(12) STANDARD PATENT (11) Application No. **AU 2014255311 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
A package for plant antimicrobial treatment

(51) International Patent Classification(s)
A01N 25/04 (2006.01) ***A01N 63/00*** (2006.01)
A01N 25/10 (2006.01) ***A01N 65/00*** (2006.01)
A01N 25/34 (2006.01) ***A01P 1/00*** (2006.01)

(21) Application No: **2014255311** (22) Date of Filing: **2014.04.10**

(87) WIPO No: **WO14/170894**

(30) Priority Data

(31) Number	(32) Date	(33) Country
225825	**2013.04.18**	**IL**

(43) Publication Date: **2014.10.23**
(44) Accepted Journal Date: **2017.11.23**

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(56) Related Art
US 2012/083412 A1
LANTEIGNE ET AL: "Production of DAPG and HCN by Pseudomonas sp. LBUM300 contribute to the biological control of bacterial canker of tomato". Phytopathology, 2012, vol. 102, pages 967-973

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 October 2014 (23.10.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/170894 A1

(51) International Patent Classification:
A01N 25/04 (2006.01) *A01N 63/00* (2006.01)
A01N 25/10 (2006.01) *A01N 65/00* (2009.01)
A01N 25/34 (2006.01) *A01P 1/00* (2006.01)

(21) International Application Number:
PCT/IL2014/050348

(22) International Filing Date:
10 April 2014 (10.04.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
225825 18 April 2013 (18.04.2013) IL

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: A PACKAGE FOR PLANT ANTIMICROBIAL TREATMENT

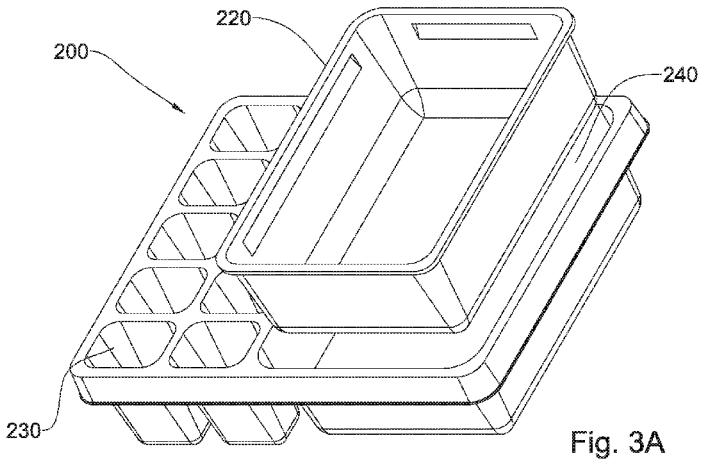

Fig. 3A

(57) Abstract: The present invention provides a package (200) comprising at least one first component in the form a well (220), the first component comprising particulate matter comprising at least one natural oil; at least one second component, in the form of a plurality of second wells (230) comprising antagonist(s) of a microbial pathogen; wherein the at least one first component and the at least one second component are contained in separate compartments of said package. Also provided by the present invention is a method for providing an anti- bacterial agent, the method comprising mixing the first component comprising particulate matter carrying at least one natural oil; and at least one second component comprising at least one antagonist of a microbial pathogen, and allowing said mixture to form into an emulsion with anti-bacterial activity. Further provided by the present invention is a method of treating or preventing a pathogen infection in a plant, the method comprises applying to said plant an amount of an emulsion comprising particulate matter, at least one natural oil and at least one antagonist of a microbial pathogen that causes said pathogen infection. Yet further, there are provided some isolated antagonistic bacteria that may be used, inter alia, in the package and methods disclosed herein.

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A PACKAGE FOR PLANT ANTIMICROBIAL TREATMENT

TECHNOLOGICAL FIELD

The present disclosure is in the field of products for antimicrobial use and in particular, plant protection and biological control of plants.

PRIOR ART

References considered to be relevant as background to the presently disclosed subject matter are listed below:

US patent application publications No. US2011028500

US patent application publication No. US2011014596

Indian Patent Application No. IN03603CH2010

US patent No. 7,485,451

Ait Ben Aoumar A. et al. J. Med. Plants Res. 6(17):4332-4338, 2011

Pouvova D. et al. Zemdirbyste-Agrucyktyre 95(3):440-446, 2008

Lanteigne C, et al. Phytopathology.102(10):967-73, 2012

Slusarski C. Vegetable Crop Research Bulletin 69:125-134 2008

Acknowledgement of the above references herein is not to be inferred as meaning that these are in any way relevant to the patentability of the presently disclosed subject matter.

BACKGROUND

Agricultural crops are susceptible to a large variety of microbial pathogens, which results in annual losses and economical damages. Methods developed to protect crops from plant diseases include plant breeding for resistance, cultural practices, application of chemical agents, and biological control.

As the use of chemical pesticides resulted in severe environmental pollution, and many pathogens are developing resistance to existing chemicals, many pesticides are

now banned for use, and organic farming is not allowed to rely on such substances at all. Thus, a major goal, therefore, is to develop new, environmentally-friendly tools to control pathogens, namely, biological control techniques.

US Patent No. 6,495,133 describes a strain of Glicladium roseum exhibiting antagonistic effects against plant pathogens. The biocontrol agent is used in treatment o seeds, soil or plants to protect against fungal pathogens of various plants, including tomato.

US patent applications publications Nos. US2011028500 and US2011014596 describe a plant pathogen inhibitor combination comprising a plant extract containing one or more anthraquinone; an anti-phytopathogenic agent which may include natural oil or oil product having fungicidal activity.

Indian Patent Application No. IN03603CH2010 describes an invert-emulsion formulation of fungal organisms as biological control. The formulation is in the form of an invert emulsion formulation. The process described includes production of fungal spores either by solid state or liquid fermentation; preparation of conidial suspension or cell suspension; preparation of aqueous phase by mixing the conidial suspension, water, emulsifier and glycerol; preparation of oil phase by adding vegetable fat mixture to a warm vegetable oil mixture; mixing of aqueous phase with oil phase to get water in oil or invert-emulsion formulation using homogenizers The product can be used for seed treatment, soil application and foliar spray.

US patent No. 7,485,451 also describes an invert emulsions (water in oil emulsions) comprising cellular material selected from living and/or dormant prokaryotic and/or eukaryotic cells and tissues, the cellular material being compatible with water-in-oil emulsions. Examples of cellular material included fungi, watermolds, algae, yeasts, bacteria, plant, inset and animal cells. The inverted emulsion also comprises an oil, such as vegetable oil and/or fish oil as well as an oil soluble non-ionic surfactant, and water. Optionally, the composition contains a thickener, such as fumed silica or bentonite.

Essential oils having an antagonistic effect have also been investigated. For example, the antibacterial activity of Moroccan plants extracts against *Clavibacter michiganensis subsp. Michiganensis* (*CBM*), the cause of tomato bacterial canker, was described [Ait Ben Aoumar A. et al. J. Med. Plants Res. 6(17):4332-4338, 2011]. In

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addition, the effectivity against *CBM* of plant essential oils from 34 aromatic plants was examined [Pouvova D. et al. Zemdirbyste-Agrucyktyre 95(3):440-446, 2008].

Recently, the simultaneous production of DAPG and HCN by Pseudomonas sp. LBUM300 was found to be beneficial for the biological control of tomato bacterial canker caused by Clavibacter michiganensis subsp. michiganensis [Lanteigne C, et al. Phytopathology. 102(10):967-73, 2012].

In addition, attempts at biological control of *CBM* Rockwool-grown greenhouse tomatoes was described. Specifically, artificial inoculation of two and three years old rockwool slabs with *CBM* bacteria dead plants reduces death rate of the plants [Slusarski C. Vegetable Crop Research Bulletin 69: 125-134 2008].

GENERAL DESCRIPTION

According to a first aspect of the present invention there is provided a package comprising:

i) a first component comprising particulate matter comprising at least one natural oil;

ii) at least one second component comprising an antagonist of a microbial pathogen;

wherein said first component and said second component are separate in the package and are mixed together prior to use.

According to a second aspect of the present invention there is provided a method for providing an anti-bacterial agent, the method comprises:

(a) providing a package according to the first aspect; and

(b) mixing the first component comprising the particulate matter carrying at least one natural oil with at least one said second component comprising at least one antagonist of a microbial pathogen, to form an emulsion with anti-bacterial activity.

According to a third aspect of the present invention there is provided a method of treating or preventing a pathogen infection in a plant, the method comprises mixing a first component comprising particulate matter comprising at least one natural oil and at least one second component comprising an antagonist of a microbial pathogen to form an emulsion and applying to said plant an amount of the emulsion in two or more doses, with a time interval between applications of between hours to days.

Biological control of plant diseases generally is defined as suppression of pathogens by application of one or more organisms that exhibit antagonistic activity towards the pathogens. The organisms that act as antagonists are regarded as biological control agents (BCAs) and the mechanisms of the antagonistic effects are based on a variety of biological properties of BCAs. These comprise production of antibiotic compounds, expression of enzymes that catalyze the decomposition of cell components of pathogens, competition for space and nutrients, the ability to parasitize pathogens, and the induction of plant defense.

The present disclosure is aimed at providing a ready for use package for bio-control of crops against microbial infection as well as for preventing such infection from developing. As will be evident from the following description, there are provided packages such that the bio-control components are isolated from each other during long term storage and are easily mixed, at the pre-determined concentrations, upon need, without any risk of environmental contamination by the package's components. It has been found that the package configuration ensures chemical stability of the components, i.e. without any damage after long term storage.

Accordingly, and in accordance with a first of its aspect, the present disclosure provides a package comprising:

- at least one first component comprising particulate matter comprising at least one natural oil;
- at least one second component comprising at least one antagonist of a microbial pathogen;

wherein the at least one first component and the at least one second component are contained in separate compartments of said package.

In some embodiments, the compartments are defined as wells within a carrier element forming part of the package.

According to some embodiments, the package comprises:

(i) one or more first wells (compartment) holding a first component comprising particulate matter comprising at least one natural oil;

(ii) one or more second wells (compartment) holding a second component comprising at least one antagonist of a microbial pathogen;

the one or more first wells and the one or more second wells each having a top opening and a recess extending downwardly from said top opening, the wells being held together in an essentially planar matrix; and

(iii) a first film sealing the openings of the one or more first wells; and

(iv) a second film sealing the openings of the one or more second wells.

In accordance with a second of its aspects, there is provided a method for providing an anti-bacterial agent, the method comprises mixing one or more first components comprising particulate matter, the particulate matter carrying at least one natural oil with one or more second components comprising at least one antagonist of a microbial pathogen, and allowing the mixture (cocktail) thus obtained to form into an emulsion. The thus formed emulsion poses anti-bacterial activity. The emulsion prepared by the components disclosed herein is stable emulsion, i.e. where no phase separation is apparent for at least several hours, for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and even up to 24 hours.

In some embodiments, the method makes use of the packages disclosed herein. In some embodiments, the use of the package provides a composition or an emulsion comprising particulate matter, a surfactant, at least one natural oil, and at least one

bacterial antagonist of a plant pathogen. In some embodiments, the antagonist is of a time that is capable of growing on sesame oil as a sole carbon source.

In accordance with yet a third of its aspects, the present disclosure provides a method of treating or preventing a pathogen infection in a plant, the method comprises applying onto said plant an amount of an emulsion comprising particulate matter, at least one natural oil and at least one antagonist of the pathogen that causes the infection.

In accordance with a fourth of its aspects, the present disclosure provides isolated antagonistic bacteria having a representative sample deposited at the CBS-KNAW institute and bearing the accession No. selected from the group consisting of CBS133252, CBS133254, CBS133255, CBS133256, CBS133257, CBS133258, CBS133259, CBS134566, CBS134567 and CBS134568. In some embodiments, these antagonistic bacteria are for use in protecting or treating plants against a pathogen induced infection.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to better understand the subject matter that is disclosed herein and to exemplify how it may be carried out in practice, embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Figures 1A-1D show a carrier element forming part of a package according to an embodiment of the invention, from different views, with Figure 1A providing an isometric view, Figure 1B providing a top view and Figures 1C and 1D providing views from side X and side Y of Figure 1B.

Figures 2A-2B show views of a carrier element in accordance with Figures 1A-1D, including films sealing the different wells of the carrier element, in accordance with an embodiment of the invention.

Figures 3A-3E show a carrier element according to an embodiment of the invention, from different views with Figure 3A providing an isometric view, Figure 3B providing a top view and Figures 3C, 3D and 3E, providing views from sides Z, X and Y of Figure 3B.

Figure 4A-4C show the effect of two selected antagonists on growth of *CBM* (Figure 4A) and *Xanthromonas* (Figure 4B) as compared to control (Figure 4C).

Figures 5A to **5E** show petri dishes of various pathogens after treatment with a plant pathogen, with or without an antagonistic bacteria

Figure 6 is a graph showing the mortality rate of tomato plants following exposure to *Clavibacter michiganensis subsp. Michiganensis* (CBM), the plants were either treated with combinations according to some embodiments of the present disclosure, or with controls before inoculation with CBM.

DETAILED DESCRIPTION OF EMBODIMENTS

The present invention is aimed at providing a package that is suitable for long term storage and upon need, provides a safe and easy method for preparing anti-microbial compositions for various applications, such as for crop protection. To this end, a package has been developed, having two (or more) wells or set of wells, each well or set of wells carrying a different component. The package is configured such that the two (or more) wells are separately and differently sealed, due, *inter alia*, to different physical and chemical characteristics of the matter in the compartments, as will be further discussed hereinafter.

Generally, the package comprises:

- at least one first component comprising particulate matter comprising at least one natural oil;
- at least one second component comprising an antagonist of a microbial pathogen;

wherein the at least one first component and the at least one second component are contained in separate compartments of said package. In other words, as long as the kit is sealed, the two components are not mixed or are not in contact with each other.

Upon mixing of the first and second components (i.e. taking out the two components from their separate compartments), a stable emulsion is obtained, suitable for application onto the crop to be treated or protected. The emulsion is applied in a form of fine droplets.

In the context of the present disclosure, the term "***particulate matter***" is used to denote a substance in the form of plurality of particle. The particles may be in any particulate form, including, without limited thereto, from finely rounded beads to amorphous structures. The particulate matter includes any form of a powder.

In some embodiments, the particulate matter comprise silica dioxide (SiO_2, in short referred to herein as silica). The silica may be naturally occurring silica particles such as bentonite clay beads, as well as synthetic silica beads.

In some embodiments, the particulate matter comprises synthetic silica particles. There are a variety of synthetic silica particles that may be used in the context of the present disclosure. For example, the particulate matter may comprise precipitated synthetic amorphous silica beads, such as the commercially available products Tixosil and Aerosil 200.

In some other embodiments, the particulate matter comprises synthetic or nature derived beads with the capacity to absorb the natural oils. Such beads may include, without being limited thereto Latex beads; calcium carbonate sorbent particle; cellulose beads; polystyrene adsorbents beads e.g. Amberlite® XAD®-2 which is a hydrophobic crosslinked polystyrene copolymer absorbent resin; charcoal; Sepharose™ beads; emulsan-alginate beads; chitosan beads; sodium alginate; styrene-maleic acid copolymer beads and styrene-divinylbenzene beads; cellulose paper beads.

To allow good distribution of the final emulsion and in accordance with some embodiments the particulate matter (particles) has a size distribution in the range of 10-25μm.

The particulate matter may also be characterized, without being limited thereto, by one or more of a surface area, in some embodiments, in the range of 400-500m^2 N_2/g and oil capacity in the range of 300-350 DBP/100 gram particulate.

The first component comprises the particulate matter that holds one or a combination of natural oils. In the context of the present disclosure it is to be understood that "***natural oil***" encompasses any organic oil obtained from nature.

The natural oil is preferably oil derived from a plant. In some embodiments, the natural oils are known as essential oils. The essential oils are preferably those known to exhibit antimicrobial (e.g. antibacterial, antifungal, antinematodal) properties.

When referring to anti-microbial properties it is to be understood as being effective against any microbial pathogen, as further discussed below.

Without being limited thereto, essential oils to be used in accordance with the present disclosure, may be those derived from the plants Origanum vulgare and Origanum spp., (e.g. Oregano), Mentha spp. (mint), Thymus spp. (Thyme), Myrtus spp., Ocimun spp. (e.g. Ocimun basilicum, also klnown as Basil), Lavandula spp. (e.g. Lavender), Micromeria spp., Coriandum spp. (e.g. Coriander/Parsley), Aloysia spp., Melissa spp., Salvia spp., Petoselinum spp., Rosmarinus spp. (e.g. Rosemary), Prunella spp., Cuminum spp (e.g. Cumin).

In some other embodiments, the natural oils are plant derived oils that is used as carbon source, e.g. as food/nutrient for the antagonistic microorganisms. These are referred to herein the term "***carbon-base oil***" or "***carbon-rich nutrient oil***". In some embodiments, the carbon-base oils are vegetable oils. Without being limited thereto, the carbon-base oil is selected from the group consisting of Sesame oil, Olive oil, Peanut oil, Cottonseed oil, Soybean oil, Palm oil, sunflower oil, safflower oil, canola oil, castor oil, coconut oil, groundnut oil.

In some embodiments, the term "natural oil", when used in plurality, encompasses a combination of at least one essential oil and at least one carbon-base oil, both being of natural source.

In some embodiments, the natural oil comprises at least Oregano oil in combination with at least one carbon-base oil. The Oregano oil is combined, at times, with at least Sesame oil.

It has been unexpectedly found that the antagonistic bacteria may be distinguished from other bacteria with no exhibited antagonistic activity towards at least CBM by their capability to grow on carbon base oil, such as sesame oil. In one embodiment, the carbon base oil on which all antagonistic bacteria grow (while non-antagonistic bacteria tested do not) is sesame oil.

The amount of the natural oil within the first component (e.g. held by the particulate matter) may vary, depending on the type(s) of the natural oil used, the amount at loading, the type of particulate matter, the conditions of loading the natural oil onto the particulate matter, the surfactants or solvents used for loading etc.

When referring to loading of the oil onto the particulate matter, it is to be understood as meaning any form of association between the oil and the particulate matter (e.g. silica particles). Without being limited thereto, the oil is held by the particulate matter by absorption onto and/or into the particles. The association between the particles and the oil is reversible, namely, under suitable conditions, such as when brought into contact with water, the oil is easily released from the particles to form an emulsion.

In some embodiments, the particulate matter holds between 20% to 50% w/w natural oil out of the total weight of the particulate matter (after loading). This is determined by conventional techniques such as HPLC or GC chromatography, as also exemplified below. In some other embodiments, the particulate matter holds about 30% w/w natural oil ("about" encompasses a range of between 25-35%, at times between 28% to 32% or around 30%).

In some embodiments, the natural oil comprises either only the essential oil(s) or a combination of at least one essential oil and at least one carbon-base oil. As such, when referring to natural oils it is to be understood as encompassing essential oil(s) as well as carbon-base oil(s). The ratio between the at least one essential oil and at least one carbon-base oil is in the range of 60:40 and 100:0, at times the range is about 80:20.

When a combination of oils is used it is to be understood that they may be absorbed onto the particulate matter together, i.e. the same particulate matter holds more than one type of oil. In some embodiments, for ease of handling, each oil type is held separately on particulate matter such that different types of particulate matter are formed, each being characterized by the type of oil it is holding.

Thus, when referring to particular matter providing Oregano and Sesame at a ratio of 80:20 it is to be understood as a mixture of two populations of particulate matter, 80% carrying Oregano oil and 20% of a type carrying Sesame oil or a single population of particles, each particle being absorbed with the two oils at the defined or desired ratio (i.e. the oils are a priori mixed and then brought into contact with the absorbing carrier/particle). Irrespective of the oil type, the particulate matter between 20% to 50% w/w of its total weight it provided by the oil loaded thereon.

The particulate matter may also comprise at least one surfactant. As appreciated, a surfactant is a compound that lowers the surface tension of a liquid and as such, the

interfacial tension between two liquids to allow the formation of, e.g. an emulsion. The surfactant may be of any kind known in the art as safe for use (e.g. non-toxic to plants or animals), including ionic surfactants, anionic surfactants, cationic surfactants as well as zwitterionic (or non-ionic) surfactants.

In some embodiments, the surfactant is of a type acceptable in organic agriculture. A non-limiting list of possible surfactants to be used in accordance with the present disclosure includes Polyethylene glycol sorbitan trioleates (Tween, e.g. Tween 85, Tween 65), sorbitan fatty acid esters (e.g. Span 40).

In some other embodiments, the surfactant comprises a salt of a fatty acid. The salt may comprise an alkaline such as potassium, calcium, sodium salts, as well as an ammonium salt.

In some embodiments, the salt of a fatty acid comprises potassium salts of fatty acids (also known as soap salts), which are at times used as insecticides, herbicides, fungicides, and/or algaecides. In some embodiments, potassium salts of fatty acids may be obtained by adding potassium hydroxide to natural fatty acids such as those found in animal fats and in plant oils. Fatty acids may be extracted from olives, cotton seeds, soya beans, peanuts, sun flowers, coconuts Palm , Rapeseed , Sesame oil, Amaranth, Corn , Jatropha.

The fatty acid forming the surfactant may also be a synthetic fatty acid as well as a semi-synthetic (e.g. a natural fatty acid that underwent a modification).

In accordance with some embodiments, the at least one surfactant is one being recognized or is labeled as having an insecticide and/or fungicide activity. Without being limited thereto, pesticidal and/or fungicidal surfactants may include, the commercial products Zohar PT-50 and Zohar LQ-215, both produced by Zohar Dalia, Israel.

In one particular embodiment, the surfactant is selected from Zohar PT-50 and Zohar LQ-215.

The compositions of these surfactants are available from Zohar Dalia. For instance, Zohar PT-50 is known to have the following composition:

Vegetable oils							
Type	**Saturated fatty acids**	**Mono-unsaturated fatty acids**	**Polyunsaturated fatty acids**			**Oleic acid (ω-9)**	**Smoke point**
			Total poly	**linolenic acid (ω-3)**	**Linoleic acid (ω-6)**		
Not hydrogenated							
Canola (rapeseed)	7.365	63.276	28.14	9-11	19-21	—	204°C
Coconut	91.00	6.000	3.000	—	2	6	177°C
Corn	12.948	27.576	54.67	1	58	28	232°C
Cottonseed	25.900	17.800	51.90	1	54	19	216°C
Flaxseed/ Linseed (European)	6–9	10–22	68–89	56–71	12–18	10–22	107°C
Olive	14.00	72.00	14.00	<1.5	9–20	—	193 °C
Palm	49.300	37.000	9.300	—	10	40	235 °C
Peanut	16.900	46.200	32.00	—	32	48	225 °C
Safflower (>70% linoleic)	8.00	15.00	75.00	—	—	—	210 °C
Safflower (high oleic)	7.541	75.221	12.82	—	—	—	210 °C
Soybean	15.650	22.783	57.74	7	50	24	238°C
Sunflower (<60% linoleic)	10.100	45.400	40.10	0.200	39.800	45.300	227°C
Sunflower (>70% oleic)	9.859	83.689	3.798	—	—	—	227°C
Fully hydrogenated							
Cottonseed (hydrog.)	93.600	1.529	.587		.287		
Palm (hydrogenated)	47.500	40.600	7.50				

Soybean (hydrogen.)	21.100	73.700	.400	.096			
Values as percent (%) by weight of total fat.							

The results provided herein show that a salt of a fatty acid as disclosed herein had some advantage in terms of stability and/or emulsification properties of the powder, and anti-microbial activity, over other known surfactants, such as the commercially known Tween 20 or Tween 80.

The amount of the surfactant in the first component may vary. However, in some embodiments, the particulate matter comprises between 5% to 10% w/w of the surfactant or combination of surfactants.

The first component comprising the particulate matter is in an essentially dry form. When referring to "***essential dry***" it is to be understood that the first component may contain low amounts of water, in some embodiments not more than 10% (w/w). In some other or additional embodiments, the water content in the first component is within the range of 1% to 7% (w/w). In yet some other embodiments, the "*essential dry*" is to be understood as encompassing no water being detected by conventional methods (i.e. no detectable amount of water).

The first component may also contain some trace amounts of an organic solvent. As will be further discussed below, a solvent may be required for the preparation of the particulate matter and some residual amounts may remain, as long as the solvent is not toxic. In some embodiments, the first component is either solvent free (i.e. contains no detectable amounts of an organic solvent) or comprises trace amount, i.e. not more than 5%, 4%, 3% or even 2% w/w organic solvent. The solvent is typically an organic volatile polar solvent, such as, without being limited thereto, a solvent selected from the group consisting of acetone, isopropyl alcohol, acetonitrile, ethanol and methanol.

In some embodiments, trace amounts of alcohol are detected in the first component.

The particulate matter of the first component is unique in its capability of forming a stable emulsion, once the particulate matter is brought into contact with water. This is achieved, inter alia, due to the presence of a surfactant in the first

component. The surfactant is added to the particles with the oil, before bringing the components into dryness.

In the context of the present disclosure, when referring to a ***stable emulsion*** it is to be understood as referring to dispersion of oil (the dispersed phase) in water (the dispersion medium) for a period of at least 1hour, at times, at least 2, 3, 4, 5, 10 or even 24 hours following the formation of the emulsion. In other words, the stability is determined by the lack of separation into an oil phase and a water phase. The lack of separation may be determined by any means known in the art, including visible inspection.

Without being bound by theory, it is the inventor's position that the incorporation of a surfactant in the particulate matter contributes to the stability of the emulsion formed. This is also evident from the non-limiting examples provided hereinbelow, where the use of potassium salts of fatty acids showed an advantage in terms of stability and safety over other types of commercially available surfactants.

To form the emulsion, the particulate matter is mixed water. The amount of water depends on the amount of particulate matter. In some embodiments, for each gram of particulate matter (30% of which is oil), water is added to provide a one liter emulsion. As such, in a 1 liter emulsion, 0.1 gr particulate matter provides an oil concentration of 0.03% v/v). In some embodiments, the percentage of oil in the final emulsion is in the range of 0.03% and 2% v/v.

In some embodiments, the mixing of the particulate matter with water provides an emulsion with a droplet size in the range of between 1 to 20μm and in some embodiments in the range between 3 to 10μm.

In some embodiments, the emulsion is an anti-microbial emulsion.

The package contains a second component comprising at least one antagonist of a microbial pathogen.

In some embodiments, the at least one antagonist of a microbial pathogen held in a gel or gel-like carrier. Various materials may be used in order to form the gel form for carrying the antagonists.

For example, the gel may be formed from a polysaccharide or combination of polysaccharides with other substances.

According to some embodiments, the gel is selected from the group consisting of agar gel (agar-agar) Guar gum, gelatin, xanthan gum, methyl cellulose gel (cellulose gum), pectin base gel, gelatin gel, and others, as known in the art.

In some embodiments, the second component comprises agar-agar thereby forming a gel holding the microbial antagonists.

In the context of the present disclosure, when referring to antagonists of a microbial pathogen or a pathogen antagonist, it is to be understood as a biological entity that inhibits the plant pathogen (a plant pathogen may also be referred to as a phytopathogen). *Inhibiting*, in the context of the present disclosure is to be understood as reducing growth of the pathogen by at least 50%, at least 70%, at least 90% or even by essentially eliminating the pathogen. The plant pathogen, in the context of the present invention may be any prokaryotic or eukaryotic organism, including, without being limited thereto bacteria, a fungi, protozoa, nematodes, or any other disease causing parasite. As such, the microbial activity of the at least one antagonist, may any one of antibacterial, antifungal, antiprotoxoal, antinematodal etc.

In some embodiments, the second component comprises at least one antagonists. In some other embodiments, the second component comprises a cocktail of antagonists. The cocktail is to be understood as a combination of two or more, at times, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15, antagonists combined together in the same or different concentrations.

In some embodiments, the at least one antagonist is of a type capable of growing on sesame oil as a sole carbon source, as shown in Table 3 hereinbelow. Such antagonists may be easily identified by conducting a conventional cultivation assay using sesame as the sole carbon source and identifying those cultivars that survived the experimental growing period.

In some embodiments, the antagonist may be referred to as a bacteriostate, i.e., that slows down growth of organisms, and in some other embodiments, the antagonist may be referred to as a bactriocide, namely, that kills the organism.

In some embodiments, the at least one antagonist of a microbial pathogen is a soil born antagonist. In this context it is to be understood that the at least one antagonist

may be obtained and isolated from the roots, soil and/or rhizophere of a plant that was shown to be tolerant (e.g. partially resistant) or resistant to the microbial pathogen.

In some other embodiments, the at least one antagonist of a microbial pathogen is a plant derived antagonist, e.g. isolated from a plant part, such as the leaves, the stem, the flower, the vascular system.

The at least one antagonist of a microbial pathogen may also be present and thus derived from the soil (i.e. soil born) and from a plant part (e.g. the vascular system).

In accordance with some embodiments, the antagonists are of a pathogen causing infection in tomato plants.

In yet some embodiments, the antagonists are of the pathogen for which treatment is desired.

The second component may include one or more antagonists. In some embodiments, the second component includes a combination of several antagonists in the same gel. However, in some other embodiments, when combinations of antagonists are to be used, they are each carried by a separate gel and mixed only prior to use. In other words, the second component is a combination of several "second components", where each antagonist is maintained separately and depending on the type of pathogen to be treated, are combined prior to exposure of the plant.

When a combination of antagonists is used, the antagonists may be provided/applied to the plant in the same amounts (CFU/ml) or in different amounts.

In accordance with some embodiments, the amount of an antagonist in the second component (either as a single antagonist or as a cocktail of antagonists) may be in the range between 500 to 5,000CFU/ml/. The ratio between the antagonists, when used as a cocktail may vary, depending on the type of pathogen to be treated and may be a priori determined by conventional laboratory methods, e.g. best bacteriostatic/bactriocidal effect in a cultivation dish.

The type of antagonist will depend on the type of pathogen to be treated by the package.

In some additional embodiments, the pathogen is *Clavibacter michiganensis subsp. Michiganensis* (CBM). In this embodiment, some antagonists that have been isolated from tomato plants that exhibited tolerance to CBM are selected from the non-

limiting group consisting of *Pseudomonas species* (Accession No. CBS133252), *Pseudomonas alcaliphila* (Accession No. CBS133254), *Bacillus subtilis* (Accession No. CBS133255), *Pseudomonas cedrina* (Accession No. CBS133256), *Pseudomonas species* (Accession No. CBS133257), *Pseudomonas species* (Accession No.CBS133258), *Pseudomonas spanius* (Accession No. CBS133259).

Other antagonists are known in the art such as those provided in Table 4 of the Report by the International Organization for Biological and Integrated Control of Noxious Animals and Plants [Edited by Philippe C. Nicot 2011], the content of which is incorporated herein by reference.

In some other embodiments, antagonists of other plants may form part of the second component of the package disclosed herein. These may include other agricultural crops, such as, without being limited thereto, those derived from the Solanaceae family, e.g. tomato, pepper, eggplant, potato; from the Cucurbitaceae family, e.g. squash, melon, gourd, cucumber, pumpkin, luff and watermelons, but also any other seed bearing plants including horticultural plants, trees etc.The package typically comprises instructions for use of the first component and the second component to form an emulsion which is to be applied onto a plant. The instructions comprise, at least mixing the first component with the second component, optionally with the addition of an amount of water, to form an emulsion. In some embodiments, the concentration of the at least one antagonist in the emulsion is at minimum 1,000CFU/ml, or in the range of 500CFU/ml to 5,000CFU/ml.

In addition, antagonists may be found in various literatures, such as, without being limited thereto, the following, which are incorporated herein by reference:

The Pathogen	**The infected plant**	**The Antagonists**	**Source of information**
Ralstonia Solanacearum	Tomato, Pepper	*Bacillus megaterium, Enterobacter cloacae, Pichia guillermondii* and *Candida ethanolica*	Journal of Plant Pathology 92(2):395-406 (2010)
E. carotovora subsp.		E-65 as a *Bacillus* sp. and E-45 as a	The Scientific World Journal (2012), Article

The Pathogen	**The infected plant**	**The Antagonists**	**Source of information**
carotovora P-138		*Lactobacillus* sp.	ID 723293.
Leptosphaeria maculans	canola	*Pseudomonas chlororaphis* and *P. aurantiaca*	Biocontrol Science and Technology 16(5/6):567582 (2006)
Ralstonia solanacearum (Pseudomonas solanacearum)	Fungi in peanut	*Pseudomonas fluorescens* RH4003 and *Bacillus subtilis* AB89	J. ISSAAS Vol. 18(1):185-192 (2012)
Ralstonia solanacearum	Wilt disease of potato	*Pseudomonas solanacearum isolates: B82;w163;wp95 and P. fluorescens*	http://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1983Articles/PlantDisease67n05_499.pdf
Rhizoctonia solani	potato	commercial products of Bacillus subtilis (Kodiak)	Crop Protection 24(11):939-950,(2005)
		Actinomycetes	Phytoprotection 82:85–102 (2001)
Xanthomonas oryzae pv.*oryzae*	Bacterial Leaf Blight Disease in Rice	*Streptomyces spp*	American Journal of Agricultural and Biological Sciences 7(2):217-223 (2012)
Xanthomonas oryzae pv.*oryzae*		Isolates from soil and water	Rice Indstry, culture, and environment 549-553
Streptomyces spp	Potato scab		Phytopathology 85:261-268 (1995); Can J Microbiol. 47(4):332-40 (2001)
Sclerotinia sclerotiorum,	Soybeans Potato	*Bacillus amyloliquefaciens*	MSU AgBioResearch 2011 Annual Report, (2012) URL:

The Pathogen	**The infected plant**	**The Antagonists**	**Source of information**
Streptomyces sp. and *Phytophthora capsici*	scab Vegetable crops	(BAC03)	http://research.msu.edu/stories/getting-root-soil-borne-diseases
Rhizoctonia solani and *Fusarium sambucinum*,	Black Scurf Dry rot of Potato		Egypt. J. Phytopathol., 36(1-2):45-56 (2008)
Rhizoctonia solani	Lettuce	Endophytic strains, *Serratia plymuthica* 3Re4-18 and *Pseudomonas trivialis* 3Re2-7, *rhizobacterium Pseudomonas fluorescens* L13-6-12	FEMS Microbiol Ecol 64:106–116 (2008)
Rhizoctonia solani	Potato	*Pseudomonas fluorescens*	Acta biol.Colomb. 12(1) pages XXX (2007)
Xanthomonas campestris pv. vesicatoria	Tomato	*Rahnella aquatilis*	Microbiological Research, 160(4):343–352 (2005)
Erwinia amylovora (Fire Blight)		*Pseudomonas fluorescens* A506, *Pantoea agglomerans* C9-1, and *Pantoea*	Plant Disease 93(4):386 URL: http://apsjournals.apsnet.org/doi/ pdf/10.1094/PDIS-93-4-0386
Erwinia amylovora (Fire Blight)		*Erwinia herbicola*	ISHS Acta Horticulturae 117: II Symposium on Fireblight URL: http://www.actahort.org/bo

The Pathogen	The infected plant	The Antagonists	Source of information
			oks/117/117_21.htm
Clavibacter michiganensis subsp. michiganensis		*Bacillus subtilis*; *Rhodosporidium diobovatum*	BioControl 49: 305–313, 2004

As noted above, in some embodiments, the antagonist is of a type that is capable of growing on sesame oil as a sole carbon source.

At times, for preparing the emulsion, water may be added. When water is added, the amount of water will depend on the amount of the first component. In some embodiments, for each gram of first component (e.g. 30% of which are oil), water is added to provide one liter emulsion. As such, in 1 liter emulsion, 0.1 gr particulate matter provides an oil concentration of 0.03% v/v.

In some embodiments, the percentage of oil in the final emulsion is in the range of 0.03% and 2% v/v.

According to the above, a final formulation may be provided by a package containing 20 grams of a powder of the first component (30% of which is the oil), and 120 ml of an antagonist gel of the second component and the instructions may include mixing the first component and the second component with water to form 20 liter emulsion containing 0.3%v/v of oil.

Similarly, a package may contain 50 grams or 100 grams of a powder of the first component, and 120 ml of an antagonist gel of the second component and the instructions may include mixing the first component and the second component with water to form a 50 or 100 liter emulsion.

In some embodiments, the second component is provided in the package in separate units, each unit carrying a different antagonist in gel. For example, a package containing in total 120 ml gel, the gel may constitute several gel units, each gel unit carrying the same or different antagonist, e.g. 8 gels of 15ml gel each. This allows

selecting the types of antagonists to be used and changing the combinations in the cocktail as needed, so as to provide a better biocontrol of the pathogen.

The package of the disclosure is suitable for the providence of a novel emulsion. In this respect, the present disclosure also provides an emulsion comprising particulate matter, a surfactant, at least one natural oil and at least one bacterial antagonist, all as defined herein as components of the package, wherein the at least one antagonist is of a type that will grow on sesame oil as a sole carbon source.

The package disclosed herein is particularly suitable for use in preventing or treating pathogenic infection in a plant. Treatment may include inhibition of pathogen growth (bacteriostatic effect) as well as total elimination of the pathogen infection (bacteriocidal effect).

In some embodiments, the treatment or prevention is of an infection caused by *Clavibacter michiganensis subsp. Michiganensis* (CBM).

In some embodiments, the treatment or prevention is of an infection caused by *xanthomonas vesicatoria*. In some other embodiments, the treatment or prevention is of an infection caused by *Streptomyces scabies.*

In some embodiments, the treatment or prevention is of an infection in a plant belonging to the Solanaceae family, e.g. tomato, pepper, eggplant, potato. In one embodiment, the plant is a tomato plant.

In some embodiments, the package in accordance with the present disclosure, comprises

- a carrier element comprising an essentially planar matrix holding together one or more first compartments/wells for carrying at least one first component comprising particulate matter including at least one natural oil; and one or more second wells/compartments for carrying at least one second component comprising at least one antagonist of a microbial pathogen; the one or more first wells and the one or more second wells each being defined by a top opening and a recess extending downwardly from the top opening;
- a first film sealing the openings of the one or more first wells; and
- a second film sealing the openings of the one or more second wells.

As appreciated, each well is a separate entity and mixing of content of one well with the other is possible only after opening the sealing films.

A non-limiting example of a carrier element is illustrated in **Figures 1A-1D** providing an isometric view (Figure 1A) and top and side views(Figures 1B to 1D) of element **100** including a planar matrix **110** including a single first well **120** and a plurality of, spaced apart, second wells **130**. In this non-limiting example, first well **120** is reversibly mountable in a depression **140** provided in planar matrix **110.** In some other embodiments, a mountable well, such as well **120**, may be fitted within an opening (hole) in the planar matrix **110** (not illustrated)**.** Alternatively, the first well may be formed as an integral part of planar matrix **110** (not illustrated).

The plurality of second wells **130** are formed, in this particular embodiment, as an integral part of planar matrix **110.** In an alternative embodiment, any of second well(s) **130** may be of a kind that can be reversibly mountable in a depression **140**, as illustrated for first well **120** or in an opening provided in planar matrix (not shown). In accordance with this embodiment, first well **120** is in fact a type of a cup that can be removed from the depression (in the form of a holding well) the planar matrix prior to use.

Carrier element **100** also includes a gripping unit **150**, in the form of a handle. The gripping unit may have other forms and shapes, so as to facilitated stable and firm holding of the carrier element when the sealing films are pulled away and removed from the package so as to release content of the first and second wells.

According to this non-limiting embodiment, inner diameter **A** of first well **110** that is configured for holding antagonist bacteria may be 80-300mm, and at times within the range between 100mm-200mm, or 120-150mm. Inner diameter **B** of second wells **130** which are configured to hold the same or different bacteria antagonist, is in the range of 20-40mm, at times within the range between 25-35mm. The wells have typically a depth of 20-60mm, at times in the range of 20-30mm.

In some embodiments, the total dimensions of the package are 500-550mm length, and 30-40mm width.

Figures 1C, which is view of **Figure 1B** also shows depression (recess or holding well) **140** for holding first well **120**, depression **140** extending downwardly with

respect to top side **160** of the carrier element. **Figure 1D** is another view of element **100** from side Y of **Figure 1B**.

The first film and the second film may each separately cover and seal the respective first wells and second wells. In accordance with some embodiments, and as also illustrated in **Figures 2A** and **2B**, carrier element **100** as illustrated in **Figures 1A** to **1D**, is combined with a first film **170** partially covering the opening of first well **120** and a second film **180** superimposed over first film **170** and covering (sealing) second wells **130**. The first film **170** and the second film **180** may be fixedly attached to each other such that upon pulling away second film **180**, the first film **170** is also pulled, thus allowing the opening of first wells **120** and second wells **130** essentially simultaneously. However, in a similar manner, first film **170** may be separate from second film **180** to allow independent opening of the first wells **120** and second wells **130**.

Turning now to **Figures 3A-3E** there is provided an element for use in a package in accordance with another embodiment of the present disclosure. Therefore, for simplicity, like reference numerals to those used in **Figures 1A-1D**, shifted by **100** are used to identify components having a similar function in **Figures 3A-to 3E**. For example, component **110** in **Figure 1A** is a well having the same function as well **210** in **Figure 2A**.

Specifically, **Figures 2A** is an isometric view, **Figure 3B** is a top view and **Figures 3C to 3E** are side views of an element **200** from sides Z, X and Y, the element **200** having a general oblong (rectangular) shape, with the planar matrix **210** including a single first well **220,** being reversibly mountable in a depression **240** provided in planar matrix **210** and a plurality of, spaced apart, second wells **230**. In accordance with this non-limiting embodiment, the first well **220** and the second wells **230** are polygonal in shape, in this particular embodiment, quadrilateral.

Further, in accordance with this non-limiting embodiment, second well **230** is shown as an integral part of the carrier element **200** but it may equally be a releasable well, as shown in the non-limiting example of Figure 1A.

The dimensions of carrier element in accordance with this embodiment may be different from that provided with respect to element 100, and in this particular embodiment may have total dimensions of 200-300mm length, and 150-250mm width, with the wells being deeper, i.e. having a depth in the range of between 50-60nm.

As appreciated, the dimensions of the carrier element and in particular the wells provided thereby may vary depending on the particular application of the package and the material to be carried thereby. Those skilled in the art would readily appreciate the adaptations required in the dimensions in order to fit the carrier to the particular application.

As detailed above, the first well(s) of the package in accordance with the present disclosure holds a composition of matter containing oil. As such at least the one or more first wells are formed from oil compatible polymers. When referring to oil compatible polymers it is to be understood as referring to polymers that are inert and do not change the properties of the composite material including the natural oil. For the same reason, the first film covering the first well(s) is composed of oil compatible polymer.

In some embodiments, the first film of the respective first wells is or comprises oil compatible thermoplastic polymers.

In some embodiments, it is required to maintain the composite material in dry form. To this end, the first film is a fluid impermeable polymer. This are referred to in the art as high barrier (HB) films. HB films may be defined by permeability to H_2O in the range of 3-4g/m^3/24hr and permeability to O_2 in the range of 6-8 cm^3/m^2/24hr.

Some non-limiting examples of HB polymers that may form the first film are those derived from any one of polyolefins, polyvinyls, polyesters.

The HB film is typically of a type that can easily peel-off the carrier.

In some embodiments, the first sealing film is a laminate having a thickness in the range of 40-100μm, at times, between 60-80μm.

Turning to the one or more second wells, in accordance with some embodiments, since the second wells carry living matter, it is desirable that the sealing film of these second wells, namely, the second film, be gas permeable. In accordance with some embodiment, the second film is permeable to oxygen or oxygen containing gas. To this end, the permeability of the second film to H_2O may be in the range of 8-10g/m^3/24hr and permeability to O_2 of about 1,200 cm^3/m^2/24hr.

The first and second films may be comprised of thermoplastic films comprising a single or blends of polymers selected from the group consisting of biaxially-oriented

polyethylene terephthalate (BOPET), biaxially oriented polypropylene (BOPP), polyvinylidene chloride (PVDC), polyethylene polypropylene, polyvinyl alcohol.

In some embodiments, the films comprise a biaxial oriented polypropylene (BOPP). In some other embodiments, the films are a laminate of BOPP with another polymer, such as polyethylene (PE). The laminate may comprise two, three of more laminated layers. The films to be used are commercially available, e.g. from Glob-Plast (Plastart , Israel) and widely used in the art.

The present disclosure also provides a method of providing a composition for treating or prevention of a pathogen infection in a plant, the method comprise mixing a first component comprising particulate matter comprising at least one natural oil, with a second component comprising at least one antagonist of a microbial pathogen, and allowing said mixture to form into an emulsion. The resulting emulsion may be referred to herein as an anti-microbial (e.g. bactericidal, fungicidal etc) effective emulsion.

In accordance with this method aspect, the first component and the second component are as defined herein.

Mixing may also require the addition of water to form the emulsion. In some embodiments, the mixing provides an emulsion with a droplet size in the range of between 1 to 20μm and in some embodiments in the range between 3 to 10μm.

The present disclosure also provides a method of treating or preventing a pathogen infection in a plant, the method comprises applying to said plant an amount of an emulsion comprising particulate matter, at least one natural oil and at least one antagonist of a microbial pathogen. In accordance with this aspect of the present disclosure, the emulsion is obtained by mixing, preferably closely prior to application, a first component and a second component as defined herein.

In some embodiments, the method of treatment or prevention comprises applying the emulsion onto a plant. The application of the emulsion may be by any means known in agriculture, including, without being limited thereto, spraying the plant, irrigation.

In yet some other embodiments, the treatment or prevention may include application onto the plant tubers, such as spraying of potato tubers (at times referred to as low spraying of tubers).

The emulsion may be applied to the plant once to obtain the anti-microbial effect, or two or more times. When more than one dose is applied, the doses may be provided with time intervals between applications of between one day interval to more than one day (two, three and more days interval). The different doses may be the same or different in the amount of the antagonists, the amount of the natural oil or both, as well as the same or different in the type of antagonists and/or natural oils provided.

In some embodiments, when more than one dose is applied, the various applications are in intervals of between 2 to 10 days at times between 3 to 8 days.

In some embodiments, the plant is provided with at least 2, 3, 4, and at times at least 5 doses of emulsion(s) provided in accordance with the present disclosure.

The frequency of treatment and the amount of doses depends on the type of the infection (e.g. how violent/harmful/death threading it is), the severity of infection, if already developed, the environmental conditions, etc. Those versed in agriculture would be able to determine the treatment schedule based on commonly used parameters.

For preventing an infection from development, it is required, in accordance with some embodiments, to apply the emulsion to the plant from the day of planting of the plant or from a first day of a suspected infection.

For treatment of an infection, it is required, in accordance with some embodiments, to apply the emulsion to the plant from the day of detection of a suspected infection.

The present disclosure also provides for an isolated antagonistic bacterium selected from the group consisting of *Pseudomonas species* (Accession No. CBS133252), *Pseudomonas alcaliphila* (Accession No. CBS133254), *Bacillus subtilis* (Accession No. CBS133255), *Pseudomonas cedrina* (Accession No. CBS133256), *Pseudomonas species* (Accession No. CBS133257), *Pseudomonas species* (Accession No.CBS133258), *Pseudomonas species* (Accession No. CBS134568), *Pseudomonas spanius* (Accession No. CBS133259), *Pseudomonas mediterranea* (Accession No. CBS134566), *Pseudomonas chlororahis* (Accession No. CBS134567) and *Pseudomonas species* (Accession No. CBS134568).

In some embodiments, the isolated antagonistic bacterium is within a carrier. In some embodiments, the carrier is a gel or gel-like carrier as defined hereinabove.

The isolated bacterium may have various applications. In some embodiments, the isolated bacterium, alone, or as a bacterial cocktail may be used for treating or protecting plant against a disease caused by a pathogen, such as those disclosed hereinabove. The following are some non-limiting examples for performing the invention. As will be shown, the formulations for the control of plant diseases the oils ingredients and the ratio among the sesame oil or its substitutes and oregano oil and its substitutes are about constant (1:0.25(w/w) ratio of oregano oil:sesame oil) whereas the quantities of the other supplemented to the oils formulation are changed according to the plant, pest and the environmental conditions. At least one antagonistic bacterium out of the possible bacteria is in use in combination with the oils formulation for the control of plant diseases.

DESCRIPTION OF NON-LIMITING EXAMPLES

The study consisted of three parts: (i) isolation and multiplication of the pathogens and potential antagonists; (ii) *in vitro* screening of potential antagonists against *Clavibacter michiganensis subsp. Michiganensis* (CBM), and (iii) in vivo evaluation of selected antagonists for the control of bacterial wilt disease on tomatoes.

Isolating of Pathogens and Potential Antagonistic Bacterial Strains

Tomato plants (*Daniela*, Hazera, Israel) were grown in greenhouses in double rows of plants on each bed with a drip irrigation system. The seedlings were planted in about 3 plants per meter row and were cultivated according the Dutch (Holland) method. The plots were under surveillance for development of infection by CBM. Upon infection, plants that survived the infection were identified, and samples containing root parts, soil (at depth of 30cm) and the rhizophere were taken into sterile bags and transported to the laboratory.

In the laboratory, samples of 10 grams each containing root parts, soil, and the rhizosphere were introduce into a stomacher bag containing 90ml of agar solution 0.1% w/v (diluted with double distilled water (DDW), sealed and sterilized in an autoclave 121°C, 1 atm, 20minutes). Following sterilization, the samples were shaked for 2 hours at a speed of 20 rpm and room temperature. Then, the bags were transferred into a stomacher device for 1 minute, at medium speed.

After stomaching the samples, each bag was opened by sterile scissors, and various dilutions of the samples were prepared in sterile tubes containing each 9ml of agar solution (0.1%w/v). The first dilution was 1ml sample into 9 ml agar ($x10^{-2}$ dilution), and continued up to $x10^{-8}$ dilution.

From each diluted sample, 100µl were transferred into Petri dishes supplemented with a media selected from one of the following agars: potato dextrose agar (PDA, Difco), a Nutrient Agar, Pseudomonas agar F (Difco) and incubated in an incubator (28°C) for 5 days. Those of interest colonies were marked and transferred using a bacterial needle into a fresh medium (the same as used for growing before isolation) to obtain an isolated single type of microbial colonies of potential antagonists.

Multiplication of isolated antagonists

The microbial colonies suspected of having antagonistic effects were purified from the microorganisms growing on the agar plates under a laminar flow hood and re-cultured in Erlenmeyer flaskon in a culturing broth containing peptone (10 gr/litre), yeast extract (20 gr/litre), glycerol (10 gr/litre), $MgSO_4$ (0.1 gr/litre), $CaCO_3$ (2 gr/litre), Each flaskon received a single isolated colony for cultivation as a pure antagonist and each flaskon was covered with a plastic cap, sterilized in an autoclave for 20min at 121°C, and kept at room temperature for one day.

Screening of effective antagonists of CBM and/or Xanthromonas.

Cultures showing antagonistic activity were screened for effective *in vitro* suppression of the pathogen by transferring each of the suspected antagonist using a bacterial needle into a Petri dish containing NA medium (nutrient Agar medium), supplemented with 0.1% yeast extract and 1% glycerol. The bacteria were placed on a straight line on the dish and the dish was then incubated for 24 hours at 28°C. After incubation time, CBM pathogen was seeded on an imaginary line perpendicular to the bacteria line and the dish was returned to incubation for an additional period of 3 days. If the bacteria has antagonistic activity, a gap between the bacterial line and the pathogen line was formed during the incubation days those having the greater gap formed, were selected as potential antagonists. These were further identified by the center of Centraalbureau voor Schimmelcultures (Fungal Biodiversity Centre, Institute of Royal Netherlands Academy of Arts and Science (CBS-KNAW).

Table 1 provides the list of species deposited at the CBS-KNAW institute on November 19, 2012 and used in the final combination/cocktail. The species were stored at -80°C in a glycerol solution (15%).

Table 1: Deposited antagonists

Antagonist	Accession No.	Name
Pseudomonas species	CBS133252	BN12-27A
Pseudomonas alcaliphila	CBS133254	BN12-28
Bacillus subtilis	CBS133255	BN12-29
Pseudomonas cedrina	CBS133256	BN12-30
Pseudomonas species	CBS133257	BN-12-31
Pseudomonas species	CBS133258	BN12-32
Pseudomonas spanius	CBS133259	BN12-33
Pseudomonas mediterranea AN1	CBS134566	BN13-01
Pseudomonas chlororahis AN10	CBS134567	BN13-02
Pseudomonas species AN21	CBS134568	BN13-03

In addition, **Figures 4A-4C** show the effect of antagonists *Pseudomonas cedrina* (CBS1333256, "AN4") (**Figure 4A**) and *Pseudomonas species* (CBS1333258, "AN19") (**Figure 4B**) on the growth of *CBM* and *Xanthromonas* (two separate spreads/lines on the plate) as compared to the effect of a control being a non-antagonistic bacteria isolated from the same source of the antagonistic bacteria and grown on media without sesame oil (Control bacteria, **Figure 4C**). Specifically, in Figure 4A and Figure 4B a non-growing zone is shown where the pathogen spreads of *CBM* and *Xanthromonas* could not grow towards the antagonistic line. In the control Figure 4C, the pathogen spreads of *CBM* and *Xanthromonas* reached the control bacteria line. These results show that AN4 and AN19 have antagonistic activity towards at least *CBM* and *Xanthromonas*.

The same experiment was conducted for each isolated bacteria which led to the list of antagonistic bacteria of Table 1.

Preparing antagonistic microbial gel

Isolated antagonists were separately transferred to Erlenmeyer flask containing the medium used for multiplication (peptone (10 gr/litre), yeast extract (20 gr/litre), glycerol (10 gr/litre), $MgSO_4$ (0.1 gr/litre), $CaCO_3$ (2 gr/litre) supplemented with 0.15% granulated Agar (Difco) and each antagonist at a concentration of between 10^7 to 10^8 CFU/ml and shaked for 72 h at 28°C. The resulting gel like cocktail was then kept in gel form, at room temperature until use. It has been shown that the antagonists can be preserved in this form for up to 12 months with a decrease in the bacterial population in logarithmic order of no more than 2.

Determining media for maintaining the antagonist

In order to determine the most appropriate media for storing the antagonistic bacteria, the following possible storage media were tested:

The tested antagonistic bacteria:

The tested antagonistic bacteria were CBS 133252; CBS 133255 and CBS 134567.

Each antagonistic bacteria was grown for 48h on Petri dishes containing the AN media. The colonies of each bacterium were collected from the surface of the growing media into sterile distilled water to a final concentration of 10^9CFU/ml.

The tested storage media:

a. Distilled water (DW)

b. Oregano oil 79% and sesame oil 19% and DW 2%.

c. Oregano oil 4% and sesame oil 0.8% in DW.

d. An emulsion comprising oregano oil 4%, sesame oil 0.8% in DW + 0.05% Tween 80 (surfactant TWEEN® 80(Product Number: P4780 Brand: Sigma).

e. Antagonistic media ("AN media") as a soft gel comprising yeast extract (20 gr/litre), glycerol (10 gr/litre), $MgSO_4$ (0.1 gr/litre), $CaCO_3$ (2 gr/litre) supplemented with 0.15% granulated Agar (Difco).

Preparing the storage media:

Each tested storage media (9 ml) was poured under aseptic condition into 15 ml sterile tubes. In total 120 tubes for each tested storage media.

To each tube, 1ml of the pre made antagonistic bacteria preparation (10^9 cells/ml) was added, were inoculating to each tube to a final concentration of 10^8CFU of the tested bacteria in each tube. The inoculated tubes were incubated for a period of 300 days at room temperature around 25°C.

Estimating the survival of the tested bacteria in the various storage media:

During the 294 days of incubation, samples from each tube (5 replications each) were taken periodically. From each tube a tenfold dilution in sterile distilled water was made from 1 to 10^{-8} .Then, from each dilution, 100 micro liters were spared on the surface AN media. After incubation for 5 days at 25°C, the population was estimated as CFU/ ml of the original test tube that were taken at a certain time. The results are the mean of the 5 replications at each time.

Results:

The survival results are summarized in Tables 2A-2E below:

Table 2A – Survival of antagonistic bacteria in distilled water

Days from inoculation	CBS 133252	CBS133255	CBS133255
1	$3.1X10^8$	$2.6X10^8$	$1.8X10^8$
14	$1.6X10^3$	$1X10^6$	$4X10^3$
28	40	$6X10^3$	10
42	0	$2X10^2$	0
56	0	20	0
70	0	0	0
84	0	0	0
98	-	-	-
112	-	-	-
126	-	-	-
140	-	-	-
154	-	-	-
168	-	-	-
189	-	-	-
210	-	-	-

231	-	-	-
252	-	-	-
273	-	-	-
294	-	-	-

Table 2B – Survival of antagonistic bacteria in Oregano oil 79%, sesame oil 19% and DW 2%

Days from inoculation	CBS 133252	CBS133255	CBS133255
1	**6X103**	**8.5X103**	**1X103**
14	**0**	**20**	**0**
28	**0**	**0**	**0**
42	**0**	**0**	**0**
56	-	-	-
70	-	-	-
84	-	-	-
98	-	-	-
112	-	-	-
126	-	-	-
140	-	-	-
154	-	-	-
168	-	-	-
189	-	-	-
210	-	-	-
231	-	-	-
252	-	-	-
273	-	-	-
294	-	-	-

Table 2C – Survival of antagonistic bacteria in Oregano oil 4% and sesame oil 0.8% in DW

Days after inoculation	CBS 133252	CBS133255	CBS133255
1	$2.4X10^8$	$2.5X10^8$	$1.7X10^3$
14	0	10	0
28	0	0	0
42	0	0	0
56	-	-	-
70	-	-	-
84	-	-	-
98	-	-	-
112	-	-	-
126	-	-	-
140	-	-	-
154	-	-	-
168	-	-	-
189	-	-	-
210	-	-	-
231	-	-	-
252	-	-	-
273	-	-	-
294	-	-	-

Table 2D – Survival of antagonistic bacteria in an emulsion (oregano oil 4%, sesame oil 0.8% in DW + 0.05% Tween 80)

Days after inoculation	CBS 133252	CBS133255	CBS133255
1	$2X10^8$	$3X10^8$	$2X10^8$
14	0	30	0
28	0	10	0
42	0	0	0
56	0	0	0
70	0	0	0
84	-	-	-
98	-	-	-
112	-	-	-
126	-	-	-
140	-	-	-
154	-	-	-
168	-	-	-
189	-	-	-
210	-	-	-
231	-	-	-
252	-	-	-
273	-	-	-
294	-	-	-

Table 2E – Survival of antagonistic bacteria in gel (yeast extract, glycerol, $MgSO_4$, $CaCO_3$, granulated Agar)

Days after inoculation	CBS 133252	CBS133255	CBS133255
1	$3.2X10^8$	$2.6X10^8$	$1.9X10^8$
14	$3.6X10^8$	$3.0X10^8$	$1.6X10^8$
28	$3.5X10^8$	$3.0X10^8$	$1.5X10^8$
42	$3.6X10^8$	$3.0X10^8$	$1.6X10^8$
56	$3.5X10^8$	$2.6X10^8$	$1.5X10^8$
70	$4.2X10^7$	$2.2X10^8$	$3.5X10^7$
84	$1.0X10^7$	$1.0X10^8$	$1.0X10^7$
98	$3.0X10^6$	$3.3X10^7$	$1.0X10^6$
112	$3.0X10^6$	$3.0X10^7$	$1.0X10^6$
126	$4.0X10^6$	$4.0X10^7$	$9.0X10^5$
140	$8.0X10^5$	$9.3X10^6$	$6.0X10^5$
154	$8.0X10^5$	$8.8X10^6$	$4.0X10^5$
168	$6.0X10^5$	$6.0X10^6$	$5.0X10^5$
189	$3.0X10^5$	$3.0X10^6$	$1.0X10^5$
210	$1.0X10^5$	$9.0X10^5$	$1.0X10^5$
231	$8.0X10^4$	$8.1X10^5$	$6.1X10^4$
252	$7.4X10^4$	$8.1X10^5$	$7.4X10^4$
273	$7.6X10^4$	$8.1X10^5$	$6.4X10^4$
294	$7.6X10^4$	$8.1X10^5$	$5.8X10^4$

Specifically, the results presented in Tables 2A to 2E show that the survival of the three tested antagonistic bacteria in the four first tested storage media was significantly different from the gel based media.

Specifically, only the gel (agar containing) media supported the long term (294 days) survival of the antagonistic bacteria. At the end of the experiment the population of CBS 133252 and CBS 133255 was above $5.0X10^4$ CFU/ml, while CBS 133255 survived to a level of about $8.0X10^5$. In all other tested media the antagonistic bacteria did not survive after the about 40 days.

Further characterization of antagonistic bacteria

To further characterize the antagonistic bacteria, the difference in growth of the bacterial (as well as non-antagonists) on various carbon source material has been investigated. Specifically, the growth of 10 antagonistic bacteria and 6 non-antagonistic bacteria on sesame oil, glucose or glycerol as separate carbon sources was examined. A media without carbon source was used as the control.

Materials

The growth media included:

Distilled water	500ml
$NH_4H_2PO_4$	0.10gr
$MgSO_4*7H_2O$	0.01gr
KCl	0.10gr
Tested carbon source	4.20gr

The bacteria stock solution included:

The bacteria was collected from 24h bacterial culture from which a solution of 0.65 Absorbance at 480nm, diluted 1:100 with distilled water was prepared.

Method

Ten mililiters of the growth media with or without the tested carbon source was poured into 30ml Erlenmeyer flasks and inoculated with 100µl of the selected bacterial solution. The inoculated flasks were incubated at 26°C, for 24h and then subjected to a ten-fold dilution and counted. Water was used as control.

Table 3 provides the colony forming units/ml for each texted carbon source and bacteria. In Table 3, the isolated and deposited bacteria from Table 1 were compared with bacteria isolated from the soil as described above, but found to have no antagonistic activity, these being referred to as *Pseudomonas spp 12 (P. spp 12)*, *Pseudomonas spp 13 (P. spp 13)*, *Pseudomonas spp 14 (P. spp 14)*, *Pseudomonas spp 15 (P. spp 15)*, *Bacilus spp 36*, *E. coli 4.*

The results in Table 3 show that bacteria with no identified antagonistic activity were not able to grow on sesame oil as a sole carbon source.

Table 3: Bacterial growth

Bacteria deposit number	Control*	Sesame oil	Glucose	Glycerol
CBS134566	60	$5X10^8$	10^7	$2X10^9$
CBS133252	80	$3X10^8$	10^2	$4X10^8$
CBS133254	100	$1X10^9$	$6X10^2$	$1X10^9$
CBS133255	50	$6X10^8$	$7X10^2$	$7X10^8$
CBS133256	110	$9X10^7$	$6X10^8$	$5X10^8$
CBS134567	90	$3X10^8$	$1X10^9$	$5X10^8$
CBS133257	130	$2X10^9$	120	$4X10^9$
CBS133258	140	$7X10^7$	156	$1X10^8$
CBS133259	60	$1X10^8$	150	$1X10^8$
CBS134568	70	$6X10^7$	$3X10^8$	$7X10^7$
P. spp 12	60	75	$4X10^9$	$8X10^8$
P. spp 13	70	54	$3X10^8$	$1X10^8$
P. spp 14	90	100	$8X10^8$	$5X10^8$
P. spp 15	90	86	$3X10^9$	$6X10^8$
Bacilus spp 36	90	100	$3X10^9$	$7X10^8$
E. coli 4	110	98	$8X10^8$	$4X10^8$
Water		0	0	0

Verifying anti-bacterial effect of essential oils

Materials and Methods

To verify the anti-bacterial effect of essential oil, the following assay was conducted.

Oil: Oregano oil with the following particulars: country of origin: Bulgaria; plant parts: flowering plant; cultivation method: certified organics, method of extraction: steam distilled.

Bacterial strains: Escherichia coli, Staphylococcus aureus, Salmonella, Clavibacter and Xanthomonas campestris were obtained from the collection of Prof. G. Kritzman Israel.

Disc Diffusion method: Bacteria were grown in nutrient broth test tubes at 27^0C for 24hrs. The paper discs were sterilized by autoclave in preparation for the disc diffusion method. Each bacteria (100 µl), was placed on Nutrient agar (NA) plates and allowed to dry for 3-5 minutes. The paper discs were saturated in 100% concentration of the oregano essential oil (20ul), and then placed onto each NA plate freshly coated with bacteria. The positive control used was 3% H_2O_2 solution and the negative control was DI water. The plates were incubated at 27^0C for 48 hours. The zone of inhibition was measured by standard ruler.

Results

The anti-bacterial effect of the commercial organic oils on bacteria is summarized in Table 4, showing a greater inhibition zone for the oregano oils treated bacteria as compared to the controls.

Table 4: Anti-bacterial effect of oregano oil

The tested bacteria	**Inhibition zone (mm)**
E. coli	**15**
S. aureus	**19**
Salmonela	**21**
Clavibacter	**24**
Xanthomonas campestris	**22**
Positive control	**8**
Negative control	**0**

Preparation of Essential Oil Powder

Materials

For preparing the oil powder, the following materials were used:

Natural oils:

Oregano oil 100% (essential oil) and Sesame oil 100% (carbon-base oil), both purchased from *Makes Scents Natural SPA line*, Lancaster PA, USA.

Surfactants:

Thymol, Carvacrol, Tween80, Tween 65, Tween R85 and Egg Lecithin all purchased from Sigma-Aldrich.

Span 40 purchased from Fluka, Israel.

Zohar LQ-215 (Potassium fatty acids) and Zohar PT-50 (Potassium fatty acids) purchased from Zohar Dalia.

Silica beads:

Tixosil (SiO_2) purchased from Rhodia group.

Aerosil 200 and Sipernat 50S (SiO_2, 20 μm) purchased from Evonik Industries AG.

Solvent:

Acetone and Acetonitrile purchased from J.T. Becker, Isopropanol (IPA), Gadot.

Methods

Powder preparation

For laboratory scale production the powders containing the natural oils, surfactants and the silica beads were prepared using common lab glassware set up including laboratory bottles of 20-50ml sizes, spatulas, magnetic stirrers and heating plates. Generally, the natural oil was weight and each was separately mixed with the selected surfactant in a 20ml vial, to which the solvent was added. The mixture of each oil were mixed and heated to a temperature of about 40°C until homogeneous solutions were obtained. To the homogenous solutions the silica beads were added until the liquid was absorbed by the beads. The bottles were left in the fuming hood overnight until all solvent has evaporated.

Loading of each of the oil in the final dry powders was 30-42%. The dry powders contained 2%-7% water.

All ratios of ingredients for powders preparation are provided in Tables 5A-5D:

Table 5A: Oregano oil based powder

Form. No.	Oregano oil	Surfactant					Silica beads		Solvent
		Tween 80	Lecithin	Tween 85	Tween 65	Span 40	Tixosil	Aerosil 200	Acetone
ORG-18A	0.5g	0.5g	0.1g					0.8g	1g
ORG-18B	0.5g	0.5g				0.1g		0.8g	1g
ORG-18C	0.5g	0.5g		0.1g				0.8g	1g
ORG-18D	0.5gg	0.5g			0.1g			0.8g	1g
ORG-20C	0.5g	0.5g			0.1g		0.56g	0.24g	1g
ORG-20D	0.5g	0.5g			0.1g		0.4g	0.4g	1g

Table 5B: Sesame oil based powder

Form. No.	Sesame oil	Surfactant					Silica beads		Solvent
		Tween 80	Lecithin	Tween 85	Tween 65	Span 40	Tixosil	Aerosil 200	Acetone
SES-19A	0.5g	0.5g	0.1g					0.8g	1g
SES-19B	0.5g	0.5g				0.1g		0.8g	1g
SES-19C	0.5g	0.5g		0.1g				0.8g	1g
SES-19D	0.5gg	0.5g			0.1g			0.8g	1g
ORG-21C	0.5g	0.5g			0.1g		0.56g	0.24g	1g
ORG-21D	0.5g	0.5g			0.1g		0.4g	0.4g	1g

Table 5C: Self emulsified Oregano oil based powder using anionic surfactants

Form. No.	Oregano oil	Surfactant		Silica beads		Solvent	
		Zohar PT-50	Zohar LQ 215	Tixosil	Aerosil 200	Isopropyl alcohol	Acetone
ORG-22A	0.5g		0.5g	0.56g	0.24g		0
ORG-22B	0.5g		0.5g	0.4g	0.4g		1g
ORG-24A	0.5g		0.25g	0.4g	0.4g		0
ORG-24B	0.5g		0.25g	0.4g	0.4g		0.5g
ORG-24C	0.75g		0.25g	0.4g	0.4g		1g
ORG-28	0.5g	0.25g		0.56	0.24g	1g	

Table 5D: Self emulsified Sesame oil based powder using anionic surfactants

Form. No.	Sesame oil	Surfactant		Silica beads		Solvent	
		Zohar PT-50	Zohar LQ 215	Tixosil	Aerosil 200	Isopropyl alcohol	Acetone
ORG-23A	0.5g		0.5g	0.56g	0.24g		0
ORG-23B	0.5g		0.5g	0.4g	0.4g		1g
ORG-25A	0.5g		0.25g	0.4g	0.4g		0
ORG-25B	0.5g		0.25g	0.4g	0.4g		0.5g
ORG-25C	0.75g		0.25g	0.4g	0.4g		1g
ORG-29	0.5g	0.25g		0.56	0.24g	1g	

For greater amounts, laboratory electro-mechanical means similar to those used in the industry were employed. These included:

1. Vertical Mechanical Stirrer DC Hsiangtai equipped with propeller;

2. Peristaltic pump 4.4 Carter 4/6 cassette manostat and tubing;

3. Dynamic Exim 5 L powder mixer equipped with ribbon type mixing blades

4. Balances

5. Beakers 1-2 L and containers 1-3 L

The preparation included weighting and mixing the oil with the surfactant(s) in a 1L beaker, to which isopropyl alcohol was added while mixing until a homogenous solution was obtained. The silica beads (Sipernat 50S) were added to a 2L beaker to which the homogenous solution was slowly (rate of 10ml/min) added while mixing (30rpm) until all liquid was absorbed into the beads.

All ratios of ingredients for powders preparation are provided in Tables 6A and 6B. The loading of the oil in the range of about 30%-42% was maintained.

Table 6A: Oregano oil based powder

Form. No.	**Oregano Oil**	**Surfactant**	**Silica beads**	**Solvent**
		Zohar PT 50	**Sipernat 50S**	**IPA**
33	20g	10g	30g	20g
34	200g	100g	300g	200g
37A	20g	15g	30g	20g
37B	20g	10g	300g	15g
38	2x200g	2x125g	2x300g	2x125g

Table 6B: Oregano oil based powder

Form. No.	Oregano Oil	Surfactant	Silica beads	Solvent
		Zohar PT 50	Sipernat 50S	IPA
35	200g	100g	300g	200g
39	200g	125g	300g	125g

In addition, also mixtures of powders (those containing Oregano oil and those containing Sesame oil) were prepared. Specifically, 400g of formulation ORG-34 (beads carrying oregano oil) was mixed with 100g of formulation SES-35 (beads carrying Sesame oil) in Dynamic Exim 5 L powder mixer at 10 rpm producing the mix O&S-A.

Each type of oil based powder was dried in the vacuum oven at 40°C for 24 hr prior to mixing the two populations together. [

In a different process, 800g of formulation ORG-38 was mixed with 200 g of formulation SES-39 in Dynamic Exim 5 L powder mixer at 10 rpm producing the mixed beads formulation O&S-B.

The mixed bead powders were used as is.

Characterization

Determination of Water Content in Powder

Water content was determined using Mettler Toledo DL-38 Karl Fisher titrator according to USP <921> method.

Determination of Isopropanol Content in Powder

IPA content was determined using a headspace analysis according to the parameters bellow:

Gas chromatograph	-	Agilent 7890 A
Column	-	BPX Volatiles, 60m×0.25mm, 1.4µm, SGE
Oven Program	-	45ºC for 2 min, then 10ºC/min to 100ºC, then 25ºC/min to 240ºC, for 5 min.
Split	-	1:25
Mass spectrometer	-	Agilent 5975C

Autosampler program	-	CTC Combi PAL Pre-incubation time: 300s Incubation temp.: 80ºC Syringe temp: 100ºC Volume of injection: 500 µl
Headspace vial	-	20 ml
Volume of sample (water)	-	2 ml
Calibration points (µg/ml)	-	10, 25, 100, 500, 1000
Concentration of ISTDs (ethanol)	-	50 µg/ml

Assay of Oregano oil in dry powder using HPLC

Impurities profile were determined in accordance with the method reported by H. Hajimehdipoor "A validated high performance liquid chromatography method for the analysis of thymol and carvacrol in Thymus vulgaris L. volatile oil" in Pharmacogn Mag. 2010 Jul-Sep; 6(23): 154 158 and adopted by SoluBest. For this purpose Nucleosil 100 C18 HD, 3µ, 150x3 mm column and Ultimate 3000 Dionex (Germany) HPLC system with photodiode array (PDA) detectors and Chromeleon Version 6.80 software packages were used. The mobile phase is Acetonitrile:Water (50:50, v/v). Minimum resolution between Carvacrol and Thymol peaks is 1.5.

Standard solutions were prepared in duplicate as following:

About 3 mg Thymol and 20 mg Carvacrol were weighted into 50 mL volumetric flask, and dissolved in 40 mL of diluents, then brought up to volume with the diluent and mixed. The resulting concentration of the Thymol standard solution was about 0.06 mg/mL and Carvacrol standard solution was about 0.4 mg/mL.

Sample solutions were prepared in duplicate as following:

About 70 mg of powdered sample was weighted into a 25 mL volumetric flask, then brought up to volume with the acetone and mixed.

Assay of Sesame oil in formulation using GC

Sesame oil absorbed on silica beads was trans-methylated overnight with methanolic HCl solution at 60ºC. Heptadecanoic acid, used as an internal standard, was

added to beads before derivatization. Methyl esters of fatty acids were extracted with hexane and dried over anhydrous sodium sulfate prior to GC analysis.

Calibration standards were prepared from different concentrations of sesame oil and blank beads. Conditions of derivatization and amount of internal standard were the same as described in sample preparation.

Quantitative analysis of sesame oil in beads was performed using Agilent 7890 gas chromatograph equipped with FID detector. Compounds were separated on DB-23 capillary column.

Results

Conventional HPLC and GC analytical methods for Oregano and Sesame oils assay were employed.

The chromatograms of the tested powders showed that no degradation (according to the conventional markers, Thymol and Carvacrol aromatic compounds) of the oil was caused during the powder preparation and storage. Table 7 below provides % of Oregano oil and Sesame oil, respectively, in the powder based on Thymol and Carvacrol aromatic compounds analysis by HPLC.

Table 7 - Oregano oil content in formulations as measured by HPLC

Form. No	Sample	% via Thymol	% via Carvacrol
ORG-18A	ORG-18A-1	25.2	27.1
	ORG-18A-2	27.1	28.3
	Average	26.1	27.7
	Difference, %	7.2	4.3
ORG-28	ORG-28-1	33.8	34.9
	ORG-28-2	32.1	33.2
	Average	32.9	34.1
	Difference	5.5	5.2
ORG-32	ORG-32-1	28.0	30.3
	ORG-32-2	28.1	30.7
	Average	28.0	30.5
	Difference	0.6	0.7

ORG-34	ORG-34-1	26.9	29.8
	ORG-34-2	27.7	30.1
	Average	27.3	30.0
	Difference	2.1	2.0
ORG-38	ORG-38-1	28.8	28.7
	ORG-38-2	28.0	29.3
	Average	28.4	29.0
	Difference	4.1	2.0

Table 8 provides the % of Sesame oil in the formulation as determined by GC Chromatograph.

Table 8 - Sesame oil content in formulations as measured by GC chromatography

Form. No.	Sample	% via C16:0	% via C18:0	% via C18:2
SES-19A	SES-19A-1	-	-	28.0
	SES-19A-2	-	-	27.6
	Average	-	-	27.8
	Difference, %	-	-	1.4
SES-35	SES-35-1	29.8	30.5	37.5
	SES-35-2	30.6	30.6	33.4
	Average	30.2	30.6	35.5
	Difference, %	2.6	0.3	12.3
SES-39	SES-39-1	32.8	33.0	39.8
	SES-30-2	31.5	32.3	37.4
	Average	32.2	32.7	38.6
	Difference, %	4.1	2.2	6.4

The water content measured using Karl Fisher titration found that the powder contains 5-7% of water. It appears the source of the water is from Zohar PT 50 surfactant, which contents 50% of water.

As to IPA content, GC Headspace precise analysis demonstrate the IPA content in the formulations, which is summarized in Table 9.

Table 9 – IPA content in the powders

Form. No	Sample Amount (gr.)	IPA (µg/ml)	IPA (%)
34	19.6	1,265	12.9
35	200	1,139	11.4
38	20.3	1,117	11.0
39	19.8	492	5.0

As can be seen from the Table 9, the amount of IPA varies from 5 to 13%. However, in the field, the formulations were diluted for at least 30 times and as such, the content of IPA was reduced to 0.17-0.43%, which is negligible and very safe amount.

The different types of dry powders prepared showed stable after long term (more than a year) storage. In addition to the above, it is noted that the powders have a characteristic odor. The oregano oil based formulations have off-white color and sesame oil based powders are white.

Upon contact with water tested formulations (ORG-28 and SES-29 immediately form an emulsion, which were stable for 24h. The emulsions consisted of droplets of 3-10 microns.The spray-ability of the emulsions was good without clogging the filters.

Safety studies in the field showed that the tested oil based powders were safe. This was determined by the presence (or not) of burns on the plants, as determined by conventional phytotoxicty parameters.

Further, long term (8 weeks) stability of the powders was determined. Specifically, the Oregano and Sesame based powders were separately sealed in aluminum foil bags and placed at accelerating storing conditions (40ºC for 8 weeks). Assay of Oregano oil was measured via two major constituents- Carvacrol and Thymol- in the beginning of the stability study (initial point) and after 8 weeks using HPLC-UV technique. The obtained values were normalized to the amounts of markers in the pure oregano oil.

Assay of sesame oil was measured via two major constituents - C16:0 and C18:0 -in the beginning of the stability study (initial point) and after 8 weeks using GC-FID analysis of methylated fatty acids. *Trans* methylation was performed upon acidic

catalysis (with MeOH/HCl) using C17:0 as an internal standard. The obtained values were normalized to the amounts of markers in the pure sesame oil.

No significant changes were observed in the both formulations: amount of oregano and sesame oils were similar before and after stability studies.

Water content was tested using Karl Fisher method. The amount of water was reduced on 42% after 8 weeks of storing in accelerating conditions in both formulations.

Isopropanol content was tested using GC method. The amount of IPA was reduced on 36 % after 8 weeks of storing in accelerating conditions in oregano formulation, but it was preserved in the sesame formulation.

Without being bound by theory, it appears that the containers were leaky and in order to reduce water or IPA loss, the containers may be more hermetically sealed.

Powders stored 8 weeks at 40°C showed good ability to form a stable emulsion similar to those of the initial powders. The stability measurements are summarized in Table 10 below:

Table 10: Stability Assays

	Time point	Oregano oil (%)		Sesame oil (%)		Water, %	IPA, %
		via C16:0	via C18:0	via Thymol	via Carvacrol		
Oregano powder SORG-121-38	Initial			28.4	29.0	7.05	11
	8 weeks			29.8	29.1	4.05	7
Sesame powder SES-121-39	Initial	32.2	32.7			6.65	5
	8 weeks	33.3	34.2			3.81	5

Solubilization and anti-bacterial activity with different surfactants

In order to create stable oil-in-water emulsion a surfactant (emulsifying agent) with HLB of 8-20 is required. Thus, in the following, two surfactants were tested Tween 80 having an HLB value of 15 and potassium salt of fatty acids extracted from palm, coconut, olive, castor and cottonseed plants (potassium salt oleate having an HLB value of 20).

It has been found that with the potassium salts of fatty acids the ratio of oil/surfactant required for obtaining a stable emulsion of oregano oils is 1:0.4 while with Tween 80, the required oil/surfactant ratio was 1:1.

The correlation between HLB values and solubilization capacity of each surfactant was found in the current case of Oregano oil, i.e. better solubiilztion with potassium salts of fatty acids. Isopropanol was used as process aid compound, which also provided additional stability for producing emulsions.

For anti-bacterial effect, several emulsifiers were tested with oregano oil and sesame oil, in water.

The tested emulsifiers included: Tween 20; Tween 80; Triton X 100; Lecithin; SDS ; Sodium Stearate and Potassium fatty acid. Each emulsifiers was tested at the following concentrations (in percentage) 1;5;10;15;20 for a mixture of water containing 25% oregano oil with 5% sesame oil.

The stability during the first 24hours (i.e. lack of phase separation) and anti-bacterial activity of each emulsion were determined. Anti bacterial activity was determined by measuring the inhibition zones of 20 µl emulsion towards the following plant pathogenic bacteria: Clavi bacter; Xanthomonas and Streptomyces spp. and by spraying the emulsions on pepper plants as test plants for phytotoxicity symptoms.

The results showed that potassium fatty acid was the most suitable material in creating stable emulsion at 10% concentration; the inhibition zone of the potassium salt of fatty acid was greater than the other tested emulsions and had no phytotoxicity on pepper plants. The data obtained (not shown) clearly demonstrated phytotoxicity on pepper plants when the emulsifiers were Tween 20 Tween 80 ,Triton X100 or SDS, as

the plants sprayed with the emulsions created therewith died, while being very vital when sprayed with the sodium stearate potassium fatty acid.

In a different set of experiments, the oregano oil and surfactant were absorbed on silica beads (with the air of isopropanol, as described above). Good anti-bacterial results were obtained when 0.5% of oregano-based powder was dispersed in water. This powder contained about 25% of oregano oil and 10% of potassium fatty acids emulsifier, namely, a concentration of 0.125% oregano oil in emulsion was active enough and needed only 0.05% of surfactant to be solubilized from the powder form.

Surprisingly, even a lower concentration of powder was sufficient to produce good crop protection in a greenhouse study. Only 0.2% of powder and consequently 0.05% of oregano oil was enough to disperse in water (to form an emulsion) and showed excellent and repeatable anti-bacterial effect with the plants being treated therewith remaining completely vital as compared to infected, but untreated control.

Preparation of Biocontrol Cocktails

Three biocontrol cocktails were prepared as follows:

The antagonistic gels containing a cocktail of the bacteria listed in Table 1 above, were each used at an amount 10^7-10^8 CPU/ml from each antagonist.

Each of the antagonistic gels were then used in two concentrations, the first as obtained, i.e. with no dilution (at an amount 10^7-10^8 CPU/ml from each antagonist) mixed with beads carrying 0.03% natural oil (equivalent to 1 gr beads in 1 liter water) referred to by the abbreviation *NB*, and a second formulation diluted x2 with water to form formulation NBx2 in which the concentration of the oil is 0.06%.

Biocontrol Experiments

In the bio-control assays, the following oil powder composition was employed:

Materials and compositions

Oils: Sesame oil 100% (SPAline Lot sicP1A11/01); Oregano oil 100% (SPAline Lot 0015181)

Surfactant: Zohar PT-50 (Zohar Dalia, Batch 05511PM1142);

SiO_2 particles: Sipernet 50S (20μm, Evonik Industries, lot 1462)

Alcohol: Isopropanol (IPA, Gadot).

Formulation:

Amount	Oregano oil	Sesame oil	Zohar PT-50	Sipernat 50S	IPA	Total
Kg	40	10	31	75	21	177
%	22.6	5.6	17.5	42.4	11.9	100

Preparation of powder

First, the oregano oil and sesame oil were mixed, to which IPA was added until a homogenous solution was obtained. To the solution, the surfactant was added by mixing until a non-viscous homogenous solution was obtained. The solution was sprayed on the SiO_2 powder and mixing was peformed using low sheer force equipment until all liquid was absorbed and continued for an additional period of 15-30min. Then the powder was grind to breakdown aggregates and sieved through mesh 1,000μm. For storage, the bag containing the powder was hermetically sealed and stored. Storage was for at least 2 years, at 15-30°C.

Biocontrol Example 1 *– In vitro Effects of Biocontrol Cocktail on various pathogens*

Tested pathogens: the following four antagonistic bacteria were tested (identified by the deposit accession number): CBS 133252, CBS 133254, CBS 134567 and CBS 133259.

Clavibacter, *Xanthomonas campestris pv. Vesicatoria* and *Yeast* - were taken from the private collection of the inventor.

Method:

Pathogen preparation - Each of the tested pathogens were separately plated on an yeast extract dextrose agar (YDC) and grown for 48h at 25±1°C, after which the surface of each plate was washed with sterile distill water. The pathogen was then collected into tubes containing sterile distilled water. For the testing, the two tested pathogens were combined and plated on a petri dish.

Antagonist preparation - The antagonist was prepared by spreading a sample (100µl) of one of the above listed test antagonists, on the surface of a Petri dish containing YDC media and growing the antagonist for 48 h at 25±1°C on Antagonistic media (the agar based gel disclosed herein). Then, with a cork cutter, pieces of the media including the antagonist cocktail were removed and placed on the surface of plates carrying the two pathogens for combined incubation.

After 24h of incubation at 25±1°C of the pathogen together with each antagonist, an inhibition zone around the pieces of the antagonists were creating. The inhibition zones were exhibited by a halo around colonies. **Figure 5A** shows the growth of pathogen bacteria without treatment with an antagonist, **Figure 5B** shows the effect of CBS 133252, **Figure 5C** shows the effect of CBS 133254, **Figures 5D** shows the effect of CBS 133567 and **Figure 5E** shows the effect of CBS 133259

Biocontrol Example 2 *- Effects of Biocontrol Cocktail on Tomato Wilt*

In vivo evaluations were conducted in three net greenhouses to assess the antibacterial activity of the three biocontrol cocktails on tomato *Daniella* plants. The tomato plants were grown with trellises.

As the positive control, the Copper Hydroxide (Kocide, Milchan Bros) was used at a concentration of 0.3% (the *Copper control*)

Non-treated plants were used as *Negative Control.*

All plants were watered with either 50ml of a biocontrol cocktail, the *Copper Control* or with none (*Negative Control*) for three consecutive days after planting, followed by spraying the plants every 7 days. For spraying the essential oil powder (in the respective amount) and the antagonistic gel were mixed directly in the container of a backpack motor sprayer and the entire plant, including leaves and stems, were sprayed.

After 2 weeks, the second plant in each growing row in the greenhouse was infected with CBM inoculum comprising two CBM strains isolated from infected plants grown in the Besor area of Israel. The inoculum included 2 strains of CBM, strain number 32 (strain designation 189/1-1) and strain number 42 (stain designation 189/6-1) [Frida Kleitman et al. Characterization of Clavibacter Michiganensis Subsp. Michiganensis population in Israel *Eur. J. Plant Pathol* (2008) 121:463-475]

Inoculation was performed by creating a cut in a leaf of the plant, near the stem, and dipping the leaf in the CBM inoculum.

During growing, the plants were under surveillance and plants that were infected were observed.

Statistical test

The results were analyzed using the Student's T-test.

Results

The above field trial was repeated six times, and the results of the six repetitions are summarized in Table 11A, providing mortality rate of the tomato plant, and Table 11B providing infection level by CBM, as tested using Agria kit for CBM (Cmm ImmunoStrip Test catalog no. STX 44001 Agdia Incorporated 30380 County Road 6 Eelkhart, Indiana 46514 USA)

Table 11A: Survival rate of tomato plants

Treatment	Mortality Rate
Negative Control	18%
Copper Control	8%
NB	2%
NBx2	1%

Table 11B: Percent of CBM infection in tomato plants

Treatment	CBM infection
Negative Control	83.5±11
Copper Control	58.8±15
NB	50.5±14
NBx2	25.8±12

Table 12 provides statistical comparison between the results of Table 11A.

Table 12: Statistical comparison in mortality rate between treatment groups

Compared Groups	P<0.05
No treatment vs. NBx2	Yes
No treatment vs. NB	Yes
No treatment vs. Copper formulation	No

Copper formulation vs. NB	Yes
NB vs. NBx2	Yes

In the Table "YES" indicates that there is a statistical significant difference; while "NO" indicating that there is no statistical significant difference Table 9 shows that when comparing NB to any other treatment group, the positive effect is statistically significant. When comparing Copper Control to Negative Control, the positive effect of the Copper Hydroxide treatment is insignificant. Doubling the dose (concentration of NB vs. NBx2) show also a statistically significant increased effect.

In addition, **Table 13** and **Figure 6** show the effect of treatment on plant death.

Table 13: mortality

Treatment	**% Mortality**
Negative Control	18%
Copper Control	8%
NB	2%
NBx2	1%

Specifically, Table 13 shows that non-treatment, *Negative Control* Group (**control**) and the *Copper Group* (**Cu**) have higher percentage of mortality as compared to the treatment groups **NB** and **NBx2**. In each group, the side row was more sensitive than the centered row (not shown). The difference between the side rows and centered rows may be attributed to the location of the side rows in the greenhouse, being affected by external parameters.

The results also show synergism. Table 14 shows that at a wide temperature range the combination of the natural oils (sesame oil beads and oregano oil beads) with the antagonists increase the survival of the plants to a much greater extent than the effect of each component alone [(A+B)/C>1].

Specifically, the results show that the combined effect of antagonists only (A) and oils only (B), divided by the Control (C) is greater than 1, i.e. synergism.

Table 14 - Synergism

Temp. °C	ONLY Antagonist ("A")	% of the Control ("A")	Antagonist and Oils ("C")	% of the Control ("C")	Only Oils ("B")	% of Control ("B")	Untreated (Control)	% Untreated (Control)	A+B/C
18	3.33*	60	0	0	3.3	66	5	100	Yes
20	14.2	65.4	0	0	15.8	72.8	21.7	100	Yes
25	35.8	44.8	4.2	5.3	64.2	80.25	80	100	Yes
28	44.2	45	5	5.1	66.7	67.9	98.3	100	Yes
32	39.2	53.5	0	0	54.2	73.9	73.3	100	Yes

Biocontrol Example 2 *- Effects of Biocontrol Cocktail on Pepper*

For the prevention of pepper infection by *xanthomonas vesicatoria* (*XV*), the same biocontrol cocktail used in Example 1 is used, with the same treatment methodology. The pathogen was originally isolated by the inventor from diseased pepper plants and pepper seedlings .The isolates of *xanthomonas vesicatoria* (XV), are in the collection of G. Kritzman.

Biocontrol Example 3 *- Effects of Biocontrol Cocktail on Potato*

For the prevention of potato infection by Streptomyces spp., the causing agent of *Streptomyces scabies.*, a biocontrol cocktail comprising three antagonists isolates obtained according to the procedure provided above with some of the previous list, were used. The three antagonists are provided in Table 15 below.

Table 15: Deposited Antagonists for potato treatment

Antagonist	Accession No.	Name
Pseudomonas mediterranea AN1	CBS134566	BN13-01
Pseudomonas chlororahis AN10	CBS134567	BN13-02
Pseudomonas species AN21	CBS134568	BN13-03

The biocontrol cocktails are prepared as described above, at three concentrations 1% (no dilution); 0.5% (1:1 dilution) and 0.025% (1:4 dilution) active ingredient (of the oil formulation) with a constant concentration of the bacterial antagonists at a final concentration in the tank mix of 10^3 CFU/ml.

Before use, the antagonists are mixed in a mixing tank with one of the dry formulations contained among others ingredient Organo oil dry powder and Sesame oil dry powder (80:20 ratio as described above). The potato seeds tubers are entered into a spraying cell by rolling on a conveyer. In the cell the tubers pass through a cloud of drops (about 80u in diameter each). After each tuber is rolled about 8 times around its axis the treated tubers are collected in a container as dry treated tubers. The treated tubers now are coated with a fine layer of the complete formulation are ready to be planted not before 72h after the treatment.

In the case of the potato scab, the efficiency of the treatment is tested not by artificially inoculating tubers but by choosing potato seeds lots naturally high infested with many typical symptoms on each seed tuber. It could be of the common scab type or of the deep pitted scab symptoms. The seeds are examined in two procedures:

a) Samples of 60 tubers in replications are taken to the laboratory. In the laboratory all the tubers are peeled by a commercial potato peeler which is operated without watering the tubers during the peeling procedure. A large sample of the potato peels are collected. Ten grams are taken to a Stomacher bag containing 90 ml of 0.1% (w/v) water agar supplemented with 0.05% (w/v) ascorbic acid. This suspension is mixed for 2h on a rotary shaker and then for 1min in stomacher. After this homogenization a tenfold dilutions is done and aliquots of 100µl were inoculated on the surface of Petri dishes containing semi selective media for isolation of *Streptomyces*. After 5 incubation days at 28°C the CFU/gr peel is calculated for the treated potato seeds and in comparison with the untreated control.

b) The treated and the untreated potato seeds are planted in a trial field with at least a 4 replication each. In such experiment the growth and yield parameters as well as the scab control on the daughter tubers at the harvest time are evaluated.

The population of Streptomyces per gram of peel was calculated and the results are in Table 16:

Table 16: treatment of Potato scab

Oil component dilution	Streptomyces spp. CFU/gr peel
No dilution (1%w/v)	40
1:1	$3X10^2$
1:2	$1X10^4$
Control (untreated)	$1X10^7$

Further biocontrol Experiments

For testing treatment or prevention of other pathogenic infections, the following protocols may be used.

I. *Protocol for In vitro antagonistic activity*

Transfer each antagonistic bacteria in a separate Petri Dish (9cm) using a bacteriological transfer loop to form a single antagonist line along the diameter of the dish, and incubate the dish for 48h at 28°C.

Transfer a tested pathogen along a line perpendicular to the antagonistic line without touching the walls of the dish.

Incubate the dishes for a further period of 5 days at a temperature between 25°C and 28°C.

Measure the zone at which the antagonist inhibited growth of the pathogen (the "inhibitory zone"). The size of the inhibitory zone being indicative of the level of antagonistic activity of the bacteria.

II. *Affect of oil formulation without antagonist.*

Create a stock solution of the oil formulation (described above, without the antagonistic bacteria) by dissolving the oil powder in the medium that is suitable for culturing the tested pathogen bacteria (culture medium which includes agar that is liquid at 37°C) at a final concentration of oil of 1% w/v. From the stock solution, create a series double diluted concentrations with the culture medium (dilutions of 1:1, 1:2, 1:4, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512) of the oil formulation. Introduce into Petri dishes and allow the oil containing medium to harden.

Prepare pathogen suspension by dilution with saline colonies after 48h of cultivation and calibrate pathogen concentration in the suspension to reach an absorbance of 0.6AU at 480nm.

Dilute the pathogen bacteria up to a concentration of 10^{-8}/ml and plate 100µl on the hardened oil containing culture media.

After 5 days of incubation at 25°C-28°C of each plate at the different oil concentrations, determine the number of colonies.

III. *Bactericide or Bactriostat Activity*

Prepare antagonist formulations with different amounts of oil diluted as described above, albeit with sterilized water and each diluted oil formulation with the antagonists at constant concentration of 10^3 antagonists per ml.

An amount of 9ml antagonists formulations with the different oil dilutions was incubated in a tube with an aliquot of 1ml of a tested pathogen suspension in a concentration of 10^8CFU/ml. The pathogen suspension was prepared from cultures after 72h cultivation.

After incubation for 3h, the content of each tube is filtered on a 0.45mm mesh membrane with sterilized water after which the pathogen is washed off from the membrane using sterilized water and vortexing.

The washed off bacteria are then tenfold diluted in a series up to a concentration of 10^{-8}CFU/ml. From each dilution 100µl aliquots are platted on a Petri dish with a suitable culture media and cultured. The formation of colonies is indicative of bacteriostat activity of the formulations while absence of colonies is indicative of bacteriocidal activity.

IV. *Plant testing:*

The plant testing is conducted in a pot or in a green-house. Plants with at least 5 leaves are used for testing.

In a pot:

On day 1, the plant is sprayed with the tested oil/antagonist formulation at various concentrations and after 12h the plant is sprayed with the pathogen solution being at a concentration of about 10^6CFU/ml.

Two hours after infection, the plants are once again sprayed with the same oil/antagonist formulation again and is continuously sprayed every 6 days for a period of 6 weeks. After 6 weeks, the disease incidence is estimated and recorded.

In an open test field or greenhouse:

The plants are grown using the conventional method of their planting and growing in the field of greenhouse.

After planting, the plants are irrigated with an amount of about 100ml oil/antagonist formulation /plant and then each plant is sprayed with the same formulation, with continuous spraying every 5 -6 days.

The disease incidence is estimated and recorded.

Toxicology tests

Various toxicological tests were performed using the formulation including a cocktail of all deposited antagonists (Table 1) each at a concentration of 10^9 as follows:

(i) Acute oral toxicity in the rat:

Protocol:

Female Sprague-Dawley™ (SD™) rats were divided into two groups (*n=3*), each receiving a single dose of 2000 mg/kg of the tested formulation (Dosage Volume of 1.97 ml/kg, oral gavage (PO) administration), at an interval of administration between the two groups of 24 hours.

Results:

- No mortality occurred in any of the tested rats throughout the 14 days observation period.
- No noticeable clinical signs in reaction to dosing were evident in any of the animals during the immediate post-dosing time or throughout the entire 14-day observation period.
- Body weight gain at the end of the 14-day study period of all animals was found to be within range of normally expected values.
- No gross pathological findings were evident in any of the animals at the time of their scheduled necropsy.
- Based on the lack of adverse reactions following a single oral administration to the rats at the limit test dose level of 2000 mg/kg, the tested formulation may be regarded as not representing an acute toxic risk by this route of administration and the acute oral median Lethal Dose (LD50) is greater than 2000 mg/kg.

(ii) Acute dermal toxicity

Protocol:

A single dose level of 2000 mg/kg of the tested formulation was topically applied for 24-hr exposure duration to a 5 female and 5 male Sprague-Dawley (SD™) rats. The rats were observed for a total of 14 days.

Results:

- No mortality occurred in any of the rats throughout the entire 14-day study period.
- No noticeable clinical signs in reaction to dosing were evident in any of the rats throughout the entire 14-day observation period.
- Body weight gain of all rats at the end of the 14-day study period was found to be within the range of normally expected values.
- No gross pathological findings were evident in any of the rats at the time of their scheduled necropsy
- Based on the lack of adverse reactions following a single dermal application to the rats the tested formulation was as not representing an acute toxic risk by this route of administration and the estimated acute dermal median lethal dose (LD50) was determined to be greater than 2000 mg/kg.

Acute dermal irritation/corrosion in rabbits

Protocol:

The potential skin irritation effects of the tested formulation were assessed following a single dermal application to a group of 3 male NZW rabbits, according to the testing procedure recommended by the OECD Guideline for the Testing of Chemicals, Section 4, No. 404, "Acute Dermal Irritation/Corrosion" adopted 24th April 2002.

Specifically, all rabbits were topically applied with a single sample of absorbent gauze measured 2 × 3 cm each moistened with 0.5 ml of the tested formulation for a 4 hour exposure duration. Each gauze patch was held in contact with the skin with non-

irritant tape and a suitable semi-occlusive dressing (TUBIGRIP stockinet) to retain the gauze patches and the tested formulation throughout the exposure period.

Dermal reactions were scored and recorded at the standard time points of 1, 24, 48 and 72 hours after patch removal.

Results:

- No erythema or edema was noted throughout the 72-hour study period in any of the rabbits.
- No other dermal reactions were noted in any of the rabbits throughout the study period.
- No noticeable clinical signs in reaction to treatment were evident in any of the rabbits throughout the entire study period.
- No abnormal changes in body weight were noted in any of the rabbits throughout the entire study period.
- Consideration of the calculated Primary Irritation Index (PII) was 0, led to the conclusion that irritation response is categorized as negligible.

(iii) Skin sensitization (local lymph node assay)

Protocol:

The potential of the tested formulation to cause skin sensitization was assessed on the basis of the testing procedures by the OECD Guideline for the Testing of Chemicals, Section 4, No. 429 "Skin Sensitization: Local Lymph Node Assay", adopted 22nd July 2010.

Specifically, three dilutions of the tested formulation were prepared in Physiological Saline. Pluronic ® L92 (1% v/v) was added to each dosing solutions prior to application.

In order to ensure reproducibility and sensitivity of the test procedure, a well-known weak to moderate contact allergen, 25% HCA, was included in this study, diluted with the Negative Control used.

Three groups of BALB/c female mice (n=5) were subjected to application of the tested formulation (one dilution per group) once daily for three consecutive days, at a

dose volume of 25 µl applied on the dorsum of each outer ear. Additional two equally sized groups were subjected to application of either the Negative Control (Physiological Saline) or the Positive Control (25% HCA), under identical conditions.

Five days after the first topical application, all mice were injected with 3H-Methyl Thymidine by intravenous (IV) injection. Approximately 5 hours later, all mice were euthanized and the auricular lymph nodes were excised. A single cell suspension of both left and right lymph nodes cells from individual animals was prepared.

The incorporation of 3H-Methyl Thymidine was measured by a β-Counter and expressed as Disintegration Per Minute (DPM) /animal.

The Stimulation Index (SI) was calculated for the groups treated with the tested formulation dilutions and the Positive Control. The SI values for all dilutions were lower than 3 and the SI values for the Positive Control was higher than 3.

Results:

- No mortality occurred in any of the mice in all groups throughout the 5-day study period.
- No noticeable clinical signs in reaction to treatment were observed in any of the tested formulation or Negative Control treated mice throughout the 5-day study period. All Positive Control treated mice displayed redness at the ears.
- All mice showed an increase in body weights at the end of the 5-day study period.
- Under the conditions of the study and according to the calculated Stimulation Index values, it was concluded that the tested formulation did not cause reactions associated with skin sensitization.

(iv) Acute eye irritation/corrosion in rabbits

Protocol:

The potential eye irritation/corrosion effects of the tested formulation were assessed following a single eye instillation to a group of 3 male NZW rabbits, according to the testing procedure recommended by the OECD Guideline for the Testing of

Chemicals, Section 4, No. 405, "Acute Eye Irritation/Corrosion" adopted October 02, 2012.

Initially, single dose of 0.1 ml the tested formulation was applied to the right eye of one rabbit (Initial Test) and subsequently to two additional rabbits (Confirmatory Test). The left eye of each rabbit was not treated and served as control.

Ocular reactions were scored and recorded at the standard time points of 1, 24, 48 and 72 hours following application.

Results:

- No ocular reaction was noted throughout the 72-hour study period in any of the rabbits.
- No noticeable clinical signs in reaction to treatment were evident in any of the rabbits throughout the entire study period.
- No abnormal changes in body weight were noted in any of the rabbits throughout the entire study period.
- Under the conditions of this study, it was concluded that the tested formulation did not cause reactions associated with eye irritation/ corrosion.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as, an acknowledgement or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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The claims defining the invention are as follows:

1. A package comprising:

i) a first component comprising particulate matter comprising at least one natural oil;

ii) at least one second component comprising an antagonist of a microbial pathogen;

wherein said first component and said second component are separate in the package and are mixed together prior to use.

2. The package of Claim 1, wherein said first component comprises the particulate matter in an essentially dry form.

3. The package of Claim 1, wherein said particulate matter comprises silica (SiO_2).

4. The package of any one of Claims 1 to 3, wherein said particulate matter exhibit at least one of a size distribution in the range of 10-25μm, a surface area in the range of 400-500m^2 N_2/g and oil capacity in the range of 300-350 DBP/100 gram particulate.

5. The package of any one of Claims 1 to 4, wherein said natural oil comprises at least one essential oil.

6. The package of any one of Claims 1 to 5, wherein said natural oil is a plant oil and said plant oil is a carbon-rich nutrient oil.

7. The package of any one of Claims 1 to 6, wherein the particulate matter comprise between 20% to 50% w/w natural oil.

8. The package of any one of Claims 1 to 7, wherein said particulate matter comprises at least one surfactant.

9. The package of Claim 8, wherein said particulate matter comprises between 5 to 10% w/w of said surfactant.

10. The package of any one of Claims 1 to 9, wherein said first component comprises water content of no more than 10% (w/w).

11. The package of any one of Claims 1 to 10, wherein said second component is in a form of a gel.

12. The package of any one of Claims 1 to 11, wherein said at least one second component comprises an antagonist at a concentration of between 500CFU/ml to 5,000CFU/ml.

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13. The package of any one of Claims 1 to 12, wherein said at least one antagonist is a bacteria selected from the group consisting of *Pseudomonas species (Accession No.* CBS133252), *Pseudomonas alcaliphila* (*Accession No.* CBS133254), *Bacillus subtilis* (*Accession No.* CBS133255), *Pseudomonas cedrina* (*Accession No.* CBS133256), *Pseudomonas species* (*Accession No.* CBS133257), *Pseudomonas species* (*Accession No.*CBS133258), *Pseudomonas species (Accession No.* CBS134568*), Pseudomonas spanius* (*Accession No.* CBS133259), *Pseudomonas mediterranea (Accession No.* CBS134566), *Pseudomonas chlororahis* (*Accession No.* CBS134567).

14. The package of any one of Claims 1 to 13, comprising instructions for use of said first component and said at least one second component to form an emulsion.

15. The package of any one of Claims 1 to 14, for use in inhibiting pathogen infection in a plant.

16. A method for providing an anti-bacterial agent, the method comprises:

(a) providing a package as claimed in any one of claims 1 to 15; and

(b) mixing the first component comprising the particulate matter carrying at least one natural oil with at least one said second component comprising at least one antagonist of a microbial pathogen, to form an emulsion with anti-bacterial activity.

17. The method of Claim 16, wherein said mixing of the first component and the at least one second component provides an emulsion with a droplet size in the range of 3 to 10μm.

18. A method of treating or preventing a pathogen infection in a plant, the method comprises mixing a first component comprising particulate matter comprising at least one natural oil and at least one second component comprising an antagonist of a microbial pathogen to form an emulsion and applying to said plant an amount of the emulsion in two or more doses, with a time interval between applications of between hours to days.

19. The method of Claim 18, wherein said first component and said second component are as defined in any one of Claims 1 to 15 and wherein said emulsion is obtained by mixing a first component comprising particulate matter comprising the at least one natural oil; and at least one second component comprising the at least one antagonist of a microbial pathogen.

20. The method of Claim 19, wherein said mixture comprises at least antagonist of a CBM,

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being selected from the group consisting of Pseudomonas species (Accession No. CBS133252), Pseudomonas alcaliphila (Accession No. CBS133254), Bacillus subtilis (Accession No. CBS133255), Pseudomonas cedrina (Accession No. CBS133256), Pseudomonas species (Accession No. CBS133257), Pseudomonas species (Accession No.CBS133258), Pseudomonas species (Accession No. CBS134568), Pseudomonas spanius (Accession No. CBS133259), Pseudomonas mediterranea (Accession No. CBS134566), Pseudomonas chlororahis (Accession No. CBS134567).

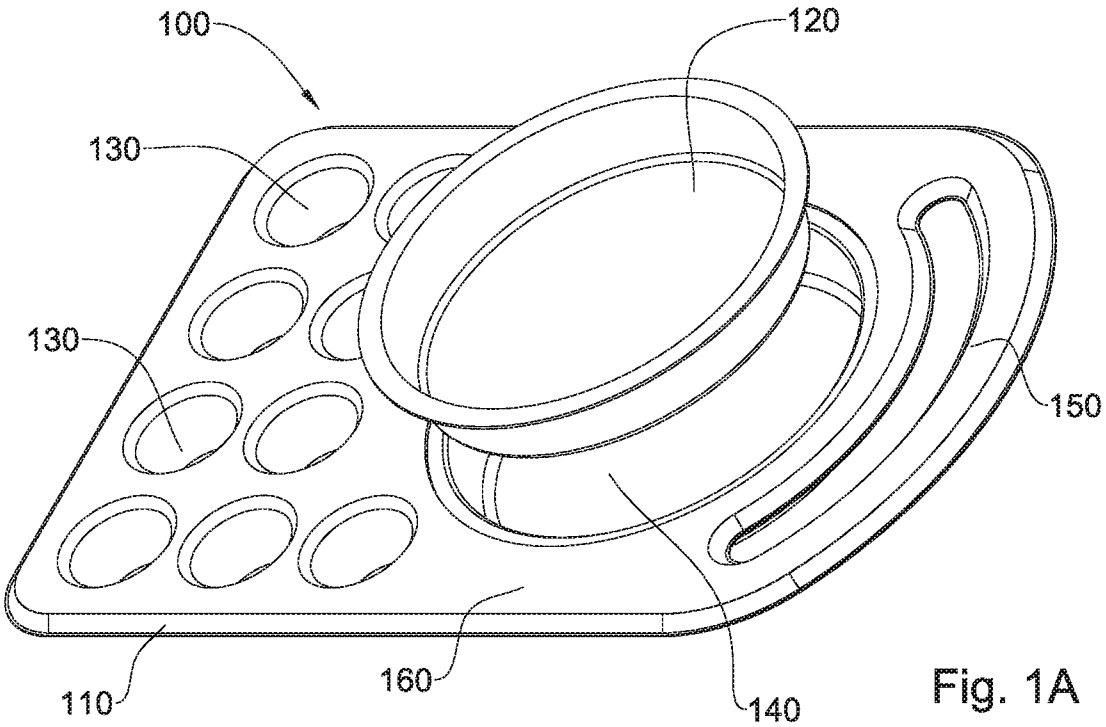

Fig. 1A

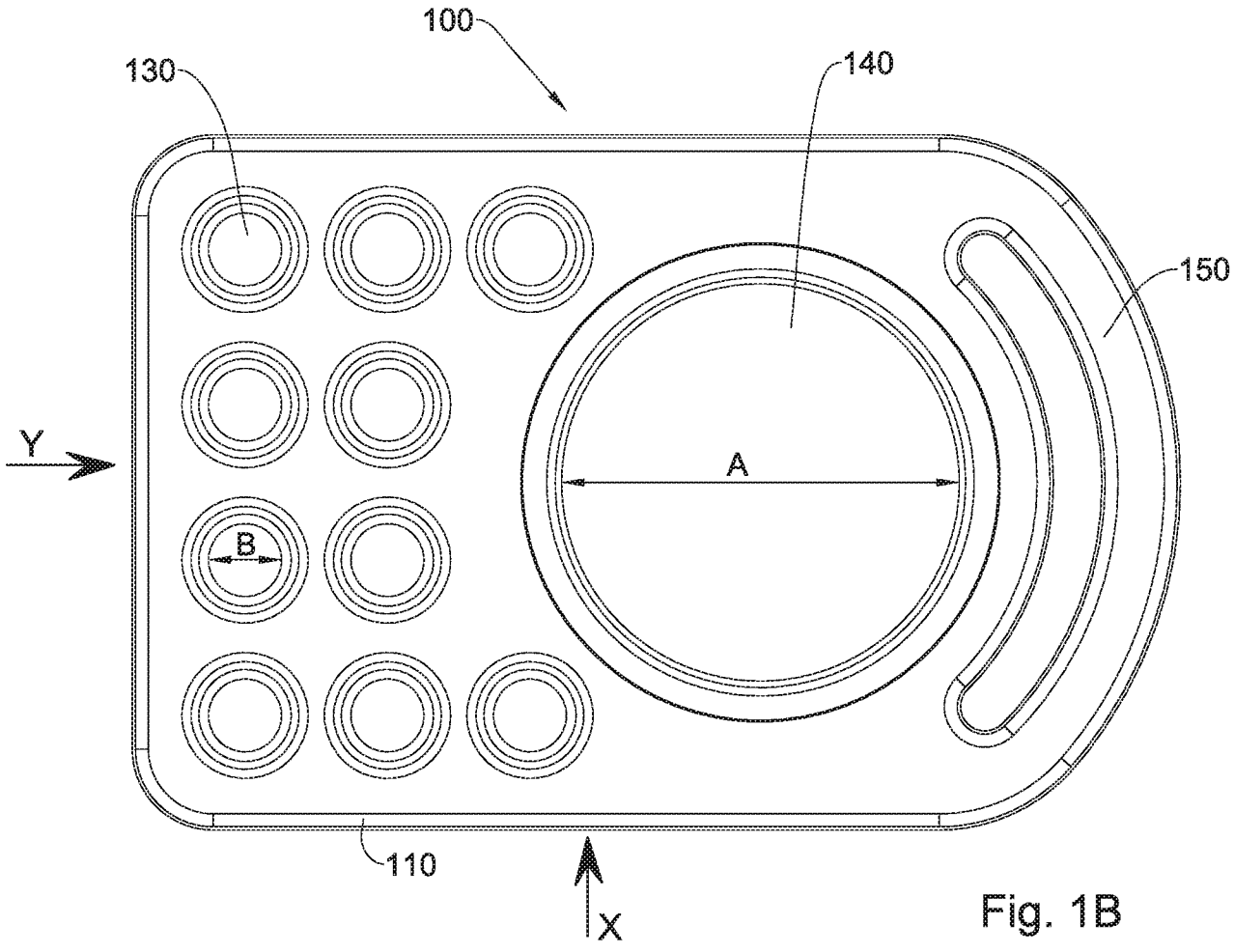

Fig. 1B

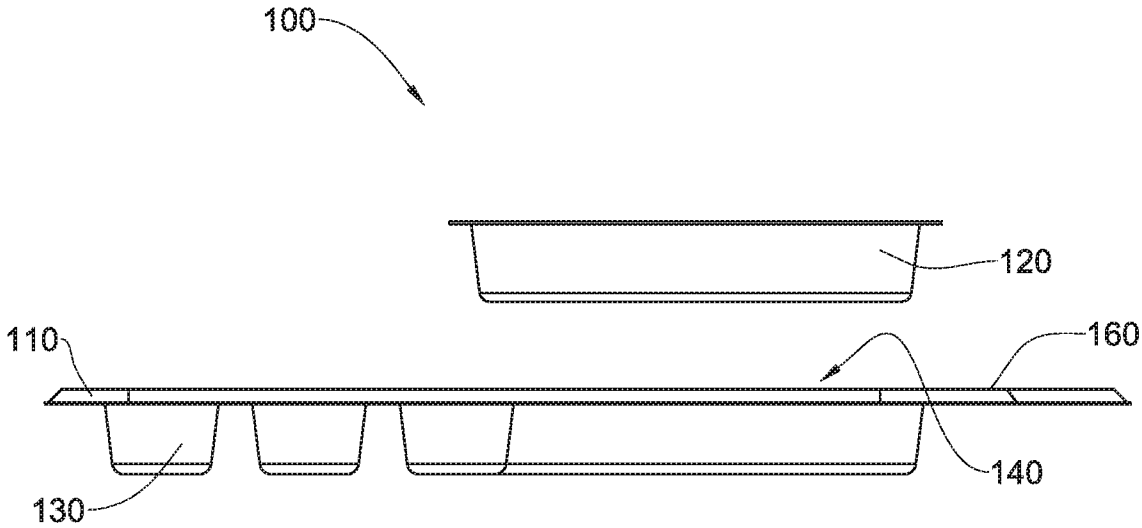

Fig. 1C

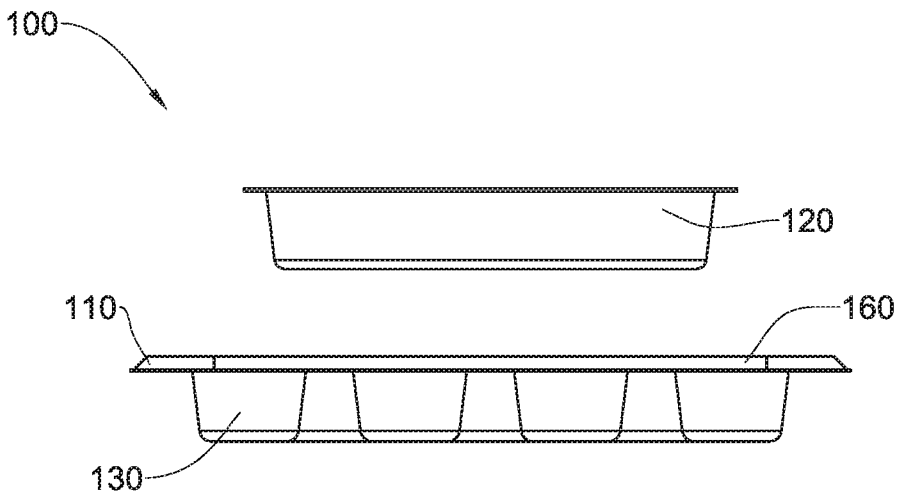

Fig. 1D

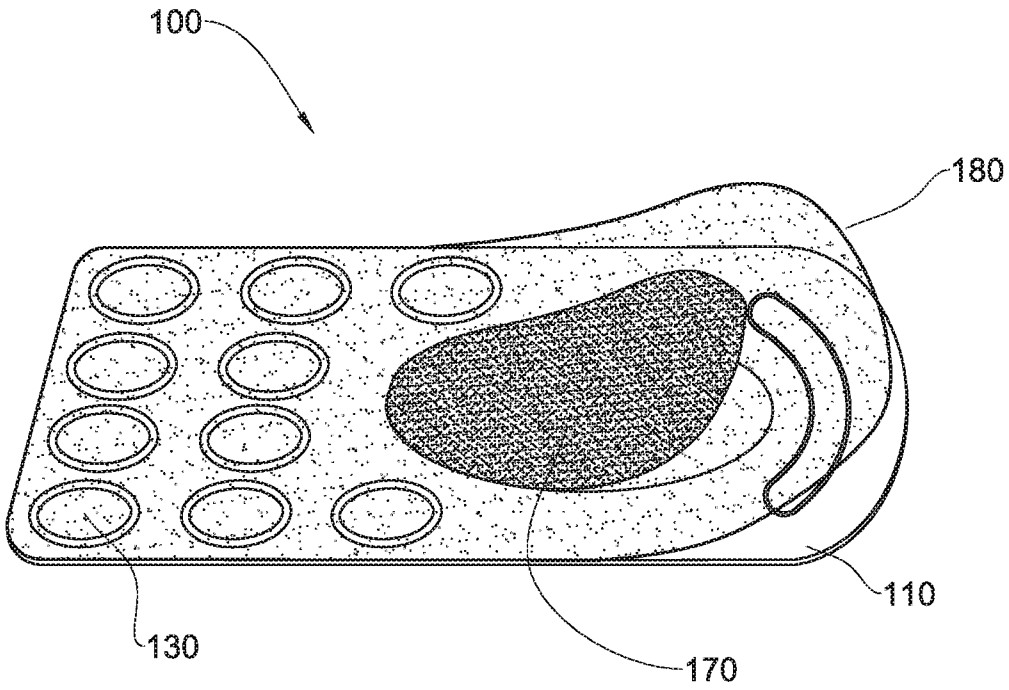

Fig. 2A

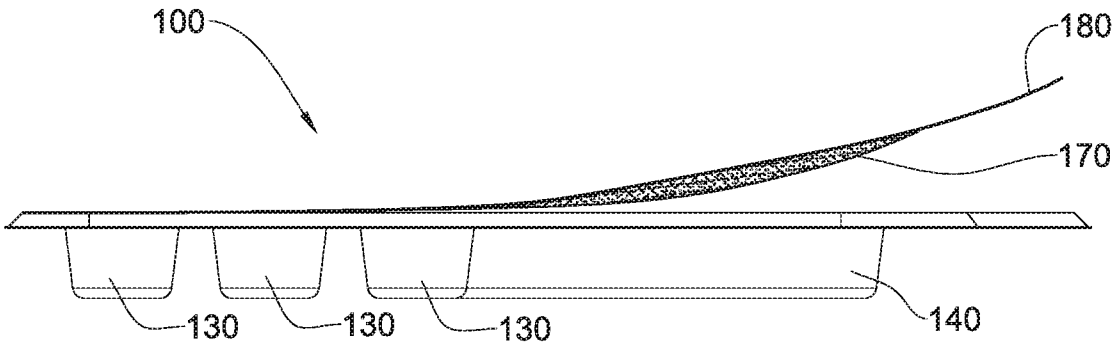

Fig. 2B

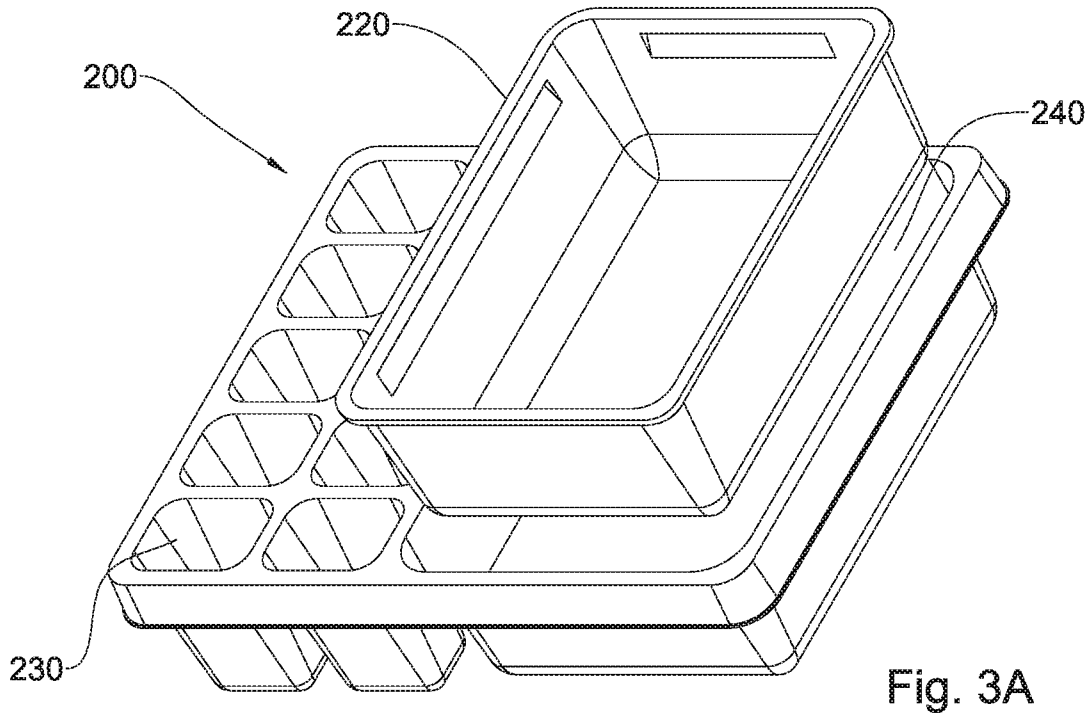

Fig. 3A

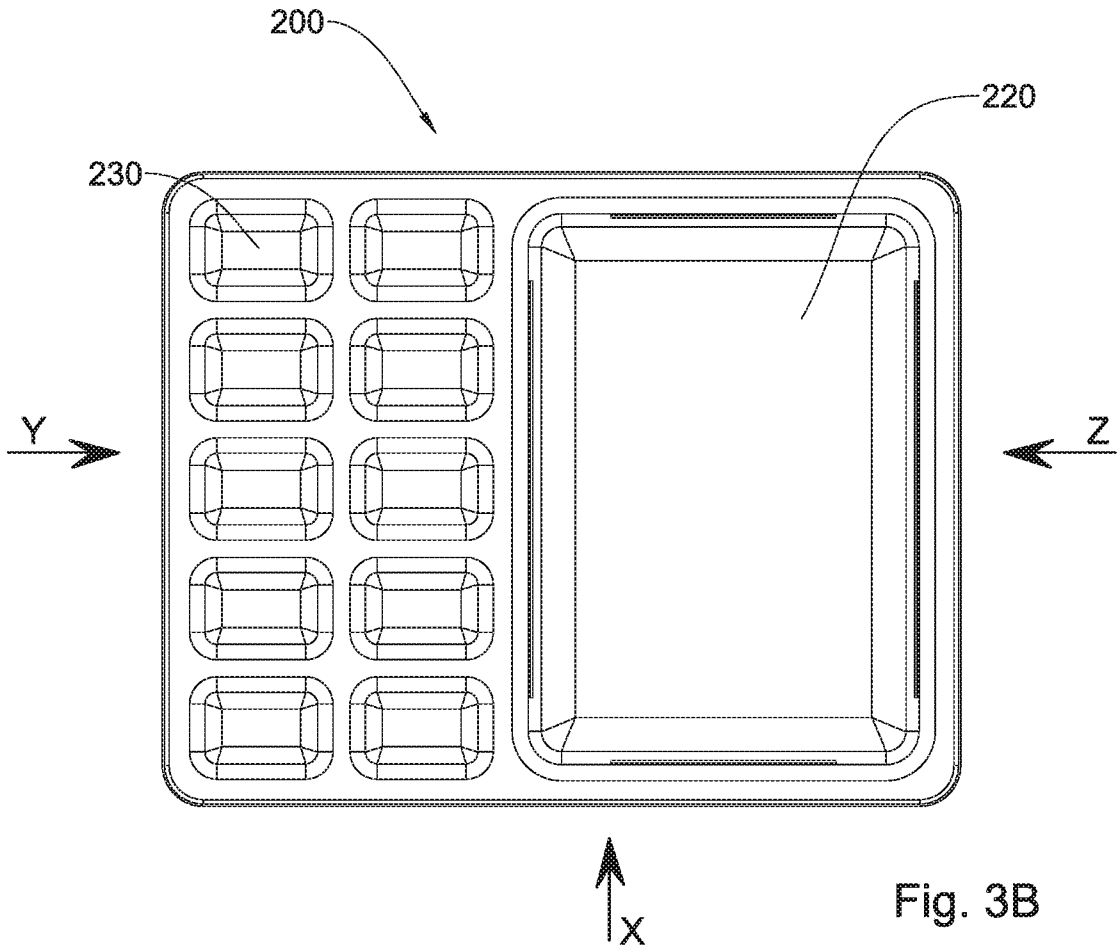

Fig. 3B

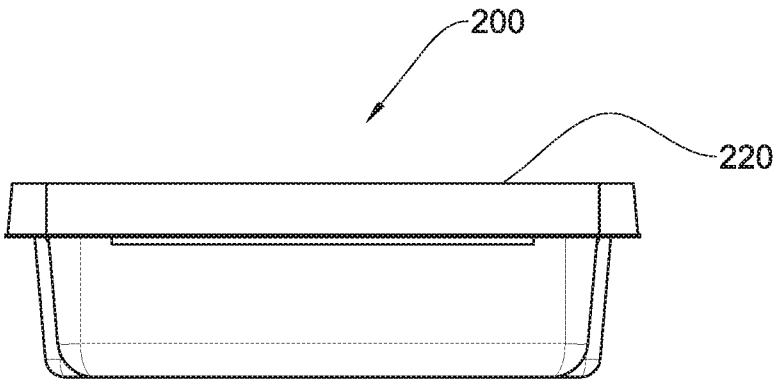

Fig. 3C

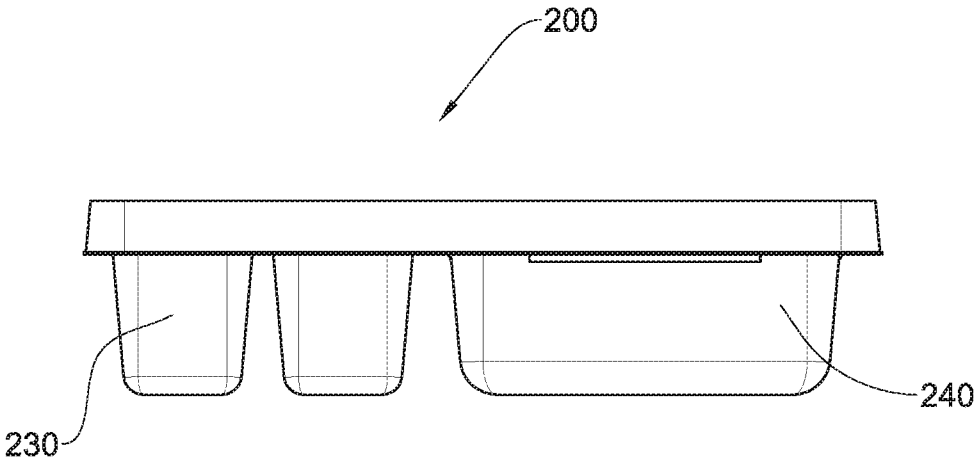

Fig. 3D

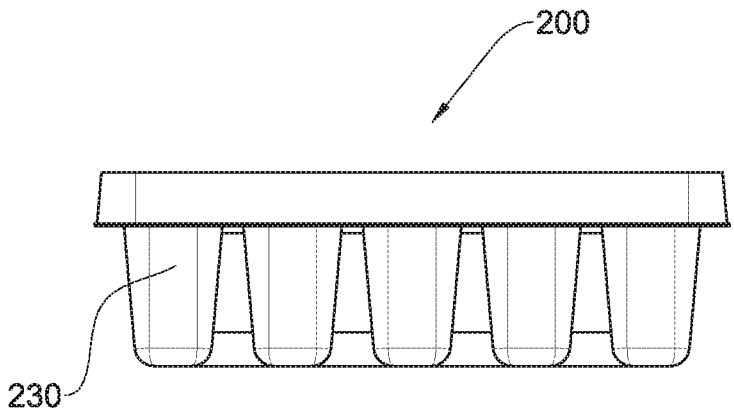

Fig. 3E

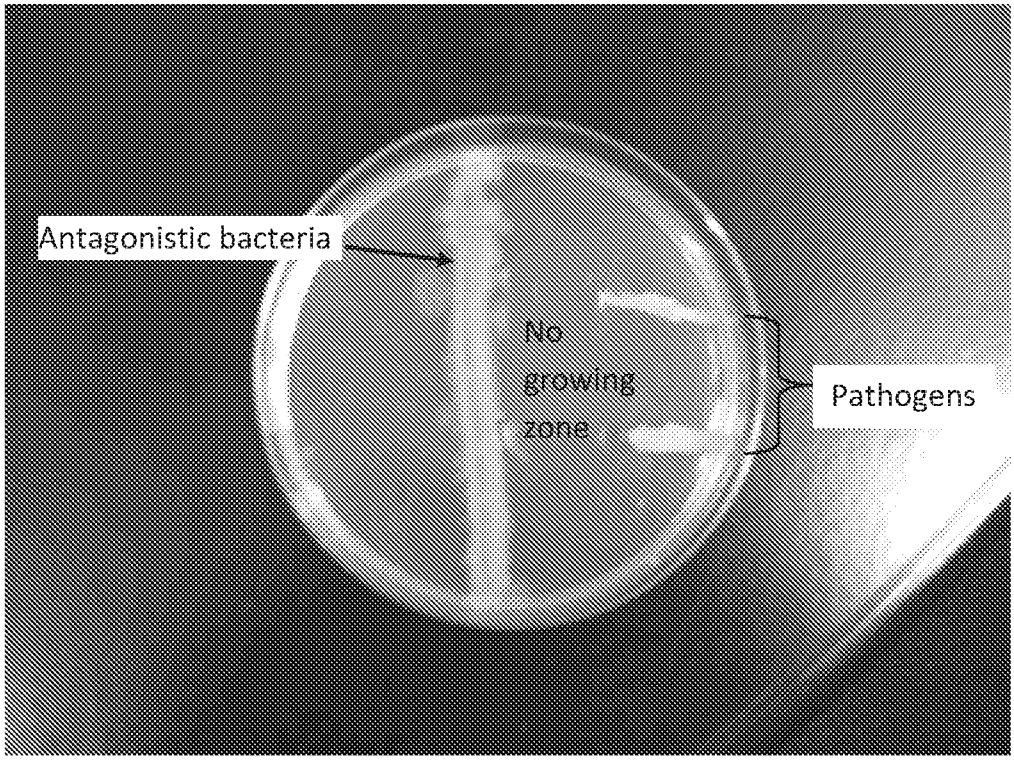

Fig. 4A

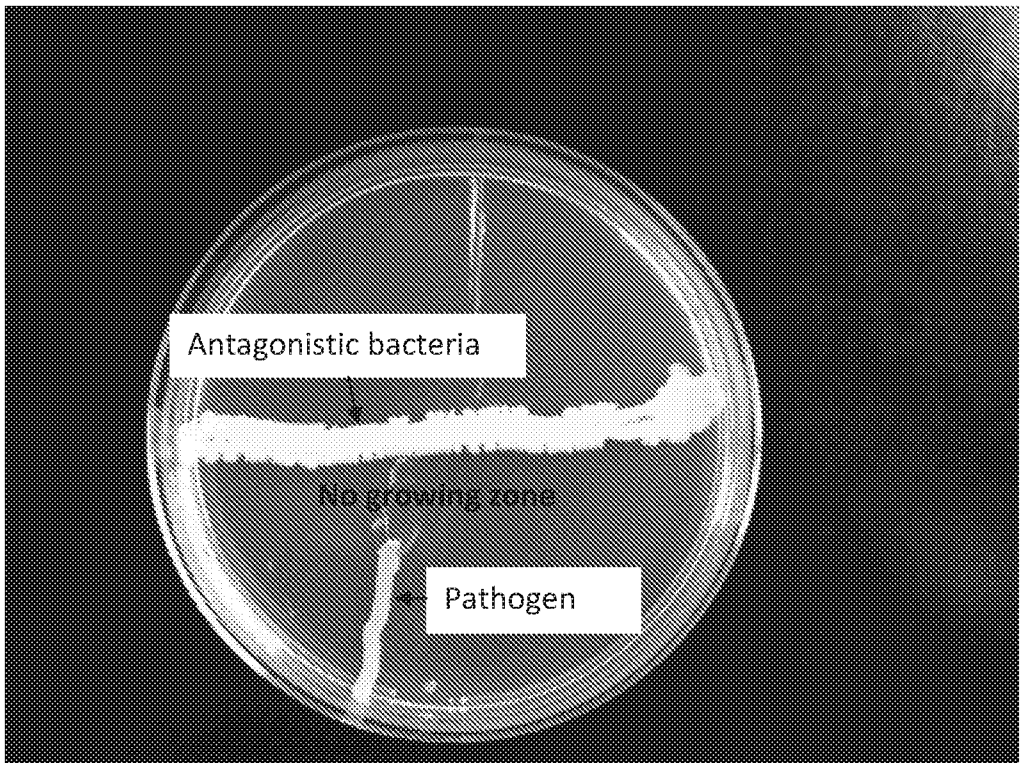
Antagonistic bacteria
No growing zone
Pathogen

Fig. 4B

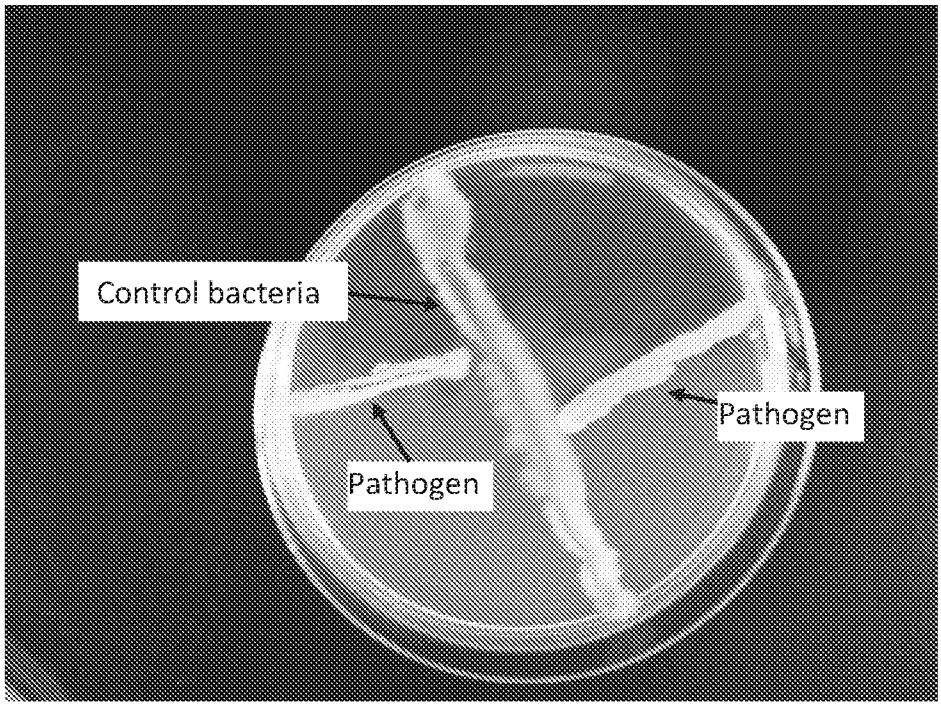
Control bacteria
Pathogen
Pathogen

Fig. 4C

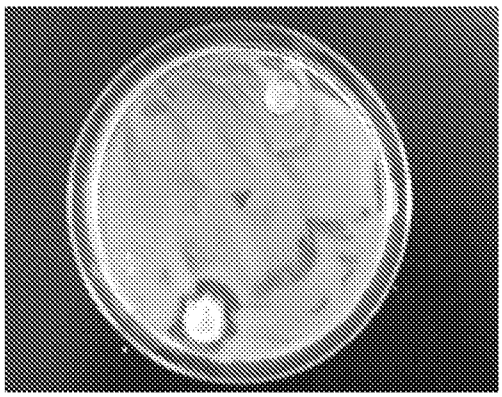

Fig. 5A

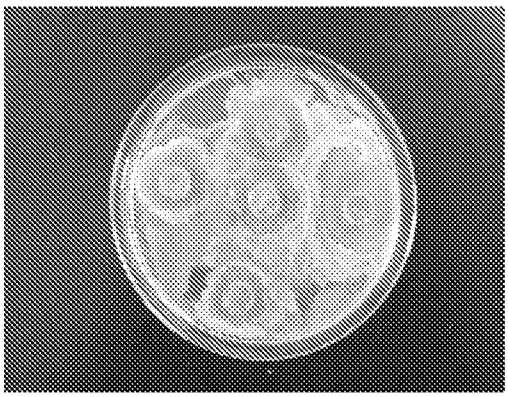

Fig. 5B

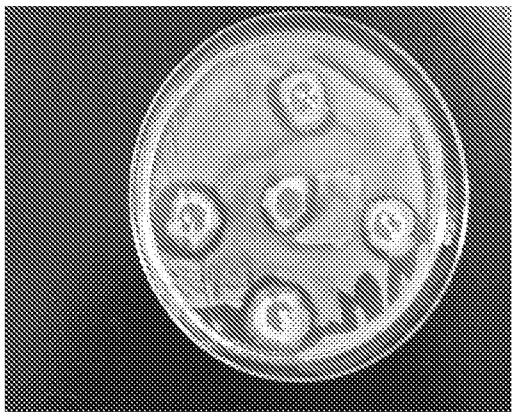

Fig. 5C

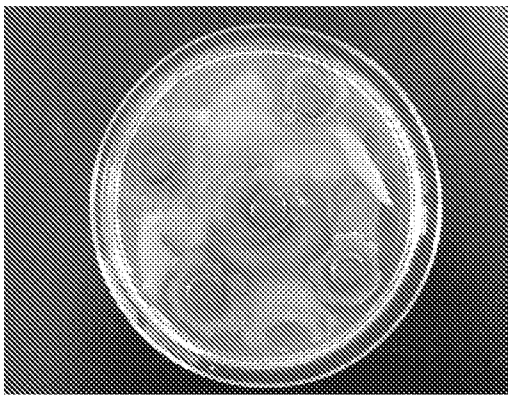

Fig. 5D

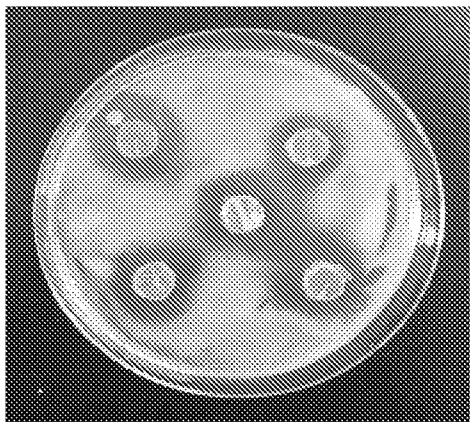

Fig. 5E

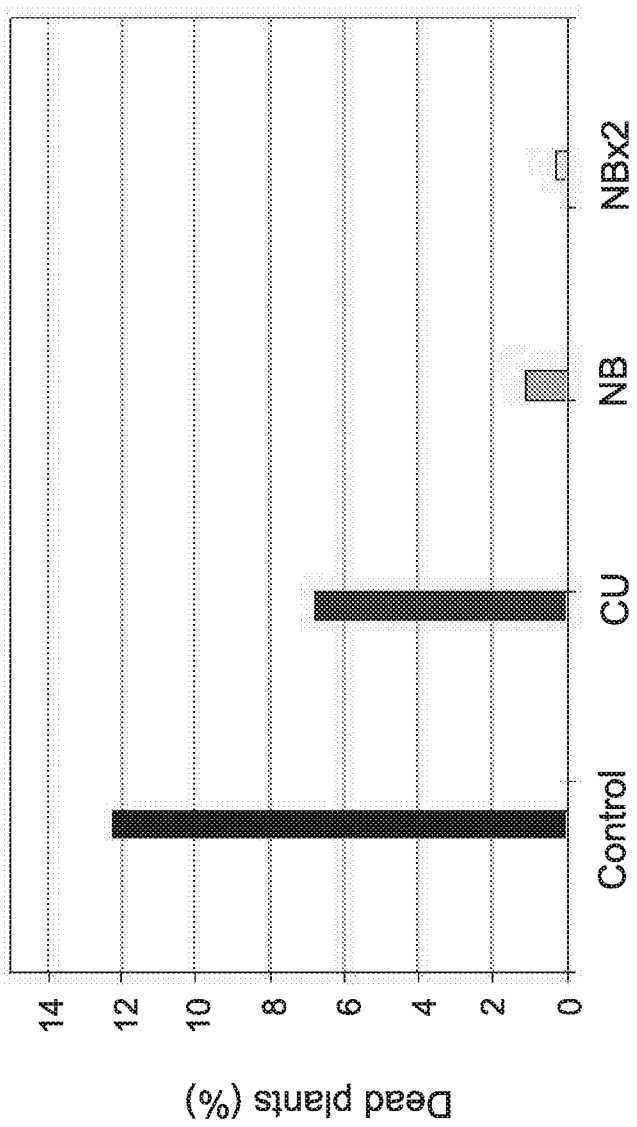

Fig. 6