing the water to an amount which is not less than 0.5 % by weight, based on the granules.

If a film-forming and water-soluble polymer is suspended for preparation of granules a), the operation is preferably carried out at a temperature of 10° to 30° C. If a solution of a film-forming and water-soluble polymer is prepared, this is effected at a temperature of 25° to 95° C, depending on the polymer type.

The improved biocontrol strains according to the invention can be added either to the polymer suspension at a temperature of less than 40° C or to the cooled polymer solution at a temperature below 40° C, preferably below 30° C.

In another process procedure, for preparation of granules b), a polysaccharide containing carboxyl or sulfate groups is dissolved in an aqueous buffer solution containing sodium or potassium ions, if appropriate at elevated temperature, for example 70° C, or two polymers which interact with one another are dissolved in the same manner. When these

solutions cool, a thermally reversible gel is formed. The microorganism is added at a temperature of less than 40° C, just before the solidification point.

All potassium or sodium-containing salts of polybasic acids can be used as buffers. Phosphate buffers such as are commercially obtainable are especially preferred. The potassium salts are preferred. A pH of about 6.5 to about 7.5 can be established, depending on the ratio of dihydrogen phosphate to monohydrogen phosphate. The pH is preferably 7.

The concentration of buffer is preferably 0.00001 mol/l to 1 mol/l, particularly preferably 0.005 mol/l to 0.05 mol/l. The water is removed as gently as possible, preferably at room temperature or at a slightly elevated temperature of up to about 35° C.

Apparatuses and processes for removing the water are known per se. The most favourable process in each case depends on the viscosity of the composition to be processed. The granules according to the invention can be prepared by known processes using customary apparatuses. Spray processes for mixing the components, for example with fluidized bed reactors, are advantageously used for the coating. In these processes, the solution or suspension of polymer and microorganism is sprayed onto the carrier suspended in the fluidized bed and is thereby dried at the same time.

In another process, the granules according to the invention are prepared by known extrusion processes. In these, all the constituents are mixed with the necessary amount of water, for example in a mixer, and the mixture is pressed through a perforated sheet. The granules are comminuted to the desired size and, if appropriate, dried. Single-screw extruders, attachable granulating machines, subgranulators, perforated diaphragms and the like can be used.

Granules in which the carrier material is enclosed with a thin layer of polymer in which the microorganisms are distributed are obtained. As a rule, discrete enclosed particles are not obtained, but agglomerates of several carrier particles which have irregular shapes are formed.

Particles of different shape are obtained, depending on the mixing and drying process chosen. Cylindrical structures in which the carrier material and microorganism are enclosed by the polymer material essentially independently of one another are thus rather formed in the extrusion process, while in the spraying-on process in a fluidized bed, agglomerates of carrier materials in which the carrier particles are enclosed by a thin layer of polymer comprising the microorganisms are rather formed. This particle shape is preferred, since a particularly rapid release of the microorganism from the thin layer of polymer occurs.

The granules are in all cases solid and free-flowing mixtures which can be employed directly as scattering granules. Their handling is easy and reliable, since they can be introduced directly into equipment for distribution in the field. The amounts applied are commonly in a range of about 1 kg to 20 kg.

They can be employed on plants, parts of plants or plant locations (fruit, blossom, foliage, stems, tubers, roots, soil) or on seeds of various crops of useful plants and the fungal diseases which occur can be suppressed or destroyed.

The granules can be used on the areas or crops to be treated at the same time as or successively with other active ingredients. Other active ingredients can be either fertilizers, trace element carriers or other preparations which influence plant growth. Selective herbicides as well as insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures of two or more of these preparations can also be used here. The invention also relates to the use of the granules according to the invention for protecting crops from disease infestation.

Formulation Examples

Example A1

10 x 250 ml of Luria broth inoculated with *Pseudomonas* biocontrol strains NRRL B-21172 or NRRL B-21173, are centrifuged after 16 hours of cell growth on a shaker, and the pellet is resuspended with 0.01 M phosphate buffer (K_2HPO_4 : KH_2PO_4 = 1 : 0.78, pH = 7) to 40 ml. 100 ml of phosphate buffer are heated to 70° C and 0.7 g of κ-carrageenan are added, so that a 0.7 % κ-carrageenan solution in 0.01 M phosphate buffer is formed. This solution

is cooled to just above the solidification point and mixed with the microorganism suspension.
This mixture is then sprayed onto 100 g of vermiculite in a fluidized bed. The following granule composition is obtained:
16 % of residual water
1.5 % of microorganism dry mass
81.9 % of vermiculite
0.6 % of k-carrageenan.

The starting charge is about 1.1×10^{10} CFU/g (colony-forming units).

To check the storage stability, the charge is determined at suitable intervals of time.

Example A2

5 g of κ-carrageenan are stirred with 40 g of 0.01 M phosphate buffer according to Example 1. 10 g of cell pellet (30 % of dry matter) of *Pseudomonas* biocontrol strains NRRL B-21172 or NRRL B-21173, prepared in a 50l fermenter, are admixed. The polymer/microorganism composition is mixed uniformly with 120 g of vermiculite powder and the mixture is then extruded. The granules thus obtained are dried to the desired water content in a fluidized bed. The following granule composition is obtained:
18 % of residual water
1.8 % of microorganism dry mass
77 % of vermiculite
3.2 % of k-carrageenan.

The starting charge is about 3.3×10^{10} CFU/g (colony-forming units).

Example A3

250 ml of Luria broth, inoculated with *Pseudomonas* biocontrol strains NRRL B-21172 or NRRL B-21173, are centrifuged after 16 hours of cell growth on a shaker, and the pellet is resuspended with 0.01 M phosphate buffer according to Example 1 to 40 ml.
The microorganism suspension is mixed with 100 ml of 3 % sodium alginate solution in 0.01 M phosphate buffer according to Example 1 and the mixture is sprayed onto 100 g of vermiculite in a fluidized bed. The following granule composition is obtained:

12 % of residual water
0.5 % of mdicroorganism dry mass
d85.5 % of vermiculite
2.5 % of sodium alginate.

The following Examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Construction of pCIB100

The plasmid pLRK*D*211 (Kroos and Kaiser, PNAS 81: 5816-5820; 1984) contains a Tn5-*lac* transposable element (encoding resistance to kanamycin) with a promoterless (in *E. coli*) *trp-lac* gene fusion inserted in IS50L of Tn5 in the correct orientation to fuse *lacZ* expression to promoters outside of Tn5. A new plasmid, pCIB100, was constructed by introducing the mobilization (*mob*) site from the plasmid pSUP5011 (Simon et al., In: Molecular Genetics of Bacteria-Plant Interaction; Puhler, A (Ed) pp 98-106; Springer Verlag, 1983) into pLRK*D*211, thus enabling the plasmid to be transferred to pseudomonads or other gram negative bacteria by conjugation. The plasmids pSUP5011 and pLRK*D*211 were digested individually with SalI and HindIII, and the resulting fragments were separated by electrophoresis in low melting point agarose. The DNA band corresponding to the SalI-HindIII fragment which contains the *trp-lac* fusion in pLRK*D*211 was cut from the gel and added to a similarly obtained SalI-HindIII fragment which contains the mob site from pSUP5011. Ligation was carried out in agarose (Methods of Enzymology; Vol. 101, Ch 3; Academic Press, New York).

The *trp-lac* fusion reporter gene in pCIB100 was found to confer constitutive *lacZ* gene expression in Pseudomonas. Applicants made a lac construct without the *trp* region and showed that it behaved satisfactorily as a promoterless reporter gene. A plasmid containing this construct was designated pCIB116.

Example 2: Construction of pCIB114

The plasmid pMC874 (Casadaban et al., J Bacteriol. 143: 971-980; 1980) carries a portion of the *lac* operon which lacks the coding region for the first 8 amino acids of *lacZ* (a promoterless, incomplete gene designated *lacZ'*). This plasmid is useful in constructing a precursor plasmid of a subsequent plasmid that may be used according to the method of the invention to prepare insertion mutants. In this embodiment of the invention, plasmid pMC874 was digested with SalI and a synthetic oligonucleotide pair of the following structure was ligated to the SalI ends:

5'-TCGAGATCTAAA-3' [SEQ ID NO 1]
3'-CTAGATTT-5' [SEQ ID NO 2]

The resulting mixture was digested with BglII and ligated with BglII digested pRK290 (Ditta *et al.*, PNAS 77: 7347-7351; 1980). This construct was designated pCIB113.

A synthetic oligonucleotide pair of the following sequence was constructed:

5'-AAAGGAGATCTGGATCCAGGAGAAGCTTGCATGCTA-3' [SEQ ID NO 3]
3'-TTTCCTCTAGACCTAGGTCCTCTTCGAACGTACGATCTAG-5' [SEQ ID NO 4]

This oligonucleotide pair, when fused to the 5' end of the promoterless *lacZ'* gene, would supply the following sites (in order): BglII-BamHI-E.coli ribosome binding site-HindIII-SphI(ATG for translation start)-BglII end.

Plasmid pCIB113 was digested with BamHI, and was ligated to the synthetic oligonucleotide pair described above. The structure of the oligonucleotide pair forces ligation in a single orientation, with the BglII end ligated to the BamHI end of *lacZ'* and resulting in loss of the BamHI site. The construct was then digested with BglII, and the small (3.0 kb) oligo + *lacZ'* + *lacY* fragment was isolated and purified from agarose. To make plasmid pCIB114, a fragment isolated and purified in this manner was ligated to BglII-digested pRK290.

Example 3: Construction of pCIB116

(1) The plasmid pCIB100 was digested with BamHI and HindIII. The large fragment carrying IS50L (the leftmost 54 base pairs), the colE1 origin of replication, and the left half of IS50R, was isolated and purified from agarose.

(2) The plasmid pCIB100 was digested with EcoR1 and HindIII. The large fragment carrying IS50R (right half), the *mob* gene, the Kan marker, and the promoter-distal portion of the *lacZ-lacY* fragment, was isolated and purified from agarose.

(3) The plasmid pCIB114 was digested with BamHI and EcoR1. The fragment carrying the *lacZ* portion of the molecule was isolated and purified from agarose gels. It was then mixed with the fragments isolated in steps (2) and (3) above. The mixture was ligated and used to transform *E. coli* HB101. Kanamycin resistant colonies were selected, and plasmid DNA was isolated from the transformants and analyzed for the correct orientation of fragments. The SalI site was removed by digestion with SalI, filling in the ends with Klenow fragment and blunt end ligation. Maniatis *et al.*, (1989) Molecular Cloning, Ch. 1, Cold Spring Harbor Laboratory Press, New York. The sequence of the region of the plasmid containing the oligonucleotide junction with IS50L was confirmed using the dideoxy chain termination procedure. The final construct was designated pCIB116.

Example 4: Construction of an Insertion Mutant Collection of Strain CGA 267356 (*i.e.* strain 11c-1-38) using pCIB116

The plasmid pCIB116 was transformed into *E.coli* strain S17-1 (R. Simon *et al.*, 1983) using standard procedures (Maniatis *et al.* 1989). The resulting strain (S17-1/pCIB116) was used as donor for the introduction of pCIB116 into the *Pseudomonas* strain CGA267256 by conjugation. The *E. coli* (S17-1/pCIB116) and *Pseudomonas* strains were grown overnight at 37°C in LB. 0.1 ml of each strain was mixed and spread onto LB plates.

The plates were incubated at 37°C for 3 hours then transferred to 28°C for overnight incubation. The mating mixture was then lifted off the plate using sterile 9 cm Whatman glass microfibre filters and was transferred to a fresh LB plate containing ampicillin (100 µg/ml) and neomycin (100 µg/ml). After 2-3 days, individual ampR NeoR trans-conjugants were picked into wells of 96-well microtiter dishes containing 100 µl minimal *Pseudomonas* media (LMG) + neomycin (100 µg/ml). The microtiter plates were incubated overnight at 28°C in an orbital shaker set at 200 rpm. After overnight incubation, 50 µl of 50% glycerol was added to each well, and the microtiter dishes were then maintained at -80°C. 10,000 individual mutants were collected and stored.

Example 5 Screening Mutants for in vitro Fungal Inhibition Activity

Individual insertion mutants were spotted on to *Neurospora* culture agar (Difco) plates (150 mm) in ordered arrays. Resuspended mycelia fragments of *Neurospora crassa* were applied to the plates using a chromatography sprayer. After incubation overnight at 28°C, fungal mycelial growth formed an opaque background on the plate except in the immediate areas of the *Pseudomonas* colonies, where zones of clearing were observed. *Pseudomonas* mutants which produced zones of clearing which were either larger or visibly different than those of the wild-type parent were selected. Parent zones of clearing had a central clear core and outer concentric zone which formed a near linear gradient from clear on the inside to indistinguishable from the mycelial background on the outside.

Example 6 Cultivation of *Rhizoctonia solani* for Greenhouse Biocontrol Assays

Rhizoctonia solani was grown on Potato Dextrose Agar (PDA, Difco), pH 5.6 in a petri dish. A 300 ml Erlenmeyer flask with 25 g millet and 50 ml distilled water was autoclaved and incubated with one agar plug (5 mm diameter) from a PDA culture of *Rhizoctonia solani*. After incubation at 20°C in the dark for 3 weeks the overgrown millet was airdried and ground in a Culatti mill (1 mm sieve, 6000 rpm).

Example 7 Biocontrol Efficacy of Insertion Mutants

Insertion mutants which produced antifungal zones visibly different than the wildtype were tested in greenhouse biocontrol assays on cotton with the pathogen *Rhizoctonia solani*. Results for two mutants are shown in Table 1. *Pseudomonas* cultures were grown overnight in Luria broth at 28°C. For Trial 1, cells were pelleted by centrifugation, then resuspended in sterile water to an optical density of 2.5 at 600 nm (approximately 2 x 10^9 colony forming units per ml). For Trial 2, however, cells were diluted to an OD of 0.25 to enable greater differentiation in the biocontrol effects of the wild-type and mutant strains. *Rhizoctonia solani* was cultured on autoclaved millet, then dried and ground into powder. Soil was prepared by mixing equal parts of potting soil (Metro-mix 360), sand and vermiculite. This is used to fill 15 cm diameter pots. A 2 cm deep circular furrow with a total length of 30 cm was formed at the perimeter of each pot. Ten cotton seeds (Stoneville 506) were placed in each furrow. *R. solani*-infested millet powder was sprinkled evenly over the seeds in the furrows at the rate of 100 mg/pot, followed by the application of 20 ml of bacterial suspension for each pot. Water was added in place of bacterial suspension in the control. Each treatment consisted of four replicate pots for a total of 40 seeds per treatment. The plants were grown in an environmentally controlled chamber with a day/night temperature regime of 26/21°C. The plants were rated for disease severity after 10 days. Two mutants, CGA 319115 [NRRL B-21172] and CGA 321730 [NRRL B-21173[, were shown to provide better disease control than the wild type parent CGA 267356. Table 1 shows results for these tests.

Table 1: Biocontrol Efficacy of CGA 319115 and CGA 321730

	Trial 1	Trial 2
Rate of Application	1.3 x 10^9 cfu/cm	1.3 x 10^8 cfu/cm
No Pathogen Check	100	100
Pathogen Check	0	0
CGA 267356 [ATCC 55169]	46	0
CGA 319115 [NRRL B-21172]	69	60
CGA 321730 [NRRL B-21173]	73	90

Numbers reflecting the lack of disease symptoms on plants were assigned for individual plants on the scale of 1-5. Following summation within each treatment, the "no pathogen check" summation was normalized to 100, and the "pathogen check" summation was normalized to zero, with the three other treatment values being assigned accordingly.

Biocontrol Assay with granulated compositions

The biological activity of the granules prepared in Example A1 is tested under greenhouse conditions after certain periods of storage at room temperature. The standardized test conditions are:
Crop plant: cotton
Pathogen: Rhizoctonia solani

The granules are added to the pot substrate in an amount of 16g/litre of pot substrate.

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the scope of the present invention.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Deposits

The following *Pseudomonas* strains have been deposited in connection with this application according to the regulations of the Budapest Treaty on January 21, 1994 with the Agricultural Research Service Culture Collection (NRRL), an International Deposit Authority recognized under the Budapest Treaty and were assigned the following Accession Numbers:

CGA 319115 NRRL B-21172
CGA 321730 NRRL B-21173

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EP-472494

EP-97571

US-4863866

WO-94/10845

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(ii) TITLE OF INVENTION: Improved Pseudomonas Biocontrol Strains

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/185,623

(B) FILING DATE: 24-JAN-1994

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: Synthetic oligonucleotide used to ligate SalI ends in construction of pCIB113.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCGAGATCTA AA 12

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..8
(D) OTHER INFORMATION: /note= "SYNTHETIC OLIGONUCLEOTIDE COMPLEMENTING RESIDUES 1 TO 8 OF SEQ. ID NO:1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTAGATC 8

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: Synthetic oligonucleotide providing restriction sites, E.coli ribosome binding site and ATG translation start used for construction of pCIB114.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAAGGAGATC TGGATCCAGG AGAAGCTTGC ATGCTA 36

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 5..40

(D) OTHER INFORMATION: /note= "SYNTHETIC OLIGONUCLEOTIDE COMPLEMENTARY TO RESIDUES 1 TO 36 OF SEQ ID NO:3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCTAGCAT GCAAGCTTCT CCTGGATCCA GATCTCCTTT 40

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 24, line 1 - 7 from the bottom.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
Agricultural Research Culture Collection (NRRL)
International Depositary Authority

Address of depositary institution *(including postal code and country)*
1815 N. University Street
Peoria, Illinois 61604
U.S.A.

Date of deposit	Accession Number
21 January 1994 (21.01.94)	NRRL B-21172

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

We request the Expert Solution where available

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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A. The indications made below relate to the microorganism referred to in the description on page 24, line 1 - 7 from the bottom.

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1815 N. University Street
Peoria, Illinois 61604
U.S.A.

Date of deposit	Accession Number
21 January 1994 (21.01.94)	NRRL B-21173

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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What is claimed is:

1. A biocontrol strain of *Pseudomonas fluorescens* designated CGA 319115 and deposited as NRRL NRRL B-21172.

2. A biocontrol strain of *Pseudomonas fluorescens* designated CGA 321730 and deposited as NRRL B-21173.

3. A method for the isolation of a biocontrol strain, comprising the steps of:
   (1) creating a library of transposon insertion mutants in a *Pseudomonas* strain;
   (2) testing said mutants for their ability to inhibit the growth of a test fungus *in vitro*;
   (3) comparing the zones of clearing in the test fungus produced by wild-type non-mutant and mutant strains;
   (4) selecting mutants which produce zones of clearing which are distinctive from the zones of clearing of the wild-type non mutant strain; and
   (5) further selecting these isolated mutants for their biocontrol properties on a plant pathogenic fungus.

4. The method of claim 3, wherein said test fungus is a strain of *Neurospora*.

5. The method of claim 3, wherein said plant pathogenic fungus is *Rhizoctonia solani*.

6. A biocontrol strain obtainable by the method of claim 3.

7. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying an effective amount of biocontrol strain CGA 319115 or CGA 321730 to an environment in which the plant pathogenic fungus may grow.

8. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying an effective amount of biocontrol strain CGA 319115 or CGA 321730 to a

plant or plant part in order to protect said plant or plant part from a plant pathogenic fungus.

9. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying an effective amount of biocontrol strain CGA 319115 or CGA 321730 to seed in order to protect a plant which develops from said seed from a plant pathogenic fungus.

10. The method according to anyone of claims 6 to 8, wherein said plant pathogenic fungus is *Rhizoctonia solani*.

11. A composition comprising a pathogenically effective amount of biocontrol strain CGA 319115 or CGA 321730 together with a suitable carrier.

12. A composition according to claim 11, wherein the carrier are granules consisting of a finely divided carrier and a polymer layer comprising the an improved *Pseudomonas* biocontrol strain according to anyone of claims 1,2 or 6, wherein the polymer is
a) a film-forming, water-soluble and essentially non-crosslinked polymer, and the granules comprise at least 0.5 % by weight of water, based on the granules, or
b) a film-forming, structurally crosslinked, water-swellable polysaccharide containing carboxyl or sulfate groups in the presence of sodium or potassium ions, and the granules comprise at least 0.5 % by weight of water, based on the granules.

13. A composition according to claim 12, wherein the granules comprise the microorganisms in an amount of 0.1 to 10% by weight, based on 1 kg of granules.

14. A composition according to claim 12, wherein the microorganism is present in a charge level of $1x10^5$ to $1x10^{11}$ cfu (colony forming units] per g of granules.

15. A composition according to claim 12, in which the film-forming, water-soluble and essentially non-crosslinked polymer is a synthetic or naturally occurring polymer.

16. A composition according to claim 12, in which the film-forming, water-soluble and essentially non-crosslinked polymer is a homo- or copolymer based on polyvinyl alcohol, polyethylene glycol or polyvinylpyrrolidone and polyacrylamides.

17. A composition according to claim 12, in which the film-forming, water-soluble and essentially non-crosslinked polymer is a polysaccharide or derivatized polysaccharide.

18. A composition according to claim 17, in which the film-forming, water-soluble and essentially non-crosslinked polymer is starch, alginate, carrageenan, k-carrageenan, t-carrageenan, xanthan, carob bean flour or methylcellulose or a mixture thereof.

19. A composition according to claim 18, in which the film-forming, water-soluble and essentially non-crosslinked polymer is k-carrageenan, t-carrageenan or alginate.

20. A composition according to claim 12, in which the film-forming, structurally crosslinked, water-swellable polymer containing carboxyl groups or sulfate groups is k-carrageenan, t-carrageenan, xanthan or a mixture of carob bean flour and xanthan.

21. A composition according to claim 12, in which the film-forming, structurally crosslinked, water-swellable polymer containing carboxyl groups or sulfate groups is k-carrageenan or t-carrageenan.

22. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying a composition of any one of claims 11 to 21 to an environment in which the plant pathogenic fungus may grow.

23. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying a composition of any one of claims 11 to 21 to a plant or plant part in order to protect said plant or plant part from a plant pathogenic fungus.

24. A method for controlling or inhibiting the growth of a plant pathogenic fungus by applying a composition of any one of claims 11 to 21 to seed in order to protect a plant which develops from said seed from a plant pathogenic fungus.

25. The method according to anyone of claims 22 to 24, wherein said plant pathogenic fungus is *Rhizoctonia solani*.

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/IB 95/00023

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N1/20 A01N63/00 //(C12N1/20,C12R1:39)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP,A,0 472 494 (CIBA GEIGY AG) 26 February 1992 see the whole document ---	1-3, 5-18, 22-25 4,19-21
Y	BIOLOGICAL ABSTRACTS, vol. 77 Philadelphia, PA, US; abstract no. 089587, VAN PEE K-H ET AL 'THE BIOSYNTHESIS OF BROMINATED PYRROLNITRIN DERIVATIVES BY PSEUDOMONAS-AUREOFACIENS' see abstract & J ANTIBIOT (TOKYO), 36 (12). 1983 (RECD. 1984). 1735-1742. ---	4
Y	US,A,4 647 537 (HARUHIRO SHIGEMITSU) 3 March 1987 see claim 1 --- -/--	19-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
19 April 1995	25.04.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/IB 95/00023

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Section Ch, Week 9029, Derwent Publications Ltd., London, GB; Class A96, AN 90-221164 & JP,A,2 150 276 (TOYOBO KK) 8 June 1990 see abstract -----	19-21

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No
PCT/IB 95/00023

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		AU-B-	655774	12-01-95
		AU-A-	8258691	28-05-92
		CA-A-	2049385	21-02-92
		JP-A-	6092815	05-04-94
		US-A-	5348742	20-09-94
US-A-4647537	03-03-87	JP-C-	1693854	17-09-92
		JP-B-	3061425	19-09-91
		JP-A-	60180589	14-09-85
		CA-A-	1232851	16-02-88