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(71) Applicants: CENTRO DE INGENIERIA GENETICA Y BIOTECNOLOGIA [CU/CU]; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). KAMBEEL, Robert, Wilhelmus [NL/NL]; Adama van Scheltemaplein 14, NL-2624 PE Delft (NL).

(72) Inventors: MENA CAMPOS, Jesús; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). DE LA RIVA DE LA RIVA, Gustavo Alberto; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). VASGUEZ MENDEZ, Ramón, P.; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). FERNANDEZ MACHUCA, Marina; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). COEGO GONZALEZ, Alberto; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). GARCIA GONZALEZ, Melba; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). PIMENTEL VASQUEZ, Eulogio; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). LOPEZ QUESADA, Alina; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). GARCIA GONZALEZ, Rolando; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). ZALDUA GUERRA, Zurima; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU). MENCHO PONCE, Juan Diego; 31 Street, '/156 & 190, Cubanacan. Playa, Havana (CU).

(74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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(54) Title: NEMATICIDIC AGENT AND METHOD FOR THE BIO-CONTROL OF NEMATODES

(57) Abstract

New bacterial strains, in particular *Sphingobacterium spiritivorum* C-926 and *Corynobacterium paurometabolum* C-924 with useful characteristics in the farming sector due to their nematicidic effect, which can be used in this context for the bio-control of nematodes. Bionematicides capable of effectively controlling phytopathogenic nematode populations in various crops of interest. Useful strains may be isolated from soil taken from areas habitually affected by a high incidence of nematodes, from banana plants showing less severe symptoms of infestation. Strains are selected which are capable of affecting the eggs of the nematode *Meloidogyne incognita*, in experiments carried out under laboratory conditions. Active strains, such as *Sphingobacterium spiritivorum* strain C-926 and *Corynobacterium paurometabolum* strain C-924, are tested to assess the protection which the bacterial preparation, applied directly, can provide to pumpkin and banana plants in relation to *Meloidogyne incognita* and *Radopholus similis*. Strains may also be tested against the zoonematodes *Haemonchus* sp. and *Trichostrongylus* sp. Control of these parasites allows veterinary use. In addition to the nematodes referred to above, and selected due to the high losses which they incur in crops, the strains may also be effective in the control of populations of *Heterodera* ssp., *Globodera* ssp., and *Pratylenchus* ssp., as well as other species of zoonematodes.

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DESCRIPTION

NEMATICIDIC AGENT AND METHOD FOR THE BIO-CONTROL OF NEMATODES

This invention is related to the field of microbiology and in particular to the isolation of two bacterial strains, a *Sphingobacterium spiritivorum* strain and a *Corynobacterium paurometabolum* strain, having useful characteristics in the farming sector due to their nematicidic effect, allowing their use for the bio-control of nematodes in crops or agricultural plants of economic interest.

Nematodes constitute one of the most significant pests in agriculture in tropical, subtropical, and temporate zones (Nickle, W.R., 1991, Manual of Agricultural Nematology, N.Y.). The most important group among the phytopathogenic species is *Meloidogyne* ssp., which causes losses estimated at between 11 and 25% of crops in most tropical areas (Sasser, J.N., 1979, in: Root knot nematodes, Ed. F. Lamberti & C.E. Taylor, Ac. Press, London). There are few options for its control, chemical nematicides being the means most frequently used to control nematode populations. Use is also made, in combination, of the introduction of less susceptible crop varieties, as well as crop rotation strategies and the integrated conduct of harvesting. Nevertheless, the control of phytopathogenic nematodes remains unsatisfactory (Atkinson, H.J., 1993, BCPC Monograph No. 65; Opportunities for Plant Molecular Biology in Crop Production, pages 257-266). The use of chemical nematicides is limited, since these are systemic substances with a wide spectrum of action as organophosphorics and carbamates, with a high level of toxicity. Another form of attack used against these parasites consists of disinfecting the field, before sowing, with products which are no less toxic, such as methyl bromide and dichloro-propene. All this has given rise to intense studies of the different natural antagonists of nematodes, with a view to their use as bio-preparations which will allow the pest to be controlled while at the same time reducing the risks derived from the excessive use of agricultural chemicals with high toxicity levels.

The nematophageous fungi which inhabit the same environment as the phytonematodes may maintain a predetermined biological balance, and limit to some extent the proliferation of the nematodes. Among other natural predators, we find *Anthrobotrys irregularis*; others are ovicides, such as *Paecilomyces lilacinus* and *Verticillium chlamydosporum*. Studies have also been made of the action of the bacteria *Pasteuria penetrans*, which forms endospores which adhere to the side of the nematode, and, on germination, invade it and cause all the organs of the white nematode to atrophy.

Despite this, the control of nematode populations by these means has continued to be ineffective. Among the reasons which have determined the low rate of effectiveness of the existing methods of biological control are the following:

- Low level of antagonism between the nematode and its antagonist;
- Elevated specificity of the antagonist for a particular kind of nematode;
- Difficulties in obtaining large volumes of the antagonist;
- Difficulties in application due to the nature of the life of the nematode;
- Diversity in the environmental distribution of the nematodes;
- Elevated proliferation of the nematodes.

An object of this invention has been the isolation and characterisation of bacterial strains in the soil which can be used as possible bio-nematicides of a broad spectrum, the use of which will control nematode populations in main agricultural crops. Another object of this invention has been to provide strains which are effective in the control of zoo-nematodes such as *Haemonchus* sp. and *Trichostrongylus* sp., which are indicators of possible use in the veterinary sector.

It was with these objects that new strains were obtained with nematicidal properties. These new strains were classified respectively as *Sphingobacterium spiritivorum* strain C-926, and *Corynobacterium paurometabolum* strain C-924, using the reference systems API 20 NE™ (Ref. 20 090, bioMerieux 69280 Marcy-l'Etoile, France), and API-50 CH (BIOMERIEUX SA, 69280 Marcy-l'Etoile, France).

The above strains were deposited on August 8, 1995, pursuant to the Budapest Treaty with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, and received deposit numbers CBS 612.95 (*Sphingobacterium spiritivorum* strain C-926) and CBS 613.95 (*Corynobacterium paurometabolum* strain C-924).

The invention therefore provides new bacterial strains, in particular the *Sphingobacterium spiritivorum* strain C-926 and the *Corynobacterium paurometabolum* strain C-924, which both have useful characteristics in the farming sector due to their nematicidic effect and can be used in this context for the bio-control of nematodes. The invention thus provides bionematicides capable of effectively controlling phytopathogenic nematode populations in various crops of interest.

The invention is not limited to the above two strains which were actually isolated, but also covers mutants thereof and derived strains having essentially similar nematicidic properties, and generally any bacterial strain which can be isolated and selected by a method similar to the method actually used for isolating strains C-926 and C-924.

Useful strains may be isolated from soil taken from areas habitually affected by a high incidence of nematodes, from plants (e.g. banana plants) showing less severe symptoms of infestation. Strains may then be selected which are capable of affecting nematode eggs, e.g. eggs of the nematode *Meloidogyne incognita*, in experiments carried out under laboratory conditions. Active strains, like *Sphingobacterium spiritivorum* strain C-926 and *Corynobacterium paurometabolum* strain C-924, may be tested to assess the protection which they, applied directly, can provide to, e.g. pumpkin and banana plants in relation to, e.g. *Meloidogyne incognita* and *Radopholus similis*. Strains may also be tested against zoonematodes, e.g. *Haemonchus* sp. and *Trichostrongylus* sp. Control of these parasites allows veterinary use. In addition to the nematodes referred to above, selected because of the high losses which they incur in crops, populations of *Heterodera* ssp., *Globodera* ssp., and *Pratylenchus* ssp., as well as other species of zoonematodes, may also be controlled effectively by the selected strains.

According to another embodiment of the invention, a nematicidically active metabolite of a selected strain is used rather than a culture of the strain. Said nematicidically active metabolite may be obtained by any useful method, for example be obtained by isolation from its natural environment, or be produced by recombinant DNA technology, or be synthesized by chemical synthesis.

The following examples merely serve to illustrate the invention and should particularly not be construed such as to limit the invention.

EXAMPLES

Example 1: Isolation of soil bacterial strains with possible effects on phytopathogenic nematodes.

In an area with a high incidence of nematodes (*Radopholus similis*), banana plants were selected which featured a high infestation in the root system, and others which, despite being in the same area, featured a much lesser infestation of the root system. The difference in the vigour of the two groups was significant. Segments of the roots of the selected plants were collected, of 1 cm in width, as well as samples of the associated soil. The soil samples were screened separately using a No. 40 mesh, and one gram of the screened material was diluted in 10 ml of sterile water, then homogenized by stirring. After being allowed to rest for five minutes, 200 µl of the supernatant was placed on Petri dishes with LB medium. The samples were then incubated for 48 hours at 28°C, and the colonies isolated were then reseeded, but separately, and cultured at 28°C for 24 hours. Comparison of the morphology of the strains derived from each of the groups of samples analyzed resulted in the observation that some of the strains were present only in the group deriving from the soil contained in the root complexes of the more vigorous banana plants, with a healthier root system. In total, four strains were isolated, which were immediately subjected to an analysis of activity with regard to the eggs of *Meloidogyne incognita*.

Example 2: Classification of the bacterial strain C-926 with nematicidal activity.

The bacterial strain isolated appeared to be a Gram negative coccobacillus, which was classified by the API-20 NE system (BIOMERIEUX SA, 69280 Marcy l'Etoile, France), and designated *Sphingobacterium spiritivorum* C-926 according to that system. The results obtained on the basis of the biochemical tests carried out are shown in Table 1.

TABLE 1

Biochemical test	24 h	48 h
Nitrate (NO_3) reduction	neg	neg
Tryptophan (TRP)	neg	neg
Glucose fermentation (GLU)	neg	neg
Arginine hydrolase (ADH)	neg	neg
Urease (URE)	neg	neg
Esculine (ESC)	pos	pos
Gelatine (GEL)	neg	neg
p-Nitrophenyl-βD-Galactopyranoside (PNPG)	pos	pos
Assimilation of glucose (GLU)	neg	pos
Assimilation of arabinose (ARA)	neg	neg
Assimilation of mannose (MNE)	neg	pos
Assimilation of mannitol (MAN)	neg	neg
Assimilation of N-Acetyl-Glucosamine (NAG)	neg	pos
Assimilation of maltose (MAL)	neg	pos
Assimilation of gluconate (GNT)	neg	neg
Assimilation of caprate (CAP)	neg	neg
Assimilation of adipate (ADI)	neg	neg
Assimilation of malate (MLT)	neg	neg
Assimilation of citrate (CIT)	neg	neg
Assimilation of phenyl acetate (PAC)	neg	neg
Oxidase activity (OX)	pos	pos

Other reactions:

Biochemical test	Notes
Fermentation with production of H_2S	pos
Kliger test	neg
Casein hydrolysis	neg
Catalase activity	pos
Voger-Proskaver test (methyl-carbinol)	neg
Starch hydrolysis	pos

Identity number in accordance with the API 20 NE system: 0462304. The identity corresponds to *Sphingobacterium spiritivorum*, the strain being designated as: *Sphingobacterium spiritivorum* C-926.

Example 3: Classification of the bacterial strain C-924 with nematicidal activity.

The bacterial strain takes the form of a short Gram-positive bacillus, and was classified by the API-50 CH system (BIOMERIEUX SA, 69280 Marcy l'Etoile/France) and was accordingly designated as *Corynobacterium paurometabolum* C-924. The results obtained on the basis of the biochemical tests carried out are shown in Table 2.

TABLE 2

Biochemical test	24 h	48 h
Glycerol	neg	neg
Erythritol	neg	neg
D-arabinose	neg	neg
L-arabinose	neg	neg
Ribose	neg	neg
D-xylose	neg	neg
L-xylose	neg	neg
Adonitol	neg	neg
β-methyl-xyloside	neg	neg
Galactose	neg	neg
D-glucose	neg	neg
D-fructose	neg	neg
D-mannose	neg	neg
L-sorbose	neg	neg
Ramnose	neg	neg
Dulcitol	neg	neg
Inositol	neg	neg
Mannitol	neg	neg
Sorbitol	neg	neg
α-methyl-D-mannoside	neg	neg
α-methyl-D-glucoside	neg	neg
N-acetyl-glucosamine	neg	neg
Amygdaline	neg	neg
Arbutin	neg	neg
Esculin	pos*	
Salicine	neg	neg
Cellobiose	neg	neg
Maltose	neg	neg
Lactose	neg	neg
Melibiose	neg	neg
Saccharose	neg	neg
Trehalose	neg	neg
Inuline	neg	neg
Melecitose	neg	neg
D-rabinose	neg	neg
Starch	neg	neg
Glucogene	neg	neg
Xylitol	neg	neg
Beta-gentibiose	neg	neg
D-turanose	neg	neg
D-lyxose	neg	neg
D-tagatose	neg	neg
D-fucose	neg	neg
L-fucose	neg	neg
D-arabitol	neg	neg
L-arabitol	neg	neg
Gluconate	neg	neg
2-keto-gluconate	pos**	
5-keto-gluconate	neg	neg

Reactions were carried out with the API Coryne system (BIOMERIEUX SA, Marcy l'Etoile/France) and designated according to this system as *Corynobacterium paurometabolum* C-924:

Biochemical test	24 h
Reduction of nitrates (NIT)	neg
Pyrazinamidase (PYZ)	pos
Pyrrolidonyl arylamidase (PyrA)	pos
Alkaline phosphatase (PAL)	neg
β-glucuronidase (β GUR)	neg
β-galactosidase (β GAL)	neg
α-glucosidase (α GAL)	neg
N-acetyl-β-glucosaminidase (β NAG)	neg
Esculin (β-glucosidase) (ESC)	neg
Urease (URE)	neg
Gelatine (hydrolase) (GEL)	neg
Glucose (GLU)	neg
Ribose (RIB)	neg
Xylose (XYL)	neg
Mannitol (MAN)	neg
Maltose (MAL)	neg
Lactose (LAC)	neg
Saccharose (SAC)	neg
Glucogene (GLY)	neg
Catalase (CAT)	pos

Identity number according to the Api Coryne system: 02400000000000000004. The identity corresponds to *Corynobacterium paurometabolum*, the strain being designated as: *Corynobacterium paurometabolum* C-924.

Example 4: Effect of the bacterial strains on the eggs and young larvae of *Meloidogyne incognita* under "in vitro" conditions.

A number of different bacterial strains were assessed with regard to their effect on the eggs and larvae of *Meloidogyne incognita*, under "in vitro" conditions. The strains to be evaluated were grown in an LB medium at 28°C for 24 hours, under continuous agitation at 100 rpm. The cells were centrifuged, washed with sterile distilled water, and the number of viable specimens was determined in each case. Dilutions of 10^6 cells/ml were prepared in the LB medium. Also included was a negative control consisting solely of the LB medium. Four variants and four replicas were taken. Each replica was held in a 25 ml capacity "Syracuse" flask. Approximately 1000 eggs of

the nematode were added to each receptacle (24 receptacles in total), and 20 ml of the dilutions of each strain, in addition to the negative controls. Periodic observations were made at 24 and 48 hours, measuring the number of eggs which had hatched and the mortality rate among the larvae.

The results of the effects of the bacterial strains C-926 and C-924, expressed as a percentage, are shown in Table 3.

TABLE 3

Strain	Eggs hatched			Effect among larvae		
	24 h	48 h	V(%)	24 h	48 h	V(%)
1 Strain C-926	0	0	0	0	0	-
2 Strain C-924	2	2	0.3	2M	2M	-
3 *E.coli*	12	20	5	12V	20V	5
4 LB+H_2O	19	25	5	19V	25V	5

Note: V is the coefficient of variation. The hatching levels are considered good for this type of "in vitro" experiment. V = alive, M = dead.

Example 5: Effect of various bacterial strains on populations of *Meloidogyne incognita* in pumpkin plants.

An experiment was conducted making use of pumpkin plants planted in pots of 7 cm in diameter, with a depth of 10 cm of soil previously infected with the eggs of *Meloidogyne incognita*. A black carbonated soil was used, which had been screened and sterilized in an autoclave at 1.5 atmospheres for one hour, and a month was allowed to elapse in order for the microflora of the soil to recover. The inoculation was carried out in each of the replicas (pots), adding 500 viable eggs of the nematode in 2 ml of distilled sterile water. The eggs were deposited uniformly at a depth of 4 cm. The application of the bacterial cultures took place 6 hours subsequent to the inoculation, 50 ml of 10^7 cells per ml being applied in each case. At 72 hours from application, a pre-germinated pumpkin seed was planted in each pot. The humidity of the soil was maintained uniformly in the pots for 60 days by watering. Once this period had elapsed, the plants were extracted, and an assessment made of the damage caused to the root system by the attack of the nematode, according to the

Zeck scale. A culture of *E.coli* MC1061 was used as a negative control, in the LB medium without any bacteria. Ten replicas were used. The strain designated as C-926 showed a higher degree of effect in the protection of the root system of the indicator plants. The results are shown in Table 4.

TABLE 4

STRAIN	Mean degree of effect	V(%)
1 Strain C-926	2.1	4
2 Strain C-924	2.0	3
3 *E.coli*	6.0	5
4 LB	6.5	5

Note: V = Coefficient of variation. The hatching levels under the conditions of this type of experiment are always greater than those which are encountered in "in vitro" hatching experiments.

Example 6: Effect of the bacterial strains on population of *Radopholus similis* in banana plants.

This experiment was carried out under similar conditions to those described above, in pots 10 cm in diameter and 20 cm in depth. 500 individual *Radolphus similis* were deposited, in 3 ml of distilled sterile water, at a depth of 4 cm. At 5 days, 50 ml of 10^7 cells/ml of each variant was applied. At 24 hours, a banana plant was planted in each pot. The plants used were derived from an "in vitro" crop, this being free of pathogens. At two months from planting, the plants were transferred to the storage facility, and the humidity of the soil was maintained with two uniform waterings a day. The final evaluation of the experiment was made at the end of five months. The experiment was conducted with seven variants and ten replicas, and the results are shown in Table 5.

TABLE 5

(Mean values per treatment)

STRAIN	Weight (g) Leaf	Weight (g) Root	Nematodes per plant Live	Dead	Total
1 *S.spiritivorum* Strain C-926	34.7	34.2	7.5	42.5	50.0
2 *C.paurometabolum* Strain C-924	36.3	36.0	15.0	15.0	20.0
3 *P.lilacinus**	36.0	34.6	105.0	-	105.0
4 *Bacillus subtilis*	38.55	40.6	1513.0	-	1513.0
5 LB + water	27.85	25.71	9275.0	-	9275.0
6 Sincocin™	2.5	43.8	322.5	-	322.5
7 B.t. kurstaki*	31.6	30.8	50.0	-	50.0

* 5 replicas.

The populations of live nematodes were subjected to a statistical analysis (ANOVA), which showed significant differences at the level of 1 to 5% of error:

TABLE 5a

TREATMENTS:

	3	6	2	7	1	5
4			+	++	++	++
3				+	++	++
6					+	++
2					+	+
7						
1						

+ Significant difference, level 5% of error

++ Highly significant difference, level 1% of error

Example 7: Control of *Radopholus similis* under field conditions, using bio-preparations of nematicidic strains.

An experiment was conducted to verify the control of bio-preparations of *Sphingobacterium spiritivorum* strain C-926 and of *Corynobacterium paurometabolum* strain C-924 on *Radopholus similis*, in a plantation of green bananas of the "Macho 3/4" variety, under field conditions. To this end, a plan was prepared of blocks located at random (7 treatments with three

replicas), in three rows of plants, divided into parcels of six plants each. The treatments applied were: *Bacillus subtilis* (negative control used because of its possible occurrence in the ecosystem, at a concentration of 10^7 cells per ml of culture), Nemacur™ (a chemical nematicide produced and marketed by Bayer AG, which was used in a concentration of 12 litres per hectare at 40%), *Sphingobacterium spiritivorum* strain C-926, *Corynobacterium paurometabolum* strain C-924, LB culture medium, and the fungus *Paecilomyces lilacinus* (a strain currently used in Cuba for controlling nematodes, and which was used in a concentration of 10^7 spores per ml of suspension, obtained from cultivation in the solid phase, the dosage recommended by the National Institute of Plant Health). The application of the strains C-924 and C-926 consisted of five litres of a dilution of 10^7 cells (UFC) per millilitre, derived from cultures previously fermented in LB medium at 28°C, for 18 hours. The phytonematological samples gave results showing variations in the nematode population at two and three months of the treatments, the initial population acting as the base (100%). A second treatment was subsequently carried out and evaluated two months later, comparing the results of the sample with those obtained prior to the first treatment. The results allow to observe the effectiveness of the strain under investigation in the bio-control of *Radopholus similis* in banana plants under field conditions (Table 6).

TABLE 6

Treatments	3 months	6 months	2 months
A. *B.subtilis*	155.36%	182.97%	128.26%
B. C-924+C-926	8.15%	398.52%	15.56%
C. C-924	0.00%	545.69%	14.98%
D. Nemacur™	0.14%	264.00%	12.35%
E. C-926	1.19%	295.70%	14.96%
F. LB medium+water	257.00%	1017.91%	900.02%
G. *P.lilacinus*	70.47%	232.00%	188.79%

Example 8: Control of *Haemonchus* sp. using bio-preparations of bacterial strains.

Collection and disinfection of the eggs:

Samples of the nematode *Haemonchus* sp. were collected from the abdomen of a sheep (ram) slaughtered for the purpose. The females of this adult nematode were treated with "Hibitane" at 0.5% for one minute, and collected in NB medium (nutrient broth) at 37°C for 24 hours. From this point on, all handling was carried out under aseptic conditions within a laminar flow.

The total volume of the NB medium containing the eggs and the remaining nematodes (20 ml) was passed through a 60 µm mesh, followed by one of 30 µm. The eggs of the nematodes were retained on the 30 µm mesh, and the mesh, with the eggs, was introduced into a solution of sodium hypochlorite at 0.1% for three minutes, followed immediately by two washes with sterile distilled water.

Handling and distribution of the eggs:

The disinfected eggs were extracted from the mesh, and carefully put back into suspension with a solution of Mss medium (minimum volume, in which the weight of the salts was replaced by sugars), consisting of one part by volume of the medium to two parts by volume of distilled sterile water. The volume was adjusted to 500 eggs per ml, and in this manner each sample of 100 µl guaranteed a mean value of 50 eggs. Two culture dishes of 96 wells were used, in which the samples of 100 µl provided were deposited, thus providing the uniform distribution of the nematode eggs.

Nematicide treatments used:

12 treatments were applied, adjusting the 100 µl samples which were mixed with the 100 µl samples containing the eggs, thus providing 200 µl for each well. Each treatment included 12 replicas.

Negative controls:
- Medium Mss diluted 2x (treatment #9)
- Sterile distilled water (treatment #5)
- *Bacillus subtilis* strain 6633 (treatment #2)
- *Escherichia coli* strain C-600 (treatment #6)

Positive controls:
- ** *B.thuringiensis* var. kurstaki H-10 (treatment #4)
- ** *B.thuringiensis* var. israeliensis "Bactimor" (treatment #1)
- ** *B.thuringiensis* var. israeliensis "Tecnar" (treatment #10)

Nematicides submitted to the test:
-*C-924 (treatment #3)
-*C-926 (treatment #8)
-*C-924+C-926 (treatment #11)
-***B.thuringiensis* 9452 (treatment #7)
-**B.t. 9499 (treatment #12)

*: Cultures (50 ml) of Mss medium with an initial d.o. = 0.05, cultured in a sieve unit at 28°C, 100 rpm for 54 hours and diluted to 10^8 UFC/ml.

**: Cultures (50 ml) of Mss medium with initial d.o. = 0.05, cultured in a sieve unit at 28°C, 100 rpm for 5 days (formation of spores) and diluted twice.

The culture dishes were sealed with parafilm, and the eggs in each replica were counted before being incubated at rest at 28°C in darkness, in anticipation of the larvae hatching. The hatched larvae were counted at 36 and 84 hours from the start of the experiment, using an inverted microscope.

TABLE 7

Treatment	T(hours)	Eggs	Larvae	% hatched
1	36	684	8	1.2
1	84	684	9	1.3
2	6	176	64	36.4
2	84	176	65	36.9
3	36	447	46	10.3
3	84	447	69	15.4
4	36	816	0	0
4	84	816	0	0
5	36	794	95	12.0
5	84	794	98	12.3
6	36	640	88	13.8
6	84	640	93	14.5
7	36	426	0	0
7	84	426	0	0
8	36	562	0	0
8	84	562	0	0
9	36	537	129	24.0
9	84	537	132	24.6
10	36	526	0	0
10	84	526	62	11.8
11	36	492	0	0
11	84	492	0	0
12	36	602	0	0
12	84	602	12.8	0

Statistical analysis of the results:

Bearing in mind that the larvae hatched between 24 and 48 hours, the statistical analysis (ANOVA) was made on the data at 36 hours.

TABLE 7A

Treatment:	9	6	5	3	1	10	12	7	4	8	11
2	++	++	++	++	++	++	++	++	++	++	++
9		++	++	++	++	++	++	++	++	++	++
6			-	+	++	++	++	++	++	++	++
5				-	++	++	++	++	++	++	++
3					++	++	++	++	++	++	++
1						-	-	-	-	-	-
10							-	-	-	-	-
12								-	-	-	-
7									-	-	-
4										-	-
8											-

- Without significant differences

* Significant differences, level of error of 5%

** Highly significant differences, level of error of 1%.

Notes:

The C-926 (treatment #8) showed a marked effect on the eggs, but this did not occur with the C-924 (treatment #3). The latter case may be due to the longer growing time to which the cultures were subjected (54 hours).

The best controls were selected from this experiment (both positive and negative), and the others were discounted, to be used in later experiments. One of the negative controls which presented difficulties was the *E.coli*, due to its continuous growth, which causes an impediment in the observation of the eggs and larvae. The distilled water was not a very good control either, due to the fact that many of the eggs "slowed down" on being collected in the water, after their treatment with hypochlorite. Sodium hypochlorite does not appear to be the ideal disinfectant for this type of disinfection (despite this having been reported), since the hatching rates (in the negative controls) should be above 50%.

Example 9: Control of *Trichostrongylus* sp. using biopreparations of the bacterial strains.

Collection and disinfection of the eggs:

Collections were made of the nematode *Trichostrongylus* sp. from the abdomen of a sheep (ram) slaughtered for this purpose. The females of this adult nematode were treated with "Hibitane" at 0.5% for one minute, and collected in NB medium (nutrient broth) at 37°C for 24 hours. From this point on, all handling was carried out under aseptic conditions within a laminar flow.

The total volume of the NB medium containing the eggs and the remaining nematodes (20 ml) was passed through a 60 µm mesh, followed by one of 30 µm. The eggs of the nematodes were retained on the 30 µm mesh, and the mesh, with the eggs, was introduced into a solution of sodium hypochlorite at 0.1% for three minutes, followed immediately by two washes with LB medium, diluted 10^{-1} with sterile distilled water.

Handling and distribution of the eggs:

The disinfected eggs were extracted from the mesh, and carefully put back into suspension with the 10^{-1} solution of the LB medium. The volume was adjusted to 100 eggs per ml, and in this manner each sample of 100 µl guaranteed a mean value of 10 eggs. One culture dish of 96 wells was used, in which the samples of 100 µl provided were deposited, thus providing the uniform distribution of the nematode eggs.

Nematicide treatments used:

12 treatments were applied, adjusting the 100 µl samples which were mixed with the 100 µl samples containing the eggs, thus providing 200 µl for each well. Each treatment included 8 replicas.

Negative controls:
- LB Medium diluted 10^{-1} (treatment #5)
- *Bacillus subtilis* strain 6633 [10^9 UFC/min] (treatment #3)
- *Bacillus subtilis* strain 6633 [10^8 UFC/min] (treatment #8)

Positive controls:
- ***B.thuringiensis* kurstaki H-10, diluted 2x (treatment #4)
- ***B.thuringiensis* kurstaki H-10, diluted 10^{-1} (treatment #9)
- ***B.thuringiensis* kurstaki H-10, diluted 10^{-2} (treatment #12)

Nematodicides submitted to the test:
-*** C-924 [10^9 UFC/ml] (treatment #1)
-*** C-924 [10^8 UFC/ml] (treatment #6)
-*** C-924 [10^7 UFC/ml] (treatment #10)
-*C-926 [10^9 UFC/ml] (treatment #2)
-*C-926 [10^8 UFC/ml] (treatment #7)
-*C-926 [10^7 UFC/ml] (treatment #11)

*: Cultures (50 ml) of LB medium with an initial d.o. = 0.05, cultured in a sieve unit at 28°C, 100 rpm for 46 hours.

**: Cultures (50 ml) of LB medium with initial d.o. = 0.05, cultured in a sieve unit at 28°C, 100 rpm until sporulation.

***: Cultures (50 ml) of LB medium with initial d.o. = 0.05, cultured in a sieve unit at 28°C, 100 rpm for 22 hours.

The culture dish was sealed with parafilm, and the eggs in each replica were counted before being incubated at rest at 28°C in darkness, in anticipation of the larvae hatching.

Results observed:

Hatched larvae were counted at 48, 120, and 192 hours (8 days) from the start of the experiment, using an inverted microscope. Observation of the larvae was continued subsequently (in the first sieve) with regard to their activity.

TABLE 8

Treatments	% of hatchings			% of larvae	
	48 h	120 h	192 h	Actives	Inactives
1	0	0	0	-	-
2	0	0	0	-	-
3	0	15.2	15.2	100	0
4	0	0	1.8	0	100
5	48.3	48.3	48.3	100	0
6	0	0	3.12	100	0
7	0	6.2	12.5	66.6	33.3
8	4.6	25.5	25.5	100	0
9	0	0	0	-	-
10	25.7	50	50	14.3	85.7
11	1.5	1.5	1.5	0	100

Notes:

The strains C-924 (treatments #1, 6 and 10) and C-926 (treatments #2, 7, and 11) showed a marked effect on the eggs in higher concentrations, but this did not occur with the lower concentration, where the inactive response of the larvae could be observed. The joined effect on the eggs and larvae of the first stage should be taken into account. Although sodium hypochlorite does not appear to be the ideal disinfectant for this type of work, the percentages of hatching of the negative controls were acceptable. The growing time of these strains is a very important factor to take into account, as can be appreciated by comparing the results of experiment #1 with those of experiment #2.

Example 10: Control of *Trichostrongylus* sp. using biopreparations of the bacterial strains taken at different growing times.

Collection and disinfection of the eggs:

Collections were made of the nematode *Trichostrongylus* sp. from the abdomen of a sheep (ram) slaughtered for this purpose. The females of this adult nematode were treated with "Hibitane" at 0.5% for one minute, and collected in an LB medium diluted at 10^{-1} at 37°C for 24 hours. From this point on, all handling was carried out under aseptic conditions within a laminar flow. The total volume of the LB medium containing the eggs and the remaining nematodes (20 ml) was passed through a 60 µm mesh, followed by one of 30 µm. The eggs of the nematodes were retained on the 30 µm mesh, and the mesh, with the eggs, was introduced into a solution of "Hibitane" at 0.5% for three minutes, followed immediately by three washes with LB medium, diluted 10^{-1}.

Handling and distribution of the eggs:

The disinfected eggs were extracted from the mesh, and carefully put back into suspension with the 10^{-1} solution of the LB medium.

The volume was adjusted to 300 eggs per ml, and in this manner each sample of 100 µl guaranteed a mean value of 30 eggs.

One culture dish of 96 wells was used, in which the samples of 100 µl provided were deposited, thus providing the uniform distribution of the nematode eggs.

Nematodicide treatments used:

16 treatments were applied, with 6 replicas, adjusting the 100 µl samples which were mixed with the 100 µl samples containing the eggs, thus providing 200 µl for each well.

Negative controls used:

- LB medium diluted 10^{-1}
- *_B.subtilis_* strain 6633 [10^7 UFC/ml], 8 hours growth
- *_B.subtilis_* strain 6633 [10^8 UFC/ml], 16 hours growth
- *_B.subtilis_* strain 6633 [10^8 UFC/ml], 20 hours growth
- *_B.subtilis_* strain 6633 [10^8 UFC/ml], 24 hours growth
- *_B.subtilis_* strain 6633 [10^8 UFC/ml], 36 hours growth

Nematicide treatments applied to the experiment:

-*C-924 [10^7 UFC/ml], 8 hours growth
-*C-924 [10^8 UFC/ml], 16 hours growth
-*C-924 [10^8 UFC/ml], 20 hours growth
-*C-924 [10^8 UFC/ml], 24 hours growth
-*C-924 [10^8 UFC/ml], 36 hours growth
-*C-926 [10^7 UFC/ml], 8 hours growth
-*C-926 [10^8 UFC/ml], 16 hours growth
-*C-926 [10^8 UFC/ml], 20 hours growth
-*C-926 [10^8 UFC/ml], 24 hours growth
-*C-926 [10^8 UFC/ml], 36 hours growth

* Cultures (50 ml) of LB medium with initial d.o. = 0.05, cultured in a sieve at 28°C at 100 rpm.

The culture dish was sealed with parafilm, and the eggs in each replica were counted before being incubated at rest at 28°C in darkness, in anticipation of the larvae hatching.

Results observed:

Counts were made of hatched larvae every 24 hours, up to 168 h (during one week), and observations made to determine the moment at which hatching occurred in each treatment.

TABLE 9

Culture time:	8h			16h			20h			24h			36h		
Treatment	H:	L:	%	H:	L:	%	H:	L:	%	H:	L:	%	H:	L:	%
C.paurometabolum															
Strain C-924*	167	6	3.5%	161	0	0%	154	0	0%	167	0	0%	266	0	0%
S.spiritivorum															
Strain C-926**	148	69	46.6%	265	149	56.2%	172	104	60.4%	149	49	32.8%	169	64	37.8%
*B.subtilis****	112	71	63.3%	166	107	64.4%	182	107	58.7%	167	113	67.6%	217	160	73.7%

CONTROL: LB diluted at 10^{-1} = Eggs: 192. Hatched larvae: 142, making 79.9 % hatching.

* Final count at 168 hours

** Hatching took place as from 110 hours of incubation

*** Hatching was prior to 48 hours.

Notes:

The strains C-924 and C-926 showed a marked effect on the eggs at different times of growth; however, it is quite clear that C-924 entirely limits hatching as from 16 hours of growth, while the effect of C-926 consists of retarding hatching up to later than 110 hours, basically in the treatments of 24 and 36 hours of growth. This delay can be effective for the control of nematodes "in vivo", by breaking the normal hatching cycle, which is between 24 and 48 hours (confirmed in the negative controls of this experiment).

It was shown by this experiment that the growth time of the strains is a very important factor to take into account, as we have already indicated by comparing the results of experiment #1 with that of #2.

"Hibitane" turns out to be an adequate disinfectant for this type of work, inasmuch as the hatching percentages in the negative controls were between 58.7 and 79.9%.

CLAIMS:

1. A method for the bio-control of nematodes, comprising contacting the soil, a plant or a seed with an effective quantity of a nematicidic agent, the said agent being a culture obtained from *Sphingobacterium spiritivorum* C-926 and/or *Corynobacterium paurometabolum* C-924, or a metabolite derived from the said strains, obtained by natural, recombinant or synthetic route, in an appropriate carrier, or using the said nematicidic agent in a veterinary composition for the control of zoonematodes.

2. A method according to Claim 1, characterised by the fact that it is used for the control of *Radopholus similis* and *Meloidogyne incognita* in plants, and for the zoonematodes *Haemonchus* ssp. and *Trichostrongylus* ssp.

3. A method according to Claim 1, characterised by the fact that, for the situation in which it is applied on the soil, the effective quantity of the nematicidic agent consists of approximately 14 L of a culture of *Sphingobacterium spiritivorum* C-926 and/or *Corynobacterium paurometabolum* C-924, which is added per hectare of ground in an approximate concentration of between 10^6 and 10^7 of colony forming units per ml of medium, which are applied to the affected ground every three months.

4. A method according to Claim 1, characterised by the fact that it is effective in the control of *Radopholus similis* and *Meloidogyne incognita* in plants and the zoonematodes *Haemonchus* ssp. and *Trichostrongylus* ssp.

5. A culture of *Sphingobacterium spiritivorum*, used as a nematodicidic agent in the method according to Claim 1, characterised by the fact that the said culture is of the strain C-926 of *Sphingobacterium spiritivorum* or any mutation derived therefrom which is active against nematodes.

6. A culture of *Corynobacterium paurometabolum*, used as a nematodicidic agent in the method according to Claim 1, characterised by the fact that the said culture is of the strain

C-924 of *Corynobacterium paurometabolum* or any mutation derived therefrom which is active against nematodes.

7. A culture according to Claim 4, characterised by the fact that it contains between approximately 10^6 and 10^7 colony forming units per ml of the culture medium.

8. A nematicidic agent used for the biological control of nematodes, characterised by the fact that the culture contains at least one strain of *Sphingobacterium spiritivorum* and/or *Corynobacterium paurometabolum*, or a mutation derived from the said strains, as well as any active principle agent or metabolite obtained on the basis of the said strains, obtained by natural, recombinant or synthetic route, and an appropriate carrier, to be used in agriculture or in the veterinary sector.

9. A nematicidic agent according to Claim 7, characterised by the fact that, for the control of phytonematodes, the said appropriate carrier is a fertilizer, a pre-packaged soil, a seed cover device, a powder, a granulate, a nebulizer, a suspension, or a liquid, or any of the variants indicated, in an encapsulated form.

10. Method for obtaining a bacterial strain having nematicidal properties, comprising collecting samples of soil and/or plant tissue from an area affected by nematodes, distinguishing samples of highly affected material from samples of less affected material, isolating bacterial strains present in said samples, comparing bacterial strains present in highly affected samples with bacterial strains present in less affected samples, selecting bacterial strains present only in said less affected samples, testing nematicidal properties of the selected strains and selecting those bacterial strains having useful nematicidal properties.

11. A bacterial strain having nematicidal activity, which is obtainable by the method of claim 10.

INTERNATIONAL SEARCH REPORT

Inte· ·ional Application No
PC·/NL 95/00271

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N63/00 C12R1/15 C12R1/01

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	JOURNAL OF CHEMICAL ECOLOGY, vol.15, no.6, June 1989, NEW YORK, US pages 1947 - 1955 B.M.ZUCKERMAN ET AL 'Suppression of Plant Parasitic Nematodes in the Chinampa Agricultural Soils' see the whole document	10,11
X	WO,A,93 19604 (RESEARCH CORPORATION TECHNOLOGIES) 14 October 1993 see page 2, line 23 - page 4, line 24 see page 11, line 20 - page 12, line 27; example 1	10,11
A		1-4,7-9
A	EP,A,0 401 560 (TODA BIOSYSTEM LABORATORY) 12 December 1990 see claims 1,3	1-9

-/--

[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

Date of the actual completion of the international search	Date of mailing of the international search report
9 November 1995	29. 11. 95
Name and mailing address of the ISA 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	Authorized officer Fletcher, A

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/NL 95/00271

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 303 426 (MYCOGEN CORPORATION) 15 February 1989 see claims -----	1-4,7-9

INTERNATIONAL SEARCH REPORT

International application No.
PC NL95/00271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1,2,4
because they relate to subject matter not required to be searched by this Authority, namely:
see attached sheet

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

Incomplete search.

Claims searched completely: 3,5-11
Claims searched incomplete: 1,2,4

In it's present wording, Claim 1 can be understood to relate in part to a method of treatment of animals using the nematicidal bacterial strains. It has been searched as if that part of the claim referred to the use of the bacterial strains for the Manufacture of a veterinary composition for the control of zoonematodes.
The same applies to dependent claims 2 and 4 which also relate, in part, to a method for control of zoonematodes.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 95/00271

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9319604	14-10-93	CA-A-	2132456	14-10-93
		EP-A-	0633725	18-01-95
		US-A-	5378460	03-01-95
EP-A-0401560	12-12-90	JP-A-	2304008	17-12-90
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		DE-D-	69006639	24-03-94
		DE-T-	69006639	23-06-94
		US-A-	5208159	04-05-93
EP-A-0303426	15-02-89	US-A-	4948734	14-08-90
		CA-A-	1335664	23-05-95
		DE-A-	3874052	01-10-92
		JP-A-	1067192	13-03-89
		US-A-	5439881	08-08-95
		US-A-	5322932	21-06-94
		US-A-	5426049	20-06-95
		US-A-	5093120	03-03-92
		US-A-	5281530	25-01-94
		US-A-	5236843	17-08-93
		US-A-	5262399	16-11-93