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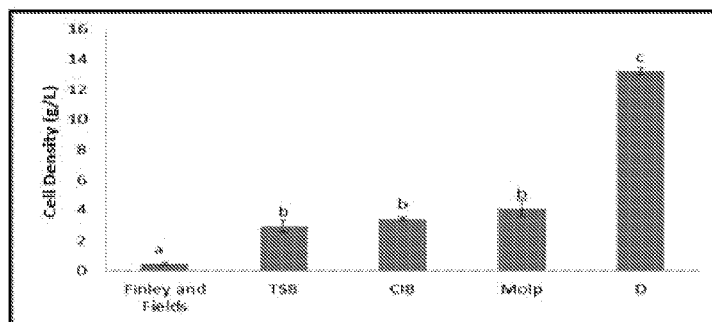
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- (54) **Title:** PRODUCTION PROCESS FOR BIOMASS AND FENGYCIN METABOLITES OF BACILLUS SPECIES AND COMPOSITIONS THEREOF FOR BIOLOGICAL PEST CONTROL

FIG. 2

Different letters denote significant differences (P value <0.05).

- (57) **Abstract:** The present invention refers to a process for increasing the production of biomass and metabolites of microorganisms of *Bacillus* sp. species. Obtained metabolites are lipopeptide compounds of the fengycin, surfactin, and iturin families, which exhibit antimicrobial activity. The invention further includes biocidal compositions comprising *Bacillus subtilis* EA- CB0015, *Bacillus amyloliquefaciens* EA-CB0959, and/or metabolites thereof, either alone or together with other biocidal agents, and the use of these compositions for the treatment of diseases caused by various phytopathogenic agents, including *Mycosphaerella fijiensis*, in a variety of crops.

**PRODUCTION PROCESS FOR BIOMASS AND METABOLITES OF *Bacillus*
SPECIES AND COMPOSITIONS THEREOF FOR BIOLOGICAL PEST
CONTROL**

5 **FIELD OF THE INVENTION**

The present invention refers to a process for increasing the production of biomass and metabolites of *Bacillus* species, including *Bacillus subtilis* and *Bacillus amyloliquefaciens*. The process includes a suitable culture medium and specific
10 environmental conditions, allowing for the production of large amounts of biomass and metabolites of the fengycin, surfactin, and iturin families, which exhibit antifungal and antibacterial activity against various phytopathogenic agents.

PRIOR ART

15 Among the microorganisms for biological control, bacteria of *Bacillus* sp. genus have received much attention due to the wide variety of antibiotic compounds they produce, their long shelf life, their fast growth in culture, and their ability to colonize leaf surfaces [1, 2, 3, 4]. In particular, certain species of *Bacillus* such as *Bacillus subtilis*,
20 *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus anthracis*, and *Bacillus thuringiensis* show antimicrobial activity [4].

The antimicrobial activity of these bacteria is due to their ability to produce lipopeptides of the surfactin, iturin, and fengycin families, which differ in the amino acid sequence
25 and the branching of the fatty acid chain. Surfactins exhibit high antibacterial activity, whereas iturins and fengycins are recognized for their antifungal activity [4].

The prior art describes the use of *B. subtilis* and *B. amyloliquefaciens* to control various disease-causing microorganisms in a wide variety of crops, including fruit and vegetable
30 crops such as blackberry, grape, raspberry, strawberry, tomato, cucumber, black pepper, orange, melon, apple, peach, custard apple, banana, papaya, mango, and kiwi.

EP2311936 discloses a *B. subtilis* strain KS1 (NITE BP-569) as a biological control agent to counteract several phytopathogenic microorganisms in vine crops. WO 98/21968 discloses an antibiotic produced by *B. subtilis* AQ153 (ATCC 55614) effective against bacterial and fungal infections and also as method for protecting plants that comprises the application of these antibiotic compounds.

WO9850422 and WO0029426 disclose other antibiotic compounds produced by the *B. subtilis* strain AQ713 (NRRL B21661) and its mutants, which exhibit insecticidal, antifungal, and antibacterial activity. WO9909819 discloses antibiotics of a *B. subtilis* strain AQ 713 (NRRL No. 21665), which produces metabolites with pesticidal activity and a high-molecular-weight metabolite, soluble in water, which exhibits insecticidal and nematicidal activity against corn rootworm and other nematodes.

US2011/0318386 describes methods for inducing systemic resistance against various pathogens through the use of biological controllers of the *Bacillus* genus, specifically of the isolated *B. mojavensis* 203-7 and isolated *B. mycoides* species. In turn, ES 2345969 describes a phytostrengtheners for application on banana and plantain pseudostems, which includes *B. subtilis*, *Trichoderma viride*, and *B. megaterium* var *phosphaticum*.

US2012/00031999 discloses a control strategy for various fungal diseases, including Black Sigatoka in banana, based on the application of synthetic fungicides with some biocontrol microorganisms and their metabolites (specifically *B. subtilis* strain QST 713, corresponding to the strain of the commercial product Serenade®).

In the production processes of microorganisms of *Bacillus* species disclosed in the prior art, the amount of biomass produced is very low, given the obtained cell density is not generally greater than 5.0 g/L [8, 9]. Thus, it is necessary to develop new processes to increase the production of biomass and its active metabolites.

Similarly, it is necessary to develop biocide compositions from these microorganisms and/or their biologically active metabolites with a greater efficiency and selectivity for controlling different phytopathogenic agents on a variety of crops.

5 **BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 Structure of the fengycins C produced by *Bacillus subtilis* EA-CB0015

FIG. 2 Cell density of *Bacillus subtilis* EA-CB0015 in different culture media.

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FIG. 3 HPLC chromatogram of the compounds of *Bacillus subtilis* EA-CB0015

FIG. 4 MS/MS spectrum of the P5 purified lipopeptide (m/z 1429.9) of *Bacillus subtilis* EA-CB0015.

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FIG. 5 MS/MS spectrum of the P5 purified lipopeptide (m/z 1447.8) after alkaline hydrolysis of the lactone ring of *Bacillus subtilis* EA-CB0015.

FIG. 6 Area under the curve of the lipopeptides produced by *Bacillus subtilis* EA-CB0015 in various culture media.

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FIG. 7 Percentages of inhibition generated by *Bacillus subtilis* EA-CB0015 on various phytopathogenic microorganisms.

FIG. 8 Growth inhibition percentages of *Mycosphaerella fijiensis* generated by *Bacillus subtilis* EA-CB0015 CFS obtained in various culture media.

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FIG. 9 Viability of *Bacillus subtilis* EA-CB0015 contained in various compositions of the invention.

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FIG. 10 Degree of severity of Black Sigatoka in banana plants treated with different compositions of the invention at a greenhouse level.

FIG. 11 Percentage of necrotic area caused by Black Sigatoka in banana leaves treated with different compositions of the invention.

5 **FIG. 12** Effect of various adjuvant UV protectors on the viability of *Bacillus subtilis* EA-CB0015.

FIG. 13 Effect of various adjuvants on the adhesion of the compositions (MH2O and P2) based on *Bacillus subtilis* EA-CB0015.

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FIG. 14 Effect of the water-based mixture composition based on *Bacillus subtilis* EA-CB0015 on the percentage of necrotic area of banana leaves at greenhouse level.

FIG. 15 Photographs of banana leaves affected by Black Sigatoka disease after treatment with various products.

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FIG. 16 Effect of water-based composition of *Bacillus subtilis* EA-CB0015 on the severity of Black Sigatoka in banana at field level.

20 **FIG. 17** Effect of *Bacillus subtilis* EA-CB0015 on the severity of the disease caused by *Botrytis cinerea* EAHP-009 in pompons.

FIG. 18 Effect of *Bacillus subtilis* EA-CB0015 on *Colletotrichum* sp. in tree tomato (*Cyphomandra betacea*).

BRIEF DESCRIPTION OF THE INVENTION

The present invention allows solving these and other disadvantages as it comprises a process for increasing the production of biomass of microorganisms of the *Bacillus* genus, including *Bacillus subtilis* EA-CB0015 and *Bacillus amyloliquefaciens* EA-CB0959, as well as their biologically active metabolites, such as lipopeptides of the surfactin, iturin, and fengycin families.

Additionally, the present invention includes agrochemical compositions that comprise microorganisms of different *Bacillus* species, including *Bacillus subtilis* EA-CB0015, *Bacillus amyloliquefaciens* EA-CB0959 and/or active metabolites thereof, either alone or in combination with biocidal agents for the control of phytopathogenic agents such as *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp. *Rhizoctonia solani* and *Fusarium solani*.

The present invention is also directed towards the use of microorganisms of *Bacillus* species, including *B. subtilis* EA-CB0015, *B. amyloliquefaciens* EA-CB0959, and/or active metabolites thereof and agrochemical compositions containing them, for inhibiting the growth of phytopathogenic microorganisms such as *Mycosphaerella fijiensis* in agricultural crops.

DETAILED DESCRIPTION OF THE INVENTION

In order to increase the production of biomass of microorganisms of *Bacillus* sp. and their metabolites (lipopeptides), the microorganism must be cultured in a suitable medium and under the environmental conditions necessary to increase the production of biomass and its metabolites. To do this, a culture medium is prepared (hereinafter medium D) comprising one or more components selected from the group consisting of carbohydrates, yeast extract, ammonium sulfate, peptone, salts containing magnesium, sulfur, manganese, chlorine, potassium, phosphorus, calcium, and sodium either in a solid, semisolid, or liquid matrix.

Environmental conditions necessary for carrying out the process of the invention include temperature, pH, stirring speed, fermentation time, and aeration. The process of the present invention can be performed on a small scale in a laboratory or at large scale
5 in a bioreactor.

In a preferred embodiment, medium D comprises, in w/v percentages, between 3.2% and 3.4% glucose, between 3.1% and 3.3% yeast extract, between $2.5 \times 10^{-3}\%$ and $4.5 \times 10^{-3}\%$ manganese sulfate, between $2 \times 10^{-3}\%$ and $4 \times 10^{-3}\%$ calcium chloride, between
10 0.08% and 0.12% ammonium sulfate, between 0.35% and 0.45% magnesium sulfate, between 0.04% and 0.12% disodium phosphate and between 0.04% y 0.12% monosodium potassium phosphate.

In another even more preferred embodiment of the invention, culture medium D
15 comprises, in w/v percentages, 3.34% glucose, 3.25% yeast extract, $4.2 \times 10^{-3}\%$ manganese sulfate, $3.1 \times 10^{-3}\%$ calcium chloride, 0.1% ammonium sulfate, 0.4% magnesium sulfate, 0.05% disodium potassium phosphate, 0.05% monosodium potassium phosphate.

20 In a preferred embodiment of the process of the invention, the microorganism is incubated for a period between 24 and 96 hours with a stirring speed of 400 to 600 rpm, aeration of 1 to 5 vvm, at a temperature between 20°C and 40°C, and pH between 5.5 and 7.5. Strong acids such as sulfuric acid and/or strong bases such as sodium hydroxide can be used to control and/or adjust the pH. Surfactants and antifoams may
25 also be added to control foam formation.

The process carried out under the above conditions allows increasing the production of biomass and active metabolites of microorganisms of *Bacillus* sp.. The biomass obtained by the process of the present invention can be separated from the culture
30 medium using conventional methods of centrifugation or microfiltration, whereas the active metabolites can be obtained by extraction with solvents, precipitation, adsorption, or chromatography.

In a preferred embodiment of the invention, the amount of biomass of microorganisms of *Bacillus* sp. obtained can range between 3.0 and 20.0 g/L, preferably between 7.0 and 18.0 g/L, while the concentration of metabolites can range between 200 and 1500 mg/L, preferably between 500 and 1000 mg/L.

5

In a preferred embodiment, the process of the invention allows increasing the production of biomass and active metabolites of *B. subtilis* EA-CB0015 and *B. amyloliquefaciens* EA-CB0959. Identification by 16S rDNA analysis established that *B. subtilis* EA-CB0015 corresponds to SEQ ID NO: 1 sequence, which is deposited in GenBank under accession number KC006063.

10

Metabolites of *B. subtilis* EA-CB0015 and *B. amyloliquefaciens* EA-CB0959 obtained by the process of the invention include lipopeptides of the surfactins, iturins, and fengycins types. Analysis by mass spectrometry and chromatographic techniques identified a new fengycin isoform produced by *B. subtilis* EA-CB0015, called fengycins C, whose amino acid sequence is (Glu1-Orn2-Tyr3-THR4-Glu5-Val6-Pro7-Gln8-Thr9-Ile10), which differs from the fengycin B sequence at position 9 and from the fengycin A sequence at positions 6 and 9.

15

Additionally, following the procedure of the invention for *B. subtilis* EA-CB0015, this strain can produce 14 different fengycin C homologues with general formula R-Glu1-Orn2-Tyr3-Thr4-Glu5-Val6-Pro7-Gln8-Thr9-Ile10, which vary according to the size of the saturated or unsaturated hydrocarbon chain (R) of 14 to 18 carbons. FIG 1. illustrates the structures of the various fengycins produced by *B. subtilis* EA-CB0015 by the process of the invention.

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The various homologues of fengycins C, as well as the surfactins and iturins, can be separated by conventional techniques such as high performance liquid chromatography (HPLC). Produced surfactins correspond to different homologues with hydrocarbon chain length between 13 and 16 carbons; iturins correspond to iturins A of 14 and 15 carbons.

30

In the case of *B. amyloliquefaciens* EA-CB0959, metabolites produced by the process of the invention correspond to various surfactin homologues (C12 to C15), two iturin A homologues (C14 and C15), and two fengycin isoforms (A and B) with 4 fengycin A homologues (C14, C15, C16, and C17) and 2 fengycin B homologues (C16 and C17).

5

In a further aspect of the invention, biomass of *B. subtilis* EA-CB0015 or biomass of *B. amyloliquefaciens* EA-CB0959 obtained by the process of the present invention inhibits the growth of various plant pathogens such as *Mycosphaerella fijiensis*, *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani*, and *Colletotrichum* sp. This inhibition can be determined using techniques such as dual plates, which involves comparing the growth of plant pathogens when cultured in a medium with and without the presence of the active substances to be assessed. Determined *in vitro* inhibition percentages are always higher than 50%.

10

After carrying out the process of the present invention, different compositions or formulations can be prepared from the obtain biomass and/or the metabolites in order to produce physicochemically stable biocidal compositions that ensure the viability of the microorganism and the activity of the metabolites in the composition for long periods.

15

These compositions can be prepared in a suitable sealed container to avoid contamination. Biomass and/or its metabolites, adjuvants and other ingredients are added to obtain a homogeneous mixture. The final product thus obtained can be collected in suitable containers and stored at room temperature.

20

In a further aspect, the present invention refers to biocidal compositions comprising *B. subtilis* EA-CB0015, *B. amyloliquefaciens* EA-CB0959, and/or their active metabolites, either alone, combined or in association with other active agents to enhance biological activity. The biocidal compositions of the present invention may contain one or more adjuvants and an agrochemically acceptable carrier.

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In a preferred embodiment, the biocidal compositions of the present invention comprise between 80.0 and 99.9% w/w of an aqueous suspension of *B. subtilis* EA-CB0015 at a

concentration of 1×10^7 a 1×10^{11} CFU/mL together with a mixture composed of 2.0% to 4.0% w/v sodium carboxymethyl cellulose (CMC), 1.0% to 5.0% v/v 3M phosphate buffer (pH 5.0), 1.0% to 4.0% v/v glycerol, 0.25% to 0.75% v/v tween 20[®], 0.25% to 0.5% v/v Triton X-100[®], 0.01% to 1.0% v/v potassium sorbate, 0.05% a 0.15% /v
5 xanthan gum, 0.2% to 1.5 % w/v skimmed milk, and 0.028% to 1.0% w/v TiO₂. This composition is of white color, has a pH of 4.0 to 6.5, and a viscosity of 20 to 80 cp.

In a further preferred embodiment, the biocidal compositions of the invention also comprise chemical pesticides such as anilinopyrimidines, bitartenols, sterols,
10 difeconazole, tebuconazole, epoxiconazole, mancozeb, chlorothalonil and other agents for the biological control of pests, together with one or more adjuvants in an agrochemically acceptable carrier.

In a further aspect, the present invention is directed to the use of microorganisms of
15 *Bacillus* sp. particularly of *B. subtilis* EA-CB0015, *B. amyloliquefaciens* EA-CB0959 and/or their metabolites, as well as their biocidal compositions, to inhibit the growth of phytopathogenic microorganisms such as *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp. *Rhizoctonia solani*, and *Fusarium solani* in agricultural crops.

20 In a further aspect, the present invention is directed to a method for treating plants against infections caused by various phytopathogens, which comprises applying an effective amount of a microorganism of *Bacillus* sp. to the plant, particularly *B. subtilis* EA-CB0015 and *B. amyloliquefaciens* EA-CB0959 and/or their metabolites, or applying
25 biocide compositions containing them, either alone or in combination with other biocidal agents.

The application can be done by spraying at a dose ranging from 0.1 to 10 liters per hectare (L/ha) in admixture with an appropriate carrier or mixed with other
30 compositions containing one or more pesticides.

The following examples further illustrate the invention, although the inventive concept is not restricted thereto.

5 **EXAMPLES**

EXAMPLE 1. Obtaining *Bacillus* sp.

10 Strains of *Bacillus* sp. were obtained from cv. Gran enano and cv. Valery cultivars, both of bananas, and cv. Harton of plantain. A plantation was selected for each cultivar and five points were established to collect composite samples of three plants before flowering using random probability sampling without replacement. Sampling was performed on leaves number 2, 5, and 10 of each plant and each leaf was split to select an area of the apex and an area of the base.

15 Bacterial isolation was carried out by washing with a sodium phosphate buffer and Tween 80[®] and performing sonication of the samples. Serial dilutions were made and plated on TSA surface (Trypticase Soy Agar, Merck at 10%). Gram positive cells were purified, cultured in Finley and Field's medium (150 rpm, 4 days, 30°C) and subjected to heat shock (80°C, 20 min). All AEFBs (Aerobic Endospore-Forming Bacteria) were stored in TSB (Tryptic Soy Broth, Merck) and glycerol (20% v/v) at -80°C, and activated in TSA at 50% prior to any experimental use.

25 **EXAMPLE 2. Obtaining biomass of *Bacillus subtilis* EA-CB0015 and *Bacillus amyloliquefaciens* EA-CB0959**

B. subtilis EA-CB0015 strain was replicated in TSA 50% and incubated for 48 hours at 30°C. A colony of the strain was inoculated in culture medium D and incubated for 12 hours at 30°C and 200 rpm. This culture was used as pre-inoculum. Fermentation was carried out in 50-mL flasks with 10 mL of culture medium D at a temperature of 30°C and 200 rpm in an orbital shaker. Each Erlenmeyer was inoculated with 1 mL of a

bacterial suspension adjusted to an OD₆₀₀ of 1 and obtained after 12 hours of growth. Cell densities of up to 13.2 ± 1.7 g/L of *B. subtilis* EA-CB0015 were obtained.

In order to assess the performance of the process in obtaining biomass of *B. subtilis* EA-CB0015, the amount of biomass obtained using the culture medium of the invention (medium D) was compared with the amount of biomass obtained using CIB, MOLP, Finley and Field's, and TSB culture media.

Cell density obtained in the culture medium of the invention was 29.3 times greater than that obtained in Finley and Field's medium (0.6 ± 0.1 g/L), 4.5 times greater than that obtained in TSB medium (2.95 ± 0.4 g/L), 3.6 times greater than that obtained in the CIB medium (3.65 ± 0.8 g/L), and 3.2 times greater than that obtained in the MOLP medium (4.1 ± 0.6 g/L), as illustrated in FIG 2.

Following the same procedure, biomass of *B. amyloliquefaciens* EA-CB0959 was obtained. As for *B. subtilis* EA-CB0015, the amount of biomass obtained using the culture medium of the invention (medium D) was higher than that obtained in the MOLP and TSB media. Cell densities obtained with the medium of the invention range between 8.0 and 10.0 g/L.

EXAMPLE 3. Extracting and determining active metabolites of *Bacillus subtilis* EA-CB0015 and *B. amyloliquefaciens* EA-CB0959

From *B. subtilis* EA-CB0015 culture obtained according to Example 2, an extraction process of their active metabolites was performed with methanol. Subsequently, a solid phase extraction (SPE) was carried out with methanol as the organic solvent and active fractions were purified by reverse phase HPLC with an UV detector at a wavelength of 214 nm.

FIG. 3 illustrates the respective chromatograms. Eluted peaks between minute 16 and 19 correspond to iturins A (FIG. 3A), peaks P1 to P14 (FIG. 3B) correspond to fengycins C, and peaks P15 to P19 (FIG. 3B) correspond to surfactins. Some of the

active metabolites were also identified by ESI-MS/MS (electrospray mass spectrometry), as shown in FIGS 4 and 5:

In order to assess the performance of the process in obtaining the two groups of active metabolites of *B. subtilis* EA-CB0015, the amount of metabolites obtained using the culture medium of the invention (medium D) was compared with the amount of metabolites obtained using CIB, MOLP, Finley and Field's, and TSB culture media. Peak areas and thus the amount of metabolites obtained were greater when using culture medium D of the invention in the process, as shown in FIG. 6.

Following the same extraction and HPLC purification procedure previously mentioned, metabolites produced by *B. amyloliquefaciens* EA-CB0959 were identified, corresponding to various surfactin homologues (C12 to C15), two iturin A homologues (C14 and C15), and two fengycin isoforms (A and B) with 4 fengycin A homologues (C14, C15, C16, and C17) and 2 fengycin B homologues (C16 and C17).

EXAMPLE 4. Evaluating the activity of *Bacillus subtilis* EA-CB0015 and *Bacillus amyloliquefaciens* EA-CB0959 against phytopathogenic microorganisms

Evaluation of antifungal activity was performed using the ring method. Briefly, a circular print (6 cm of diameter) of *B. subtilis* EA-CB0015 was made in a Petri dish (9 cm of diameter) with PDA, and then a disk (5 mm of diameter) of the fungus (grown for 10 days) was placed in the center thereof. Petri dishes inoculated only with disks of the fungus were used as absolute control, and the radial mycelial growth was measured when the fungus reached a growth equal to the diameter of the circle formed by the bacteria.

The experiments had a completely randomized univariate design with three replicates per treatment. The established response variable was the percentage of mycelial growth inhibition, which was calculated considering growth of the absolute control as 100%. As FIG. 7 shows, the percentage of inhibition generated by *B. subtilis* EA-CB0015 was approximately 20% on *Pestalotia* sp. and 80% on *Moniliophthora roreri*.

In addition, *B. subtilis* EA-CB0015 exhibits antibacterial activity against various microorganisms, including *Ralstonia solanacearum*, generating inhibition zones of up to 6 millimeters in BGTA culture medium. Quantitative antagonism tests against *R. solanacearum* were performed by surface seeding 100 µL of a *R. solanacearum* suspension adjusted to 10⁶ CFU/mL in BGTA agar. Then, TSA discs (5 mm) of *B. subtilis* EA-CB0015 were incubated for 48 hours at 30°C. Finally, the generated inhibition zone was determined after 72 hours.

With the same methodology described above, the activity of *B. amyloliquefaciens* EA-CB0959 against *F. oxysporum*, *M. fijiensis* and *R. solanacearum* was evaluated, with inhibition percentages of 58.5% and 76.0%, and an inhibition radius of 10.9 mm, respectively.

EXAMPLE 5. Evaluating *Bacillus subtilis* EA-CB0015 and *Bacillus amyloliquefaciens* EA-CB0959 against *Mycosphaerella fijiensis*

To select the antagonistic bacteria, an initial screening was performed using the microplate technique with the modified methodology of Peláez 2006 [10]. It was quickly established which cell-free supernatants (CFS) of the isolated AEFBs generated mycelial growth inhibition on *Mycosphaerella fijiensis* when incubated in liquid medium.

For the evaluation, strains of *M. fijiensis* EASGK09, *M. fijiensis* EASGK10, *M. fijiensis* EASGK11, and *M. fijiensis* EASGK14 fungi were used, isolated from cv. Gran enano banana leaves and following the methodology of Dupont, 1982[11]. CFS of *B. subtilis* UA321 were used as positive control and fresh sterile broth was used as absolute control.

A dual plates test was conducted with the modified methodology of Riveros [12]. Growth inhibition percentages of fungus colonies were evaluated on PDA (Merck, supplemented with chloramphenicol: 200 ppm and ampicillin: 250 ppm). Inhibition

tests were conducted on the germ tube using the modified varnish technique described Talavera [13].

Growth inhibition was evaluated on the germ tube of fungal ascospores discharged on leaf discs of cv. Gran enano banana previously submerged in CFS. The inhibition percentage on the germ tube was determined considering spore germination of the absolute control as 100%.

Bacteria selected as antagonists were those whose CFS showed *M. fijiensis* growth inhibition percentages higher than those of *B. subtilis* UA321 positive control. This initial selection process was carried out with 648 AEFBs. AEFBs selected as antagonists of *M. fijiensis* were tested again using the microplate technique and subjected to dual plates and ascospores inhibition tests using the CFS obtained from fermentation in MOLP culture media [14]. These tests used *M. fijiensis* EASGK14 strain and the same controls of the initial screening.

Finally, AEFBs selected as antagonists of *M. fijiensis* were subjected to three additional tests against the fungus: microplates with MOLP culture medium, dual plates, and ascospores inhibition. Then, a weighted average of the three tests was calculated, resulting in values of 60%, 20%, and 20% for the ascospores, dual plates, and microplates tests respectively.

The higher weight associated with the ascospores test relates to the importance of attacking the fungus before it enters leaf stomata. Inhibition percentages of mycelial growth and ascospores germination of *M. fijiensis* generated by *B. subtilis* EA-CB0015 and *B. amyloliquefaciens* EA-CB0959 *in vitro* are shown in Table 1.

Table 1. Inhibition percentages generated by *Bacillus* sp. against *M. fijiensis*

Microorganism	Mycelial growth inhibition/Technique:		Inhibition of ascospores growth ^c	Weighted average of the three tests ^d
	Microplates in MOLP (%) ^a	Dual plates (%) ^b		

<i>Bacillus subtilis</i> EA-CB0015	89 ± 1	78 ± 1	98 ± 1	92
<i>Bacillus amyloliquefaciens</i> EA-CB0959	89 ± 0	62 ± 2	77 ± 0	76

^a Mycelial growth inhibition of *M. fijiensis* using the microplate technique with MOLP broth; 2 replicates and 4 repetitions per treatment of the experiment performed 2 times over time.

^b Mycelial growth inhibition of *M. fijiensis* using the dual plates in PDA technique; 2 replicates and 30 repetitions per treatment of the experiment performed 2 times over time.

^c Germination inhibition of *M. fijiensis* ascospores with two replications for treatment.

^d Average of the three antagonism tests: ascospores (60%), microplates (20%), and dual plates (20%).

The growth inhibition percentage of *M. fijiensis* obtained with *B. subtilis* EA-CB0015 CFS in medium D was 1.5 higher than that obtained in CIB medium (53.0 ± 4.0 %), 80.9 times higher than that obtained in Finley and Field's medium (1.0 ± 1.6 %) (FIG. 7). Growth inhibition percentages of *M. fijiensis* obtained using the CFS in MOLP and TBS media were statistically similar to that obtained in medium D.

In vitro growth inhibition of *M. fijiensis* by the CFS of the isolated AEFBs suggests they have an impact on the cellular structures of the fungus. For this reason, the presence of morphological changes in the mycelium and ascospores of *M. fijiensis* was evaluated by light microscopy.

It was observed that the CFS of all antagonist bacteria produced morphological changes, manifested by masses on mycelial hyphae and inhibitions in the germination of the ascospores tube when compared with the absolute control.

EXAMPLE 6. Biocidal compositions comprising *Bacillus subtilis* EA-CB0015

For the development of the biocidal compositions of the invention, a pre-selection of the adjuvants was carried out and the combinations that generate the most stable mixtures and the ratios of each component in the mixture were established by evaluating such

aspects as the occurrence of phases and the presence of precipitates. Selected adjuvants and their roles are shown in Table 2.

Table 2. Adjuvants of the various compositions.

Adjuvant ^a	Product ^b	Formulation ^c	Role ^d
Oil	Sunflower oil	EM; OB	Provides substance to the formulation. Preventing microorganism desiccation.
	Soybean oil	EM; OB	
	Canola oil	EM; OB	
Surfactant	Tween 20	EM; MH2O	Improving coverage of the hydrophobic surface of the plant. Helping the mixture of hydrophobic spores with water. Helping the mixture of hydrophobic spores with water.
	Tween 80	EM; MH2O	
	Triton x-100	EM; MH2O	
Adherent	Xanthan gum	MH2O; EM	Improving adherence of the microorganism to the surface of the leaf. Stabilizing the mixture.
Dispersant	Sodium alginate	MH2O	Neutralizing interactions between similar particles to achieve a uniform suspension. Ensuring release of the microorganism after application.
	Veguum	MH2O	
	Sodium carboxymethyl cellulose (CMC)	MH2O	
Humectant	Glycerol	MH2O	Increasing moisture content of the product by absorbing water in the air. Preventing microorganism desiccation.
Antimicrobial agent	Propionic acid	MH2O; EM	Preventing product degradation due to contamination

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^a Group of adjuvants required for the mixture.

^b Adjuvants selected for the evaluation.

^c Composition of which they make part. EM: Emulsion, OB: Oil-based composition, MH₂O: Water-based mixture.

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^d Role of each adjuvant in the mixture.

For the composition in emulsion form, the best combination of surfactant and oil was established using a factorial 3 x 2 designed (factors: type of oil and type of emulsion, with three levels each) was used. A constant ratio was used to evaluate the components: 20 mL oil, 1 μ mol surfactant, and 80 mL water.

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After selecting the type of oil and surfactant, a ternary mixture with center point design was carried out in order to determine the ratios of sunflower oil, surfactant, and dispersant (xanthan gum) that should be added to obtain a highly stable emulsion.

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Evaluated ranges were 0.3 to 1.0%, 0.0 to 5.0%, and 14.0 to 19.6% for xanthan gum, oil, and surfactant, respectively. Ratios were selected as reported by Burges (1998) and Brar et al (2006) [15, 16]. In addition to the evaluated components, to the mixtures was added a 3M phosphate buffer of pH 5.0 (3%) to offset drastic changes in pH when

adding the adjuvants, propionic acid (0.5%) as an antimicrobial agent, and q.s. water 100%.

As for the water-based mixture, the most stable combination of dispersant, surfactant, and adherent was determined using a fractional factorial design that produced eight mixtures. These mixtures were then evaluated in the fractional design to select the water-based mixture adjuvants shown in Table 3. The remainder was completed with water.

Table 3. Evaluated mixtures using a fractional design for selecting water-based mixture adjuvants (MH₂O)

Mixture	X gum	(CMC)	(veggum)	(alginate)	(tween 20)	(tween 80)	(triton X-100)
1	-	-	-	+	+	+	-
2	+	-	-	-	-	+	+
3	-	+	-	-	+	-	+
4	+	+	-	+	-	-	-
5	-	-	+	+	-	-	+
6	+	-	+	-	+	-	-
7	-	+	+	-	-	+	-
8	+	+	+	+	+	+	+
- ^a	0,1%	0%	0%	0%	0%	0%	0%
+ ^b	0,5%	1,25%	1,25%	1,25%	2,50%	2,50%	2,50%

^a Minimum level for the evaluation of each factor.

^b Maximum level for the evaluation of each factor

* The + y - signs denote the level for the evaluation of each factor in the mixture.

Mixture 3 was selected as the most stable and subjected to a ternary mixture with center point design in order to determine the rations of CMC, Tween 20®, and Triton X-100®.

Evaluated ranges were 0.5% to 3.5% for the three components tested. These ranges were determined as reported by Burges (1998) and Brar et al (2006) [16, 15].

The concentration of xanthan gum remained constant at the lowest level (0.1%) evaluated in the fractional factorial design since all stable mixtures contained this additive at this concentration. In addition to the evaluated components, to the design mixtures was added a 3M phosphate buffer of pH 5.0 (3%) to offset drastic changes in

pH when adding the adjuvants, propionic acid (0.5%) as an antimicrobial agent, and q.s. water 100%.

Mixtures with improved stability over time for the emulsion and the water-based mixture were selected. Water was replaced with bacterial culture *B. subtilis* EA-CB0015, and its final bacterial concentration was adjusted to $2 \pm \times 10^8$ CFU/mL.

In addition to the two above mentioned compositions, bacterial suspensions (SB) consisting solely of the bacterial culture obtained after 72 hours of fermentation and conditioned with a 3M phosphate buffer of pH5 (3M K₂HPO₄, 3M KH₂PO₄) (3%) and propionic (0.5%) were evaluated. Table 4 illustrates the compositions of the various formulations evaluated with *B. subtilis* EA-CB0015.

Table 4. Compositions of various formulations based on *Bacillus subtilis* EA-CB0015.

FORMULATION	ACRONYM	COMPOSITION
Bacterial suspension	BS	Bacterial culture (94.25% v/v - 98.75% v/v), 3M phosphate buffer of pH 5 (1% v/v - 5% v/v), and propionic acid (0.25% v/v - 0.75% v/v)
Composition water base	MH ₂ O	Bacterial culture (86.6% v/v - 93, 2% v/v) sodium carboxymethyl cellulose (2% w/v - 4% w/v), 3M phosphate buffer of pH 5 (1% v/v - 5% v/v), glycerol (1% v/v - 4% v/v) Tween 20® (0.25% v/v - 0,75% v/v) Triton X-100 (0.25% v/v - 0,75% v/v), propionic acid (0.25% v/v - 0,75% v/v), and xanthan gum (0.05% w/v - 0,15% w/v).
Composition emulsion base	EM	Bacterial culture (71.4% w/v - 83,6% v/v), sunflower oil (14% v/v - 18% v/v), 3M phosphate buffer of pH 5 (1% v/v - 5%

		v/v), Tween 80 ® (1% v/v - 4% v/v), xanthan gum (0.2% w/v – 0.9% w/v), and propionic acid (0.25% v/v – 0.75% v/v).
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EXAMPLE 7. Evaluation of the biocidal compositions of the invention

The compositions obtained in Example 6 were used to evaluate their antagonistic capacity against ascospores of *M. fijiensis* and the viability of *B. subtilis* EA-CB0015 in the formulation for a given storage time (180 days).

Regarding the evaluation of the viability of the bacteria over time, FIG. 9 shows a gradual decrease in CFU over time for all treatments, with a more marked decrease for the emulsion from the third month. The water-based mixture and the bacterial suspension showed very similar decreases, although the former showed the lowest value.

According to the above, the best compositions in relation to the viability of *B. subtilis* EA-CB0015 in the formulation during the evaluated time period are those based on water or with a bacterial suspension.

In order to evaluate the effect of the compositions on the development of Black Sigatoka in banana plants, an experiment was conducted to assess the emulsion, the water-based mixture, and the bacterial suspension of *B. subtilis* EA-CB0015. The compositions were diluted to a concentration of 1.0×10^8 CFU/mL and applied by spraying 30 drops/cm² on the first leaf completely unfolded after the flag leaf (leaf number one), on which the evaluation was conducted.

Inoculation of the pathogen was performed through artificial inoculation by adding 20 mL of a mycelial suspension of 10-day old *M. fijiensis* on leaf number one. Inoculation of the pathogen was done 24 hours before applying the compositions of the AEFBs. The degree of severity of the disease was determined 30 days after applying the

compositions using the Fouré scale (1982)[17] and the percentage of necrotic leaf area was determined using photos of leaves and the Assess 1.0 image analysis software.

For both measurements, the results of applying sterile water were used as negative control and the data reported for the chemical fungicide Dithane® and the biological fungicide Rhapsody®, employed according to the provider's recommendations, were used as positive control. Results are illustrated in FIGS. 10 and 11, which show the differences in the degree of severity and in the percentage of necrotic area for the various treatments evaluated by analysis of variance ($p < 0.05$).

It is noteworthy that the water-based mixture composition was the only biological treatment that showed significant disease control equal to the chemical control of Dithane® for the two analyzed response variables. This bioformulation reduced the degree of severity to 97.1% and reported a necrotic area of 2.3%, a percentage similar to that obtained by chemical control (1.0%). The degree of severity and the percentage of necrotic area of the negative control was 4.2 and 16.3%, respectively.

Other evaluated treatments showed no disease control, that is, they did not show significant differences when compared with the negative control for any of the analyzed two variables.

EXAMPLE 8. Evaluating the physicochemical properties, adhesion, resistance to UV radiation, and characteristics of the compositions of the invention.

Adhesion and resistance to UV radiation of the compositions of Example 6 were lower when compared with other chemicals in the market. To improve these properties, an initial selection of adjuvants was carried out. Table 5 shows the adjuvants used to improve adhesion and UV protection, and the range used for their evaluation.

Table 5. Adjuvants evaluated to improve adhesion and UV protection of the compositions of the present invention.

Role	Adjuvant Type	Concentration (% w/v)
UV Protector	TiO ₂ (Technical)	0,5 - 1,5
UV Protector	ZnO ₂ (Technical)	0,5 - 1,5
UV Protector	Polyvinyl alcohol (PVA)	0.1 – 1.0
Adherent	Skimmed milk	0,1 - 1,5
Adherent	Pegal®	0,01 - 0,1
Adherent	Sodium caseinate (CaNa)	0.1 - 1.0

The adjuvants were appraised using cost, market availability, and compatibility with the composition of the invention as criteria. Then, an evaluation of the pre-selected adjuvants was carried out using a multifactorial experimental design where top performers were identified for each evaluation criteria in a specific concentration range (FIGS 12 and 13).

According to FIG. 12, the water-based bacterial composition (MH₂O) of the invention, supplemented with the adjuvant TiO₂ at 0.5% w/v, improved UV resistance, reducing cell death of *B. subtilis* EA-CB0015 from 55% to 21.5% after being exposed to UV radiation for 120 minutes. With this finding a new water-based composition was developed, incorporating TiO₂ at 0.5% and replacing propionic acid with potassium sorbate in a composition called P2.

FIG. 13 shows that the addition of sodium caseinate and skimmed milk significantly improved the adhesion of the product to hydrophobic surfaces. Therefore, skimmed milk was added to the water-based composition P2, forming a new composition named P3.

In order to determine whether the composition of *B. subtilis* EA-CB0015 can act in combination with the chemical fungicide mixtures, the viability of *B. subtilis* EA-CB0015 was determined before and after subjecting the composition to tank mixtures used for the control of Black Sigatoka in commercial plantations. For this purpose, a sample of 10 mL of the composition was taken and subjected to the various tank mixtures described in Table 6.

Table 6. Mixtures of the water-based composition based on *Bacillus subtilis* EA-CB005
with various chemical fungicides.

Compound/ Treatment	Water (%V/V)	Agricultural Oil (%V/V)	Dithane® (%V/V)	Pegal® (%V/V)	Chlorothalon il® (%V/V)	Formulation (%V/V)	SICO® (%V/V)	SIGANEX® (%V/V)	Baycor® (%V/V)	Impulse® (%V/V)	OPUS® (%V/V)	Atlas® (%V/V)	Calixin® (%V/V)
Mixture 1	72,53	0	0	0	7,47	20	0	0	0	0	0	0	0
Mixture 2	38,86	40,88	8,79	0,41	0	9,31	1,76	0	0	0	0	0	0
Mixture 3	38,51	40,88	8,79	0,41	0	9,22	0	2,2	0	0	0	0	0
Mixture 4	38,51	40,88	8,79	0,41	0	9,22	0	0	2,2	0	0	0	0
Mixture 5	38,86	40,88	8,79	0,41	0	9,31	0	0	0	1,76	0	0	0
Mixture 6	36,75	40,88	8,79	0,41	0	8,78	0	0	0	0	4,4	0	0
Mixture 7	38,86	40,88	8,79	0,41	0	9,31	0	0	0	0	0	1,76	0
Mixture 8	38,51	40,88	8,79	0,41	0	9,21	0		0	0	0	0	2,2

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For this evaluation, an 8 x 8 multifactorial design was used, wherein the first factor assessed was the type of tank mixtures with 8 levels, and the second factor was the exposure to the mixtures or the viability evaluation time with eight levels (0.0, 0.5, 1.5,
10 3.0, 6.0, 12.0, and 25.0 hours). The results were compared with the Rhapsody® biological control and two replicates were used per treatment.

The evaluated response variable was the number of CFU/mL in each of the evaluation times. Additionally, t_{d50} was determined, corresponding to the time when 50% of the
15 biomass of *B. subtilis* EA-CB0015 loses its viability due to the exposure to each of the mixtures, using an univariate design for data analysis, wherein the factor is the percentage (%) of cell death.

20 The percentage of cell death after three and twenty-five hours was also determined, (3 hours is the average time it takes to apply a composition after it has been prepared and 25 hours is the maximum time that a composition remains in the mixing tanks before being applied).

Table 7 shows t_{d50} (the time when 50% of the *B. subtilis* EA-CB0015 spores lose viability) and the percentage of cell death after 3 and 25 hours for the composition of *B. subtilis* EA-CB0015 in each fungicide mixture. For its production, the culture was taken to a stirred tank and mixed with the respective composition adjuvants. M1, M2, M3, M4, M5, M6, M7, and M8 denote the various fungicide mixtures to which the formulations were subjected.

Table 7. Cell death percentage and t_{d50} of *B. subtilis* EA-CB0015 after mixing with various chemical fungicide mixtures.

Mixture	t_{d50} MH ₂ O (hours)	% cell death after 3 hours	% cell death after 25 hours
M1	>25	3,7	24,6
M2	>25	18,6	10,6
M3	>25	32,1	25,4
M4	>25	25,9	22,0
M5	>25	16,3	36,4
M6	>25	7,2	28,1
M7	>25	17,0	17,4
M8	>25	6,0	6,5

The above table indicates that the composition of *B. subtilis* EA-CB0015 showed a t_{d50} greater than 25 hours for all mixtures, reaching average viability reductions of only 20.1%. Furthermore, given that viability reductions in the composition were lower than 50%, the composition of the present invention most likely will provide extra protection to *B. subtilis* EA-CB0015, allowing to maintain viability for long periods of exposure to fungicide mixtures.

Regarding the percentage of viability loss, it was observed that in ordinary conditions, (3 hours) M2 (SICO®), M3 (Siganex®), and M4 (Baycor®) mixtures showed the highest bacterial viability reduction rates in the composition, whereas in extraordinary

conditions (25 hours), M1 (Bravonil®), M3 (Siganex®), M5 (Bumper®), and M6 (Opus®) showed the highest reduction rates.

EXAMPLE 9. Evaluating the effect of the compositions of the invention on the severity of Black Sigatoka. Greenhouse and field tests.

An evaluation of the effect of the bacterial water-based composition of *B. subtilis* EA-CB0015 on Black Sigatoka in greenhouse conditions was performed. To this end, 4-month old c.v. Williams banana plants were used and pathogen inoculation was made artificially by applying a mycelial suspension of the *M. fijiensis* fungus to leaf number one of the plant. The ascospore discharge methodology used by Cenibanano was employed to obtain the mycelium of *M. fijiensis* for the inoculation of the plants [11].

The compositions were applied one day after the inoculation of the plants with the pathogen. The compositions were diluted to a concentration of $1.0 \times 10^8 \pm 0.1$ CFU/mL and applied using a *Mini Spray gun with cup K-3®* airbrush with fan sprayer connected to a 30-psi compressor and calibrated for spraying 50 drops/cm² at a distance of 30 cm. The top and underside of the infected leaves were fumigated only once at a distance of 30 cm, ensuring a minimum concentration of 50 drops/cm².

A single-factor design was used for this experiment to evaluate the water-based composition (MH₂O y P3), a chemical control: Bravonil® in water, a biological control: Serenade® ($1 \times 10^8 \pm 0.1$ CFU/ml), and sterile water as absolute control.

Disease development was measured one month after the plants were infected. The degree of infection was determined using the Fouré scale(1982) [17] and the necrotic area was determined using a 8 Mega-pixel Samsung camera and Zeiss®'s Axio Visio® 4.2 image processing software.

The analysis of variance (ANOVA) indicated that there are statistically significant differences among the treatments. To determine these differences, a multiple range test

was conducted using the Turkey method. The percentages of necrotic area for the various treatments are shown in FIG. 14.

As shown in FIG. 14, the water-based mixture composition (MH₂O and P3) of *B. subtilis* EA-CB0015 according to the present invention and the Bravonil 720® positive control significantly reduced the percentage of necrotic area of banana leaves with percentages of 1.67% and 1.26%, respectively. FIG. 15 includes photographs of leaves subjected to each of the treatments, which visually show the difference in appearance of post-treatment leaves. The photographs were selected from portions of average leaves.

To determine the effectiveness of the water-based mixture composition of *B. subtilis* EA-CB0015, the product was evaluated at field level in a lot of 1.5 ha, with three plots per treatment. Each plot of 220 m² contained 42 plants; six central plants were taken per plot in order to evaluate the disease. Treatments were applied every 11 days with a motor sprayer (Stihl® SR-420) of 15 L capacity, spraying 60 drops per cm² of leaf.

The evaluation of Black Sigatoka disease was performed using two methodologies: biological warning and severity by Stover. FIG. 16 shows the area under the curve (AUC) for the severity of Black Sigatoka obtained during 14 weeks of evaluation. This figure shows that both water-based compositions of *B. subtilis* EA-CB0015 (MH₂O y P3) reduced disease severity with no significant differences when compared with chemical controls (Bravonil®, Dithane®) and biological control (Serenade®).

EXAMPLE 10. Effect of *B. subtilis* EA-CB0015 on *Botrytis cinerea* in pompoms

The effect of *B. subtilis* EA-CB0015 on *Botrytis Cinerea* in pom poms was evaluated. The pompoms were disinfected for 1 min in sodium hypochlorite 1%, washed with sterile distilled water, and finally allowed to dry. Then, each flower was placed in a disposable cup and each treatment was sprayed with an airbrush. After 24 hours of applying the treatments, the pathogen (*B. cinerea*) at a concentration of 5×10^3

spores/mL using an atomizer (2 mL) was applied and incubated at an average temperature of 20°C and a relative humidity above 90%.

Disease measurement was performed after 7 days according to the percentage of affected petals and the severity. FIG. 17 shows that the spore suspension of *B. subtilis* EA-CB0015 (T1) decreased the severity of the disease by 84% when compared with the untreated control (C).

EXAMPLE 11. Effect of *B. subtilis* EA-CB0015 on *Colletotrichum* sp. in tree tomato (*Cyphomandra betacea*).

The effect of *B. subtilis* EA-CB0015 on *Colletotrichum* sp. in tomato tree was evaluated. To this end, tomatoes were disinfected for 2 minutes in 70% ethanol, washed with sterile distilled water, and finally allowed to dry.

Then, a puncture of less than 2 mm in depth was made in the halfway region of the fruit and 25 µL of water (C) or spores of *B. subtilis* EA-CB0015 (T1, concentration 1×10^7 CFU/mL) were applied. After 24 hours, the puncture was inoculated with 15 µL of *Colletotrichum* sp. EAHP-007 at a concentration of 400,000 spores/mL.

FIG. 18 shows the effects of the suspension of spores of *B. subtilis* EA-CB0015 on the diameter of the puncture generated by *Colletotrichum* sp. EAHP-007 in the fruit, achieving effective control of the disease.

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It should be understood that the present invention is not limited to the embodiments described and illustrated herein. As it will be apparent to one skilled in the art, there are
5 potential variations and modifications that do not depart from the spirit of the invention, which is only defined by the following claims:

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CLAIMS

- 1) A procedure for increasing the production of biomass and metabolites of microorganisms of *Bacillus* sp. species, comprising the culture of the microorganism in a suitable culture medium under specific environmental conditions.
- 2) A procedure according to Claim 1, wherein the microorganism is selected from the group consisting of *Bacillus subtilis* and *Bacillus amyloliquefaciens*.
- 3) A procedure according to Claim 1, wherein the microorganism is *Bacillus subtilis* EA-CB0015 or *Bacillus amyloliquefaciens* EA-CB0959.
- 4) A procedure according to Claim 1, wherein the suitable culture medium comprises one or more components selected from the group consisting of carbohydrates, yeast extract, ammonium sulfate, peptone, salts containing magnesium, sulfur, manganese, chlorine, potassium, phosphorus, calcium, and sodium either in a solid, semisolid, or liquid matrix.
- 5) A procedure according to Claim 4, wherein the suitable culture medium has the following composition:

COMPONENT	Concentration (g/L)
Glucose	30,0 - 35,0
Yeast extract	30,0 - 35,0
MnSO ₄	0,025 - 0,05
Calcium chloride	0,02 - 0,04
Ammonium sulfate	0,80 - 1,20
MgSO ₄	3,50 - 5,00
HPO ₄	0.40 - 0.60
KH ₂ PO ₄	0.40 - 0.60

6) A procedure according to Claim 1, wherein the specific environmental conditions include pH, temperature, stirring speed, fermentation time, and aeration.

7) A procedure according to Claim 6, wherein the specific environmental conditions of culture are:

Stirring speed	400-600 rpm
Aeration	1-5 vvm
pH	5,5 - 7,5
Temperature	20 - 40°C
Fermentation time	10-100 hours

8) A procedure according to Claim 1, wherein the obtained biomass is additionally separated by centrifugation and/or microfiltration.

9) A procedure according to Claim 1, wherein the metabolites are additionally extracted by solvent extraction, precipitation, adsorption, or chromatography.

10) Biomass of *Bacillus subtilis* or *Bacillus amyloliquefaciens* obtained by a procedure according to Claims 1 to 8.

11) Biomass of *Bacillus subtilis* EA-CB0015 obtained by a procedure according to Claims 1 to 8.

12) Biomass of *Bacillus amyloliquefaciens* EA-CB0959 obtained by a procedure according to Claims 1 to 8.

13) Metabolites of *Bacillus subtilis* and/or metabolites of *Bacillus amyloliquefaciens*, obtained by a procedure according to Claims 1 to 9.

14) Metabolites of *Bacillus subtilis* EA-CB0015 and/or metabolites of *Bacillus amyloliquefaciens* EA-CB0959, obtained by a procedure according to Claims 1 to 9.

15) Metabolites of *Bacillus subtilis* EA-CB0015 and/or *Bacillus amyloliquefaciens* EA-CB0959 according to Claim 14, characterized by being lipopeptides of the surfactin, iturin, and fengycin families.

5 16) Metabolites of *Bacillus subtilis* EA-CB0015 according to Claim 15, wherein the lipopeptides of the fengycin family correspond to fengycin C having the general formula:



10 wherein R corresponds to a saturated or unsaturated hydrocarbon chain of 14 to 18 carbons.

17) Metabolites of *Bacillus subtilis* EA-CB0015 and/or metabolites of *Bacillus amyloliquefaciens* EA-CB0959 according to Claims 13 to 16, with antimicrobial activity.

15

18) A composition comprising biomass of *Bacillus subtilis* EA-CB0015 according to Claim 11 and/or metabolites thereof, together with an agrochemically acceptable carrier.

20 19) A composition comprising biomass of *Bacillus amyloliquefaciens* EA-CB0959 according to Claim 12 and/or its metabolites, together with an agrochemically acceptable carrier.

25 20) A composition comprising metabolites of *Bacillus subtilis* EA-CB0015 and/or metabolites of *Bacillus amyloliquefaciens* EA-CB0959 according to Claim 14, together with an agrochemically acceptable carrier.

30 21) A composition according to any of Claims 18 to 20, further comprising one or more biocidal agents selected from the group consisting of anilinopyrimidines, bitartenols, sterols, difeconazole, tebuconazole, epoxiconazole, mancozeb, and cloratolonil.

22) A composition according to any of Claims 18 to 21, comprising:

COMPONENT	CONCENTRATION
Culture of <i>Bacillus</i> sp.at a concentration of 7.0 to 18.0 g/L	86.6% - 93.2% v/v
Sodium carboxymethyl cellulose	2.0 - 4.0 w/v
phosphate buffer 3M pH 5	1.0 - 5.0 v/v
Glycerol	1.0 - 4.0 v/v
Tween 20	0.25 - 0.75 v/v
Triton X100	0.25 - 0.75 v/v
Potassium sorbate	0.01 - 0.1 v/v
Xanthan gum	0.05 - 0.15 w/v
Skimmed milk	0.20 - 1.50 w/v
Titanium dioxide	0.03 - 1.00 w/v

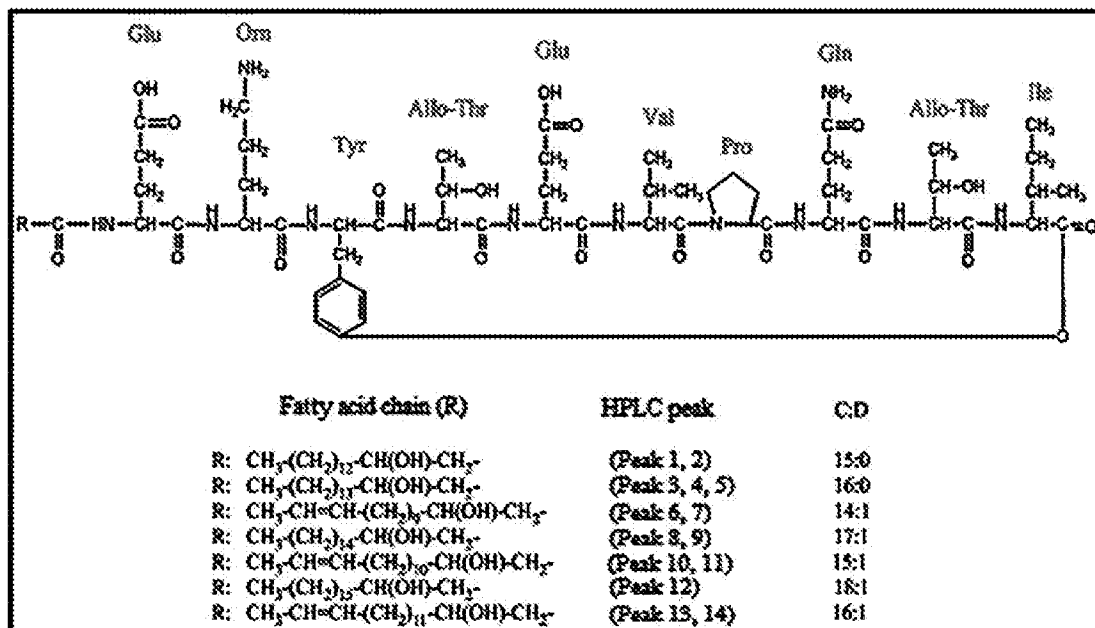
- 23) Use of biomass of *Bacillus subtilis* EA-CB0015 and/or biomass of *Bacillus amyloliquefaciens* EA-CB0959 according to Claims 11 and 12 for controlling plant pathogens such as *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp., *Rhizoctonia solani* and *Fusarium solani*.
- 24) Use of biomass of *Bacillus subtilis* EA-CB0015 and/or *Bacillus amyloliquefaciens* EA-CB0959 according to Claims 14 for controlling plant pathogens such as *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp. *Rhizoctonia solani* and *Fusarium solani*.
- 25) Use of a composition according to Claims 18 to 22 for controlling plant pathogens such as *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp., *Rhizoctonia solani* and *Fusarium solani*.
- 26) A method for the treatment of crops against phytopathogenic agents that comprises the application of an effective biomass amount of *Bacillus subtilis* EA-

CB0015 and/or biomass of *Bacillus amyloliquefaciens* EA-CB0959 according to Claims 11 and 12.

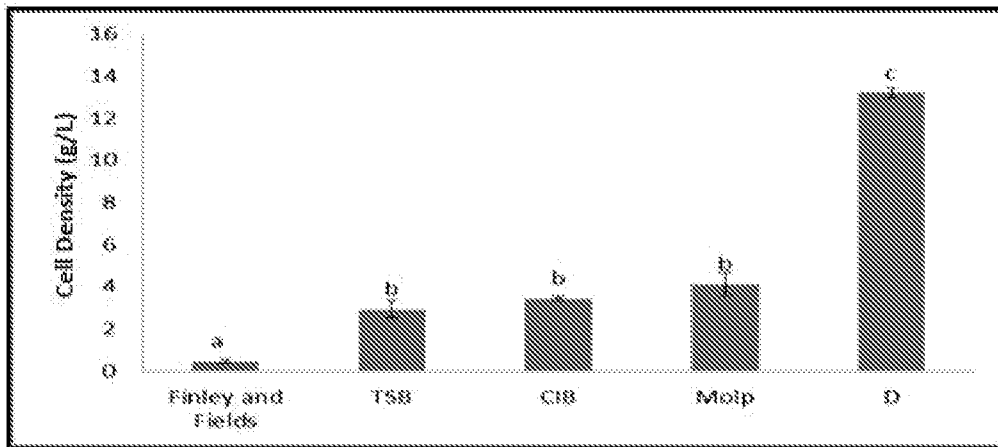
27) A method for the treatment of crops against phytopathogenic agents that
5 comprises the application of an effective amount of metabolites of *Bacillus subtilis* EA-CB0015 and/or *Bacillus amyloliquefaciens* EA-CB0959 according to Claim 14.

28) A method for the treatment of crops against phytopathogenic agents such as
10 *Mycosphaerella fijiensis*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Botrytis cinerea*, *Colletotrichum* sp., *Monilia* sp., *Rhizoctonia solani* y *Fusarium solani*, comprising the application of an effective amount of a composition according to Claims 18 to 22.

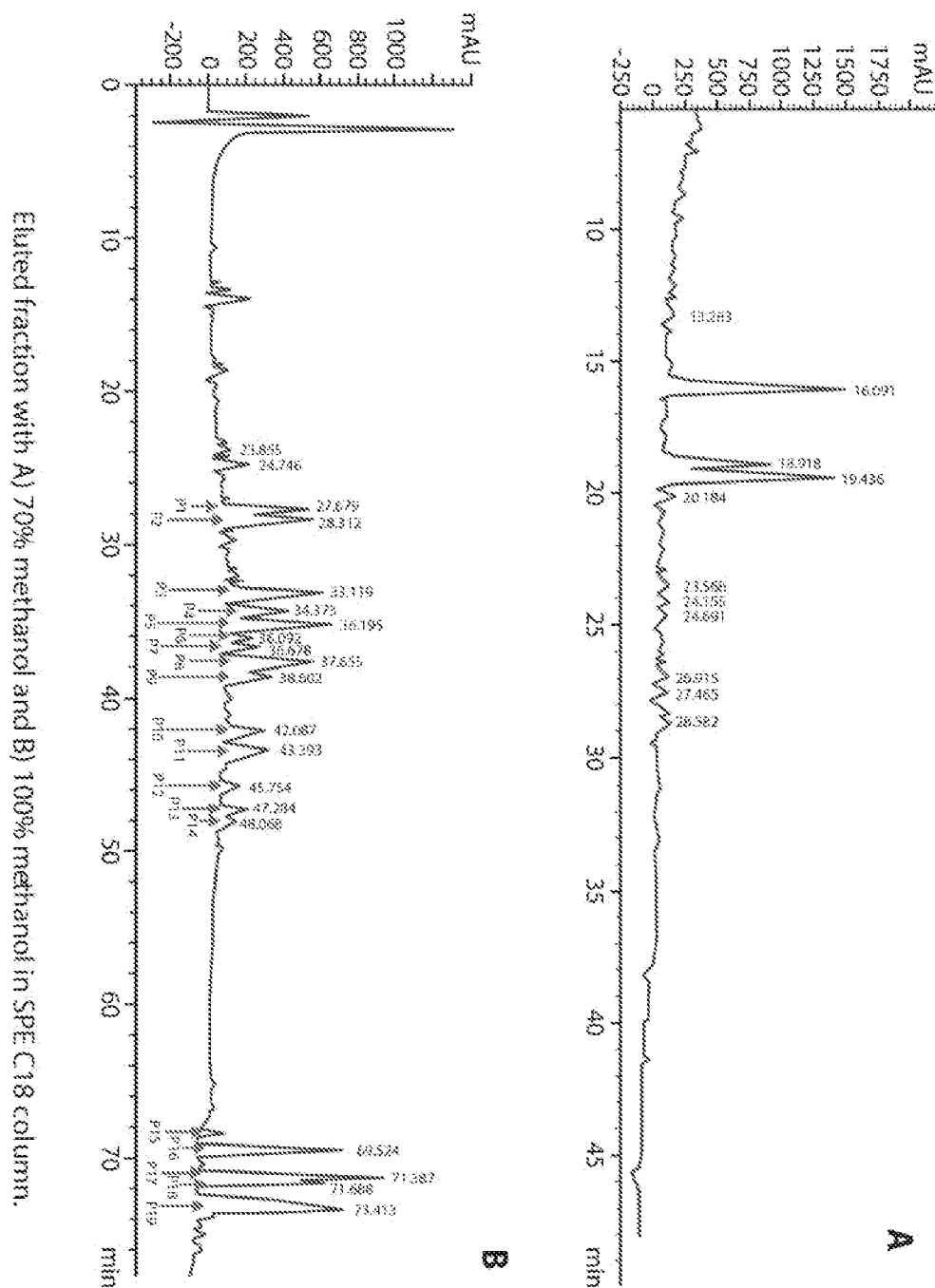
15 *****

FIG 1.

(C:D: C denotes the number of carbons and D denotes the number of unsaturations)

FIG. 2

Different letters denote significant differences (P value <0.05).

FIG. 3

Eluted fraction with A) 70% methanol and B) 100% methanol in SPE C18 column.

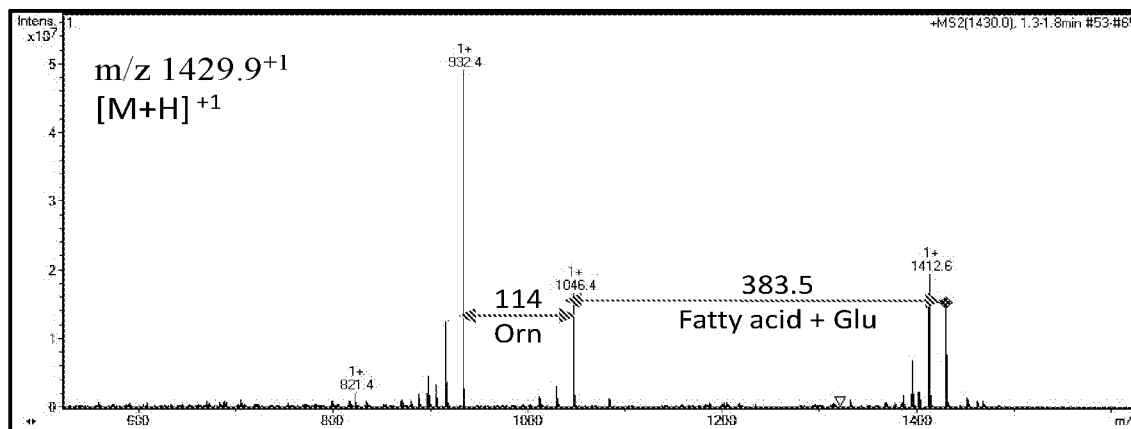
FIG. 4

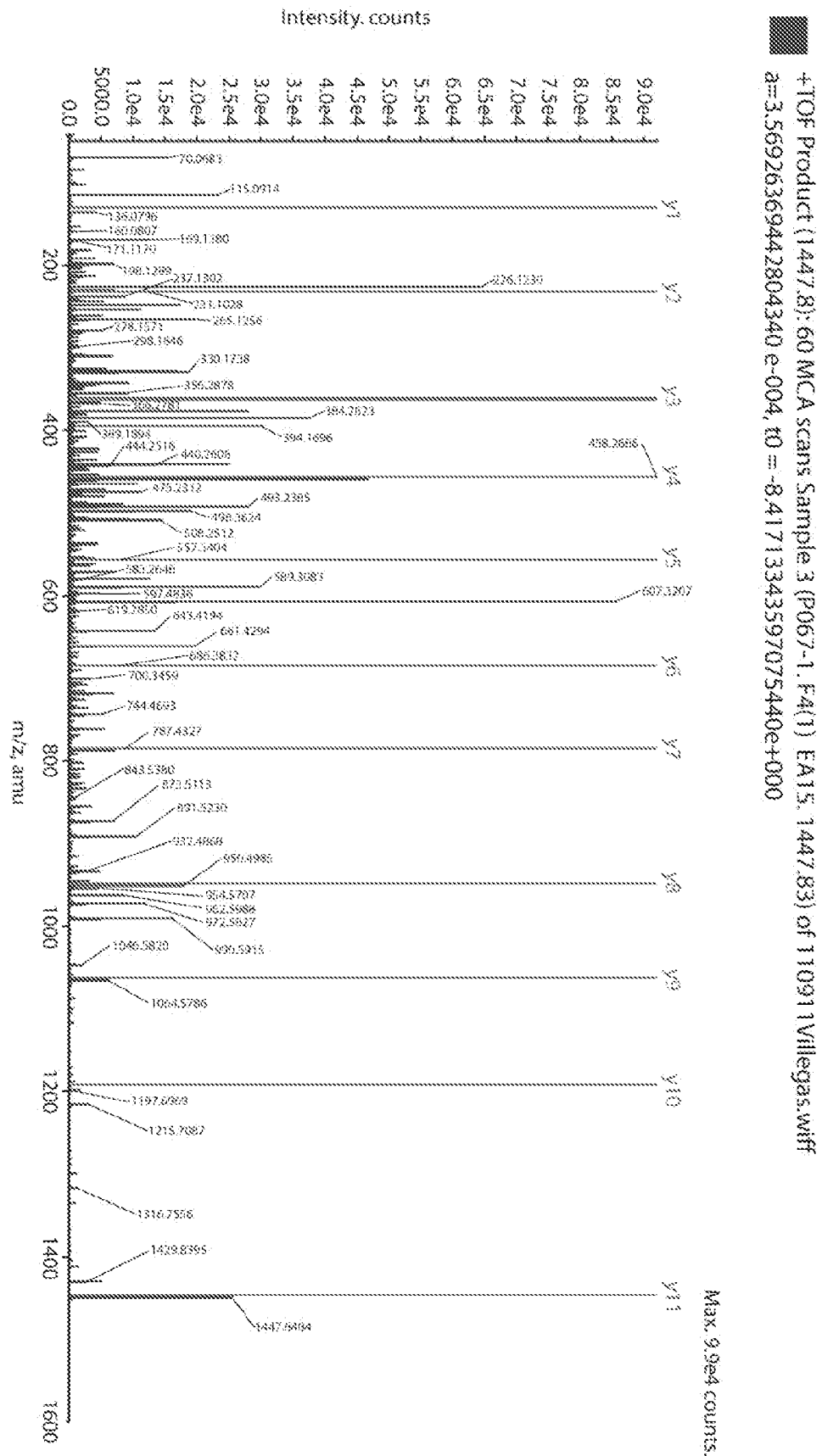
FIG. 5.

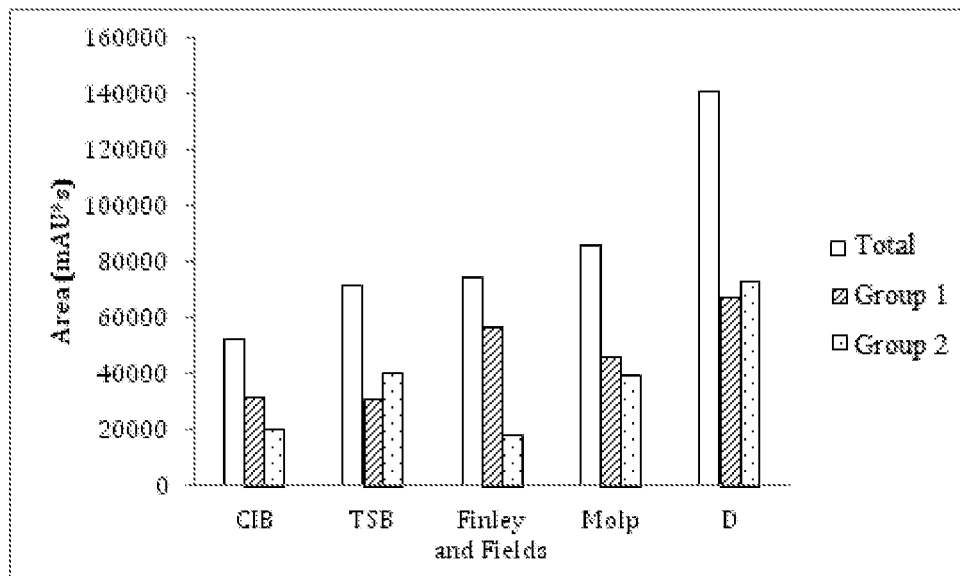
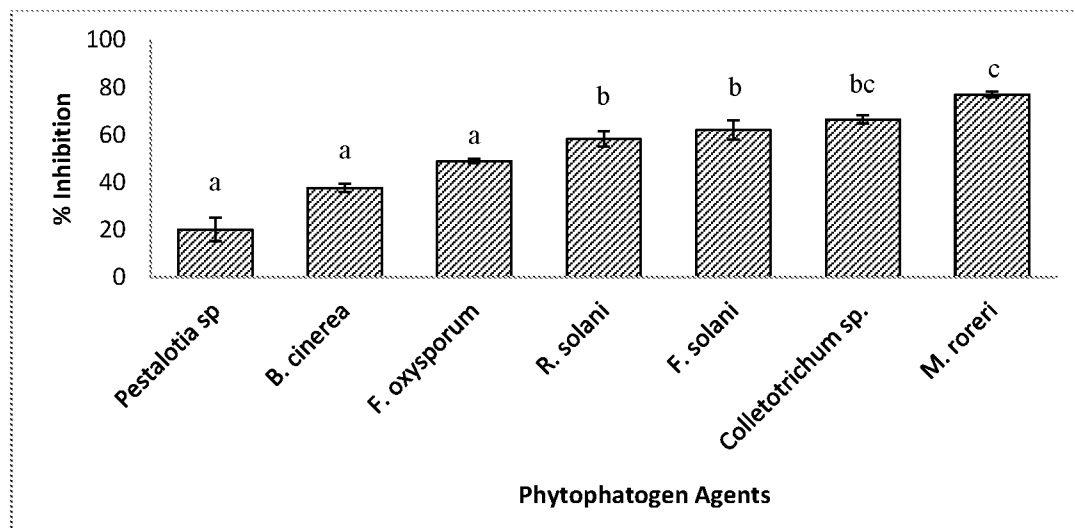
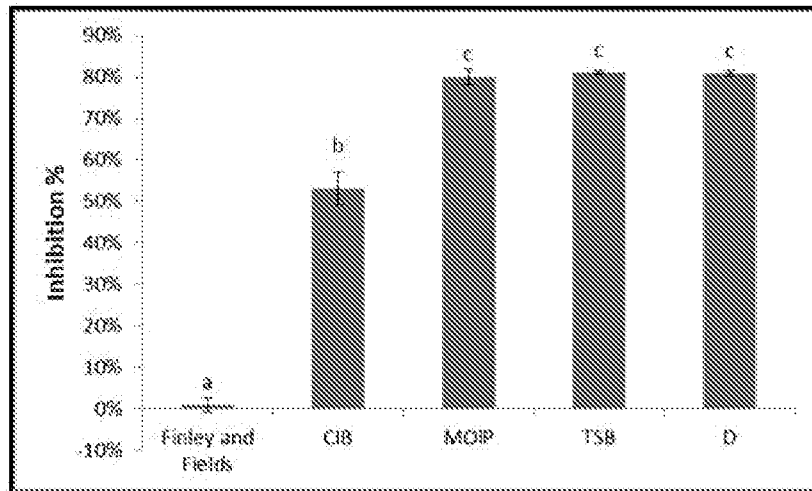
FIG. 6

FIG. 7

Different letters denote significant differences (P value <0.05).

FIG. 8

Different letters denote significant differences (P value <0.05).

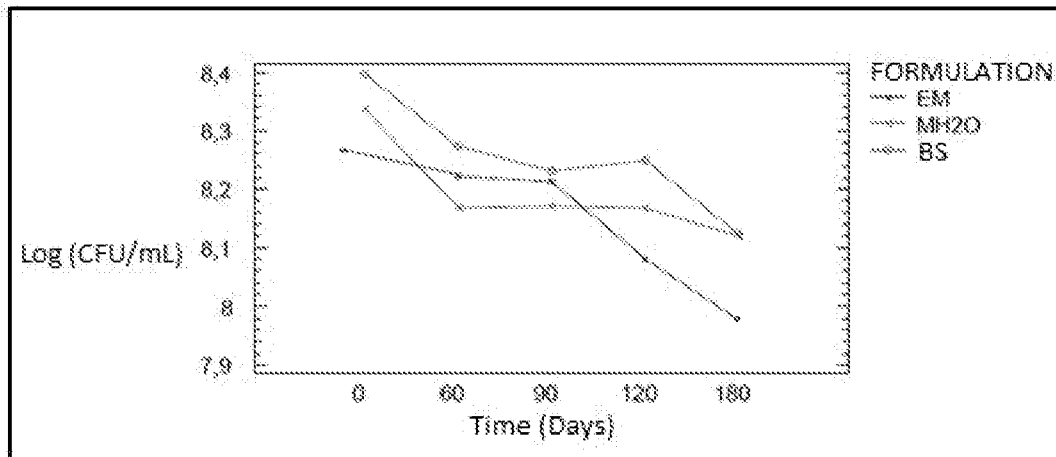
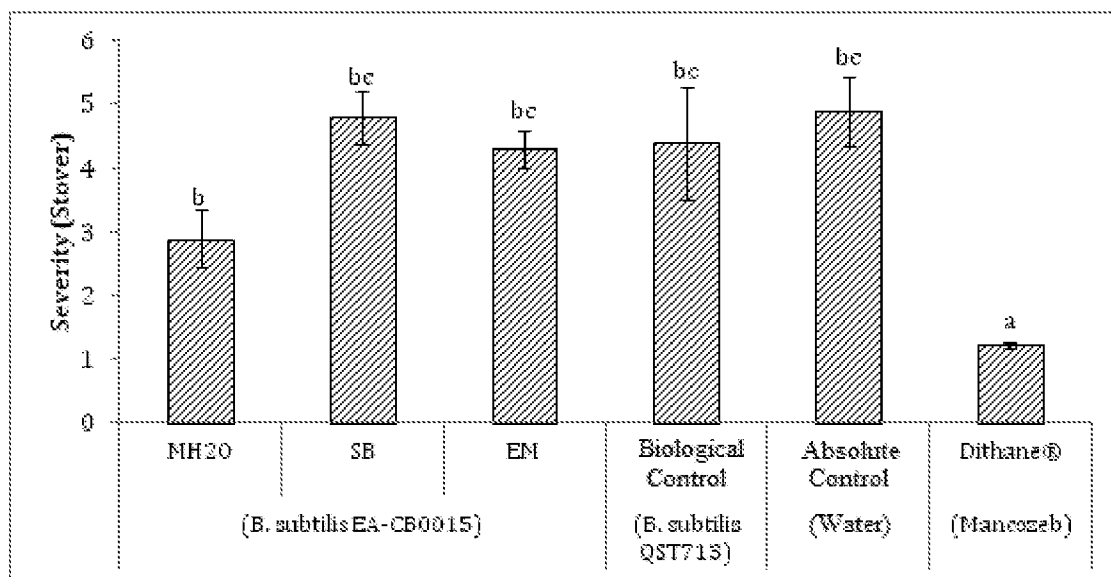
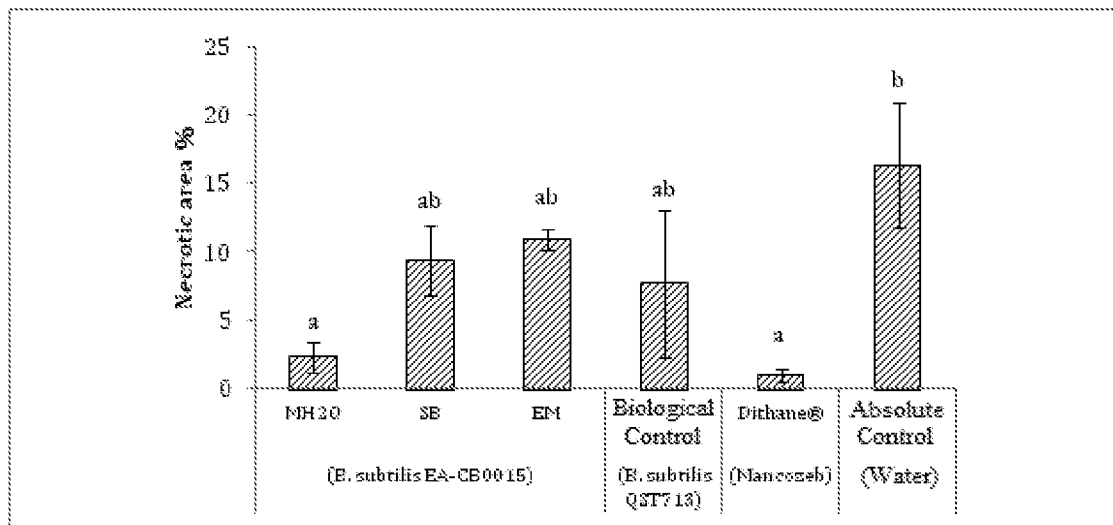
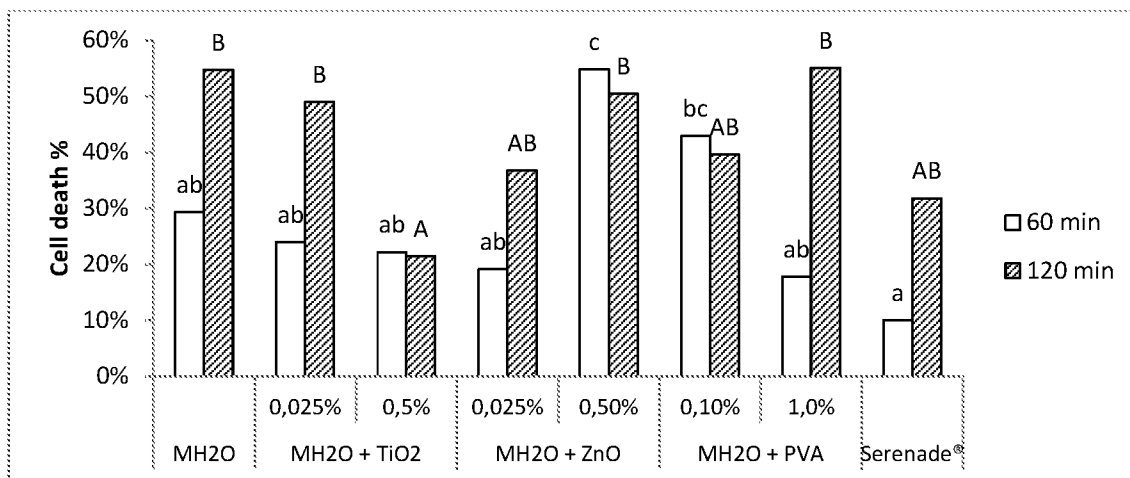
FIG. 9

FIG. 10

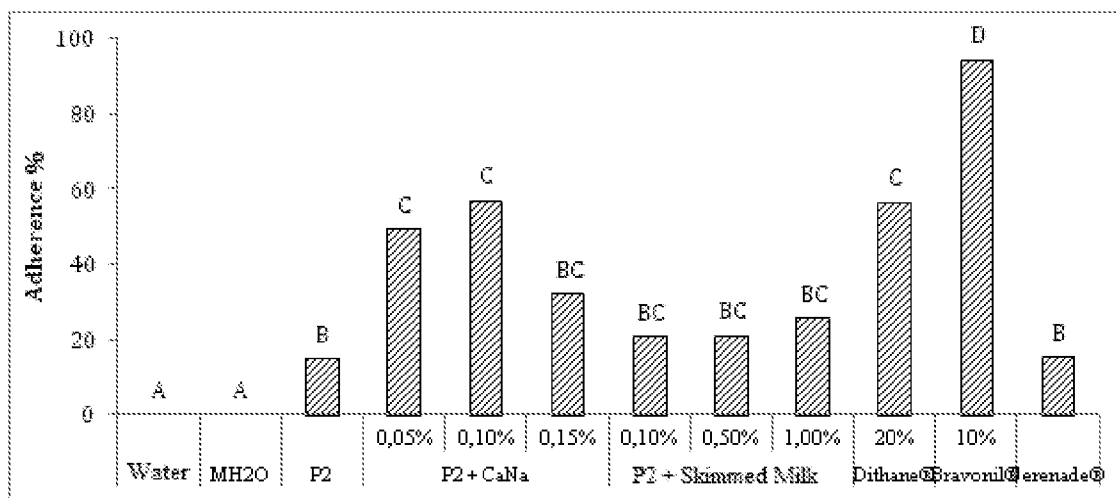
Different letters denote significant differences (P value <0.05).

FIG. 11

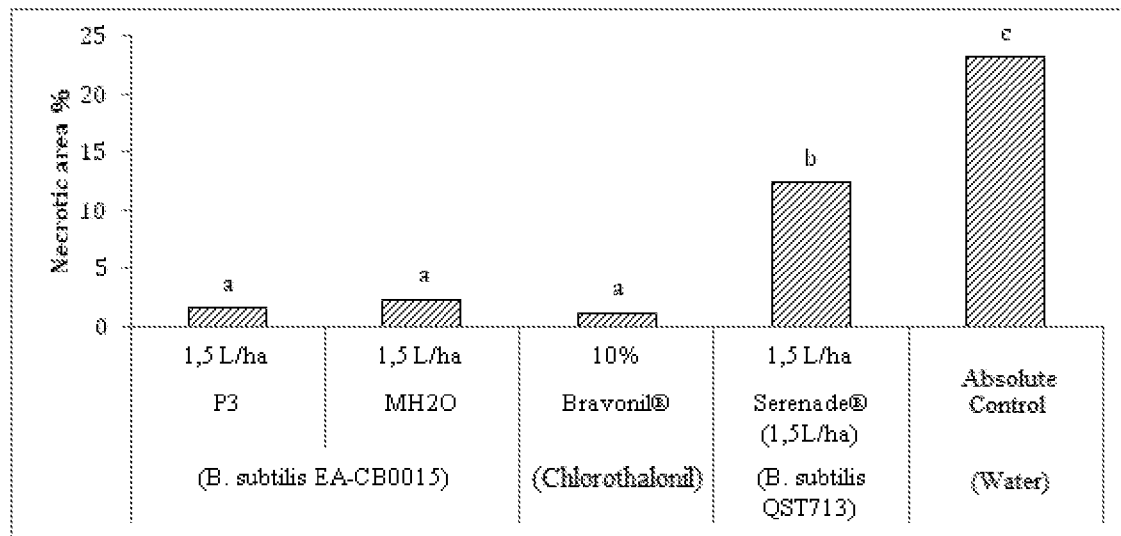
Different letters denote significant differences (P value <0.05).

FIG. 12

Different letters denote significant differences (P value < 0.05).

FIG. 13

Different letters denote significant differences (P value < 0.05).

FIG. 14

Different letters denote significant differences (P value <0.05).

FIG. 15

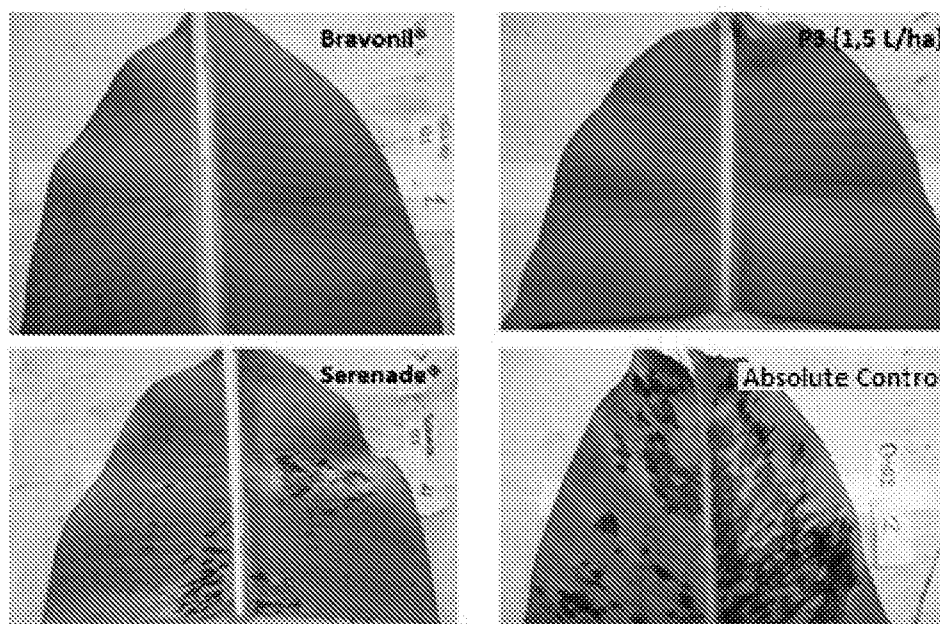
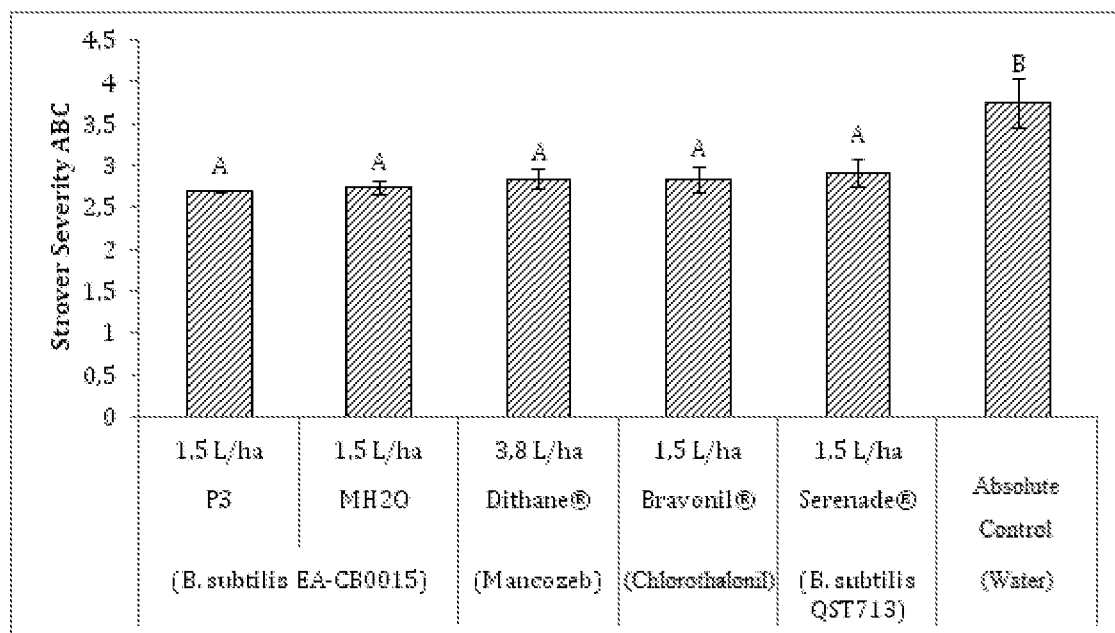
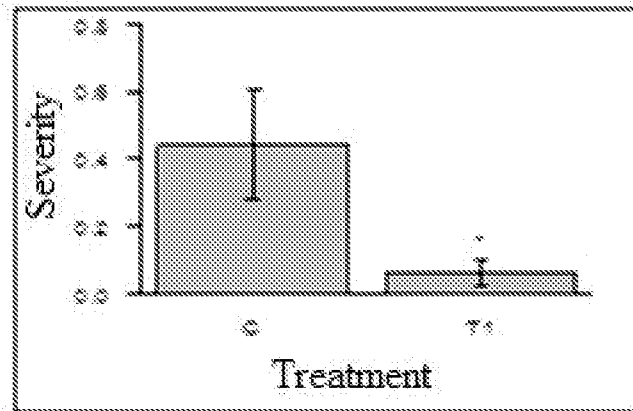
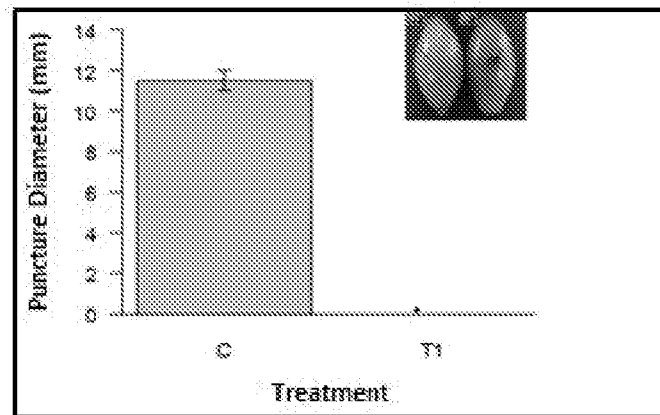


FIG. 16

Different letters denote significant differences (P value <0.05).

FIG. 17

* Denotes significant differences in relation to the control (C)
(P value < 0.05)

FIG. 18

* Denotes significant differences in relation to the control (C)
(P value <0.05)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/061167

A. CLASSIFICATION OF SUBJECT MATTER INV. A01N63/02 C07K14/32 ADD.		
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) A01N C07K		
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 practicable, search terms used) EPO-Internal, Sequence Search, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VALESKA VILLEGAS-ESCOBAR ET AL: "Fengycin C Produced by Bacillus subtilis EA-CB0015", JOURNAL OF NATURAL PRODUCTS, vol. 76, no. 4, 5 March 2013 (2013-03-05), pages 503-509, XP055142706, ISSN: 0163-3864, DOI: 10.1021/np300574v	1-4,6, 8-11, 13-20, 23-28
Y	abstract page 507 - page 508; figure 4 ----- -/--	5,7,21
☒ Further documents are listed in the continuation of Box C. ☒ See patent family 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 application or patent 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 30 September 2014	Date of mailing of the international search report 07/10/2014	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Gurdjian, Didier	

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2014/061167

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ISABEL CEBALLOS ET AL: "Cultivable Bacteria Populations Associated with Leaves of Banana and Plantain Plants and Their Antagonistic Activity Against", MICROBIAL ECOLOGY, SPRINGER-VERLAG, NE, vol. 64, no. 3, 5 May 2012 (2012-05-05), pages 641-653, XP035116074, ISSN: 1432-184X, DOI: 10.1007/S00248-012-0052-8	1-4,6,8, 10, 12-15, 18,20, 23-28
Y	abstract	5,7,21
X	----- WO 2013/050700 A2 (UNIV LILLE 1 SCIENCES ET TECHNOLOGIES USTL [FR]; COUTTE FRANCOIS [FR];) 11 April 2013 (2013-04-11)	1,2,4, 13-15
Y	page 6, line 16 - line 18 page 6, line 26 - page 7, line 10; examples 2,3	5,7,21
Y	----- WO 2010/108973 A2 (BASF SE [DE]; SCHERER MARIA [DE]; KLAPPACH KRISTIN [DE]; HADEN EGON [D]) 30 September 2010 (2010-09-30) page 7, line 37 page 8, line 12 - line 14 page 9, line 27 page 24; claims 1,10-12 page 26 - page 28	21
X	----- EEMAN M ET AL: "Influence of environmental conditions on the interfacial organisation of fengycin, a bioactive lipopeptide produced by Bacillus subtilis", JOURNAL OF COLLOID AND INTERFACE SCIENCE, ACADEMIC PRESS, NEW YORK, NY, US, vol. 329, no. 2, 15 January 2009 (2009-01-15), pages 253-264, XP025677564, ISSN: 0021-9797, DOI: 10.1016/J.JCIS.2008.10.017 [retrieved on 2008-10-14]	13-15
A	abstract	1,2,10, 18,23,26

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Information on patent family members

International application No

PCT/IB2014/061167

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WO 2010108973 A2	30-09-2010	AR 076150 A1 AU 2010227493 A1 CA 2753150 A1 CN 102361551 A CR 20110460 A EA 201101333 A1 EC SP11011349 A EP 2410840 A2 JP 2012521390 A KR 20110132461 A NZ 594887 A PE 05362012 A1 US 2012003199 A1 US 2014212401 A1 WO 2010108973 A2	18-05-2011 20-10-2011 30-09-2010 22-02-2012 24-10-2011 30-05-2012 31-10-2011 01-02-2012 13-09-2012 07-12-2011 29-11-2013 05-05-2012 05-01-2012 31-07-2014 30-09-2010