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(54) Title: BENEFICIAL COMBINATIONS WITH RECOMBINANT *BACILLUS* CELLS EXPRESSING A SERINE PROTEASE

(57) Abstract: The present invention relates to a composition comprising (a) a recombinant *Bacillus cereus* family member that expresses a fusion protein comprising (i) an enzyme having serine protease activity comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6 and (ii) a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member or exosporium fragments derived from such recombinant *Bacillus cereus* family member; and (b) at least one insecticide or plant growth promoter disclosed herein, in a synergistically effective amount. Furthermore, the present invention relates to the use of this composition as well as a method for nematode control, enhancing plant growth, promoting plant health, and/or reducing overall damage of plants and plant parts.

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BENEFICIAL COMBINATIONS WITH RECOMBINANT *BACILLUS* CELLS EXPRESSING A SERINE PROTEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/281,648, filed November 20, 2021, the contents of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] In crop protection, there is a continuous need for applications that improve the health and/or the growth of plants. Healthier plants generally result in higher yields and/or better quality of a plant or its products.

[0003] In order to promote plant health, fertilizers are employed worldwide, based on both inorganic and organic substances. A fertilizer may be a single substance or a composition, and is used to provide nutrients to plants. A major breakthrough in the application of fertilizers was the development of nitrogen-based fertilizer by Justus von Liebig around 1840. Fertilizers, however, can lead to soil acidification and destabilization of nutrient balance in soil, including depletion of minerals and enrichment of salt and heavy metals. In addition, excessive fertilizer use can lead to alteration of soil fauna as well as contaminate surface water and ground water. Further, unhealthful substances such as nitrate may become enriched in plants and fruits.

[0004] In addition, insecticides and fungicides are employed worldwide to control pests. Synthetic insecticides or fungicides often are non-specific and therefore can act on organisms other than the target organisms, including other naturally occurring beneficial organisms. Because of their chemical nature, they may also be toxic and non-biodegradable. Consumers worldwide are increasingly conscious of the potential environmental and health problems associated with the residuals of chemicals, particularly in food products. This has resulted in growing consumer pressure to reduce the use or at least the quantity of chemical (i.e., synthetic) pesticides. Thus, there is a need to manage food chain requirements while still allowing effective pest control.

[0005] A further problem arising with the use of synthetic insecticides or fungicides is that the repeated and exclusive application of an insecticide or fungicide often leads to selection of resistant animal pests or microorganisms. Normally, such strains are also cross-resistant against other active ingredients having the same mode of action. An effective control

of the pathogens with said active compounds is then not possible any longer. However, active ingredients having new mechanisms of action are difficult and expensive to develop.

[0006] The use of biological control agents (BCAs), which act as insecticides and/or plant health-enhancing and/or plant protection agents, is an alternative to fertilizers and synthetic pesticides. In some cases, the effectiveness of BCAs is not at the same level as for conventional insecticides and fungicides, especially in case of severe infection pressure. Consequently, in some circumstances, biological control agents, their mutants and metabolites produced by them are, in particular, in low application rates, not entirely satisfactory. Thus, there is a constant need for developing new insecticides, plant health-enhancing and/or plant protection compositions, including biological control agents used in conjunction with synthetic fungicides and insecticides, to strive to fulfill the above-mentioned requirements.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0007] The official copy of the sequence listing is submitted electronically via EFS-Web as an XML-formatted sequence listing with a file named "BCS219008 WO.xml" created on November 16, 2022, and having a size of 19 kilobytes, and is filed concurrently with the specification. The sequence listing contained in this XML-formatted document is part of the specification and is herein incorporated by reference in its entirety.

SUMMARY

[0008] In view of this, it was in particular an object of the present invention to provide compositions which have an enhanced ability to improve plant growth and/or to enhance plant health or which exhibit enhanced activity against insects, mites, and/or nematodes.

[0009] Accordingly, it was found that these objectives are achieved with the compositions according to the invention as defined in the following. By applying a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) at least one enzyme having serine protease activity; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells; and b) at least one particular insecticide disclosed herein, one is able to enhance preferably in a superadditive manner (i) plant growth, plant yield and/or plant health and/or (ii) the activity against insects, mites, nematodes and/or phytopathogens.

[0010] References herein to targeting sequences, exosporium proteins, exosporium protein fragments, fusion proteins, and recombinant exosporium producing *Bacillus* cells that

express such fusion proteins should not be considered to be stand-alone embodiments. Instead, throughout the present application, references to the targeting sequences, exosporium proteins, exosporium protein fragments, fusion proteins, and recombinant exosporium producing *Bacillus* cells that express such fusion proteins should be considered to be disclosed and claimed only in combination (and preferably in a synergistic combination) with one or more of the particular insecticides described herein. Furthermore, references to the "particular insecticide disclosed herein" are intended to encompass insecticides described below under the heading "Insecticides."

[0011] The present invention is directed to a composition comprising in synergistically effective amounts: a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) a *Bacillus firmus* serine protease; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells; and b) at least one insecticide selected from the group consisting of acetamiprid, aldicarb, amitraz, beta-cyfluthrin, carbaryl, clothianidin, cyfluthrin, cypermethrin, deltamethrin, endosulfan, ethion, ethiprole, ethoprophos, fenamiphos, fenobucarb, fenthion, fipronil, flubendiamide, flupyradifurone, fluopyram, formetanate, heptanophos, imidacloprid, methamidophos, methiocarb, methomyl, niclosamide, oxydemeton-methyl, phosalone, silafluofen, spirodiclofen, spiromesifen, spirotetramat, thiacloprid, thiodicarb, tralomethrin, triazophos, triflumuron, vamidothion, 1-{2-fluoro-4-methyl-5-[(R)-(2,2,2-trifluoroethyl)sulphinyl]phenyl}-3-(trifluoromethyl)-1H-1,2,4-triazol-5-amine, 1-(3-chloropyridin-2-yl)-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-2H-tetrazol-2-yl]methyl}-1H-pyrazole-5-carboxamide and pesticidal terpene mixtures comprising the three terpenes α-terpinene, p-cymene and limonene; *Bacillus firmus* I-1582; *Purpureocillium lilacinum* strain 251 (AGAL 89/030550; e.g., BIOACT® DC from Bayer CropScience Biologics GmbH); and a composition comprising one or more fatty acids or derivatives thereof selected from unsaturated and saturated C_{12-24} fatty acids, salts thereof, esters thereof or mixtures of any of the foregoing, wherein at least 95% of said fatty acids or derivatives thereof are in the rage of C14 to C20 (e.g., FLIPPER® by AlphaBio Pesticides or Bayer AG).

[00011] In some embodiments, the targeting sequence comprises: an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; a targeting sequence comprising amino acids 1–35 of SEQ ID NO: 1; a targeting sequence comprising amino acids 20–35 of SEQ ID NO: 1; a targeting sequence comprising amino acids 22–31 of SEQ ID NO: 1; a targeting

sequence comprising amino acids 22–33 of SEQ ID NO: 1; a targeting sequence comprising amino acids 20–31 of SEQ ID NO: 1; a targeting sequence comprising SEQ ID NO: 1; a targeting sequence comprising SEQ ID NO: 2; or an exosporium protein comprising an amino acid sequence having at least 85% identity with SEQ ID NO: 3.

[00012] In some embodiments, the exosporium-producing *Bacillus* cells are cells of a *Bacillus cereus* family member. The recombinant exosporium-producing *Bacillus* cells may be any one of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus samanii, Bacillus gaemokensis, Bacillus weihenstephensis*, *Bacillus toyoiensis,* and combinations thereof. In a further embodiment, the recombinant *Bacillus* cells are cells of *Bacillus thuringiensis* BT013A.

[00013] In certain aspects, the fusion protein comprises a serine protease enzyme from *Bacillus firmus*.

[00014] In certain embodiments, the insecticide is selected from the group consisting of acetamiprid, aldicarb, amitraz, beta-cyfluthrin, carbaryl, clothianidin, cyfluthrin, cypermethrin, deltamethrin, endosulfan, ethion, ethiprole, ethoprophos, fenamiphos, fenobucarb, fenthion, fipronil, flubendiamide, flupyradifurone, fluopyram, formetanate, heptanophos, imidacloprid, methamidophos, methiocarb, methomyl, niclosamide, oxydemeton-methyl, phosalone, silafluofen, spirodiclofen, spiromesifen, spirotetramat, thiacloprid, thiodicarb, tralomethrin, triazophos, triflumuron, vamidothion, 1-{2-fluoro-4-methyl-5-[(R)-(2,2,2-trifluoroethyl)sulphinyl]phenyl}-3-(trifluoromethyl)-1H-1,2,4-triazol-5-amine, 1-(3-chloropyridin-2-yl)-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-2H-tetrazol-2-yl]methyl}-1H-pyrazole-5-carboxamide, pesticidal terpene mixtures comprising the three terpenes α-terpinene, p-cymene and limonene, and a composition comprising one or more fatty acids or derivatives thereof selected from unsaturated and saturated C_{12-24} fatty acids, salts thereof, esters thereof or mixtures of any of the foregoing, wherein at least 95% of said fatty acids or derivatives thereof are in the range of C14 to C20 (e.g., FLIPPER® by AlphaBio Pesticides or Bayer AG).

[00015] In other embodiments, the insecticide is selected from the group consisting of clothianidin, cypermethrin, ethiprole, fipronil, fluopyram, flupyradifurone, imidacloprid, methiocarb, and thiodicarb.

[00016] In some embodiments, the composition of the present invention comprises a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) an enzyme having serine protease activity comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-6 or an amino acid sequence having at least 95%

identity to SEQ ID NO: 6; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells; and b) at least one insecticide selected from the group consisting of clothianidin, cypermethrin, ethiprole, fipronil, fluopyram, flupyradifurone, imidacloprid, methiocarb, and thiodicarb in a synergistically effective amount.

[00017] In a particular aspect of the above embodiments (i) the at least one insecticide is clothianidin; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00018] In a particular aspect of the above embodiments (i) the at least one insecticide is fluopyram; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) an enzyme having serine protease activity comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00019] In a particular aspect of the above embodiments (i) the at least one insecticide is clothianidin; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00020] In a particular aspect of the above embodiments (i) the at least one insecticide is fipronil; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids

25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00021] In a particular aspect of the above embodiments (i) the at least one insecticide is flupyradifurone; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00022] In a particular aspect of the above embodiments (i) the at least one insecticide is imidacloprid; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00023] In a particular aspect of the above embodiments (i) the at least one insecticide is methiocarb; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00024] In a particular aspect of the above embodiments (i) the at least one insecticide is thiodicarb; (ii) the targeting sequence comprises an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (iii) the enzyme having serine protease activity comprises an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (iv) the recombinant *Bacillus cereus* family member cells comprise cells of *Bacillus thuringiensis* or *Bacillus mycoides.* In yet another particular embodiment, the recombinant *Bacillus cereus* family member cells are cells of *Bacillus thuringiensis* BT013A.

[00025] In yet other embodiments, the composition further comprises at least one fungicide. The at least one fungicide may be synthetic.

[00026] In some aspects, the composition further comprises at least one auxiliary selected from the group consisting of extenders, solvents, spontaneity promoters, carriers, emulsifiers, dispersants, frost protectants, thickeners and adjuvants.

[00027] In other aspects, the invention is directed to a seed treated with any of the compositions disclosed herein.

[00028] Furthermore, the present invention relates to use of the disclosed compositions as an insecticide and/or biostimulant. In certain aspects, the disclosed compositions are used for reducing overall damage of plants and plant parts as well as losses in harvested fruits or vegetables caused by insects, mites, nematodes and/or phytopathogens. In other aspects, the disclosed compositions are used for enhancing plant growth and/or promoting plant health.

[00029] Additionally, the present invention is directed to a method of treating a plant, a plant part, such as a seed, root, rhizome, corm, bulb, or tuber, and/or a locus on which or near which the plant or the plant parts grow, such as soil, to enhance plant growth and/or promote plant health comprising the step of simultaneously or sequentially applying to a plant, a plant part and/or a plant loci: a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) an enzyme having serine protease activity, preferably comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells; and b) at least one insecticide selected from a particular insecticide disclosed herein that exhibits activity against insects, mites, nematodes and/or phytopathogens in a synergistically effective amount.

[00030] In another embodiment, the present invention is a method for reducing overall damage of plants and plant parts as well as losses in harvested fruits or vegetables caused by insects, mites, nematodes and/or phytopathogens comprising the step of simultaneously or sequentially applying to a plant, a plant part, such as a seed, root, rhizome, corm, bulb, or tuber, and/or a locus on which or near which the plant or the plant parts grow, such as soil: a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) an enzyme having serine protease activity, preferably comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells; and b) at least one insecticide selected from the particular insecticides disclosed herein that exhibits activity against insects, mites, nematodes and/or phytopathogens in a synergistically effective amount.

[00031] In the above paragraphs, the term "comprise" or any derivative thereof (e.g., comprising, comprises) may be replaced with "consist of" or the applicable corresponding derivative thereof.

[00032] Other objects and features will be in part apparent and in part pointed out hereinafter.

DEFINITIONS

[00033] When the articles "a", "an", "one", "the", and "said" are used herein, they mean "at least one" or "one or more" unless otherwise indicated.

[00034] The term "*Bacillus cereus* family member" as used herein refers to any *Bacillus* species that is capable of producing an exosporium. Thus, the *Bacillus cereus* family of bacteria includes the species *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus samanii, Bacillus gaemokensis*, *Bacillus weihenstephensis*, and *Bacillus toyoiensis*. *Bacillus cereus* family members are also referred to in the art as "*Bacillus cereus* sensu lato."

[00035] The terms "comprising," "including", and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[00036] The term "foliar" used herein with respect to the application of enzymes or recombinant microorganisms to plants means that the enzyme or recombinant microorganism is applied to one or more aerial portions of the plant, including stems, leaves, fruits, flowers, or other exposed aerial portions of the plant.

[00037] The term "fusion protein" as used herein refers to a protein having a polypeptide sequence that comprises sequences derived from two or more separate proteins. A fusion protein can be generated by joining together a nucleic acid molecule that encodes all or part of a first polypeptide with a nucleic acid molecule that encodes all or part of a second polypeptide to create a nucleic acid sequence which, when expressed, yields a single polypeptide having functional properties derived from each of the original proteins. As such, a fusion protein may include a polypeptide comprising a combination of polypeptide sequences that would not naturally occur together without human intervention. For example, a fusion protein may include a polypeptide that deviates from polypeptide sequences that exist in nature, a polypeptide that comprises a synthetic polypeptide sequence or a polypeptide expressed by a recombinant DNA sequence that has been incorporated into a host cell by genetic transformation or gene editing.

[00038] Reference in this application to an "isolated polypeptide", "isolated fusion protein", or an equivalent term or phrase, is intended to mean that the polypeptide or the fusion protein is one that is present alone or in combination with other compositions, but not within its natural environment. Similarly, a DNA molecule encoding a serine protease or any naturally occurring serine protease variant would be an isolated DNA molecule so long as the nucleotide sequence was not within the DNA of the bacterium from which the sequence encoding the protein is naturally found. A synthetic nucleotide sequence encoding the amino acid sequence of the naturally occurring serine protease would be considered to be isolated for the purposes of this disclosure. For the purposes of this disclosure, any transgenic nucleotide sequence, *i.e.*, the nucleotide sequence of the DNA inserted into the genome of the cells of a plant or bacterium, or present in an extrachromosomal vector, would be considered to be an isolated nucleotide sequence whether it is present within the plasmid or similar structure used to transform the cells, within the genome of the plant or bacterium, or present in detectable amounts in tissues, progeny, biological samples or commodity products derived from the plant or bacterium.

[00039] The term "germination rate" as used herein refers to the number of seeds that germinate during a particular time period. For example, a germination rate of 85% indicates that 85 out of 100 seeds germinate during a given time period.

[00040] The term "inactivate" or "inactivation" as used herein in reference to the inactivation of spores of a recombinant *Bacillus cereus* family member means that the spores are unable to germinate, or that the spores can germinate, but are damaged such that germination does not result in a living bacterium. The terms "partially inactivate" or "partial inactivation" mean that a percentage of the spores are inactivated, but that some spores retain the ability to

germinate and return to a live, replicating state. The term "genetic inactivation" refers to inactivation of spores a recombinant *Bacillus cereus* family member by a mutation of the spore's DNA that results in complete or partial inactivation of the spore. The terms "physical inactivation" and "chemical inactivation" refer to inactivation of spores using any physical or chemical means, e.g., by heat treatment, gamma irradiation, x-ray irradiation, UV-A irradiation, UV-B irradiation, or treatment with a solvent such as glutaraldehyde, formaldehyde, hydrogen peroxide, acetic acid, bleach, chloroform, phenol, or any combination thereof.

[00041] The terms "native sequence", "native amino acid sequence", "wild-type sequence", and "wild-type amino acid sequence" are used interchangeably herein to refer to an amino acid sequence as it exists in a naturally occurring protein.

[00042] A "plant growth medium" includes any material that is capable of supporting the growth of a plant.

[00043] The terms "promoting plant growth" and "stimulating plant growth" are used interchangeably herein, and refer to the ability to enhance or increase at least one of the plant's height, weight, leaf size, root size, fruit size, shoot size or stem size, and/or the ability to increase protein yield from the plant, and/or to increase crop yield, and/or to improve plant vigor. For example, this may relate to increased length and/or fresh and/or dry weights of roots and/or shoots of treated plants or crops compared to untreated plants or crops.

[00044] Increased yield of a plant, in particular of an agricultural, silvicultural and/or ornamental plant, means that the yield of a product of the respective plant is increased by a measurable amount over the yield of the same product of the plant produced under the same conditions, but without the application of the compositions disclosed herein.

[00045] Improved plant vigor includes the following: (a) improved vitality of the plant, (b) improved quality of the plant and/or of the plant products, e.g., enhanced protein content, (c) improved visual appearance, (d) delay of senescence, (e) enhanced root growth and/or more developed root system (e.g., determined by the dry mass of the root), (f) enhanced nodulation, in particular rhizobial nodulation, (g) longer panicles, (h) bigger leaf blade, (i) less dead basal leaves, (j) increased chlorophyll content, (k) prolonged photosynthetically active period, (l) increased or improved plant stand density, (m) less plant verse (lodging), (n) increased plant weight, (o) increased plant height, (p) tillering increase, (q) stronger and/or more productive tillers, (r) less non-productive tillers, (s) enhanced photosynthetic activity and/or enhanced pigment content and thus greener leaf color, (t) earlier and/or improved germination, (u) improved and/or more uniform and/or earlier emergence, (v) increased shoot growth, (w)

earlier flowering, (x) earlier fruiting, (y) earlier grain maturity, (z) less fertilizers needed, (aa) less seeds needed.

[00046] The term "recombinant" as used in reference to the bacteria described herein encompasses bacteria having any genetic modification as compared to wild-type bacteria of the same type, including bacteria that have been modified to delete of a gene or a portion of a gene (e.g., bacteria that have a "knock-out" of a gene), as well as bacteria that have been modified to express an exogenous peptide or protein.

[00047] The term "rhizosphere" is used interchangeably with "root zone" to denote that segment of the soil that surrounds the roots of a plant and is influenced by them.

[00048] The term "synergistically effective amount" as used herein refers to an amount of a first substance (e.g., a first enzyme) that when used in combination with a second substance (e.g., a second enzyme) produces a biological effect that is greater than the sum of the biological effects of each of the respective first and second substances when used alone.

[00049] The term "targeting sequence" as used herein refers to a polypeptide sequence that, when present as part of a longer polypeptide or a protein, results in the localization of the longer polypeptide or the protein to a specific subcellular location. The targeting sequences described herein result in localization of proteins to the exosporium of a *Bacillus cereus* family member.

[00050] The following abbreviations are useful for understanding the depositary institution of the microbial strains listed below.

[00051] ATCC is the abbreviation for the American Type Culture Collection, International Depository Authority for the Purposes of Depositing Biological Material for the Purposes of Patenting under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, having the address ATCC Patent Depository, 10801 University Boulevard, Manassas, Virginia 10110, U.S.A.

[00052] CBS is the abbreviation for the Centraalbureeau voor Schimmelcultures, an International Depository Authority for the Purposes of Depositing Biological Material for the Purposes of Patenting under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, having the address Uppsalalaan 8, Baarn/Utrecht, The Netherlands.

[00053] CGMCC is the abbreviation for the China General Microbiological Culture Collection Cente, an International Depository Authority for the Purposes of Depositing Biological Material for the Purposes of Patenting under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, having the

address of Institute of Microbiology, Chinese Academy of Sciences, No. 1 Beichen West Road, Chaoyang District, Beijing 100 101.

[00054] CNCM is the acronym for the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, France.

[00055] DSM is the abbreviation for Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, an International Depositary Authority for the Purposes of Depositing Biological Material for the Purposes of Patenting under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, having the address Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany.

[00056] IMI is the acronym for CABI Bioscience, Egham, UK (formerly International Mycological Institute; also known as CMI and CABI).

[00057] NRRL is the abbreviation for the Agricultural Research Service Culture Collection, International Depository Authority for the Purposes of Deposing Biological Material for the Purposes of Patenting under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, having the address National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, U.S.A.

DETAILED DESCRIPTION OF THE INVENTION

[00058] Compositions of the present invention comprise a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) an enzyme having serine protease activity from *Bacillus firmus*; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells; and b) at least one particular insecticide or biostimulant disclosed herein in a synergistically effective amount.

Fusion Proteins for Expression in *Bacillus Cereus* Family Members

[00059] The fusion proteins of the present invention comprise a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member. The fusion proteins further comprise an enzyme having serine protease activity. When expressed in *Bacillus cereus* family member bacteria, these fusion proteins are targeted to the exosporium layer of the spore and are physically oriented such that the serine protease is displayed on the outside of the spore.

[00060] This *Bacillus* exosporium display (BEMD) system can be used to deliver the serine protease to plants (e.g., to plant foliage, fruits, flowers, stems, or roots) or to a plant growth medium such as soil. Enzymes and proteins delivered to the soil or another plant growth medium in this manner persist and exhibit activity in the soil for extended periods of time. Introduction of recombinant *Bacillus cereus* family member bacteria expressing the fusion proteins described herein into soil or the rhizosphere of a plant leads to a beneficial enhancement of plant growth and/or to control pests, such as nematodes, in many different soil conditions. The use of the BEMD to create these enzymes allows them to continue to exert their beneficial results to the plant and the rhizosphere over the first months of a plant's life.

[00061] In addition, as is described further hereinbelow, the BEMD system can be modified such that the exosporium of the recombinant *Bacillus cereus* family member can be removed from the spore, generating exosporium fragments containing the fusion proteins. The exosporium fragments can also be used to deliver the serine proteases to plants in a cell-free preparation.

Targeting Sequences, Exosporium Proteins, and Exosporium Protein Fragments for Targeting Enzymes Having Serine Protease Activity to the Exosporium of a Bacillus cereus Family Member

[00062] For ease of reference, descriptions of the amino acid sequences for the targeting sequences, exosporium proteins, and exosporium protein fragments that can be used for targeting of enzymes or proteins (e.g., enzymes having serine protease activity) to the exosporium of a *Bacillus cereus* family members, are provided in **Table 1** together with their SEQ ID NOs.

Table 1. Peptide and Protein Sequences Used for Targeting of Proteins or Peptides of Interest to the Exosporium of *Bacillus cereus* Family Members

Protein, Protein Fragment, or Targeting Sequence	SEQ ID NO:
AA 1–41 of BclA (*B. anthracis* Sterne)	1*
Met + AA 20–35 of BclA (*B. anthracis* Sterne)	2
Full length BclA (*B. anthracis* Sterne)	3*

AA = amino acids

* AA 1–41 of *B. anthracis* Sterne strain BclA have 100% sequence identity with AA 1–41 of *B. thuringiensis* BclA.

[00063] *Bacillus* is a genus of rod-shaped bacteria. The *Bacillus cereus* family of bacteria includes any *Bacillus* species that is capable of producing an exosporium. Thus, the *Bacillus cereus* family of bacteria includes the species *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus samanii, Bacillus gaemokensis*, *Bacillus weihenstephensis*, and *Bacillus toyoiensis*. Under stressful environmental conditions, *Bacillus cereus* family bacteria undergo sporulation and form oval endospores that can stay dormant for extended periods of time. The outermost layer of the endospores is known as the exosporium and comprises a basal layer surrounded by an external nap of hair-like projections. Filaments on the hair-like nap are predominantly formed by the collagen-like glycoprotein BclA, while the basal layer is comprised of a number of different proteins. Another collagen-related protein, BclB, is also present in the exosporium and exposed on endospores of *Bacillus cereus* family members. BclA, the major constituent of the surface nap, has been shown to be attached to the exosporium with its amino-terminus (N-terminus) positioned at the basal layer and its carboxy-terminus (C-terminus) extending outward from the spore.

[00064] The scientific literature describes the *Bacillus cereus* "family" or "group" as a subgroup within the genus *Bacillus*. See Priest et al., "Population Structure and Evolution of the *Bacillus cereus* Group," J. Bacteriology, 2004, vol. 186. no. 23, pp. 7959–7970; Peng et al., "The Regulation of Exosporium-Related Genes in *Bacillus thuringiensis*," Nature Scientific Reports, 2016, vol. 6, no. 19005, pp. 1-12. Peng et al. states:

> Spores of the *B. cereus* group are complex, multilayered structures. The nucleoid containing core is enclosed within a peptidoglycan cortex, which is surrounded by the spore coat. Spores of all the *B. cereus* group species are encircled by an additional loose-fitting layer called the exosporium, which is not present on other species such as *Bacillus subtilis*, for which the coat constitutes the outermost layer of the mature spore. The exosporium is a balloon-like layer that acts as the outer permeability barrier of the spore and contributes to spore survival and virulence.

[00065] It was previously discovered that certain sequences from the N-terminal regions of BclA and BclB could be used to target a peptide or protein to the exosporium of a *Bacillus cereus* family member endospore (*see* U.S. Patent Application Publication Nos. 2010/0233124 and 2011/0281316, and Thompson et al., "Targeting of the BclA and BclB Proteins to the *Bacillus anthracis* Spore Surface", Molecular Microbiology 70(2):421–34

(2008)). It was also found that the BetA/BAS3290 protein of *Bacillus anthracis* localized to the exosporium. Further targeting sequences, as well as exosporium proteins and fragments of exosporium proteins, that can be incorporated into a fusion protein and used to target a peptide or protein of interest to the exosporium of a recombinant *Bacillus cereus* family member are described in U.S. Patent Application Publication Nos. 2016/0031948 and 2016/0108096, which are incorporated by reference herein in their entirety.

[00066] In particular, amino acids 20–35 of BclA from *Bacillus anthracis* Sterne strain have been found to be sufficient for targeting to the exosporium. A sequence alignment of amino acids 1–41 of BclA (SEQ ID NO: 1) with the corresponding N-terminal regions of several other *Bacillus cereus* family exosporium proteins and *Bacillus cereus* family proteins having related sequences is shown in FIGS. 1A and 1B of U.S. Patent Application Publication No. 2016/0108096. As can be seen from FIGS. 1A and 1B, there is a region of high homology among all of the proteins in the region corresponding to amino acids 20–41 of BclA. However, in these sequences, the amino acids corresponding to amino acids 36–41 of BclA contain secondary structure and are not necessary for fusion protein localization to the exosporium. The conserved targeting sequence region of BclA (amino acids 20–35 of SEQ ID NO: 1) is shown in bold in FIGS. 1A and 1B. A more highly conserved region spanning amino acids 25–35 of BclA within the targeting sequence is underlined in the sequences in FIGS. 1A and 1B, and is the recognition sequence for ExsFA/BxpB/ExsFB and homologs, which direct and assemble the described proteins on the surface of the exosporium. As can be seen from this figure, each of these sequences contains a conserved region corresponding to amino acids 20–35 of BclA (SEQ ID NO: 1; shown in bold), and a more highly conserved region corresponding to amino acids 25–35 of BclA (underlined).

[00067] Any portion of BclA which includes amino acids 20–35 can be used as to target a fusion protein to the exosporium. In addition, full-length exosporium proteins or exosporium protein fragments can be used for targeting the fusion proteins to the exosporium. Thus, full-length BclA or a fragment of BclA that includes amino acids 20–35 can be used for targeting to the exosporium. For example, full length BclA (SEQ ID NO: 3) or a midsized fragment of BclA that lacks the carboxy-terminus such as amino acids 1–196 of BclA or amino acids 1–166 of BclA can be used to target the fusion proteins to the exosporium. Midsized fragments such as these have less secondary structure than full length BclA and have been found to be suitable for use as a targeting sequence. The targeting sequence can also comprise much shorter portions of BclA which include amino acids 20–35, such as SEQ ID NO: 1 (amino acids 1–41 of BclA), amino acids 1–35 of SEQ ID NO: 1, amino acids 20–35 of SEQ ID NO: 1, or a

methionine residue linked to amino acids 20–35 of BclA. Even shorter fragments of BclA which include only some of amino acids 20–35 also exhibit the ability to target fusion proteins to the exosporium. For example, the targeting sequence can comprise amino acids 22–31 of SEQ ID NO: 1, amino acids 22–33 of SEQ ID NO: 1, or amino acids 20–31 of SEQ ID NO: 1.

[00068] Furthermore, any amino acid sequence comprising amino acids 20–35 of BclA can serve as the targeting sequence.

[00069] The targeting sequence can comprise amino acids 1–35 of SEQ ID NO: 1, amino acids 20–35 of SEQ ID NO: 1, SEQ ID NO: 1, SEQ ID NO: 2, amino acids 22–31 of SEQ ID NO: 1, amino acids 22–33 of SEQ ID NO: 1, or amino acids 20–31 of SEQ ID NO: 1. Alternatively, the targeting sequence can consist of amino acids 1–35 of SEQ ID NO: 1, amino acids 20–35 of SEQ ID NO: 1, or SEQ ID NO: 1. Alternatively, the targeting sequence can consist of amino acids 22–31 of SEQ ID NO: 1, amino acids 22–33 of SEQ ID NO: 1, or amino acids 20–31 of SEQ ID NO: 1. Alternatively, the exosporium protein can comprise full length BclA (SEQ ID NO: 3), or the exosporium protein fragment can comprise a midsized fragment of BclA that lacks the carboxy-terminus, such as amino acids 1–196 of BclA.

[00070] The targeting sequence can comprise amino acids 2–35 of SEQ ID NO: 1; amino acids 5–35 of SEQ ID NO: 1; amino acids 8–35 of SEQ ID NO: 1; amino acids 10–35 of SEQ ID NO: 1; or amino acids 15–35 of SEQ ID NO: 1.

[00071] Furthermore, it has been found that sequences shorter than amino acids 20–35 of BclA can be used to target a fusion protein to the exosporium of a recombinant *Bacillus cereus* family member. In particular, amino acids 20–33 of BclA, amino acids 20–31 of BclA, amino acids 21–33 of BclA, or amino acids 23–31 of BclA can be used to target a fusion protein to the exosporium of a recombinant *Bacillus cereus* family member. Thus, the targeting sequence can consist of amino acids 20–33 of SEQ ID NO: 1, amino acids 20–31 of SEQ ID NO: 1, amino acids 21–33 of SEQ ID NO: 1, or amino acids 23–31 of SEQ ID NO: 1.

[00072] Even shorter regions within amino acids 20–35 of BclA can also be used for targeting a fusion protein to the exosporium of a recombinant *Bacillus cereus* family member. In particular, any amino acid sequence that includes amino acids 25–30 of SEQ ID NO: 1 or the corresponding amino acids from any of the sequences shown in FIGS. 1A and 1B of U.S. Patent Application Publication No. 2016/0108096 can be used. A skilled person will recognize that starting with amino acids 25–30 of SEQ ID NO: 1 or the corresponding region of any of the sequences shown in FIGS. 1A and 1B, additional amino acids can be added to the amino-terminus, the carboxy terminus, or both the amino- and carboxy termini to create a targeting

sequence that will be effective for targeting a fusion protein to the exosporium of a recombinant *Bacillus cereus* family member.

[00073] In addition, it can readily be seen from the sequence alignment in FIGS. 1A and 1B of U.S. Patent Application Publication No. 2016/0108096 that while amino acids 20–35 of BclA are conserved, and amino acids 25–35 are more conserved, some degree of variation can occur in this region without affecting the ability of the targeting sequence to target a protein to the exosporium. The corresponding regions of any of the SEQ ID NOs. shown in FIGS. 1A and 1B can also be used to target a fusion protein to the exosporium of a recombinant *Bacillus cereus* family member. By "corresponding regions," it is meant that when the sequences are aligned with SEQ ID NO: 1, as shown in FIGS. 1A and 1B, the regions of the other amino acid sequences that align with the amino acids of SEQ ID NO: 1 are the "corresponding regions" of those sequences.

[00074] FIG. 1 lists the percent identity of the corresponding amino acids of each sequence to amino acids 20–35 of BclA ("20–35% Identity") and to amino acids 25–35 of BclA ("25–35 % Identity"). Sequences having a targeting sequence identity as low as 43.8% with amino acids 20–35 of BclA (SEQ ID NO: 1), wherein the identity with amino acids 25–35 of BclA is 54.5%, retain the ability to target fusion proteins to the exosporium. Data are provided in Table 58 in Example 59 of PCT Publication No. WO 2016/044661, which is incorporated herein by reference in its entirety. Table 58 shows the enzyme levels of phosphatidylcholine-specific phospholipase C gene (PC-PLC) and lipase on *Bacillus cereus* family member spores expressing fusion proteins containing these enzymes and various targeting sequences with sequence identity to amino acids 20-35 of BclA ranging from 50.0% to 68.8% and with sequence identity to amino acids 25-35 ranging from 63.6% to 81.8%.

[00075] These data show that targeting of a protein of interest (e.g., an enzyme) to the exosporium proteins can be achieved using targeting sequences having 50–68.8% identity to amino acids 20–35 of BclA (SEQ ID NO: 1), wherein the identity to amino acids 25–35 of BclA is 63.6% to 81.8%. Such motif is present in a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of the recombinant *Bacillus* bacterium and comprises the sequence X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-X_{15}-X_{16}, wherein:

X_1 is any amino acid or absent;

X_2 is phenylalanine (F), leucine (L), isoleucine (I), or methionine (M);

X_3 is any amino acid;

X_4 is proline (P) or serine (S);

X_5 is any amino acid;

X_6 is leucine (L), asparagine (N), serine (S), or isoleucine (I);

X_7 is valine (V) or isoleucine (I);

X_8 is glycine (G);

X_9 is proline (P);

X_{10} is threonine (T) or proline (P);

X_{11} is leucine (L) or phenylalanine (F);

X_{12} is proline (P);

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is proline (P), glutamine (Q), or threonine (T); and

X_{16} is proline (P), threonine (T), or serine (S).

[00076] Any of the targeting sequences, exosporuim proteins, or exosporium protein fragments can be used to target any protein or peptide of interest, including the proteins having serine protease activity described herein, to the exosporium of a recombinant *Bacillus cereus* family member.

[00077] FIGS. 1A and 1B of U.S. Patent Application Publication No. 2016/0108096 list the percent identity of each of the corresponding amino acids of each sequence to amino acids 20–35 of BclA ("20–35% Identity") and to amino acids 25–35 of BclA ("25–35% Identity"). Thus, for example, as compared to amino acids 20–35 of BclA, the corresponding amino acids of BetA/BAS3290 are about 81.3% identical, the corresponding amino acids of BAS4623 are about 50.0% identical, the corresponding amino acids of BclB are about 43.8% identical, the corresponding amino acids of BAS1882 are about 62.5% identical, the corresponding amino acids of the KBAB4 2280 gene product are about 81.3% identical, and the corresponding amino acids of the KBAB4 3572 gene product are about 81.3% identical. The sequence identities over this region for the remaining sequences are listed in FIGS. 1A and 1B.

[00078] With respect to amino acids 25–35 of BclA, the corresponding amino acids of BetA/BAS3290 are about 90.9% identical, the corresponding amino acids of BAS4623 are about 72.7% identical, the corresponding amino acids of BclB are about 54.5% identical, the corresponding amino acids of BAS1882 are about 72.7% identical, the corresponding amino acids of the KBAB4 2280 gene product are about 90.9% identical, and the corresponding amino acids of the KBAB4 3572 gene product are about 81.8% identical. The sequence identities over this region for the remaining sequences are listed in FIGS. 1A and 1B of U.S. Patent Application Publication No. 2016/0108096.

[00079] Thus, the targeting sequence can comprise an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%.

[00080] The targeting sequence can also comprise an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[00081] The targeting sequence can also comprise an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[00082] The targeting sequence can also comprise an amino acid sequence having at least about 56% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 56% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[00083] The targeting sequence can comprise an amino sequence having at least about 62% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%. Alternatively, the targeting sequence can consist of an amino acid sequence consisting of 16 amino acids and having at least about 62% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 of SEQ ID NO: 1 is at least about 72%.

[00084] The targeting sequence can comprise an amino acid sequence having at least 68% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%. Alternatively, the targeting sequence consists of an amino acid

sequence consisting of 16 amino acids and having at least 68% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[00085] The targeting sequence can also comprises an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 of SEQ ID NO: 1 is at least about 72%.

[00086] The targeting sequence can also comprise an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 of SEQ ID NO: 1 is at least about 81%.

[00087] The targeting sequence can also comprise an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[00088] The targeting sequence can comprise an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 90%. Alternatively, the targeting sequence consists of an amino acid sequence consisting of 16 amino acids and having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 90%.

[00089] The skilled person will recognize that variants of the above sequences can also be used as targeting sequences, so long as the targeting sequence comprises amino acids 20–35 of BclA, the corresponding amino acids of BetA/BAS3290, BAS4263, BclB, BAS1882, the KBAB4 2280 gene product, or the KBAB 3572 gene product, or a sequence comprising any of the above noted sequence identities to amino acids 20–35 and 25–35 of BclA is present.

[00090] Moreover, exosporium proteins having a high degree of sequence identity with any of the full-length exosporium proteins or the exosporium protein fragments described above can also be used to target a peptide or protein to the exosporium of a *Bacillus cereus*

family member. Thus, the fusion protein can comprise an exosporium protein or exosporium protein fragment comprising an amino acid sequence having at least 85% identity with SEQ ID NO: 3.

[00091] Alternatively, the fusion protein can comprise an exosporium protein having at least 90% identity with SEQ ID NO: 3.

[00092] The fusion protein can comprise an exosporium protein having at least 95% identity with SEQ ID NO: 3.

[00093] The fusion protein can comprise an exosporium protein having at least 98% identity with SEQ ID NO: 3.

[00094] The fusion protein can comprise an exosporium protein having at least 99% identity with SEQ ID NO: 3.

[00095] The fusion protein can comprise an exosporium protein having 100% identity with SEQ ID NO: 3.

[00096] The targeting sequence, exosporium protein or exosporium protein fragment of the present invention may also be described in terms of a motif that provides the targeting function. FIGS. 1A and 1B show a sequence alignment of the amino-terminal region of BclA (SEQ ID NO: 1) with the corresponding amino-terminal regions of a number of other *Bacillus cereus* family member exosporium proteins. As can be seen from FIG. 1, there is a conserved motif at amino acids 20–35 of BclA (shown in bold in FIG. 1), with a more highly conserved motif at amino acids 25–35 of BclA (shown in bold and underlined in FIG. 1). This more highly conserved region is the recognition sequence for ExsFA/BxpB/ExsFB and homologs, which direct and assemble the described exosporium proteins on the surface of the exosporium.

[00097] During sporulation of a recombinant *Bacillus cereus* family member expressing any of the fusion proteins described herein, the targeting motif, exosporium protein, or exosporium protein fragment is recognized by the spore exosporium assembly machinery and directed to the exosporium, resulting in display of the protein or peptide of interest portion of the fusion protein (e.g., the enzyme having serine protease activity) on the outside of the spore.

[00098] The use of different targeting sequences allows for control of the expression level of the fusion protein on the surface of the *Bacillus cereus* family member spore. Use of certain of the targeting sequences described herein will result in a higher level of expression of the fusion protein, whereas use of others of the targeting sequences will result in lower levels of expression of the fusion protein on the surface of the spore.

[00099] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment can comprise the amino acid sequence GXT at its carboxy terminus, wherein X is any amino acid.

[000100] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment, can comprise an alanine residue at the position of the targeting sequence that corresponds to amino acid 20 of SEQ ID NO: 1.

[000101] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment can further comprise a methionine, serine, or threonine residue at the amino acid position immediately preceding the first amino acid of the targeting sequence, exosporium protein, or exosporium protein fragment or at the position of the targeting sequence that corresponds to amino acid 20 of SEQ ID NO: 1.

Fusion Proteins for Expression in Recombinant Bacillus cereus *Family Members*

[000102] Fusion proteins comprising a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member are provided. The fusion proteins further comprise an enzyme having serine protease activity.

[000103] In any of the fusion proteins described herein, the fusion protein can comprise: (1) a targeting sequence comprising an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (2) a targeting sequence comprising amino acids 1–35 of SEQ ID NO: 1; (3) a targeting sequence comprising amino acids 20–35 of SEQ ID NO: 1; (4) a targeting sequence comprising SEQ ID NO: 1; (5) an exosporium protein comprising an amino acid sequence having at least 85% identity with SEQ ID NO: 3; (6) a targeting sequence comprising amino acids 2–35 of SEQ ID NO: 1; (7) a targeting sequence comprising amino acids 5–35 of SEQ ID NO: 1; (8) a targeting sequence comprising amino acids 8–35 of SEQ ID NO: 1; (9) a targeting sequence comprising amino acids 10–35 of SEQ ID NO: 1; (10) a targeting sequence comprising amino acids 15–35 of SEQ ID NO: 1; (11) a targeting sequence consisting of amino acids 20–33 of SEQ ID NO: 1; (12) a targeting sequence consisting of amino acids 21–33 of SEQ ID NO: 1; (13) a targeting sequence consisting of amino acids 23–31 of SEQ ID NO: 1.

[000104] For example, the targeting sequence can comprise an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[000105] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[000106] The targeting sequence can comprise an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000107] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 50% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000108] The targeting sequence can comprise an amino acid sequence having at least about 56% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[000109] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 56% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 63%.

[000110] The targeting sequence can comprise an amino sequence having at least about 62% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000111] Alternatively, the targeting sequence can consist of an amino sequence having at least about 62% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000112] The targeting sequence can comprise an amino acid sequence having at least about 68% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000113] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 68% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000114] The targeting sequence can comprise an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000115] Alternatively, the targeting sequence can consist of an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 72%.

[000116] The targeting sequence can comprise an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000117] Alternatively, the targeting sequence can consist of an amino sequence having at least about 75% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000118] The targeting sequence can comprise an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000119] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 81%.

[000120] The targeting sequence can comprise an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 90%.

[000121] Alternatively, the targeting sequence can consist of an amino acid sequence having at least about 81% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 90%.

[000122] For example, the targeting sequence can consist of: (a) an amino acid sequence consisting of 16 amino acids and having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%; (b) amino acids 1–35 of SEQ ID NO: 1; (c) amino acids 20–35 of SEQ ID NO: 1; (d) SEQ ID NO: 1; (e) SEQ ID NO: 2.

[000123] In any of the fusion proteins described herein, the fusion protein can comprise an exosporium protein or an exosporium protein fragment comprising an amino acid sequence having at least 90% identity with SEQ ID NO: 3.

[000124] The fusion protein can comprise an exosporium protein or an exosporium protein fragment comprising an amino acid sequence having at least 95% identity with SEQ ID NO: 3.

[000125] The fusion protein can comprise an exosporium protein or an exosporium protein fragment comprising an amino acid sequence having at least 98% identity with SEQ ID NO: 3.

[000126] The fusion protein can comprise an exosporium protein or an exosporium protein fragment comprising an amino acid sequence having at least 99% identity with SEQ ID NO: 3.

[000127] The fusion protein can comprise an exosporium protein or an exosporium protein fragment comprising an amino acid sequence having 100% identity with SEQ ID NO: 3.

[000128] The fusion protein can comprise a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of the recombinant *Bacillus* bacterium, wherein the targeting sequence, exosporium protein, or exosporium protein fragment comprises the sequence X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-X_{15}-X_{16}, wherein:

X_1 is any amino acid or absent;

X_2 is phenylalanine (F), leucine (L), isoleucine (I), or methionine (M);

X_3 is any amino acid;

X_4 is proline (P) or serine (S);

X_5 is any amino acid;

X_6 is leucine (L), asparagine (N), serine (S), or isoleucine (I);

X_7 is valine (V) or isoleucine (I);

X_8 is glycine (G);

X_9 is proline (P);

X_{10} is threonine (T) or proline (P);

X_{11} is leucine (L) or phenylalanine (F);

X_{12} is proline (P);

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is proline (P), glutamine (Q), or threonine (T); and

X_{16} is proline (P), threonine (T), or serine (S)

[000129] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment can comprise the amino acid sequence GXT at its carboxy terminus, wherein X is any amino acid.

[000130] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment can comprise an alanine residue at the position of the targeting sequence that corresponds to amino acid 20 of SEQ ID NO: 1.

[000131] In any of the fusion proteins described herein, the targeting sequence, exosporium protein, or exosporium protein fragment can further comprise a methionine, serine,

or threonine residue at the amino acid position immediately preceding the first amino acid of the targeting sequence, exosporium protein, or exosporium protein fragment or at the position of the targeting sequence that corresponds to amino acid 20 of SEQ ID NO: 1.

Fusion Proteins Comprising an Enzyme Having Serine Protease Activity

[000132] Fusion proteins comprising a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member and an enzyme having serine protease activity are provided.

[000133] The fusion proteins can comprise an enzyme having serine protease activity.

[000134] Serine proteases are one of the largest and mostly widely distributed class of proteases. Serine proteases cleave peptide bonds at serine residues within a specific recognition site in a protein. These proteases are frequently used by bacteria for nutrient scavenging in the environment. Serine proteases have also been show to exhibit nematicidal activity through digestion of intestinal tissue in nematodes. Studies of *Bacillus firmus* strain DS-1, which shows nematicidal activity against *Meloidogyne incognita* and soybean cyst nematode, revealed that the serine protease produced by that strain has serine protease activity and degraded the intestinal tissues of nematodes. Geng, C., et al., "A Novel Serine Protease, Sep1, from *Bacillus firmus* DS-1 Has Nematicidal Activity and Degrades Multiple Intestinal-Associated Nematode Proteins", Scientific Reports, 2016, vol. 6, no. 25012.

[000135] Other studies have shown that serine proteases have activity against pathogens such as fungal plant pathogens and oomycetes, such as *Pythium*. See Dunne et al., "Overproduction of an Inducible Extracellular Serine Protease Improves Biological Control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81," Microbiology, 2000, vol. 146, pp. 2069-2078, and Yen, Y., et al., "An Antifungal Protease Produced by *Pseudomonas aeruginosa* M-1001 with Shrimp and Crab Shell Powder as a Carbon Source," Enzyme and Microbial Technology, 2006, vol. 39, pp. 311-317.

[000136] In **Table 2**, SEQ ID NOs: 4-6 are amino acid sequences for wild-type enzymes and a variant enzyme that exhibit or are predicted to exhibit serine protease activity. Thus, for example, SEQ ID NOs: 4 and 5 provide the amino acid sequence for wild-type serine protease enzymes from two different *Bacillus firmus* strains and have 98% sequence similarity. SEQ ID NO: 6 provides the amino acid sequence for the same enzyme as in SEQ ID NO: 4, except for a deletion of amino acids 181-240 of SEQ ID NO: 4, such that SEQ ID NOs: 4 and 6 have 81% sequence similarity. The catalytic residues referenced in Geng, et al., 2016, above, are maintained in the variant serine protease amino acid sequence of SEQ ID NO: 6.

Table 2. Amino Acid Sequences for Serine Protease and Variant

Enzyme	SEQ ID NO:
Serine Protease from *Bacillus firmus* DS-1 (Sep1)	4
Serine Protease from *Bacillus firmus* Strain 1 (Sep1)	5
Serine Protease Variant with Deletion	6

[000137] The enzyme having serine protease activity can comprise a serine protease from *Bacillus firmus*, also referred to as a *Bacillus firmus* serine protease enzyme. In yet another embodiment, the serine protease from *Bacillus firmus* can be Sep1 from a *Bacillus firmus* strain. In yet another embodiment, the serine protease can be Sep1 from *Bacillus firmus* DS-1, which is SEQ ID NO: 4. See Geng, et al., 2016, above. In yet another embodiment, the serine protease can be Sep1 from another *Bacillus firmus* strain, such as SEQ ID NO: 5.

[000138] For serine protease enzymes described herein, "sequence identity" or "percent sequence identity" or "% sequence identity" is determined by aligning the entire length of the sequences in such a way as to obtain optimal matching so that the minimal number of edit operations (e.g., inserts, deletions and substitutions) are needed in order to transform the one sequence into an exact copy of the other sequence being aligned. The Needleman-Wünsch Global Alignmment of Protein Sequences, which is an algorithm that is available through the U.S National Library of Medicine's National Center for Biotechnology Information ("NCBI") website, is one example of such analysis.

[000139] Alternatively or in addition, the enzyme having serine protease activity can comprise an amino acid sequence having at least 80% identity to any one of SEQ ID NOs: 4-5.

[000140] The enzyme having serine protease activity can comprise an amino acid sequence having at least 85% identity to any one of SEQ ID NOs: 4-5.

[000141] The enzyme having serine protease activity can comprise an amino acid sequence having at least 90% identity to any one of SEQ ID NOs: 4-5.

[000142] The enzyme having serine protease activity can comprise an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5.

[000143] The enzyme having serine protease activity can comprise an amino acid sequence having at least 98% identity to any one of SEQ ID NOs: 4-5.

[000144] The enzyme having serine protease activity can comprise an amino acid sequence having at least 99% identity to any one of SEQ ID NOs: 4-5.

[000145] The enzyme having serine protease activity can comprise an amino acid sequence having 100% identity to any one of SEQ ID NOs: 4-5.

[000146] For example, the enzyme can comprise SEQ ID NOs: 4-5.

[000147] Alternatively, the enzyme can consist of SEQ ID NOs: 4-5.

[000148] Additionally, or alternatively, the enzyme having serine protease activity can comprise an amino acid sequence having at least one amino acid deletion relative to the sequence of a wild-type serine protease enzyme from a *Bacillus firmus* bacterium, wherein the amino acid deletion retains the catalytic residues of the wild-type enzyme and results in the same or increased serine protease activity as compared to the serine protease activity of the wild-type serine protease enzyme under the same conditions. In one embodiment the wild-type serine protease enzyme is Sep1 from *Bacillus firmus* DS-1. See Geng, et al., 2016, above.

[000149] In one embodiment, the enzyme has increased serine protease activity as compared to the serine protease activity of the wild-type serine protease enzyme under the same conditions.

[000150] For example, the amino acid sequence of the enzyme can comprise SEQ ID NO: 6.

[000151] Alternatively or in addition, the enzyme having serine protease activity can comprise an amino acid sequence having at least 80% identity to SEQ ID NO: 6.

[000152] The enzyme having serine protease activity can comprise an amino acid sequence having at least 85% identity to SEQ ID NO: 6.

[000153] The enzyme having serine protease activity can comprise an amino acid sequence having at least 90% identity to SEQ ID NO: 6.

[000154] The enzyme having serine protease activity can comprise an amino acid sequence having at least 95% identity to SEQ ID NO: 6.

[000155] The enzyme having serine protease activity can comprise an amino acid sequence having at least 98% identity to SEQ ID NO: 6.

[000156] The enzyme having serine protease activity can comprise an amino acid sequence having at least 99% identity to SEQ ID NO: 6.

[000157] The enzyme having serine protease activity can comprise an amino acid sequence having 100% identity to SEQ ID NO: 6.

[000158] Alternatively, the enzyme can consist of SEQ ID NO: 6.

[000159] In addition, the enzyme having serine protease activity and having 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 6 maintains the deletion in SEQ ID NO: 6 (of amino acid 181-240 of SEQ ID NO: 5).

Optional Inclusion of Signal Peptides in the Fusion Proteins

[000160] When a fusion protein comprises an enzyme whose native sequence includes a signal peptide, the enzyme can be used without the signal peptide. Alternatively, the native signal peptide (or another signal peptide) can optionally be included at the amino terminus of the enzyme, immediately preceding the first amino acid of the enzyme sequence.

[000161] In addition, a signal peptide can optionally be included at the amino terminus of the enzymes whose native sequences do not include a signal peptide.

[000162] In any of the fusion proteins described herein, the enzyme having serine protease activity can further comprise a signal peptide.

[000163] Where the signal peptide is present, it is preferably present at the amino terminus of the enzyme having serine protease activity.

[000164] The signal peptide preferably immediately precedes the first amino acid of the enzyme having serine protease activity.

[000165] Where the fusion protein comprises a signal peptide, the signal peptide can be present at the amino terminus of the enzyme having serine protease activity.

Methods for Making the Fusion Proteins

[000166] Any of the fusion proteins described herein can be made using standard cloning and molecular biology methods known in the art. For example, a gene encoding a protein or peptide of interest (e.g., an enzyme having serine protease activity) can be amplified by polymerase chain reaction (PCR) or, alternatively, *de novo* synthesized, and ligated to DNA coding for any of the targeting sequences, exosporium proteins, or exosporium protein fragments described herein, to form a DNA molecule that encodes the fusion protein. The DNA molecule encoding the fusion protein can be cloned into any suitable vector, for example a plasmid vector. The vector suitably comprises a multiple cloning site into which the DNA molecule encoding the fusion protein can be easily inserted. The vector also suitably contains a selectable marker, such as an antibiotic resistance gene, such that bacteria transformed, transfected, or mated with the vector can be readily identified and isolated. Where the vector is a plasmid, the plasmid suitably also comprises an origin of replication. Alternatively, DNA coding for the fusion protein can be integrated into the chromosomal DNA of the *B. cereus* family member or spore-forming bacterium host.

Tags, Markers, and Linkers that Can Be Included in the Fusion Proteins

[000167] Any of the fusion proteins described herein can also comprise additional polypeptide sequences that are not part of the targeting sequence, exosporium protein, exosporium protein fragment, or the enzyme having serine protease activity. For example, the fusion protein can include tags or markers to facilitate purification or visualization of the fusion protein (e.g., a polyhistidine tag or a fluorescent protein such as GFP or YFP) or visualization of recombinant *Bacillus cereus* family member spores expressing the fusion protein.

[000168] Expression of fusion proteins on the exosporium of a *Bacillus cereus* family member using the targeting sequences, exosporium proteins, and exosporium protein fragments described herein is enhanced due to a lack of secondary structure in the amino-termini of these sequences, which allows for native folding of the fused proteins and retention of activity. Proper folding can be further enhanced by the inclusion of a short amino acid linker between the targeting sequence, exosporium protein, exosporium protein fragment, spore coat protein, and the enzyme having serine protease activity.

[000169] Thus, any of the fusion proteins described herein can comprise an amino acid linker between the targeting sequence, the exosporium protein, or the exosporium protein fragment and the enzyme having serine protease activity.

[000170] The linker can comprise a polyalanine linker or a polyglycine linker. A linker comprising a mixture of both alanine and glycine residues can also be used.

[000171] For example, in a fusion protein where the targeting sequence comprises SEQ ID NO: 1, a fusion protein can have one of the following structures:

No linker: SEQ ID NO: 1 – POI

Alanine Linker: SEQ ID NO: 1 –A_n–POI

Glycine Linker: SEQ ID NO: 1 –G_n–POI

Mixed Alanine and Glycine Linker: SEQ ID NO: 1 – $(A/G)_n$ – POI

where A_n, G_n, and $(A/G)_n$ are any number of alanines, any number of glycines, or any number of a mixture of alanines and glycines, respectively. For example, n can be 1 to 25, and is preferably 5 to 10. Where the linker comprises a mixture of alanine and glycine residues, any combination of glycine and alanine residues can be used. In the above structures, "POI" stands for "protein of interest" and represents the enzyme having serine protease activity.

[000172] Alternatively or in addition, the linker can comprise a protease recognition site. Inclusion of a protease recognition site allows for targeted removal, upon exposure to a protease that recognizes the protease recognition site, of the fusion protein containing the enzyme having serine protease activity.

[000173] Where the fusion protein comprises both a linker and signal peptide, the linker would typically be amino-terminal to the signal peptide. For example, where the fusion protein comprises SEQ ID NO: 2, a polyalanine linker, a signal sequence, and the serine protease of SEQ ID NO: 4, these elements would typically be arranged in the following order within the fusion protein, going from the amino-terminus of the fusion protein to the carboxy-terminus: SEQ ID NO: 2 – A_n–signal sequence–SEQ ID NO: 4.

Recombinant *Bacillus cereus* Family Members Hosts for Expression of the Fusion Proteins

[000174] The invention further relates to recombinant *Bacillus cereus* family members that express a fusion protein. The fusion protein can be any of the fusion proteins described above.

[000175] The recombinant *Bacillus cereus* family member can comprise any *Bacillus* species that is capable of producing an exosporium. For example, the recombinant *Bacillus cereus* family member can comprise *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus samanii, Bacillus gaemokensis, Bacillus weihenstephensis*, *Bacillus toyoiensis*, or a combination of any thereof. The recombinant *Bacillus cereus* family member suitably comprises *Bacillus thuringiensis* or *Bacillus mycoides*.

[000176] To generate a recombinant *Bacillus cereus* family member expressing a fusion protein, any *Bacillus cereus* family member can be conjugated, transduced, or transformed with a vector encoding the fusion protein using standard methods known in the art (e.g., by electroporation). The bacteria can then be screened to identify transformants by any method known in the art. For example, where the vector includes an antibiotic resistance gene, the bacteria can be screened for antibiotic resistance. Alternatively, DNA encoding the fusion protein can be integrated into the chromosomal DNA of a *B. cereus* family member host. The recombinant *Bacillus cereus* family member can then exposed to conditions which will induce sporulation. Suitable conditions for inducing sporulation are known in the art. For example, the recombinant *Bacillus cereus* family member can be plated onto agar plates, and incubated at a temperature of about 30°C for several days (e.g., 3 days).

[000177] Thus, the recombinant *Bacillus cereus* family member can be in the form of a spore.

[000178] Inactivated strains, non-toxic strains, or genetically manipulated strains of any of the above species can also suitably be used. For example, a *Bacillus thuringiensis* that lacks the Cry toxin can be used. Alternatively or in addition, once the recombinant *B. cereus* family member spores expressing the fusion protein have been generated, they can be inactivated to

prevent further germination once in use. Any method for inactivating bacterial spores that is known in the art can be used. Suitable methods include, without limitation, heat treatment, gamma irradiation, x-ray irradiation, UV-A irradiation, UV-B irradiation, chemical treatment (e.g., treatment with glutaraldehyde, formaldehyde, hydrogen peroxide, acetic acid, bleach, or any combination thereof), or a combination thereof. Alternatively, spores derived from nontoxigenic strains, or genetically or physically inactivated strains, can be used.

[000179] Thus, the recombinant *Bacillus cereus* family member can be in the form of a spore, wherein the spore is inactivated.

[000180] The recombinant *Bacillus cereus* family member can coexpress two or more of any of the fusion proteins described herein. For example, the recombinant *Bacillus cereus* family member can coexpress at least one fusion protein that comprises SEQ ID NO: 4 together with a fusion protein that comprises SEQ ID NO: 6.

[000181] Many *Bacillus cereus* family member strains have inherent beneficial attributes. For example, some strains have plant-growth promoting effects. Other strains are endophytic. Some strains are both endophytic and have plant-growth promoting effects.

[000182] Thus, any of the recombinant *Bacillus cereus* family members described herein can comprise a plant-growth promoting strain of bacteria, an endophytic strain of bacteria, or a strain of bacteria that is both plant-growth promoting and endophytic.

[000183] The plant-growth promoting strain of bacteria can comprise a strain of bacteria that produces an insecticidal toxin (e.g., a Cry toxin), produces a fungicidal compound (e.g., a β-1,3-glucanase, a chitosanase, a lyticase, or a combination of any thereof), produces a nematocidal compound (e.g., a Cry toxin), produces a bacteriocidal compound, is resistant to one or more antibiotics, comprises one or more freely replicating plasmids, binds to plant roots, colonizes plant roots, forms biofilms, solubilizes nutrients, secretes organic acids, or any combination thereof.

[000184] The recombinant *Bacillus cereus* family member can comprises an endophytic strain of bacteria.

[000185] The recombinant *Bacillus cereus* family member can comprise an inactivating mutation in its BclA gene, its CotE gene, or its CotO gene (e.g., a knock-out of the BclA gene, CotE gene, or CotO gene). For example, the recombinant *Bacillus cereus* family member can comprise an inactivating mutation in its BclA gene (e.g., a knock-out of the BclA gene). It has been found that expression of fusion proteins in a recombinant *Bacillus cereus* family member having such a mutation results in increased expression levels of the fusion protein.

[000186] Compositions of the present invention include cultures, such as whole broth cultures, of the strains described herein. The term culture refers to a population of cells growing in the absence of other species in a predetermined culture media under controlled laboratory or manufacturing conditions. Biologically pure cultures of the recombinant *Bacillus cereus* family members of the present invention may be obtained according to methods well known in the art.

[000187] Conventional large-scale microbial culture processes include submerged fermentation, solid state fermentation, or liquid surface culture. During the fermentation, as nutrients are depleted, cells begin the transition from growth phase to sporulation phase, such that the final product of fermentation is largely spores, metabolites and residual fermentation medium. Sporulation is part of the natural life cycle of *Bacillus cereus* family members and is generally initiated by the cell in response to stressful environmental conditions, such as nutrient limitation. Fermentation is configured to obtain high levels of colony forming units and to promote sporulation. The bacterial cells, spores and metabolites in culture media resulting from fermentation may be used directly or concentrated by conventional industrial methods, such as centrifugation or filtration such as tangential-flow filtration or depth filtration, and evaporation.

[000188] Compositions of the present invention include the products of the microbial culture processes described herein. In embodiments in which submerged fermentation is used as the culture process, the product is referred to as a "fermentation broth" or a "whole broth culture." Such broth may be concentrated, as described above. The concentrated fermentation broth may be washed, for example, via a diafiltration process, to remove residual fermentation broth and metabolites. The term "broth concentrate," as used herein, refers to fermentation broth that has been concentrated by conventional industrial methods, as described above, but remains in liquid form. The term "fermentation product," as used herein, refers to fermentation broth or whole broth culture, broth concentrate and/or dried fermentation broth or broth concentrate.

[000189] The fermentation broth or broth concentrate can be dried with or without the addition of carriers using conventional drying processes or methods such as spray drying, freeze drying, tray drying, fluidized-bed drying, drum drying, or evaporation. The term "fermentation product," as used herein, refers to fermentation broth or whole broth culture, broth concentrate and/or dried fermentation broth or broth concentrate.

[000190] The resulting dry products may be further processed, such as by milling or granulation, to achieve a specific particle size or physical format. Carriers, described below, may also be added post-drying.

[000191] Cell-free preparations of fermentation broth of the strains of the present invention can be obtained by any means known in the art, such as extraction, centrifugation and/or filtration of fermentation broth. Those of skill in the art will appreciate that so-called cell-free preparations may not be devoid of cells but rather are largely cell-free or essentially cell-free, depending on the technique used (e.g., speed of centrifugation) to remove the cells. The resulting cell-free preparation may be dried and/or formulated with components that aid in its application to plants or to plant growth media. Concentration methods and drying techniques described above for fermentation broth are also applicable to cell-free preparations.

[000192] As described further below, the recombinant *Bacillus cereus* family member can comprise a mutation or other modification that allows for collection of exosporium fragments comprising the fusion proteins from spores of the recombinant *Bacillus cereus* family member.

Promoters for Expression of Fusion Proteins in Recombinant *Bacillus cereus* Family Members

[000193] The DNA encoding the fusion proteins used in the recombinant *Bacillus cereus* family members, exosporium fragments, formulations, plant seeds, and methods, described herein is suitably under the control of a sporulation promoter which will cause expression of the fusion protein on the exosporium of a *B. cereus* family member endospore (e.g., a native *bclA* promoter from a *B. cereus* family member).

[000194] Thus, any of the fusion proteins described above can be expressed in the recombinant *Bacillus cereus* family member under the control of a sporulation promoter that is native to the targeting sequence, exosporium protein, or exosporium protein fragment of the fusion protein, or a portion of such a promoter.

[000195] Any of the fusion proteins can be expressed under the control of a high-expression sporulation promoter.

[000196] The high-expression sporulation promoter can comprise a sigma-K sporulation-specific polymerase promoter sequence.

[000197] For ease of reference, illustrative nucleotide sequences for promoters that can be used to express any of the fusion proteins in a recombinant *Bacillus cereus* family member are provided in **Table 3** below, together with their SEQ ID NOs. **Table 3** also provides illustrative minimal promoter sequences for many of the promoters. In **Table 3**, sigma-K sporulation-specific polymerase promoter sequences in the promoters are indicated by bold and underlined text. The promoter sequences are immediately upstream of the start codon for each

of the indicated genes. In other words, in the sequences shown in **Table 3** below, the last nucleotide of the promoter sequence immediately precedes the first nucleotide of the start codon for the coding region of the gene encoding the indicated protein.

Table 3. Promoter Sequences for Expression of Fusion Proteins in Recombinant *Bacillus cereus* Family Members

Promoter (SEQ ID NO:)	Promoter Sequence
ExsY promoter (*B. cereus* F837/76) (SEQ ID NO: 7)	TTTCTTAATCCTTTACCCTTTACTTTTGTAAAAGTTGATACACTTCCATCCGGCTCTGTAATTTCTAA TTCATCAATAAATGGTCTTCGCAAAAAGCCTGTAATTTTATCATAAACAATTAAACGAGTGAGCCT AAAAGCAGCTAACGCGAAAATAAAAAATAAAAGCCAGCTTGTAAACAGCATAATTCCACCTTCCC TTATCCTCTTTCGCCTATTTAAAAAAAGGTCTTGAGATTGTGACCAAATCTCCTCAACTCC**AATATC TTA**TTAATGTAAATACAAACAAGAAGATAAGGA
ExsY minimal promoter (*B. cereus* F837/76) (SEQ ID NO: 8)	ACCAAATCTCCTCAACTCC**AATATCTTA**TTAATGTAAATACAAACAAGAAGATAAGGA
CotY/CotZ promoter (*B. anthracis* Sterne) (SEQ ID NO: 9)	TAGAAGAAGAACGCCGACTACTTTATGTCGCAATTACACGGGCGAAAGAAGAACTTTACATTTCCT CTCCGCAATTTTTTAGAGGAAAAAAATTAGATATATCTCGTTTTTTATACACTGTGCGAAAAGATTT ACCTGAAAAGACATCCACTAAATAAGGATGTCTTTTTTTATATTGTATTATGTACATCCCTACTATA TAAATTCCCTGCTTTTATCGTAAGAATTAACGTAATATCAACCATATCCCGTT**CATATTGTA**GTAGT GTATGTCAGAACTCACGAGAAGGAGTGAACATA
CotY/CotZ minimal promoter (*B. anthracis* Sterne) (SEQ ID NO: 10)	TCAACCATATCCCGTT**CATATTGTA**GTAGTGTATGTCAGAACTCACGAGAAGGAGTGAACATA
BclA promoter (*B. cereus* F837/76) (SEQ ID NO: 11)	ATCGATGGAACCTGTATCAACCACTATAATTTCATCCACAATTTTTTCAACTGAGTCTAAACAACG GGCTATTGTCTTCTCCTCATCTCGAACAAT**CATACATAAACTA**ATTGTAATTCCTTGCTTGTTCAAC ATAATCACCCTCTTCCAAATCAAT**CATATGTTA**TA**CATATACTA**AACTTTCCATTTTTTTAAATTGT TCAAGTAGTTTAAGATTTCTTTTCAATAATTCAAATGTCCGTGTCATTTTCTTTCGGTTTTG**CATCTA CTA**TATAATGAACGCTTTATGGAGGTGAATTT
BclA minimal promoter (*B. cereus* F837/76)	AATCAAT**CATATGTTA**TA**CATATACTA**AACTTTCCATTTTTTTAAATTGTTCAAGTAGTTTAAGATT TCTTTTCAATAATTCAAATGTCCGTGTCATTTTCTTTCGGTTTTG**CATCTACTA**TATAATGAACGCT TTATGGAGGTGAATTT

Promoter (SEQ ID NO:)	**Promoter Sequence**
(SEQ ID NO: 12)	
BclA promoter (*B. anthracis* Sterne) (SEQ ID NO: 13)	TAATCACCCTCTTCCAAATCAAT**CATATGTTA**TA**CATATACTA**AACTTTCCATTTTTTTAAATTGT TCAAGTAGTTTAAGATTTCTTTTCAATAATTCAAATGTCCGTGTCATTTTCTTTCGGTTTTG**CATCT ACTA**TATAATGAACGCTTTATGGAGGTGAATTT

[000198] The sigma-K sporulation-specific polymerase promoter sequences in the promoter sequences shown in **Table 3** result in high expression levels of the fusion protein during late sporulation. The consensus sequence for the sigma-K sporulation-specific polymerase promoter sequence is CATANNNTN (SEQ ID NO: 14); however, this sequence can comprise up to two mutations and still be functional. The sigma-K sporulation-specific polymerase promoter sequence is generally found upstream of the ribosome binding site (RBS).

[000199] Promoters having a high degree of sequence identity to any of the sequences shown above in **Table 3** can also be used to express the fusion proteins.

[000200] For example, fusion protein can be expressed under the control of a BclA promoter, a CotY promoter, an ExsY promoter, or a promoter having a high degree of sequence identity to any of these promoters.

[000201] Thus, for example, the fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 80% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000202] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 85% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000203] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 90% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000204] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 95% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000205] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 98% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000206] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having at least 99% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000207] The fusion protein can be expressed under the control of a promoter comprising a nucleic acid sequence having 100% identity with a nucleic acid sequence of any one of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000208] The fusion protein can be expressed under the control of a promoter comprising a sigma-K sporulation specific polymerase promoter sequence, wherein the sigma-K

sporulation-specific polymerase promoter sequence or sequences have 100% identity with the corresponding nucleotides of any of SEQ ID NOs: 7, 8, 9, 10, 11, 12, or 13.

[000209] The fusion proteins can be expressed under the control of a promoter that is native to the targeting sequence, exosporium protein, or exosporium protein fragment of the fusion protein. Thus, for example, where the targeting sequence is derived from BclA, the fusion protein can be expressed under the control of a native BclA promoter (e.g., SEQ ID NO: 11, or 12).

[000210] **Table 3** also provides illustrative minimal promoter sequences. The fusion proteins can be expressed under any of these minimal promoter sequences.

[000211] Furthermore, the fusion protein can be expressed under a portion of any of the promoters listed above in **Table 3**, so long as the portion of the promoter includes a sigma-K sporulation-specific polymerase promoter sequence. For example, the fusion protein can be expressed under a promoter region that comprises the first 25, 50, 100, 150, 200, 250, or 300 nucleotides upstream of the start codon, so long as that region comprises a sigma-K sporulation-specific polymerase promoter sequence.

Mutations and Other Genetic Alterations to Recombinant *Bacillus cereus* Family Members that Allow for Collection of Free Exosporium and Exosporium Fragments Derived from Such Recombinant *Bacillus cereus* Family Members

[000212] As is described further hereinbelow, the recombinant *Bacillus cereus* family members that express fusion proteins comprising a protein or peptide of interest (e.g., an enzyme having serine protease activity) and a targeting sequence, an exosporium protein, or an exosporium protein fragment that targets the fusion protein to the exosporium of the recombinant *Bacillus cereus* family member can be used for various purposes, including delivering the proteins or peptides of interest plants, seeds, a plant growth medium, or an area surrounding a seed or a plant (e.g., via soil drench, foliar application, or as a seed treatment). However, in some cases, the presence of the living microorganisms may not be desirable, and instead, it would be desirable to separate the living spore from the fusion proteins in the exosporium on the outside surface of the spore. For example, in some applications it will be desirable to increase enzyme activity without concern for spore integrity. In such situations, use of exosporium fragments that have been separated from the spores may be preferred over the use of living microorganisms having the enzyme on their exosporium.

[000213] In addition, for some uses, it may be desirable to reduce the density of the product. In such instances, it would be desirable to separate the dense spore from the

exosporium (containing the fusion proteins). Furthermore, under some circumstances the presence of live spores would lead to potential for bacterial growth in a product, which would be undesirable for some applications.

[000214] Mutations or other genetic alterations (e.g., overexpression of a protein) can be introduced into the recombinant *Bacillus cereus* family members that allow free exosporium to be separated from spores of the recombinant *Bacillus cereus* family member. This separation process yields exosporium fragments that contain the fusion proteins but that are substantially free of the spores themselves. By "substantially free of spores" it is meant that once the free exosporium is separated from the spores, a preparation is obtained that contains less than 5% by volume of spores, preferably less than 3% by volume of spores, even more preferably less than 1% by volume of spores, and most preferably contains no spores or if spores are present, they are undetectable. These exosporium fragments can be used in place of the recombinant *Bacillus cereus* family members themselves in any of the formulations, plant seeds, and methods described herein.

[000215] Exosporium fragments derived from spores of a recombinant *Bacillus cereus* family member can be used in any of the formulations, plant seeds, and methods described herein. The recombinant *Bacillus cereus* family member expresses any of the fusion proteins described herein. The recombinant *Bacillus cereus* family member also comprises a mutation or expresses a protein, wherein the expression of the protein is increased as compared to the expression of the protein in a wild-type *Bacillus cereus* family member under the same conditions. The mutation or the increased expression of the protein results in *Bacillus cereus* family member spores having an exosporium that is easier to remove from the spore as compared to the exosporium of a wild-type spore.

[000216] The recombinant *Bacillus cereus* family member: (i) can comprise a mutation in a CotE gene; (ii) can express an ExsY protein, wherein the expression of the ExsY protein is increased as compared to the expression of the ExsY protein in a wild-type *Bacillus cereus* family member under the same conditions, and wherein the ExsY protein comprises a carboxy-terminal tag comprising a globular protein; (iii) can express a BclB protein, wherein the expression of the BclB protein is increased as compared to the expression of the BclB protein in a wild-type *Bacillus cereus* family member under the same conditions; (iv) can express a YjcB protein, wherein the expression of the YjcB protein is increased as compared to the expression of the YjcB protein in a wild-type *Bacillus cereus* family member under the same conditions; (v) can comprise a mutation in an ExsY gene; (vi) can comprise a mutation in

a CotY gene; (vii) can comprise a mutation in an ExsA gene; or (viii) can comprise a mutation in a CotO gene.

[000217] The recombinant *Bacillus cereus* family member can comprise a mutation in the CotE gene, such as a knock-out of the CotE gene or a dominant negative form of the CotE gene. The mutation in the CotE gene can partially or completely inhibit the ability of CotE to attach the exosporium to the spore.

[000218] The recombinant *Bacillus cereus* family member can express an ExsY protein. The ExsY protein comprises a carboxy-terminal tag comprising a globular protein (e.g., a green fluorescent protein (GFP) or a variant thereof), and the expression of the ExsY protein is increased as compared to the expression of the ExsY protein in a wild-type *Bacillus cereus* family member under the same conditions. The globular protein can have a molecular weight of between 25 kDa and 100 kDa. Expression of the ExsY protein comprising the carboxy-terminal tag comprising a globular protein can inhibit binding of the ExsY protein to its targets in the exosporium.

[000219] The recombinant *Bacillus cereus* family member can express a BclB protein. Expression of the BclB protein can result in the formation of a fragile exosporium. The expression of the BclB protein can be increased as compared to the expression of the BclB protein in a wild-type *Bacillus cereus* family member under the same conditions.

[000220] The recombinant *Bacillus cereus* family member can express a YjcB protein. Expression of the YjcB protein can cause the exosporium to form in pieces rather than in a complete structure. The expression of the YjcB protein can be increased as compared to the expression of the YjcB protein in a wild-type *Bacillus cereus* family member under the same conditions.

[000221] The recombinant *Bacillus cereus* family member can comprise a mutation an ExsY gene, such as a knock-out of the ExsY gene. The mutation in the ExsY gene can partially or completely inhibit the ability of ExsY to complete the formation of the exosporium or attach the exosporium to the spore.

[000222] The recombinant *Bacillus cereus* family member can comprise a mutation a CotY gene, such as a knock-out of the CotY gene. The mutation in the CotY gene can result in the formation of a fragile exosporium.

[000223] The recombinant *Bacillus cereus* family member can comprise a mutation an ExsA gene, such as a knock-out of the ExsA gene. The mutation in the ExsA gene can result in the formation of a fragile exosporium.

[000224] The recombinant *Bacillus cereus* family member can comprise a mutation a CotO gene, such as a knock-out of the CotO gene or a dominant negative form of the CotO gene. The mutation in the CotO gene can cause the exosporium to form in strips.

[000225] For ease of reference, descriptions of illustrative sequences for CotE, ExsY, BclB, YjcB, CotY, ExsA, and CotO are provided in **Table 4** below.

Table 4. Sequences of Proteins that Can be Mutated or Otherwise Genetically Altered to Allow for Collection of Free Exosporium

Protein	SEQ ID NO:
CotE, *Bacillus cereus* group	15
ExsY, *Bacillus thuringiensis*	16
CotY, *Bacillus cereus*	17
CotO, *Bacillus anthracis*	18

[000226] Exosporium fragments can be prepared from any of these recombinant *Bacillus cereus* family members and used for various purposes as described further herein below. Where the recombinant *Bacillus cereus* family member expresses a fusion protein, the exosporium fragments will comprise the fusion proteins. Upon purification of the exosporium fragments that contain the fusion proteins from the spores, a cell-free protein preparation is obtained in which the fusion proteins are stabilized and supported through covalent bonds to the exosporium fragments.

[000227] To remove the exosporium from spores of the recombinant *Bacillus cereus* family members that have mutations or other genetic alterations that allow for collection of free exosporium, a suspension or fermentation broth of the spores can be subjected to centrifugation or filtration to produce fragments of exosporium that are separated from the spores. Where the recombinant *Bacillus cereus* family member expresses a fusion protein, the exosporium fragments will comprise the fusion protein.

[000228] A suspension or fermentation broth comprising the spores can be subjected to centrifugation, followed by collection of the supernatant. The supernatant comprises the fragments of the exosporium and is substantially free of spores.

[000229] Alternatively, a suspension or fermentation broth comprising the spores can be subjected to filtration, followed by collection of the filtrate. The filtrate comprises the fragments of the exosporium and is substantially free of spores.

[000230] The suspension or fermentation broth of spores can be agitated or mechanically disrupted prior to centrifugation or filtration.

[000231] The exosporium fragments can also be separated from the spores by gradient centrifugation, affinity purification, or by allowing the spores to settle out of the suspension.

[000232] Due to the strong covalent bonds between the fusion proteins and the exosporium fragments, the fusion proteins become resistant to heat. The heat resistance of the fusion proteins bound to the exosporium fragments allows them to be used for applications that require heat-resistant proteins or enzymes.

[000233] Exosporium fragments derived from a recombinant *Bacillus cereus* family member are provided.

[000234] The exosporium fragments can be derived from any of the recombinant *Bacillus cereus* family members that comprise any of the mutations or other genetic alterations described herein that allow for collection of free exosporium.

[000235] The exosporium fragments can comprise any of the fusion proteins described above.

Insecticides

[000236] The composition according to the present invention comprises at least one particular insecticide disclosed herein.

[000237] "Insecticides" as well as the term "insecticidal" refers to the ability of a substance to increase mortality or inhibit growth rate of insects. As used herein, the term "insects" includes all organisms in the class "Insecta". The term "pre-adult" insects refers to any form of an organism prior to the adult stage, including, for example, eggs, larvae, and nymphs. As used herein, the terms "insecticide" and "insecticidal" also encompass "nematicide" and "nematicidal" and "acaricide" and "acaricidal."

[000238] "Nematicides" and "nematicidal" refers to the ability of a substance to increase mortality or inhibit the growth rate of nematodes. In general, the term "nematode" comprises eggs, larvae, juvenile and mature forms of said organism.

[000239] "Acaricide" and "acaricidal" refers to the ability of a substance to increase mortality or inhibit growth rate of ectoparasites belonging to the class Arachnida, sub-class Acari.

[000240] The active ingredients specified herein by their "common name" are known and described, for example, in the Pesticide Manual ("The Pesticide Manual", 14th Ed., British Crop Protection Council 2006) or can be searched in the internet (e.g., http://www.alanwood.net/pesticides).

[000241] In some embodiments, insecticides are selected from the group consisting of (1) Acetylcholinesterase (AChE) inhibitors, for example carbamates, e.g., Alanycarb, Aldicarb, Bendiocarb, Benfuracarb, Butocarboxim, Butoxycarboxim, Carbaryl, Carbofuran, Carbosulfan, Ethiofencarb, Fenobucarb, Formetanate, Furathiocarb, Isoprocarb, Methiocarb, Methomyl, Metolcarb, Oxamyl, Pirimicarb, Propoxur, Thiodicarb, Thiofanox, Triazamate, Trimethacarb, XMC and Xylylcarb or organophosphates, e.g., Acephate, Azamethiphos, Azinphos-ethyl, Azinphos-methyl, Cadusafos, Chlorethoxyfos, Chlorfenvinphos, Chlormephos, Chlorpyrifos, Chlorpyrifos-methyl, Coumaphos, Cyanophos, Demeton-S-methyl, Diazinon, Dichlorvos/DDVP, Dicrotophos, Dimethoate, Dimethylvinphos, Disulfoton, EPN, Ethion, Ethoprophos, Famphur, Fenamiphos, Fenitrothion, Fenthion, Fosthiazate, Heptenophos, Imicyafos, Isofenphos, Isopropyl O-(methoxyaminothio-phosphoryl)salicylate, Isoxathion, Malathion, Mecarbam, Methamidophos, Methidathion, Mevinphos, Monocrotophos, Naled, Omethoate, Oxydemeton-methyl, Parathion, Parathion-methyl, Phenthoate, Phorate, Phosalone, Phosmet, Phosphamidon, Phoxim, Pirimiphos-methyl, Profenofos, Propetamphos, Prothiofos, Pyraclofos, Pyridaphenthion, Quinalphos, Sulfotep, Tebupirimfos, Temephos, Terbufos, Tetrachlorvinphos, Thiometon, Triazophos, Trichlorfon and Vamidothion;

[000242] (2) GABA-gated chloride channel antagonists, for example cyclodiene organochlorines, e.g., Chlordane and Endosulfan, or phenylpyrazoles (fiproles), e.g., Ethiprole and Fipronil;

[000243] (3) Sodium channel modulators / voltage-dependent sodium channel blockers, for example pyrethroids, e.g,. Acrinathrin, Allethrin, d-cis-trans Allethrin, d-trans Allethrin, Bifenthrin, Bioallethrin, Bioallethrin S-cyclopentenyl isomer, Bioresmethrin, Cycloprothrin, Cyfluthrin, beta-Cyfluthrin, Cyhalothrin, lambda-Cyhalothrin, gamma-Cyhalothrin, Cypermethrin, alpha-Cypermethrin, beta-Cypermethrin, theta-Cypermethrin, zeta-Cypermethrin, Cyphenothrin [(1R)-trans isomers], Deltamethrin, Empenthrin [(EZ)-(1R) isomers), Esfenvalerate, Etofenprox, Fenpropathrin, Fenvalerate, Flucythrinate, Flumethrin, tau-Fluvalinate, Halfenprox, Imiprothrin, Kadethrin, Momfluorothrin, Permethrin, Phenothrin [(1R)-trans isomer), Prallethrin, Pyrethrine (pyrethrum), Resmethrin, Silafluofen, Tefluthrin, Tetramethrin, Tetramethrin [(1R) isomers)], Tralomethrin and Transfluthrin or DDT or Methoxychlor;

[000244] (4) Nicotinic acetylcholine receptor (nAChR) agonists, for example neonicotinoids, e.g., Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid and Thiamethoxam or Nicotine or Sulfoxaflor or Flupyridafurone;

[000245] (5) Nicotinic acetylcholine receptor (nAChR) allosteric activators, for example spinosyns, e.g., Spinetoram and Spinosad;

[000246] (6) Chloride channel activators, for example avermectins/milbemycins, e.g., Abamectin, Emamectin benzoate, Lepimectin and Milbemectin;

[000247] (7) Juvenile hormone mimics, for example juvenile hormon analogues, e.g., Hydroprene, Kinoprene and Methoprene or Fenoxycarb or Pyriproxyfen;

[000248] (8) Miscellaneous non-specific (multi-site) inhibitors, for example alkyl halides, e.g., Methyl bromide and other alkyl halides; or Chloropicrin or Sulfuryl fluoride or Borax or Tartar emetic;

[000249] (9) Selective homopteran feeding blockers, e.g., Pymetrozine or Flonicamid;

[000250] (10) Mite growth inhibitors, e.g., Clofentezine, Hexythiazox and Diflovidazin or Etoxazole;

[000251] (11) Microbial disruptors of insect midgut membranes, e.g., *Bacillus thuringiensis* subspecies *israelensis, Bacillus sphaericus, Bacillus thuringiensis* subspecies *aizawai, Bacillus thuringiensis* subspecies *kurstaki, Bacillus thuringiensis* subspecies *tenebrionis* and *Bt* crop proteins: Cry1Ab, Cry1Ac, Cry1Fa, Cry2Ab, mCry3A, Cry3Ab, Cry3Bb, Cry34/35Ab1;

[000252] (12) Inhibitors of mitochondrial ATP synthase, for example Diafenthiuron or organotin miticides, e.g., Azocyclotin, Cyhexatin and Fenbutatin oxide or Propargite or Tetradifon;

[000253] (13) Uncouplers of oxidative phoshorylation via disruption of the proton gradient, for example Chlorfenapyr, DNOC and Sulfluramid;

[000254] (14) Nicotinic acetylcholine receptor (nAChR) channel blockers, for example Bensultap, Cartap hydrochloride, Thiocyclam and Thiosultap-sodium;

[000255] (15) Inhibitors of chitin biosynthesis, type 0, for example Bistrifluron, Chlorfluazuron, Diflubenzuron, Flucycloxuron, Flufenoxuron, Hexaflumuron, Lufenuron, Novaluron, Noviflumuron, Teflubenzuron and Triflumuron;

[000256] (16) Inhibitors of chitin biosynthesis, type 1, for example Buprofezin;

[000257] (17) Moulting disruptors, for example Cyromazine;

[000258] (18) Ecdysone receptor agonists, for example Chromafenozide, Halofenozide, Methoxyfenozide and Tebufenozide;

[000259] (19) Octopamine receptor agonists, for example Amitraz;

[000260] (20) Mitochondrial complex III electron transport inhibitors, for example Hydramethylnon or Acequinocyl or Fluacrypyrim;

[000261] (21) Mitochondrial complex I electron transport inhibitors, for example METI acaricides, e.g., Fenazaquin, Fenpyroximate, Pyrimidifen, Pyridaben, Tebufenpyrad and Tolfenpyrad or Rotenone (Derris);

[000262] (22) Voltage-dependent sodium channel blockers, e.g., Indoxacarb or Metaflumizone;

[000263] (23) Inhibitors of acetyl CoA carboxylase, for example tetronic and tetramic acid derivatives, e.g., Spirobudiclofen, Spirodiclofen, Spiromesifen and Spirotetramat;

[000264] (24) Mitochondrial complex IV electron transport inhibitors, for example phosphines, e.g., Aluminium phosphide, Calcium phosphide, Phosphine and Zinc phosphide or Cyanide;

[000265] (25) Mitochondrial complex II electron transport inhibitors, for example Cyenopyrafen and Cyflumetofen;

[000266] (26) Ryanodine receptor modulators, for example diamides, e.g., Chlorantraniliprole, Cyantraniliprole, Flubendiamide and Tetrachloroantraniliprole.

[000267] Further insecticides with unknown or uncertain mode of action are, for example, Afidopyropen, Afoxolaner, Azadirachtin, Benclothiaz, Benzoximate, Bifenazate, Broflanilide, Bromopropylate, Chinomethionat, Cryolite, Cyclobutrifluram, Cyclaniliprole, Cycloxaprid, Cyhalodiamide Dicloromezotiaz, Dicofol, Diflovidazin, Flometoquin, Fluazaindolizine, Fluensulfone, Flufenerim, Flufenoxystrobin, Flufiprole, Fluhexafon, Fluopyram, Fluralaner, Fluxametamide, Fufenozide, Guadipyr, Heptafluthrin, Imidaclothiz, Iprodione, Lotilaner, Meperfluthrin, Paichongding, Pyflubumide, Pyridalyl, Pyrifluquinazon, Pyriminostrobin, Sarolaner, Tetramethylfluthrin, Tetraniliprole, Tetrachlorantraniliprole, Tioxazafen, Thiofluoximate, Triflumezopyrim and Iodomethane; furthermore products based on *Bacillus firmus* (including but not limited to strain CNCM I-1582, such as, for example, VOTIVO™, BioNem) or one of the following known active compounds: 1-{2-fluoro-4-methyl-5-[(2,2,2-trifluorethyl)sulfinyl]phenyl}-3-(trifluoromethyl)-1H-1,2,4-triazol-5-amine (known from WO 2006/043635), {1'-[(2E)-3-(4-chlorophenyl)prop-2-en-1-yl]-5-fluorospiro[indole-3,4'-piperidin]-1(2H)-yl}(2-chloropyridin-4-yl)methanone (known from WO 2003/106457), 2-chloro-N-[2-{1-[(2E)-3-(4-chlorophenyl)prop-2-en-1-yl]piperidin-4-yl}-4-(trifluoromethyl)phenyl]isonicotinamide (known from WO 2006/003494), 3-(2,5-dimethylphenyl)-4-hydroxy-8-methoxy-1,8-diazaspiro[4.5]dec-3-en-2-one (known from WO

2009/049851), 3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1,8-diazaspiro[4.5]dec-3-en-4-yl ethyl carbonate (known from WO 2009/049851), 4-(but-2-yn-1-yloxy)-6-(3,5-dimethylpiperidin-1-yl)-5-fluoropyrimidine (known from WO 2004/099160), 4-(but-2-yn-1-yloxy)-6-(3-chlorophenyl)pyrimidine (known from WO 2003/076415), PF1364 (CAS-Reg.No. 1204776-60-2), methyl 2-[2-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-5-chloro-3-methylbenzoyl]-2-methylhydrazinecarboxylate (known from WO 2005/085216), methyl 2-[2-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-5-cyano-3-methylbenzoyl]-2-ethylhydrazinecarboxylate (known from WO 2005/085216), methyl 2-[2-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-5-cyano-3-methylbenzoyl]-2-methylhydrazinecarboxylate (known from WO 2005/085216), methyl 2-[3,5-dibromo-2-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)benzoyl]-2-ethylhydrazinecarboxylate (known from WO 2005/085216), N-[2-(5-amino-1,3,4-thiadiazol-2-yl)-4-chloro-6-methylphenyl]-3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazole-5-carboxamide (known from CN 102057925), 8-chloro-N-[(2-chloro-5-methoxyphenyl)sulfonyl]-6-(trifluoromethyl)imidazo[1,2-a]pyridine-2-carboxamide (known from WO 2009/080250), N-[(2E)-1-[(6-chloropyridin-3-yl)methyl]pyridin-2(1H)-ylidene]-2,2,2-trifluoroacetamide (known from WO 2012/029672), 1-[(2-chloro-1,3-thiazol-5-yl)methyl]-4-oxo-3-phenyl-4H-pyrido[1,2-a]pyrimidin-1-ium-2-olate (known from WO 2009/099929), 1-[(6-chloropyridin-3-yl)methyl]-4-oxo-3-phenyl-4H-pyrido[1,2-a]pyrimidin-1-ium-2-olate (known from WO 2009/099929), 4-(3-{2,6-dichloro-4-[(3,3-dichloroprop-2-en-1-yl)oxy]phenoxy}propoxy)-2-methoxy-6-(trifluoromethyl)pyrimidine (known from CN 101337940), N-[2-(tert-butylcarbamoyl)-4-chloro-6-methylphenyl]-1-(3-chloropyridin-2-yl)-3-(fluoromethoxy)-1H-pyrazole-5-carboxamide (known from WO 2008/134969), butyl [2-(2,4-dichlorophenyl)-3-oxo-4-oxaspiro[4.5]dec-1-en-1-yl] carbonate (known from CN 102060818), 3E)-3-[1-[(6-chloro-3-pyridyl)methyl]-2-pyridylidene]-1,1,1-trifluoro-propan-2-one (known from WO 2013/144213), N-(methylsulfonyl)-6-[2-(pyridin-3-yl)-1,3-thiazol-5-yl]pyridine-2-carboxamide (known from WO 2012/000896), N-[3-(benzylcarbamoyl)-4-chlorophenyl]-1-methyl-3-(pentafluoroethyl)-4-(trifluoromethyl)-1H-pyrazole-5-carboxamide (known from WO 2010/051926), 5-bromo-4-chloro-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-2-(3-chloro-2-pyridyl)pyrazole-3-carboxamido (known from CN 103232431), Tioxazafen, 4-[5-(3,5-dichlorophenyl)-4,5-dihydro-5-(trifluoromethyl)-3-isoxazolyl]-2-methyl-*N*-(*cis*-1-oxido-3-thietanyl)-benzamide, 4-[5-(3,5-dichlorophenyl)-4,5-dihydro-5-(trifluoromethyl)-3-isoxazolyl]-2-methyl-*N*-(*trans*-1-oxido-3-thietanyl)-benzamide and 4-[(5*S*)-5-(3,5-dichlorophenyl)-4,5-dihydro-5-(trifluoromethyl)-3-isoxazolyl]-2-methyl-*N*-(*cis*-1-oxido-3-thietanyl)benzamide (known from WO 2013/050317

A1), *N*-[3-chloro-1-(3-pyridinyl)-1*H*-pyrazol-4-yl]-*N*-ethyl-3-[(3,3,3-trifluoropropyl)sulfinyl]-propanamide, (+)-*N*-[3-chloro-1-(3-pyridinyl)-1*H*-pyrazol-4-yl]-*N*-ethyl-3-[(3,3,3-trifluoropropyl)sulfinyl]-propanamide and (-)-*N*-[3-chloro-1-(3-pyridinyl)-1*H*-pyrazol-4-yl]-*N*-ethyl-3-[(3,3,3-trifluoropropyl)sulfinyl]-propanamide (known from WO 2013/162715 A2, WO 2013/162716 A2, US 2014/0213448 A1), 5-[[(2*E*)-3-chloro-2-propen-1-yl]amino]-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile (known from CN 101337937 A), 3-bromo-*N*-[4-chloro-2-methyl-6-[(methylamino) thioxomethyl]phenyl]-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide, (Liudaibenjiaxuanan, known from CN 103109816 A); *N*-[4-chloro-2-[[(1,1-dimethylethyl) amino]carbonyl]-6-methylphenyl]-1-(3-chloro-2-pyridinyl)-3-(fluoromethoxy)-1*H*-Pyrazole-5-carboxamide (known from WO 2012/034403 A1), *N*-[2-(5-amino-1,3,4-thiadiazol-2-yl)-4-chloro-6-methylphenyl]-3-bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide (known from WO 2011/085575 A1), 4-[3-[2,6-dichloro-4-[(3,3-dichloro-2-propen-1-yl)oxy]phenoxy] propoxy]-2-methoxy-6-(trifluoromethyl)-pyrimidine (known from CN 101337940 A); (2*E*)- and 2(*Z*)-2-[2-(4-cyanophenyl)-1-[3-(trifluoromethyl)phenyl]ethylidene]-*N*-[4-(difluoromethoxy) phenyl]-hydrazinecarboxamide (known from CN 101715774 A); 3-(2,2-dichloroethenyl)-2,2-dimethyl-4-(1*H*-benzimidazol-2-yl)phenyl-cyclopropanecarboxylic acid ester (known from CN 103524422 A); (4a*S*)-7-chloro-2,5-dihydro-2-[[(methoxycarbonyl)[4-[(trifluoromethyl)thio] phenyl]amino]carbonyl]-indeno[1,2-*e*][1,3,4]oxadiazine-4a(3*H*)-carboxylic acid methyl ester (known from CN 102391261 A).

[000268] Other insecticides that are used in the compositions of the present invention are biological products, such as the following.

[000269] *Bacillus thuringiensis subsp. aizawai*, in particular strain ABTS-1857 (SD-1372; e.g., XENTARI® from Valent BioSciences); *Bacillus mycoides*, isolate J. (e.g., BmJ from Certis USA LLC, a subsidiary of Mitsui & Co.); *Bacillus sphaericus*, in particular Serotype H5a5b strain 2362 (strain ABTS-1743) (e.g., VECTOLEX® from Valent BioSciences, US); *Bacillus thuringiensis* subsp. kurstaki strain BMP 123 from Becker Microbial Products, IL; *Bacillus thuringiensis* subsp. *aizawai*, in particular serotype H-7 (e.g., FLORBAC® WG from Valent BioSciences, U.S.); *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (e.g., DIPEL® ES from Valent BioSciences, U.S.); *Bacillus thuringiensis* subsp. *kurstaki* strain BMP 123 by Becker Microbial Products, IL; *Bacillus thuringiensis israelensis* strain BMP 144 (e.g., AQUABAC® by Becker Microbial Products IL); *Burkholderia* spp., in particular *Burkholderia rinojensis* strain A396 (also known as *Burkholderia rinojensis* strain MBI 305) (Accession No. NRRL B-50319; WO 2011/106491 and WO 2013/032693; e.g., MBI-206 TGAI and ZELTO®

from Marrone Bio Innovations or BIO$_{ST}$ from Albaugh); *Chromobacterium subtsugae*, in particular strain PRAA4-1T (MBI-203; e.g., GRANDEVO® from Marrone Bio Innovations); *Paenibacillus popilliae* (formerly *Bacillus popilliae*; e.g., MILKY SPORE POWDER™ and MILKY SPORE GRANULAR™ from St. Gabriel Laboratories); *Bacillus thuringiensis* subsp. *israelensis* (serotype H-14) strain AM65-52 (Accession No. ATCC 1276) (e.g., VECTOBAC® by Valent BioSciences, U.S.)

[000270] *Beauveria bassiana* strain ATCC 74040 (e.g., NATURALIS® from Biofa); *Beauveria bassiana* strain GHA (Accession No. ATCC74250; e.g., BOTANIGUARD® ES and MYCONTROL-O® from Laverlam International Corporation); *Beauveria bassiana* strain CG 716 (e.g., BOVEMAX® from Novozymes); *Beauveria bassiana* strain 147 (e.g., product OSTRINIL®); *Beauveria bassiana* strain NPP111B005; *Beauveria bassiana* strain PPRI 5339 (Accession No. NRRL 50757) (e.g., VELIFER® and BROADBAND® from BASF SE); *Beauveria bassiana* strain R444 (e.g., BB-PROTEC® from Andermatt Biocontrol), *Metarhizium anisopliae*, strain F52 (DSM3884/ ATCC 90448; e.g. Met52 by Novozymes); *Metarhizium anisopliae var acridum* strain ARSEF324 (e.g., GREEN MUSCLE® and GREEN GUARD® from BASF SE)); *Metarhizium anisopliae* var *acridum* isolate IMI 330189 (ARSEF7486); *Isaria fumosorosea* strain FE 9901 (e.g., NOFLY® from Koppert); *Beauveria brongniartii* (e.g., BEAUPRO® from Andermatt Biocontrol AG); *Lecanicillium lecanii* (formerly known as *Verticillium lecanii*) strain KV01 (e.g., MYCOTAL® from Koppert); *Metarhizium anisopliae* 3213-1 (deposited under NRRL accession number 67074) (WO 2017/066094; Pioneer Hi-Bred International).

[000271] *Bacillus subtilis*, in particular strain QST713/AQ713 (having NRRL Accession No. B-21661; available as SERENADE® OPTI or SERENADE® ASO from Bayer CropScience LP, US); *Bacillus pumilus*, in particular strain QST2808 (having Accession No. NRRL No. B-30087).

[000272] *Bacillus firmus*, in particular, strain CNMC I-1582 (e.g., VOTIVO® from BASF SE); *Bacillus amyloliquefaciens*, in particular strain FZB42 (e.g., RHIZOVITAL® from ABiTEP, DE); *Bacillus amyloliquefaciens* strain PTA-4838 (AVEO EZ® from Valent/Sumitomo; VARNIMO® ST from LidoChem); *Bacillus amyloliquefaciens* MBI600 and cis-Jasmone (2-Cyclopenten-1-one, 3-methyl-2-(2Z)-2-pentenyl) (TRUNEMCO® from Nufarm Americas, Inc.); *Bacillus cereus*, in particular spores of *Bacillus cereus* strain CNCM I-1562 (cf. U.S. Patent No. 6,406,690); *Bacillus laterosporus* (also known as *Brevibacillus laterosporus*; e.g., BIO-TODE® from Agro-Organics, ZA); *Bacillus megaterium*, strain YFM3.25 (e.g., BIOARC® from BioArc); *Bacillus mojavensis*, strain SR11 (CECT-7666 by Probelte S.A);

Bacillus nematocida B16 (CGMCC Accession No. 1128); a mixture of *Bacillus licheniformis* FMCH001 and *Bacillus subtilis* FMCH002 (available as QUARTZO® (WG), PRESENCE® (WP) from FMC Corporation); *Pasteuria nishizawae* (e.g., OYACYST® LF/ST from Pasteuria Bioscience; CLARIVA® PN from Syngenta/ChemChina); *Burkholderia rinojensis*, e.g., strain A396 (also known as *Burkholderia rinojensis* strain MBI 305) (Accession No. NRRL B-50319; WO 2011/106491 and WO 2013/032693; MAJESTENE® from Marrone Bio Innovations; also, e.g., BIO_{ST} from Albaugh); *Pasteuria penetrans* (formerly *Bacillus penetrans*; e.g., PASTEURIA™ Wettable Powder from Pasteuria Bioscience); *Pasteuria usgae* (e.g., ECONEM™ from Pasteuria Bioscience); *Streptomycete* sp., such as *Streptomyces lydicus* strain WYEC108 (also known as *Streptomyces lydicus* strain WYCD108US) (ACTINO-IRON® and ACTINOVATE® from Novozymes); *Streptomyces saraceticus* (e.g., CLANDA® from A & A Group (Agro Chemical Corp.)

[000273] *Purpureocillium lilacinum* strain 251 (AGAL 89/030550; e.g., BIOACT® DC from Bayer CropScience Biologics GmbH).

[000274] *Myrothecium verrucaria*, strain AARC-0255 (e.g., DITERA™ by Valent Biosciences); *Purpureocillium lilacinum* strain 580 (BIOSTAT® WP (ATCC No. 38740) by Laverlam), strain in the product BIO-NEMATON® (T.Stanes and Company Ltd.), strain in the product MYSIS® (Varsha Bioscience and Technology India Pvt Ltd.), strain in the product BIOICONEMA® (Nico Orgo Maures, India), strain in the product NEMAT® (Ballagro Agro Tecnologia Ltda, Brazil), and a strain in the product SPECTRUM PAE L® (Promotora Tecnica Industrial, S.A. DE C.V., Mexico).

[000275] Biological insecticides may also include non-microbial products, such as a terpene blend comrpsing as active ingredients substantially pure α-terpinene, substantially pure p-cymene and substantially pure limonene in a relative ratio of about 35-45:12-20:10-15 (e.g., REQUIEM® by Bayer CropScience LP, U.S.) and a composition comprising one or more fatty acids or derivatives thereof selected from unsaturated and saturated C_{12-24} fatty acids, salts thereof, esters thereof or mixtures of any of the foregoing, wherein at least 95% of said fatty acids or derivatives thereof are in the rage of C14 to C20 (e.g., FLIPPER® by AlphaBio Pesticides or Bayer AG).

Plant-Growth Promoting Agents

[000276] The composition according to the present invention comprises at least one particular biostimulant disclosed herein. Plant growth promoting active ingredients that can be used in the compositions of the present invention are listed below.

[000277] *Bacillus pumilus*, in particular strain QST2808 (having Accession No. NRRL No. B-30087); *Bacillus subtilis*, in particular strain QST713/AQ713 (having NRRL Accession No. B-21661 and described in U.S. Patent No. 6,060,051; available as SERENADE® OPTI or SERENADE® ASO from Bayer CropScience LP); *Bacillus subtilis*, in particular strain AQ30002 (having Accession Nos. NRRL B-50421 and described in U.S. Patent Application No. 13/330,576); *Bacillus subtilis*, in particular strain AQ30004 (and NRRL B-50455 and described in U.S. Patent Application No. 13/330,576); *Sinorhizobium meliloti* strain NRG-185-1 (NITRAGIN® GOLD from Bayer CropScience).

[000278] *Bacillus subtilis* strain BU1814, (available as TEQUALIS® from BASF SE); *Bacillus subtilis* rm303 (RHIZOMAX® from Biofilm Crop Protection); *Bacillus amyloliquefaciens* pm414 (LOLI-PEPTA® from Biofilm Crop Protection); *Bacillus mycoides* BT155 (Accession No. NRRL B-50921), *Bacillus mycoides* EE118 (Accession No. NRRL B-50918), *Bacillus mycoides* EE141 (Accession No. NRRL B-50916), *Bacillus mycoides* BT46-3 (Accession No. NRRL B-50922), *Bacillus cereus* family member EE128 (Accession No. NRRL B-50917), *Bacillus thuringiensis* BT013A (Accession No. NRRL B-50924) also known as *Bacillus thuringiensis* 4Q7, *Bacillus cereus* family member EE349 (Accession No. NRRL B-50928), *Bacillus amyloliquefaciens* SB3281 (Accession No. ATCC PTA-7542; WO 2017/205258), *Bacillus amyloliquefaciens* TJ1000 (available as QUIKROOTS® from Novozymes); *Bacillus firmus*, in particular strain CNMC I-1582 (e.g., VOTIVO® from BASF SE); *Bacillus pumilus*, in particular strain GB34 (e.g., YIELD SHIELD® from Bayer Crop Science, DE); *Bacillus amyloliquefaciens*, in particular strain IN937a; *Bacillus amyloliquefaciens*, in particular strain FZB42 (e.g., RHIZOVITAL® from ABiTEP, DE); *Bacillus amyloliquefaciens* BS27 (Accession No. NRRL B-5015); a mixture of *Bacillus licheniformis* FMCH001 and *Bacillus subtilis* FMCH002 (available as QUARTZO® (WG), PRESENCE® (WP) from FMC Corporation); *Bacillus cereus*, in particular strain BP01 (Accession No. ATCC 55675; e.g., MEPICHLOR® from Arysta Lifescience, US); *Bacillus subtilis*, in particular strain MBI 600 (e.g., SUBTILEX® from BASF SE); *Bradyrhizobium japonicum* (e.g., OPTIMIZE® from Novozymes); *Mesorhizobium cicer* (e.g., NODULATOR® from BASF SE); *Rhizobium leguminosarium biovar viciae* (e.g., NODULATOR® from BASF SE); *Delftia acidovorans*, in particular strain RAY209 (e.g., BIOBOOST® from Brett Young Seeds); *Lactobacillus* sp. (e.g., LACTOPLANT® from LactoPAFI); *Paenibacillus polymyxa*, in particular strain AC-1 (e.g., TOPSEED® from Green Biotech Company Ltd.); *Pseudomonas proradix* (e.g., PRORADIX® from Sourcon Padena); *Azospirillum brasilense* (e.g., VIGOR® from KALO, Inc.); *Azospirillum lipoferum* (e.g., VERTEX-IF™ from TerraMax, Inc.); a

mixture of *Azotobacter vinelandii* and *Clostridium pasteurianum* (available as INVIGORATE® from Agrinos).

[000279] *Purpureocillium lilacinum* (previously known as *Paecilomyces lilacinus*) strain 251 (AGAL 89/030550; e.g., BIOACT® DC from Bayer CropScience Biologics GmbH), *Penicillium bilaii*, strain ATCC 22348 (e.g., JUMPSTART® from Acceleron BioAg), *Talaromyces flavus*, strain V117b; *Trichoderma atroviride* strain CNCM I-1237 (e.g., ESQUIVE® WP from Agrauxine, FR), *Trichoderma viride*, e.g., strain B35 (Pietr et al., 1993, Zesz. Nauk. A R w Szczecinie 161: 125-137).

[000280] *Trichoderma atroviride* strain LC52 (also known as *Trichoderma atroviride* strain LU132; e.g., SENTINEL® from Agrimm Technologies Limited); *Trichoderma atroviride* strain SC1, having Accession No. CBS 122089, WO 2009/116106 and U.S. Patent No. 8,431,120 (e.g., VINTEC® from Bi-PA); *Trichoderma asperellum* strain kd (e.g., T-GRO® from Andermatt Biocontrol); *Trichoderma asperellum* strain Eco-T (Plant Health Products, ZA); *Trichoderma harzianum* strain T-22 (having Accession No. ATCC 20847; e.g., TRIANUM-P® from Andermatt Biocontrol or Koppert); *Myrothecium verrucaria* strain AARC-0255 (e.g., DITERA™ from Valent Biosciences); *Penicillium bilaii* strain ATCC 20851; *Pythium oligandrum* strain M1 (ATCC 38472; e.g., POLYVERSUM® from Biopreparty, CZ); *Trichoderma virens* strain GL-21 having Accession No. NRRL 15948 (e.g., SOILGARD® from Certis, USA); *Verticillium albo-atrum* (formerly *V. dahliae*) strain WCS850 (CBS 276.92; e.g., DUTCH TRIG® from Tree Care Innovations).

[000281] Examples of plant growth regulators that can be used in the compositions of the present invention are listed below.

[000282] Abscisic acid and related analogues [e.g., (2Z,4E)-5-[6-Ethynyl-1-hydroxy-2,6-dimethyl-4-oxocyclohex-2-en-1-yl]-3-methylpenta-2,4-dienoic acid, methyl-(2Z,4E)-5-[6-ethynyl-1-hydroxy-2,6-dimethyl-4-oxocyclohex-2-en-1-yl]-3-methylpenta-2,4-dienoate, (2Z,4E)-3-ethyl-5-(1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl)penta-2,4-dienoic acid, (2E,4E)-5-(1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl)-3-(trifluoromethyl)penta-2,4-dienoic acid, methyl (2E,4E)-5-(1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl)-3-(trifluoromethyl)penta-2,4-dienoate, (2Z,4E)-5-(2-hydroxy-1,3-dimethyl-5-oxobicyclo[4.1.0]hept-3-en-2-yl)-3-methylpenta-2,4-dienoic acid], acibenzolar, acibenzolar-S-methyl, S-adenosylhomocysteine, allantoin, 2-Aminoethoxyvinylglycine (AVG), aminooxyacetic acid and related esters [e.g., (Isopropylidene)-aminooxyacetic acid-2-(methoxy)-2-oxoethylester, (Isopropylidene)-aminooxyacetic acid-2-(hexyloxy)-2-oxoethylester, (Cyclohexylidene)-aminooxyacetic acid-2-(isopropyloxy)-2-oxoethylester], 1-aminocycloprop-1-yl carboxylic acid and derivatives thereof, e.g., disclosed in DE 3335514, EP 30287, DE 2906507 or U.S. Patent No. 5,123,951, 5-aminolevulinic acid, ancymidol, 6-benzylaminopurine, bikinin, brassinolide, brassinolide-ethyl, L-canaline, catechin and catechines (e.g., (2S,3R)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-chromen-3,5,7-triol), chitooligosaccharides (CO; COs differ from LCOs in that they lack the pendant fatty acid chain that is characteristic of LCOs. COs, sometimes referred to as N-acetylchitooligosaccharides, are

also composed of GlcNAc residues but have side chain decorations that make them different from chitin molecules [$(C_8H_{13}NO_5)_n$, CAS No. 1398-61-4] and chitosan molecules [$(C_5H_{11}NO_4)_n$, CAS No. 9012-76-4]), chitinous compounds, chlormequat chloride, cloprop, cyclanilide, 3-(Cycloprop-1-enyl)propionic acid, 1-[2-(4-cyano-3,5-dicyclopropylphenyl)acetamido]cyclohexanecarboxylic acid, 1-[2-(4-cyano-3-cyclopropylphenyl)acetamido]cyclohexanecarboxylic acid, daminozide, dazomet, dazomet-sodium, n-decanol, dikegulac, dikegulac-sodium, endothal, endothal-dipotassium, -disodium, and mono(N,N-dimethylalkylammonium), ethephon, flumetralin, flurenol, flurenol-butyl, flurenol-methyl, flurprimidol, forchlorfenuron, gibberellic acid, inabenfide, indol-3-acetic acid (IAA), 4-indol-3-ylbutyric acid, isoprothiolane, probenazole, jasmonic acid, Jasmonic acid or derivatives thereof (e.g., jasmonic acid methyl ester, jasmonic acid ethyl ester), lipo-chitooligosaccharides (LCO, sometimes referred to as symbiotic nodulation (Nod) signals (or Nod factors) or as Myc factors, consist of an oligosaccharide backbone of β-l,4-linked *N*-acetyl-D-glucosamine ("GlcNAc") residues with an N-linked fatty acyl chain condensed at the non-reducing end. As understood in the art, LCOs differ in the number of GlcNAc residues in the backbone, in the length and degree of saturation of the fatty acyl chain and in the substitutions of reducing and non-reducing sugar residues), linoleic acid or derivatives thereof, linolenic acid or derivatives thereof, maleic hydrazide, mepiquat chloride, mepiquat pentaborate, 1-methylcyclopropene, 3-methylcyclopropene, 1-ethylcyclopropene, 1-n-propylcyclopropene, 1-cyclopropenylmethanol, methoxyvinylglycin (MVG), 3'-methyl abscisic acid, 1-(4-methylphenyl)-N-(2-oxo-1-propyl-1,2,3,4-tetrahydroquinolin-6-yl)methanesulfonamide and related substituted tetrahydroquinolin-6-yl)methanesulfonamides, (3E,3aR,8bS)-3-({[(2R)-4-Methyl-5-oxo-2,5-dihydrofuran-2-yl]oxy}methylen)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one and related lactones as outlined in EP 2248421, 2-(1-naphthyl)acetamide, 1-naphthylacetic acid, 2-naphthyloxyacetic acid, nitrophenolate-mixture, 4-Oxo-4[(2-phenylethyl)amino]butyric acid, paclobutrazol, 4-phenylbutyric acid and its related salts (e.g., sodium-4-phenylbutanoate, potassium-4-phenylbutanoate), phenylalanine, N-phenylphthalamic acid, prohexadione, prohexadione-calcium, putrescine, prohydrojasmon, rhizobitoxin, salicylic acid, salicylic acid methyl ester, sarcosine, sodium cycloprop-1-en-1-yl acetate, sodium cycloprop-2-en-1-yl acetate, sodium-3-(cycloprop-2-en-1-yl)propanoate, sodium-3-(cycloprop-1-en-1-yl) propanoate, sidefungin, spermidine, spermine, strigolactone, tecnazene, thidiazuron, triacontanol, trinexapac, trinexapac-ethyl, tryptophan, tsitodef, uniconazole, uniconazole-P, 2-fluoro-N-(3-methoxyphenyl)-*9H*-purin-6-amine.

Compositions According to the Present Invention

[000283] According to the present invention the composition comprises a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) at least one plant growth stimulating protein or peptide selected from the group consisting of an enzyme involved in the production or activation of a plant growth stimulating compound; an enzyme that degrades or modifies a bacterial, fungal, or plant nutrient source; and a protein or peptide that

protects a plant from a pathogen; and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells; and b) at least one particular insecticide disclosed herein in a synergistically effective amount.

[000284] A "synergistically effective amount" according to the present invention represents a quantity of a combination of a recombinant exosporium-producing *Bacillus* cells that express a fusion protein and at least one insecticide as described herein that is more effective against insects, mites, nematodes and/or phytopathogens than a recombinant exosporium-producing *Bacillus* cells that express a fusion protein or the insecticide alone. A "synergistically effective amount" according to the present invention also represents a quantity of a combination of a recombinant exosporium-producing *Bacillus* cells that expresses a fusion protein and at least one particular insecticide disclosed herein that is more effective at enhancing plant growth and/or promoting plant health than the a recombinant exosporium-producing *Bacillus* cells that express a fusion protein or the insecticide alone.

[000285] The present invention comprises each and every combination of each of the particular insecticides and/or plant growth promoting active ingredients (i.e., biostimulants) disclosed herein with the recombinant exosporium-producing *Bacillus* cells.

[000286] In a highly preferred embodiment the present invention relates to a composition comprising: a) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) an enzyme with serine protease activity from *Bacillus firmus*, preferably having an amino acid sequence with at least 95% identity to SEQ ID NOs: 4-6 or exosporium fragments derived from such recombinant exosporium-producing *Bacillus* cells and (ii) a targeting sequence that localizes the fusion protein to the exosporium of the *Bacillus* cells; and b) at least one particular insecticide disclosed herein in a synergistically effective amount and the at least one insecticide is selected from the group consisting of acetamiprid, aldicarb, amitraz, beta-cyfluthrin, carbaryl, clothianidin, cyfluthrin, cypermethrin, deltamethrin, endosulfan, ethion, ethiprole, ethoprophos, fenamiphos, fenobucarb, fenthion, fipronil, flubendiamide, fluopyram, flupyradifurone, formetanate, heptanophos, imidacloprid, methamidophos, methiocarb, methomyl, niclosamide, oxydemeton-methyl, phosalone, silafluofen, spirodiclofen, spiromesifen, spirotetramat, thiacloprid, thiodicarb, tralomethrin, triazophos, triflumuron, vamidothion, 1-{2-fluoro-4-methyl-5-[(R)-(2,2,2-trifluoroethyl)sulphinyl]phenyl}-3-(trifluoromethyl)-1H-1,2,4-triazol-5-amine, and 1-(3-chloropyridin-2-yl)-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-3-{[5-(trifluoromethyl)-2H-tetrazol-2-yl]methyl}-1H-pyrazole-5-carboxamide in a synergistically effective amount.

[000287] In a preferred embodiment the composition according to the present invention further comprises at least one fungicide.

[000288] In general, "fungicidal" means the ability of a substance to increase mortality or inhibit the growth rate of fungi. The term "fungus" or "fungi" includes a wide variety of nucleated sporebearing organisms that are devoid of chlorophyll. Examples of fungi include yeasts, molds, mildews, rusts, and mushrooms.

Further Additives

[000289] One aspect of the present invention is to provide a composition as described above additionally comprising at least one auxiliary selected from the group consisting of extenders, solvents, spontaneity promoters, carriers, emulsifiers, dispersants, frost protectants, thickeners and adjuvants. Those compositions are referred to as formulations.

[000290] Accordingly, in one aspect of the present invention such formulations, and application forms prepared from them, are provided as crop protection agents and/or pesticidal agents, such as drench, drip and spray liquors, comprising the composition of the invention. The application forms may comprise further crop protection agents and/or pesticidal agents, and/or activity-enhancing adjuvants such as penetrants, examples being vegetable oils such as, for example, rapeseed oil, sunflower oil, mineral oils such as, for example, liquid paraffins, alkyl esters of vegetable fatty acids, such as rapeseed oil or soybean oil methyl esters, or alkanol alkoxylates, and/or spreaders such as, for example, alkylsiloxanes and/or salts, examples being organic or inorganic ammonium or phosphonium salts, examples being ammonium sulphate or diammonium hydrogen phosphate, and/or retention promoters such as dioctyl sulphosuccinate or hydroxypropylguar polymers and/or humectants such as glycerol and/or fertilizers such as ammonium, potassium or phosphorous fertilizers, for example.

[000291] Examples of typical formulations include water-soluble liquids (SL), emulsifiable concentrates (EC), emulsions in water (EW), suspension concentrates (SC, SE, FS, OD), water-dispersible granules (WG), granules (GR) and capsule concentrates (CS); these and other possible types of formulation are described, for example, by Crop Life International and in Pesticide Specifications, Manual on Development and Use of FAO and WHO Specifications for Pesticides, FAO Plant Production and Protection Papers – 173, prepared by the FAO/WHO Joint Meeting on Pesticide Specifications, 2004, ISBN: 9251048576. The formulations may comprise active agrochemical compounds other than one or more active compounds of the invention.

[000292] The formulations or application forms in question preferably comprise auxiliaries, such as extenders, solvents, spontaneity promoters, carriers, emulsifiers, dispersants,

frost protectants, biocides, thickeners and/or other auxiliaries, such as adjuvants, for example. An adjuvant in this context is a component which enhances the biological effect of the formulation, without the component itself having a biological effect. Examples of adjuvants are agents which promote the retention, spreading, attachment to the leaf surface, or penetration.

[000293] These formulations are produced in a known manner, for example by mixing the active compounds with auxiliaries such as, for example, extenders, solvents and/or solid carriers and/or further auxiliaries, such as, for example, surfactants. The formulations are prepared either in suitable plants or else before or during the application.

[000294] Suitable for use as auxiliaries are substances which are suitable for imparting to the formulation of the active compound or the application forms prepared from these formulations (such as, e.g., usable crop protection agents, such as spray liquors or seed dressings) particular properties such as certain physical, technical and/or biological properties.

[000295] Suitable extenders are, for example, water, polar and nonpolar organic chemical liquids, for example from the classes of the aromatic and non-aromatic hydrocarbons (such as paraffins, alkylbenzenes, alkylnaphthalenes, chlorobenzenes), the alcohols and polyols (which, if appropriate, may also be substituted, etherified and/or esterified), the ketones (such as acetone, cyclohexanone), esters (including fats and oils) and (poly)ethers, the unsubstituted and substituted amines, amides, lactams (such as N-alkylpyrrolidones) and lactones, the sulphones and sulphoxides (such as dimethyl sulphoxide).

[000296] If the extender used is water, it is also possible to employ, for example, organic solvents as auxiliary solvents. Essentially, suitable liquid solvents are: aromatics such as xylene, toluene or alkylnaphthalenes, chlorinated aromatics and chlorinated aliphatic hydrocarbons such as chlorobenzenes, chloroethylenes or methylene chloride, aliphatic hydrocarbons such as cyclohexane or paraffins, for example petroleum fractions, mineral and vegetable oils, alcohols such as butanol or glycol and also their ethers and esters, ketones such as acetone, methyl ethyl ketone, methyl isobutyl ketone or cyclohexanone, strongly polar solvents such as dimethylformamide and dimethyl sulphoxide, and also water.

[000297] In principle it is possible to use all suitable solvents. Suitable solvents are, for example, aromatic hydrocarbons, such as xylene, toluene or alkylnaphthalenes, for example, chlorinated aromatic or aliphatic hydrocarbons, such as chlorobenzene, chloroethylene or methylene chloride, for example, aliphatic hydrocarbons, such as cyclohexane, for example, paraffins, petroleum fractions, mineral and vegetable oils, alcohols, such as methanol, ethanol, isopropanol, butanol or glycol, for example, and also their ethers and esters, ketones such as

acetone, methyl ethyl ketone, methyl isobutyl ketone or cyclohexanone, for example, strongly polar solvents, such as dimethyl sulphoxide, and water.

[000298] All suitable carriers may in principle be used. Suitable carriers are in particular: for example, ammonium salts and ground natural minerals such as kaolins, clays, talc, chalk, quartz, attapulgite, montmorillonite or diatomaceous earth, and ground synthetic minerals, such as finely divided silica, alumina and natural or synthetic silicates, resins, waxes and/or solid fertilizers. Mixtures of such carriers may likewise be used. Carriers suitable for granules include the following: for example, crushed and fractionated natural minerals such as calcite, marble, pumice, sepiolite, dolomite, and also synthetic granules of inorganic and organic meals, and also granules of organic material such as sawdust, paper, coconut shells, maize cobs and tobacco stalks.

[000299] Liquefied gaseous extenders or solvents may also be used. Particularly suitable are those extenders or carriers which at standard temperature and under standard pressure are gaseous, examples being aerosol propellants, such as halogenated hydrocarbons, and also butane, propane, nitrogen and carbon dioxide.

[000300] Examples of emulsifiers and/or foam-formers, dispersants or wetting agents having ionic or nonionic properties, or mixtures of these surface-active substances, are salts of polyacrylic acid, salts of lignosulphonic acid, salts of phenolsulphonic acid or naphthalenesulphonic acid, polycondensates of ethylene oxide with fatty alcohols or with fatty acids or with fatty amines, with substituted phenols (preferably alkylphenols or arylphenols), salts of sulphosuccinic esters, taurine derivatives (preferably alkyltaurates), phosphoric esters of polyethoxylated alcohols or phenols, fatty acid esters of polyols, and derivatives of the compounds containing sulphates, sulphonates and phosphates, examples being alkylaryl polyglycol ethers, alkylsulphonates, alkyl sulphates, arylsulphonates, protein hydrolysates, lignin-sulphite waste liquors and methylcellulose. The presence of a surface-active substance is advantageous if one of the active compounds and/or one of the inert carriers is not soluble in water and if application takes place in water.

[000301] Further auxiliaries that may be present in the formulations and in the application forms derived from them include colorants such as inorganic pigments, examples being iron oxide, titanium oxide, Prussian Blue, and organic dyes, such as alizarin dyes, azo dyes and metal phthalocyanine dyes, and nutrients and trace nutrients, such as salts of iron, manganese, boron, copper, cobalt, molybdenum and zinc.

[000302] Stabilizers, such as low-temperature stabilizers, preservatives, antioxidants, light stabilizers or other agents which improve chemical and/or physical stability may also be present. Additionally present may be foam-formers or defoamers.

[000303] Furthermore, the formulations and application forms derived from them may also comprise, as additional auxiliaries, stickers such as carboxymethylcellulose, natural and synthetic polymers in powder, granule or latex form, such as gum arabic, polyvinyl alcohol, polyvinyl acetate, and also natural phospholipids, such as cephalins and lecithins, and synthetic phospholipids. Further possible auxiliaries include mineral and vegetable oils.

[000304] There may possibly be further auxiliaries present in the formulations and the application forms derived from them. Examples of such additives include fragrances, protective colloids, binders, adhesives, thickeners, thixotropic substances, penetrants, retention promoters, stabilizers, sequestrants, complexing agents, humectants and spreaders. Generally speaking, the active compounds may be combined with any solid or liquid additive commonly used for formulation purposes.

[000305] Suitable retention promoters include all those substances which reduce the dynamic surface tension, such as dioctyl sulphosuccinate, or increase the viscoelasticity, such as hydroxypropylguar polymers, for example.

[000306] Suitable penetrants in the present context include all those substances which are typically used in order to enhance the penetration of active agrochemical compounds into plants. Penetrants in this context are defined in that, from the (generally aqueous) application liquor and/or from the spray coating, they are able to penetrate the cuticle of the plant and thereby increase the mobility of the active compounds in the cuticle. This property can be determined using the method described in the literature (Baur, et al., 1997, Pesticide Science, 51, 131-152). Examples include alcohol alkoxylates such as coconut fatty ethoxylate (10) or isotridecyl ethoxylate (12), fatty acid esters such as rapeseed or soybean oil methyl esters, fatty amine alkoxylates such as tallowamine ethoxylate (15), or ammonium and/or phosphonium salts such as ammonium sulphate or diammonium hydrogen phosphate, for example.

[000307] The formulations preferably comprise between 0.0001% and 98% by weight of active compound or, with particular preference, between 0.01% and 95% by weight of active compound, more preferably between 0.5% and 90% by weight of active compound, based on the weight of the formulation. The content of the active compound is defined as the sum of the recombinant exosporium-producing *Bacillus* cells and the at least one particular insecticide disclosed herein.

[000308] The active compound content of the application forms (crop protection products) prepared from the formulations may vary within wide ranges. The active compound concentration of the application forms may be situated typically between 0.0001% and 95% by weight of active compound, preferably between 0.0001% and 1% by weight, based on the weight of the application form. Application takes place in a customary manner adapted to the application forms.

[000309] Furthermore, in one aspect of the present invention a kit of parts is provided comprising a recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and at least one particular insecticide disclosed herein in a synergistically effective amount in a spatially separated arrangement.

[000310] In a further embodiment of the present invention the above-mentioned kit of parts further comprises at least one additional fungicide and/or at least one particular insecticide disclosed herein. The fungicide and/or the insecticide can be present either in the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom component of the kit of parts or in the insecticide component of the kit of parts being spatially separated or in both of these components. Preferably, the fungicide and/or the insecticide are present in the recombinant exosporium-producing *Bacillus* cells or exosporium fragments component.

[000311] Moreover, the kit of parts according to the present invention can additionally comprise at least one auxiliary selected from the group consisting of extenders, solvents, spontaneity promoters, carriers, emulsifiers, dispersants, frost protectants, thickeners and adjuvants as mentioned below. This at least one auxiliary can be present either in the recombinant exosporium-producing *Bacillus* cells or exosporium fragment component of the kit of parts or in the insecticide component of the kit of parts being spatially separated or in both of these components.

[000312] In another aspect of the present invention the composition as described above is used for reducing overall damage of plants and plant parts as well as losses in harvested fruits or vegetables caused by insects, mites, nematodes and/or phytopathogens.

[000313] Furthermore, in another aspect of the present invention the composition as described above increases the overall plant health.

[000314] The term "plant health" generally comprises various sorts of improvements of plants that are not connected to the control of pests. For example, advantageous properties that may be mentioned are improved crop characteristics including: emergence, crop yields, protein content, oil content, starch content, more developed root system, improved root growth, improved root size maintenance, improved root effectiveness, improved stress tolerance (e.g.,

against drought, heat, salt, UV, water, cold), reduced ethylene (reduced production and/or inhibition of reception), tillering increase, increase in plant height, bigger leaf blade, less dead basal leaves, stronger tillers, greener leaf color, pigment content, photosynthetic activity, less input needed (such as fertilizers or water), less seeds needed, more productive tillers, earlier flowering, early grain maturity, less plant verse (lodging), increased shoot growth, enhanced plant vigor, increased plant stand and early and better germination.

[000315] With regard to the use according to the present invention, improved plant health preferably refers to improved plant characteristics including: crop yield, more developed root system (improved root growth), improved root size maintenance, improved root effectiveness, tillering increase, increase in plant height, bigger leaf blade, less dead basal leaves, stronger tillers, greener leaf color, photosynthetic activity, more productive tillers, enhanced plant vigor, and increased plant stand.

[000316] With regard to the present invention, improved plant health preferably especially refers to improved plant properties selected from crop yield, more developed root system, improved root growth, improved root size maintenance, improved root effectiveness, tillering increase, and increase in plant height.

[000317] The effect of a composition according to the present invention on plant health as defined herein can be determined by comparing plants which are grown under the same environmental conditions, whereby a part of said plants is treated with a composition according to the present invention and another part of said plants is not treated with a composition according to the present invention. Instead, said other part is not treated at all or treated with a placebo (i.e., an application without a composition according to the invention such as an application without all active ingredients (i.e., without the recombinant exosporium-producing *Bacillus cereus* family member-based biological control agent as described herein and without an insecticide as described herein), or an application without the recombinant exosporium-producing *Bacillus cereus* family member-based biological control agent as described herein, or an application without an insecticide as described herein.

[000318] The composition according to the present invention may be applied in any desired manner, such as in the form of a seed coating, soil drench, and/or directly in-furrow and/or as a foliar spray and applied either pre-emergence, post-emergence or both. In other words, the composition can be applied to the seed, the plant or to harvested fruits and vegetables or to the soil wherein the plant is growing or wherein it is desired to grow (plant's locus of growth).

[000319] Reducing the overall damage of plants and plant parts often results in healthier plants and/or in an increase in plant vigor and yield.

[000320] Preferably, the composition according to the present invention is used for treating conventional or transgenic plants or seed thereof.

[000321] The present invention also relates to methods for stimulating plant growth using any of the compositions described above comprising recombinant exosporium-producing *Bacillus* cells that express a fusion protein and at least one particular insecticide disclosed herein. The method for stimulating plant growth comprises applying to a plant, a plant part, to the locus surrounding the plant or in which the plant will be planted (e.g., soil or other growth medium) a composition comprising recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising: (i) at least one plant growth stimulating protein or peptide; and (ii) a targeting sequence, exosporium protein, or exosporium protein fragment, and at least one further particular insecticide disclosed herein in a synergistically effective amount.

[000322] In another aspect of the present invention a method for reducing overall damage of plants and plant parts as well as losses in harvested fruits or vegetables caused by insects, mites, nematodes and/or phytopathogens is provided comprising the step of simultaneously or sequentially applying the recombinant exosporium-producing *Bacillus* cells and at least one particular insecticide disclosed herein in a synergistically effective amount.

[000323] In another embodiment of the present invention, the composition comprises at least one fungicide and/or at least one insecticide in addition to the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the particular insecticide disclosed herein. In one embodiment, the at least one fungicide is a synthetic fungicide.

[000324] The method of the present invention includes the following application methods, namely both of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein may be formulated into a single, stable composition with an agriculturally acceptable shelf life (so called "solo-formulation"), or being combined before or at the time of use (so called "combined-formulations").

[000325] If not mentioned otherwise, the expression "combination" stands for the various combinations of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least insecticide, and optionally the at least one fungicide, in a solo-formulation, in a single "ready-mix" form, in a combined spray mixture composed from solo-formulations, such as a "tank-mix", and especially in a combined use of the

single active ingredients when applied in a sequential manner, i.e., one after the other within a reasonably short period, such as a few hours or days, e.g., 2 hours to 7 days. The order of applying the composition according to the present invention is not essential for working the present invention. Accordingly, the term "combination" also encompasses the presence of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide on or in a plant to be treated or its surrounding, habitat or storage space, e.g., after simultaneously or consecutively applying the recombinant exosporium-producing *Bacillus* cells and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide to a plant its surrounding, habitat or storage space.

[000326] If the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide are employed or used in a sequential manner, it is preferred to treat the plants or plant parts (which includes seeds and plants emerging from the seed), harvested fruits and vegetables according to the following method: Firstly applying the at least one particular insecticide disclosed herein and optionally the at least one fungicide and/or the at least one additional insecticide on the plant or plant parts, and secondly applying the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom to the same plant or plant parts. By this application manner the amount of residues of insecticides/fungicides on the plant upon harvesting is as low as possible. The time periods between the first and the second application within a (crop) growing cycle may vary and depend on the effect to be achieved. For example, the first application is done to prevent an infestation of the plant or plant parts with insects, mites, nematodes and/or phytopathogens (this is particularly the case when treating seeds) or to combat the infestation with insects, mites, nematodes and/or phytopathogens (this is particularly the case when treating plants and plant parts) and the second application is done to prevent or control the infestation with insects, mites, nematodes and/or phytopathogens and/or to promote plant growth. Control in this context means that the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom are not able to fully exterminate the pests or phytopathogenic fungi but are able to keep the infestation on an acceptable level.

[000327] The present invention also provides methods of enhancing the killing, inhibiting, preventative and/or repelling activity of the compositions of the present invention by multiple applications. In some other embodiments, the compositions of the present invention are applied to a plant and/or plant part for two times, during any desired development stages or

under any predetermined pest pressure, at an interval of about 1 hour, about 5 hours, about 10 hours, about 24 hours, about two days, about 3 days, about 4 days, about 5 days, about 1 week, about 10 days, about two weeks, about three weeks, about 1 month or more. Still in some embodiments, the compositions of the present invention are applied to a plant and/or plant part for more than two times, for example, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, or more, during any desired development stages or under any predetermined pest pressure, at an interval of about 1 hour, about 5 hours, about 10 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 1 week, about 10 days, about 2 weeks, about 3 weeks, about 1 month or more. The intervals between each application can vary if it is desired. One skilled in the art will be able to determine the application times and length of interval depending on plant species, plant pest species, and other factors.

[000328] By following the before mentioned steps, a very low level of residues of the at least one fungicide and/or at least one particular insecticide disclosed herein and/or additional insecticide on the treated plant, plant parts, and the harvested fruits and vegetables can be achieved.

[000329] If not mentioned otherwise the treatment of plants or plant parts (which includes seeds and plants emerging from the seed), harvested fruits and vegetables with the composition according to the invention is carried out directly or by action on their surroundings, habitat or storage space using customary treatment methods, for example dipping, spraying, atomizing, irrigating, evaporating, dusting, fogging, broadcasting, foaming, painting, spreading-on, watering (drenching), drip irrigating. It is furthermore possible to apply the recombinant exosporium-producing *Bacillus* cells, the at least one particular insecticide disclosed herein, and optionally the at least one fungicide as solo-formulation or combined-formulations by the ultra-low volume method, or to inject the composition according to the present invention as a composition or as sole-formulations into the soil (in-furrow).

[000330] The term "plant to be treated" encompasses every part of a plant including its root system and the material - e.g., soil or nutrition medium - which is in a radius of at least 10 cm, 20 cm, 30 cm around the caulis or bole of a plant to be treated or which is at least 10 cm, 20 cm, 30 cm around the root system of said plant to be treated, respectively.

[000331] The amount of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom which is used or employed in combination with at least one particular insecticide disclosed herein, optionally in the presence of at least one fungicide, depends on the final formulation as well as size or type of the plant, plant parts, seeds, harvested fruits and vegetables to be treated. Usually, the recombinant exosporium-producing *Bacillus*

cells or exosporium fragments derived therefrom to be employed or used according to the invention is present in about 1% to about 80% (w/w), preferably in about 1% to about 60% (w/w), more preferably about 10% to about 50% (w/w) of its solo-formulation or combined-formulation with the at least one particular insecticide disclosed herein, and optionally the fungicide.

[000332] Also the amount of the at least one particular insecticide disclosed herein which is used or employed in combination with the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom, optionally in the presence of at least one fungicide, depends on the final formulation as well as size or type of the plant, plant parts, seeds, harvested fruit or vegetable to be treated. Usually, the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom to be employed or used according to the invention is present in about 0.1% to about 80% (w/w), preferably 1% to about 60% (w/w), more preferably about 10% to about 50% (w/w) of its solo-formulation or combined-formulation with the at least one particular insecticide disclosed herein, and optionally the at least one fungicide.

[000333] Application of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom may be effected as a foliar spray, as a soil treatment, and/or as a seed treatment/dressing. When used as a foliar treatment, in one embodiment, about 1/16 to about 5 gallons of whole broth are applied per acre. When used as a soil treatment, in one embodiment, about 1 to about 5 gallons of whole broth are applied per acre. When used for seed treatment about 1/32 to about 1/4 gallons of whole broth are applied per acre. For seed treatment, the end-use formulation contains 1×10^3,1×10^4, at least 1×10^5, at least 1×10^6, 1×10^7, at least 1×10^8, at least 1×10^9, or at least 1×10^{10} colony forming units per gram.

[000334] The recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and at least one particular insecticide disclosed herein, and if present preferably also the fungicide are used or employed in a synergistic weight ratio. The skilled person is able to find out the synergistic weight ratios for the present invention by routine methods. The skilled person understands that these ratios refer to the ratio within a combined-formulation as well as to the calculative ratio of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom described herein and the at least one particular insecticide disclosed herein when both components are applied as mono-formulations to a plant to be treated. The skilled person can calculate this ratio by simple mathematics since the volume and the amount of the recombinant exosporium-producing *Bacillus* cells or exosporium

fragments derived therefrom and the at least one particular insecticide disclosed herein, respectively, in a mono-formulation is known to the skilled person.

[000335] The ratio can be calculated based on the amount of the at least one particular insecticide disclosed herein, at the time point of applying said component of a combination according to the invention to a plant or plant part and the amount of recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom shortly prior (e.g., 48 h, 24 h, 12 h, 6 h, 2 h, 1 h) or at the time point of applying said component of a combination according to the invention to a plant or plant part.

[000336] The application of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein to a plant or a plant part can take place simultaneously or at different times as long as both components are present on or in the plant after the application(s). In cases where the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and insecticide are applied at different times and insecticide is applied noticeable prior to the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom, the skilled person can determine the concentration of insecticide on/in a plant by chemical analysis known in the art, at the time point or shortly before the time point of applying the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom. Vice versa, when the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom are applied to a plant first, the concentration of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom can be determined using tests which are also known in the art, at the time point or shortly before the time point of applying the insecticide.

[000337] In particular, in one embodiment the synergistic weight ratio of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein lies in the range of 1:1000 to 1000:1, preferably in the range of 1:500 to 500:1, more preferably in the range of 1:300 to 500:1. Especially preferred ratios are between 20:1 and 1:20, such as 10:1, 5:1 or 2:1. It has to be noted that when these ratio ranges refer to the recombinant *Bacillus cereus* family member-based biological control agent (to be combined with at least one particular insecticide or a preparation of at least one particular insecticide disclosed herein), for example, a ratio of 100:1 means 100 weight parts of a spore preparation of the recombinant exosporium-producing *Bacillus*-based biological control agent and 1 weight part of insecticide are combined (either as a solo formulation, a combined formulation or by separate applications to plants so that the

combination is formed on the plant). In one aspect of this embodiment, the spore preparation of the recombinant exosporium-producing *Bacillus* cells is a dried spore preparation containing at least about 1×10^4 cfu/g, at least about 1×10^5 cfu/g, at least about 1×10^6 cfu/g at least about 1×10^7 cfu/g, at least about 1×10^8 cfu/g, at least about 1×10^9 cfu/g, at least about 1×10^{10} cfu/g, or at least about 1×10^{11} cfu/g.

[000338] In another embodiment, the synergistic weight ratio of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein is in the range of 1:100 to 20,000:1, preferably in the range of 1:50 to 10,000:1 or even in the range of 1:50 to 1,000:1.

[000339] In one embodiment of the present invention, the concentration of the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom after dispersal is at least 50 g/ha, such as 50 – 7500 g/ha, 50 – 2500 g/ha, 50 – 1500 g/ha; at least 250 g/ha (hectare), at least 500 g/ha or at least 800 g/ha.

[000340] The application rate of composition to be employed or used according to the present invention may vary. The skilled person is able to find the appropriate application rate by way of routine experiments.

[000341] In another aspect of the present invention a seed treated with the composition as described above is provided.

[000342] The control of insects, mites, nematodes and/or phytopathogens by treating the seed of plants has been known for a long time and is a subject of continual improvements. Nevertheless, the treatment of seed entails a series of problems which cannot always be solved in a satisfactory manner. Thus, it is desirable to develop methods for protecting the seed and the germinating plant that remove the need for, or at least significantly reduce, the additional delivery of crop protection compositions in the course of storage, after sowing or after the emergence of the plants. It is desirable, furthermore, to optimize the amount of active ingredient employed in such a way as to provide the best-possible protection to the seed and the germinating plant from attack by insects, mites, nematodes and/or phytopathogens, but without causing damage to the plant itself by the active ingredient employed. In particular, methods for treating seed ought also to take into consideration the intrinsic insecticidal and/or nematicidal properties of pest-resistant or pest-tolerant transgenic plants, in order to achieve optimum protection of the seed and of the germinating plant with a minimal use of crop protection compositions.

[000343] The present invention therefore also relates in particular to a method for protecting seed and germinating plants from attack by pests, by treating the seed with the

recombinant exosporium-producing *Bacillus* cells as defined above and at least one particular insecticide disclosed herein in a synergistically effective amount. The method of the invention for protecting seed and germinating plants from attack by pests encompasses a method in which the seed is treated simultaneously in one operation with the recombinant exosporium-producing *Bacillus* cells and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide. It also encompasses a method in which the seed is treated at different times with the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide.

[000344] The invention likewise relates to the use of the composition of the invention for treating seed for the purpose of protecting the seed and the resultant plant against insects, mites, nematodes and/or phytopathogens.

[000345] The invention also relates to seed which at the same time has been treated with a recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and at least one particular insecticide disclosed herein, and optionally at least one fungicide. The invention further relates to seed which has been treated at different times with the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein and optionally the at least one fungicide and/or the at least one insecticide. In the case of seed which has been treated at different times with the recombinant exosporium-producing *Bacillus* cells or exosporium fragments derived therefrom and the at least one particular insecticide disclosed herein, and optionally the at least one fungicide, the individual active ingredients in the composition of the invention may be present in different layers on the seed.

[000346] Furthermore, the invention relates to seed which, following treatment with the composition of the invention, is subjected to a film-coating process in order to prevent dust abrasion of the seed.

[000347] One of the advantages of the present invention is that, owing to the particular systemic properties of the compositions of the invention, the treatment of the seed with these compositions provides protection from insects, mites, nematodes and/or phytopathogens not only to the seed itself but also to the plants originating from the seed, after they have emerged. In this way, it may not be necessary to treat the crop directly at the time of sowing or shortly thereafter.

[000348] A further advantage is to be seen in the fact that, through the treatment of the seed with composition of the invention, germination and emergence of the treated seed may be promoted.

[000349] It is likewise considered to be advantageous composition of the invention may also be used, in particular, on transgenic seed.

[000350] It is also stated that the composition of the invention may be used in combination with agents of the signaling technology, as a result of which, for example, colonization with symbionts is improved, such as rhizobia, mycorrhiza and/or endophytic bacteria, for example, is enhanced, and/or nitrogen fixation is optimized.

[000351] The compositions of the invention are suitable for protecting seed of any variety of plant which is used in agriculture, in greenhouses, in forestry or in horticulture. More particularly, the seed in question is that of cereals (e.g., wheat, barley, rye, oats and millet), maize, cotton, soybeans, rice, potatoes, sunflower, coffee, tobacco, canola, oilseed rape, beets (e.g., sugar beet and fodder beet), peanuts, vegetables (e.g., tomato, cucumber, bean, brassicas, onions and lettuce), fruit plants, lawns and ornamentals. Particularly important is the treatment of the seed of cereals (such as wheat, barley, rye and oats) maize, soybeans, cotton, canola, oilseed rape and rice.

[000352] As already mentioned above, the treatment of transgenic seed with the composition of the invention is particularly important. The seed in question here is that of plants which generally contain at least one heterologous gene that controls the expression of a polypeptide having, in particular, insecticidal and/or nematicidal properties. These heterologous genes in transgenic seed may come from microorganisms such as *Bacillus, Rhizobium, Pseudomonas, Serratia, Trichoderma, Clavibacter, Glomus* or *Gliocladium*. The present invention is particularly suitable for the treatment of transgenic seed which contains at least one heterologous gene from *Bacillus* sp. With particular preference, the heterologous gene in question comes from *Bacillus thuringiensis*.

[000353] For the purposes of the present invention, the composition of the invention is applied alone or in a suitable formulation to the seed. The seed is preferably treated in a condition in which its stability is such that no damage occurs in the course of the treatment. Generally speaking, the seed may be treated at any point in time between harvesting and sowing. Typically, seed is used which has been separated from the plant and has had cobs, hulls, stems, husks, hair or pulp removed. Thus, for example, seed may be used that has been harvested, cleaned and dried to a moisture content of less than 15% by weight. Alternatively, seed can also be used that after drying has been treated with water, for example, and then dried again.

[000354] When treating seed it is necessary, generally speaking, to ensure that the amount of the composition of the invention, and/or of other additives, that is applied to the seed is selected such that the germination of the seed is not adversely affected, and/or that the plant which emerges from the seed is not damaged. This is the case in particular with active ingredients which may exhibit phytotoxic effects at certain application rates.

[000355] The compositions of the invention can be applied directly, in other words without comprising further components and without having been diluted. As a general rule, it is preferable to apply the compositions in the form of a suitable formulation to the seed. Suitable formulations and methods for seed treatment are known to the skilled person and are described in, for example, the following documents: U.S. Patent Nos. 4,272,417; 4,245,432; 4,808,430; 5,876,739; U.S. Patent Publication No. 2003/0176428 A1; and PCT Patent Publication Nos. WO 2002/080675 A1; WO 2002/028186 A2.

[000356] The combinations which can be used in accordance with the invention may be converted into the customary seed-dressing formulations, such as solutions, emulsions, suspensions, powders, foams, slurries or other coating compositions for seed, and also ULV formulations.

[000357] These formulations are prepared in a known manner, by mixing composition with customary adjuvants, such as, for example, customary extenders and also solvents or diluents, colorants, wetters, dispersants, emulsifiers, antifoams, preservatives, secondary thickeners, stickers, gibberellins, and also water.

[000358] Colorants which may be present in the seed-dressing formulations which can be used in accordance with the invention include all colorants which are customary for such purposes. In this context it is possible to use not only pigments, which are of low solubility in water, but also water-soluble dyes. Examples include the colorants known under the designations Rhodamin B, C.I. Pigment Red 112, and C.I. Solvent Red 1.

[000359] Wetters which may be present in the seed-dressing formulations which can be used in accordance with the invention include all of the substances which promote wetting and which are customary in the formulation of active agrochemical ingredients. Use may be made preferably of alkylnaphthalenesulphonates, such as diisopropyl- or diisobutyl-naphthalenesulphonates.

[000360] Dispersants and/or emulsifiers which may be present in the seed-dressing formulations which can be used in accordance with the invention include all of the nonionic, anionic and cationic dispersants that are customary in the formulation of active agrochemical ingredients. Use may be made preferably of nonionic or anionic dispersants or of mixtures of

nonionic or anionic dispersants. Suitable nonionic dispersants are, in particular, ethylene oxide-propylene oxide block polymers, alkylphenol polyglycol ethers and also tristryrylphenol polyglycol ethers, and the phosphated or sulphated derivatives of these. Suitable anionic dispersants are, in particular, lignosulphonates, salts of polyacrylic acid, and arylsulphonate-formaldehyde condensates.

[000361] Antifoams which may be present in the seed-dressing formulations which can be used in accordance with the invention include all of the foam inhibitors that are customary in the formulation of active agrochemical ingredients. Use may be made preferably of silicone antifoams and magnesium stearate.

[000362] Preservatives which may be present in the seed-dressing formulations which can be used in accordance with the invention include all of the substances which can be employed for such purposes in agrochemical compositions. Examples include dichlorophen and benzyl alcohol hemiformal.

[000363] Secondary thickeners which may be present in the seed-dressing formulations which can be used in accordance with the invention include all substances which can be used for such purposes in agrochemical compositions. Those contemplated with preference include cellulose derivatives, acrylic acid derivatives, xanthan, modified clays and highly disperse silica.

[000364] Stickers which may be present in the seed-dressing formulations which can be used in accordance with the invention include all customary binders which can be used in seed-dressing products. Preferred mention may be made of polyvinylpyrrolidone, polyvinyl acetate, polyvinyl alcohol and tylose.

[000365] Gibberellins which may be present in the seed-dressing formulations which can be used in accordance with the invention include preferably the gibberellins A1, A3 (= gibberellic acid), A4 and A7, with gibberellic acid being used with particular preference. The gibberellins are known (cf. R. Wegler, "Chemie der Pflanzenschutz- und Schädlingsbekämpfungsmittel", Volume 2, Springer Verlag, 1970, pp. 401-412).

[000366] The seed-dressing formulations which can be used in accordance with the invention may be used, either directly or after prior dilution with water, to treat seed of any of a wide variety of types. Accordingly, the concentrates or the preparations obtainable from them by dilution with water may be employed to dress the seed of cereals, such as wheat, barley, rye, oats and triticale, and also the seed of maize, rice, oilseed rape, peas, beans, cotton, sunflowers and beets, or else the seed of any of a very wide variety of vegetables. The seed-dressing formulations which can be used in accordance with the invention, or their diluted preparations,

may also be used to dress seed of transgenic plants. In that case, additional synergistic effects may occur in interaction with the substances formed through expression.

[000367] For the treatment of seed with the seed-dressing formulations which can be used in accordance with the invention, or with the preparations produced from them by addition of water, suitable mixing equipment includes all such equipment which can typically be employed for seed dressing. More particularly, the procedure when carrying out seed dressing is to place the seed in a mixer, to add the particular desired amount of seed-dressing formulations, either as such or following dilution with water beforehand, and to carry out mixing until the distribution of the formulation on the seed is uniform. This may be followed by a drying operation.

[000368] The application rate of the seed-dressing formulations which can be used in accordance with the invention may be varied within a relatively wide range. It is guided by the particular amount of the recombinant exosporium-producing *Bacillus cereus* family member-based biological control agent and the at least one particular insecticide disclosed herein in the formulations, and by the seed. The application rates in the case of the composition are situated generally at between 0.001 and 50 g per kilogram of seed, preferably between 0.01 and 15 g per kilogram of seed.

[000369] The compositions according to the invention, in case they exhibit insecticidal and miticidal and/or nematicidal activity, in combination with good plant tolerance and favourable toxicity to warm-blooded animals and being tolerated well by the environment, are suitable for protecting plants and plant organs, for increasing harvest yields, for improving the quality of the harvested material and for controlling animal pests, in particular insects, mites, arachnids, helminths, nematodes and molluscs, which are encountered in agriculture, in horticulture, in animal husbandry, in forests, in gardens and leisure facilities, in protection of stored products and of materials, and in the hygiene sector. They can be preferably employed as plant protection agents. In particular, the present invention relates to the use of the composition according to the invention as insecticide and/or fungicide.

[000370] They are active against normally sensitive and resistant species and against all or some stages of development. The abovementioned pests include:

[000371] pests from the phylum *Arthropoda*, especially from the class *Arachnida*, for example, *Acarus* spp., *Aceria sheldoni*, *Aculops* spp., *Aculus* spp., *Amblyomma* spp., *Amphitetranychus viennensis*, *Argas* spp., *Boophilus* spp., *Brevipalpus* spp., *Bryobia graminum, Bryobia praetiosa, Centruroides* spp., *Chorioptes* spp., *Dermanyssus gallinae*, *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Dermacentor* spp., *Eotetranychus*

spp., *Epitrimerus* pyri, *Eutetranychus* spp., *Eriophyes* spp., *Glycyphagus domesticus, Halotydeus destructor, Hemitarsonemus* spp., *Hyalomma* spp., *Ixodes* spp., *Latrodectus* spp., *Loxosceles* spp., *Metatetranychus* spp., *Neutrombicula autumnalis, Nuphersa* spp., *Oligonychus* spp., *Ornithodorus* spp., *Ornithonyssus* spp., *Panonychus* spp., *Phyllocoptruta oleivora, Polyphagotarsonemus latus*, *Psoroptes* spp., *Rhipicephalus* spp., *Rhizoglyphus* spp., *Sarcoptes* spp., *Scorpio maurus, Steneotarsonemus* spp., *Steneotarsonemus spinki*, *Tarsonemus* spp., *Tetranychus* spp., *Trombicula alfreddugesi*, *Vaejovis* spp., *Vasates lycopersici*;

[000372] in particular clover mite, brown mite, hazelnut spider mite, asparagus spider mite, brown wheat mite, legume mite, oxalis mite, boxwood mite, Texas citrus mite, Oriental red mite, citrus red mite, European red mite, yellow spider mite, fig spider mite, Lewis spider mite, six-spotted spider mite, Willamette mite, Yuma spider mite, web-spinning mite, pineapple mite, citrus green mite, honey-locust spider mite, tea red spider mite, southern red mite, avocado brown mite, spruce spider mite, avocado red mite, Banks grass mite, carmine spider mite, desert spider mite, vegetable spider mite, tumid spider mite, strawberry spider mite, two-spotted spider mite, McDaniel mite, Pacific spider mite, hawthorn spider mite, four-spotted spider mite, Schoenei spider mite, Chilean false spider mite, citrus flat mite, privet mite, flat scarlet mite, white-tailed mite, pineapple tarsonemid mite, West Indian sugar cane mite, bulb scale mite, cyclamen mite, broad mite, winter grain mite, red-legged earth mite, filbert big-bud mite, grape erineum mite, pear blister leaf mite, apple leaf edgeroller mite, peach mosaic vector mite, alder bead gall mite, Perian walnut leaf gall mite, pecan leaf edgeroll mite, fig bud mite, olive bud mite, citrus bud mite, litchi erineum mite, wheat curl mite, coconut flower and nut mite, sugar cane blister mite, buffalo grass mite, bermuda grass mite, carrot bud mite, sweet potato leaf gall mite, pomegranate leaf curl mite, ash sprangle gall mite, maple bladder gall mite, alder erineum mite, redberry mite, cotton blister mite, blueberry bud mite, pink tea rust mite, ribbed tea mite, grey citrus mite, sweet potato rust mite, horse chestnut rust mite, citrus rust mite, apple rust mite, grape rust mite, pear rust mite, flat needle sheath pine mite, wild rose bud and fruit mite, dryberry mite, mango rust mite, azalea rust mite, plum rust mite, peach silver mite, apple rust mite, tomato russet mite, pink citrus rust mite, cereal rust mite, rice rust mite;

[000373] from the class Chilopoda, for example, *Geophilus* spp., *Scutigera* spp.;

[000374] from the order or the class Collembola, for example, *Onychiurus armatus*;

[000375] from the class Diplopoda, for example, *Blaniulus guttulatus*;

[000376] from the class Insecta, e.g., from the order *Blattodea*, for example, *Blattella asahinai, Blattella germanica, Blatta orientalis*, *Leucophaea maderae*, *Panchlora* spp., *Parcoblatta* spp., *Periplaneta* spp., *Supella longipalpa*;

[000377] from the order Coleoptera, for example, *Acalymma vittatum*, *Acanthoscelides obtectus*, *Adoretus* spp., *Agelastica* alni, *Agriotes* spp., *Alphitobius diaperinus*, *Amphimallon solstitialis*, *Anobium punctatum, Anoplophora* spp., *Anthonomus* spp., *Anthrenus* spp., *Apion* spp., *Apogonia* spp., *Atomaria* spp., *Attagenus* spp., *Bruchidius obtectus*, *Bruchus* spp., *Cassida* spp., *Cerotoma trifurcata*, *Ceutorrhynchus* spp., *Chaetocnema* spp., *Cleonus mendicus, Conoderus* spp., *Cosmopolites* spp., *Costelytra zealandica, Ctenicera* spp., *Curculio* spp., *Cryptolestes ferrugineus, Cryptorhynchus lapathi*, *Cylindrocopturus spp., Dermestes spp., Diabrotica* spp., *Dichocrocis* spp., *Dicladispa armigera, Diloboderus* spp., *Epilachna* spp., *Epitrix* spp., *Faustinus* spp., *Gibbium psylloides, Gnathocerus cornutus, Hellula undalis*, *Heteronychus arator, Heteronyx* spp., *Hylamorpha elegans, Hylotrupes bajulus, Hypera postica*, *Hypomeces squamosus, Hypothenemus* spp., *Lachnosterna consanguinea, Lasioderma serricorne, Latheticus oryzae, Lathridius* spp., *Lema* spp., *Leptinotarsa decemlineata*, *Leucoptera* spp., *Lissorhoptrus oryzophilus, Lixus* spp., *Luperodes* spp., *Lyctus* spp., *Megascelis* spp., *Melanotus* spp., *Meligethes aeneus, Melolontha* spp., *Migdolus* spp., *Monochamus* spp., *Naupactus xanthographus*, *Necrobia* spp., *Niptus hololeucus*, *Oryctes rhinoceros, Oryzaephilus surinamensis*, *Oryzaphagus oryzae*, *Otiorrhynchus spp.*, *Oxycetonia jucunda, Phaedon cochleariae, Phyllophaga* spp., *Phyllophaga helleri, Phyllotreta* spp., *Popillia japonica, Premnotrypes* spp., *Prostephanus truncatus*, *Psylliodes* spp., *Ptinus* spp., *Rhizobius ventralis, Rhizopertha dominica, Sitophilus* spp., *Sitophilus* oryzae, *Sphenophorus* spp., *Stegobium paniceum, Sternechus* spp., *Symphyletes* spp., *Tanymecus* spp., *Tenebrio molitor, Tenebrioides mauretanicus, Tribolium* spp., *Trogoderma* spp., *Tychius* spp., *Xylotrechus* spp., *Zabrus* spp.;

[000378] preferably from Banded cucumber beetle (*Diabrotica balteata*), Northern corn rootworm (Diabrotica barberi), Southern corn rootworm (*Diabrotica undecimpunctata howardi*), Western cucumber beetle (*Diabrotica undecimpunctata tenella*), Western spotted cucumber beetle (*Diabrotica undecimpunctata undecimpunctata*), Western corn rootworm (*Diabrotica virgifera virgifera*), Mexican corn rootworm (*Diabrotica virgifera zeae*);

[000379] from the order Diptera, for example, *Aedes* spp., *Agromyza* spp., *Anastrepha* spp., *Anopheles* spp., *Asphondylia* spp., *Bactrocera* spp., *Bibio hortulanus, Calliphora erythrocephala, Calliphora vicina, Ceratitis capitata, Chironomus* spp., *Chrysomyia* spp., *Chrysops* spp., *Chrysozona pluvialis*, *Cochliomyia* spp., *Contarinia* spp., *Cordylobia anthropophaga, Cricotopus sylvestris, Culex* spp., *Culicoides* spp., *Culiseta* spp., *Cuterebra* spp., *Dacus oleae*, *Dasyneura* spp., *Delia* spp., *Dermatobia hominis, Drosophila* spp., *Echinocnemus* spp., *Fannia* spp., *Gasterophilus* spp., *Glossina* spp., *Haematopota* spp., *Hydrellia* spp., *Hydrellia griseola*, *Hylemya* spp., *Hippobosca* spp., *Hypoderma* spp., *Liriomyza*

spp., *Lucilia* spp., *Lutzomyia* spp., *Mansonia* spp., *Musca* spp., *Oestrus* spp., *Oscinella frit*, *Paratanytarsus* spp., *Paralauterborniella subcincta, Pegomyia* spp., *Phlebotomus* spp., *Phorbia* spp., *Phormia* spp., *Piophila* casei, *Prodiplosis* spp., *Psila rosae*, *Rhagoletis* spp., *Sarcophaga* spp., *Simulium* spp., *Stomoxys* spp., *Tabanus* spp., *Tetanops* spp., *Tipula* spp.;

[000380] from the order Heteroptera, for example, *Anasa* tristis, *Antestiopsis* spp., *Boisea* spp., *Blissus* spp., *Calocoris* spp., *Campylomma livida, Cavelerius* spp., *Cimex* spp., *Collaria* spp., *Creontiades dilutus, Dasynus piperis, Dichelops furcatus, Diconocoris hewetti*, *Dysdercus* spp., *Euschistus* spp., Eurygaster spp., *Heliopeltis* spp., *Horcias nobilellus, Leptocorisa* spp., *Leptocorisa varicornis, Leptoglossus phyllopus*, *Lygus* spp., *Macropes excavatus, Miridae, Monalonion atratum, Nezara* spp., *Oebalus* spp., *Pentomidae, Piesma quadrata, Piezodorus* spp., *Psallus* spp., *Pseudacysta persea, Rhodnius* spp., *Sahlbergella singularis, Scaptocoris castanea, Scotinophora* spp., *Stephanitis nashi, Tibraca* spp., *Triatoma* spp.;

[000381] from the order Homoptera, for example, *Acizzia acaciaebaileyanae*, *Acizzia dodonaeae, Acizzia uncatoides, Acrida turrita*, *Acyrthosipon* spp., *Acrogonia* spp., *Aeneolamia* spp., *Agonoscena* spp., *Aleyrodes proletella, Aleurolobus barodensis, Aleurothrixus floccosus, Allocaridara malayensis, Amrasca* spp., *Anuraphis cardui*, *Aonidiella* spp., *Aphanostigma piri, Aphis* spp., *Arboridia apicalis, Arytainilla* spp., *Aspidiella* spp., *Aspidiotus* spp., *Atanus* spp., *Aulacorthum solani, Bemisia tabaci, Blastopsylla occidentalis, Boreioglycaspis melaleucae*, *Brachycaudus helichrysi, Brachycolus* spp., *Brevicoryne brassicae*, *Cacopsylla* spp., *Calligypona marginata, Carneocephala fulgida, Ceratovacuna lanigera, Cercopidae, Ceroplastes* spp., *Chaetosiphon fragaefolii, Chionaspis tegalensis, Chlorita onukii, Chondracris rosea, Chromaphis juglandicola, Chrysomphalus ficus, Cicadulina mbila, Coccomytilus halli, Coccus* spp., *Cryptomyzus ribis*, *Cryptoneossa* spp., *Ctenarytaina* spp., *Dalbulus* spp., *Dialeurodes citri, Diaphorina citri*, *Diaspis* spp., *Drosicha* spp., *Dysaphis* spp., *Dysmicoccus* spp., *Empoasca* spp., *Eriosoma* spp., *Erythroneura* spp., *Eucalyptolyma* spp., *Euphyllura* spp., *Euscelis bilobatus, Ferrisia* spp., *Geococcus coffeae*, *Glycaspis* spp., *Heteropsylla cubana, Heteropsylla spinulosa, Homalodisca coagulata, Hyalopterus arundinis, Icerya* spp., *Idiocerus* spp., *Idioscopus* spp., *Laodelphax striatellus, Lecanium* spp., *Lepidosaphes* spp., *Lipaphis erysimi, Macrosiphum* spp., *Macrosteles facifrons, Mahanarva* spp., *Melanaphis sacchari, Metcalfiella* spp., *Metopolophium dirhodum, Monellia costalis*, *Monelliopsis pecanis, Myzus* spp., *Nasonovia ribisnigri, Nephotettix* spp., *Nettigoniclla spectra*, *Nilaparvata lugens, Oncometopia* spp., *Orthezia praelonga, Oxya chinensis, Pachypsylla* spp., *Parabemisia myricae, Paratrioza* spp., *Parlatoria* spp., *Pemphigus* spp., *Peregrinus maidis*, *Phenacoccus*

spp., *Phloeomyzus passerinii, Phorodon humuli, Phylloxera* spp., *Pinnaspis aspidistrae, Planococcus* spp., *Prosopidopsylla flava, Protopulvinaria pyriformis, Pseudaulacaspis pentagona, Pseudococcus* spp., *Psyllopsis* spp., *Psylla* spp., *Pteromalus* spp., *Pyrilla* spp., *Quadraspidiotus* spp., *Quesada* gigas, *Rastrococcus* spp., *Rhopalosiphum* spp., *Saissetia* spp., *Scaphoideus titanus, Schizaphis graminum, Selenaspidus articulatus, Sogata* spp., *Sogatella furcifera, Sogatodes* spp., *Stictocephala festina, Siphoninus phillyreae, Tenalaphara malayensis, Tetragonocephela* spp., *Tinocallis caryaefoliae, Tomaspis* spp., *Toxoptera* spp., *Trialeurodes vaporariorum, Trioza* spp., *Typhlocyba* spp., *Unaspis* spp., *Viteus vitifolii, Zygina* spp.;

[000382] from the order Hymenoptera, for example, *Acromyrmex* spp., *Athalia* spp., *Atta* spp., *Diprion* spp., *Hoplocampa* spp., *Lasius* spp., *Monomorium pharaonis, Sirex* spp., *Solenopsis invicta, Tapinoma* spp., *Urocerus* spp., *Vespa* spp., *Xeris* spp.;

[000383] from the order Isopoda, for example, *Armadillidium vulgare, Oniscus asellus, Porcellio scaber*;

[000384] from the order Isoptera, for example, *Coptotermes* spp., *Cornitermes cumulans, Cryptotermes* spp., *Incisitermes* spp., *Microtermes obesi, Odontotermes* spp., *Reticulitermes* spp.;

[000385] from the order Lepidoptera, for example, *Achroia grisella, Acronicta major, Adoxophyes* spp., *Aedia leucomelas, Agrotis* spp., *Alabama* spp., *Amyelois transitella, Anarsia* spp., *Anticarsia* spp., *Argyroploce* spp., *Barathra brassicae, Borbo cinnara, Bucculatrix thurberiella, Bupalus piniarius, Busseola* spp., *Cacoecia* spp., *Caloptilia theivora, Capua reticulana, Carpocapsa pomonella, Carposina niponensis, Cheimatobia brumata, Chilo* spp., *Choristoneura* spp., *Clysia ambiguella, Cnaphalocerus* spp., *Cnaphalocrocis medinalis, Cnephasia* spp., *Conopomorpha* spp., *Conotrachelus* spp., *Copitarsia* spp., *Cydia* spp., *Dalaca noctuides, Diaphania* spp., *Diatraea saccharalis, Earias* spp., *Ecdytolopha aurantium, Elasmopalpus lignosellus, Eldana saccharina, Ephestia* spp., *Epinotia* spp., *Epiphyas postvittana, Etiella* spp., *Eulia* spp., *Eupoecilia ambiguella, Euproctis* spp., *Euxoa* spp., *Feltia* spp., *Galleria mellonella, Gracillaria* spp., *Grapholitha* spp., *Hedylepta* spp., *Helicoverpa* spp., *Heliothis* spp., *Hofmannophila pseudospretella, Homoeosoma* spp., *Homona* spp., *Hyponomeuta padella, Kakivoria flavofasciata, Laphygma* spp., *Laspeyresia molesta, Leucinodes orbonalis, Leucoptera* spp., *Lithocolletis* spp., *Lithophane antennata, Lobesia* spp., *Loxagrotis albicosta, Lymantria* spp., *Lyonetia* spp., *Malacosoma neustria, Maruca testulalis, Mamstra brassicae, Melanitis leda, Mocis* spp., *Monopis obviella, Mythimna separata, Nemapogon cloacellus, Nymphula* spp., *Oiketicus* spp., *Oria* spp., *Orthaga* spp., *Ostrinia* spp., *Oulema oryzae, Panolis flammea, Parnara* spp., *Pectinophora* spp., *Perileucoptera* spp., *Phthorimaea* spp., *Phyllocnistis*

citrella, Phyllonorycter spp., *Pieris* spp., *Platynota stultana*, *Plodia interpunctella, Plusia* spp., *Plutella xylostella, Prays* spp., *Prodenia* spp., *Protoparce* spp., *Pseudaletia* spp., *Pseudaletia unipuncta, Pseudoplusia includens, Pyrausta nubilalis, Rachiplusia nu, Schoenobius* spp., *Scirpophaga* spp., *Scirpophaga innotata, Scotia segetum, Sesamia* spp., *Sesamia inferens, Sparganothis* spp., *Spodoptera* spp., *Spodoptera praefica, Stathmopoda* spp., *Stomopteryx subsecivella, Synanthedon* spp., *Tecia solanivora, Thermesia gemmatalis*, *Tinea cloacella*, *Tinea pellionella*, *Tineola bisselliella*, *Tortrix* spp., *Trichophaga tapetzella, Trichoplusia* spp., *Tryporyza incertulas, Tuta absoluta, Virachola* spp.;

[000386] from the order Orthoptera or Saltatoria, for example, *Acheta domesticus, Dichroplus* spp., *Gryllotalpa* spp., *Hieroglyphus* spp., *Locusta* spp., *Melanoplus* spp., *Schistocerca gregaria*;

[000387] from the order Phthiraptera, for example, *Damalinia* spp., *Haematopinus* spp., *Linognathus* spp., *Pediculus* spp., *Ptirus* pubis, *Trichodectes* spp.;

[000388] from the order Psocoptera for example *Lepinatus* spp., *Liposcelis* spp.;

[000389] from the order Siphonaptera, for example, *Ceratophyllus* spp., *Ctenocephalides* spp., *Pulex irritans, Tunga penetrans, Xenopsylla cheopsis*;

[000390] from the order Thysanoptera, for example, *Anaphothrips obscurus, Baliothrips biformis, Drepanothrips reuteri, Enneothrips flavens, Frankliniella spp., Heliothrips* spp., *Hercinothrips femoralis, Rhipiphorothrips cruentatus, Scirtothrips* spp., *Taeniothrips cardamomi, Thrips* spp.;

[000391] from the order Zygentoma (=Thysanura), for example, *Ctenolepisma* spp., *Lepisma saccharina, Lepismodes inquilinus, Thermobia domestica*;

[000392] from the class Symphyla, for example, *Scutigerella* spp.;

[000393] pests from the phylum Mollusca, especially from the class Bivalvia, for example, *Dreissena* spp., and from the class Gastropoda, for example, *Arion* spp., *Biomphalaria* spp., *Bulinus* spp., *Deroceras* spp., *Galba* spp., *Lymnaea* spp., *Oncomelania* spp., *Pomacea* spp., *Succinea* spp.;

[000394] animal pests from the phylums Plathelminthes and Nematoda, for example, *Ancylostoma duodenale, Ancylostoma ceylanicum, Acylostoma braziliensis, Ancylostoma* spp., *Ascaris* spp., *Brugia malayi, Brugia timori, Bunostomum* spp., *Chabertia* spp., *Clonorchis* spp., *Cooperia* spp., *Dicrocoelium* spp., *Dictyocaulus filaria, Diphyllobothrium latum, Dracunculus medinensis, Echinococcus granulosus, Echinococcus multilocularis, Enterobius vermicularis*, *Faciola* spp., *Haemonchus* spp., *Heterakis* spp., *Hymenolepis nana, Hyostrongulus* spp., *Loa Loa, Nematodirus* spp., *Oesophagostomum* spp., *Opisthorchis* spp., *Onchocerca volvulus*,

Ostertagia spp., *Paragonimus* spp., *Schistosomen* spp., *Strongyloides fuelleborni, Strongyloides stercoralis*, *Stronyloides* spp., *Taenia saginata*, *Taenia solium, Trichinella spiralis, Trichinella nativa, Trichinella britovi, Trichinella nelsoni, Trichinella pseudopsiralis, Trichostrongulus* spp., *Trichuris trichuria, Wuchereria bancrofti*;

[000395] phytoparasitic pests from the phylum Nematoda, for example, *Aphelenchoides* spp., *Bursaphelenchus* spp., *Ditylenchus* spp., *Globodera* spp., *Heterodera* spp., *Longidorus* spp., *Meloidogyne* spp., *Pratylenchus* spp., *Radopholus* spp., *Trichodorus* spp., *Tylenchulus* spp., *Xiphinema* spp., *Helicotylenchus* spp., *Tylenchorhynchus* spp., *Scutellonema* spp., *Paratrichodorus* spp., *Meloinema* spp., *Paraphelenchus* spp., *Aglenchus* spp., *Belonolaimus* spp., *Nacobbus* spp., *Rotylenchulus* spp., *Rotylenchus* spp., *Neotylenchus* spp., *Paraphelenchus* spp., *Dolichodorus* spp., *Hoplolaimus* spp., *Punctodera* spp., *Criconemella* spp., *Quinisulcius* spp., *Hemicycliophora* spp., *Anguina* spp., *Subanguina* spp., *Hemicriconemoides* spp., *Psilenchus* spp., *Pseudohalenchus* spp., *Criconemoides* spp., *Cacopaurus* spp., *Hirschmaniella* spp, *Tetylenchus* spp.

[000396] The fact that the composition is well tolerated by plants at the concentrations required for controlling plant diseases and pests allows the treatment of above-ground parts of plants, of propagation stock and seeds, and of the soil.

[000397] According to the invention all plants and plant parts can be treated. By plants is meant all plants and plant populations such as desirable and undesirable wild plants, cultivars and plant varieties (whether or not protectable by plant variety or plant breeder's rights). Cultivars and plant varieties can be plants obtained by conventional propagation and breeding methods which can be assisted or supplemented by one or more biotechnological methods such as by use of double haploids, protoplast fusion, random and directed mutagenesis, molecular or genetic markers or by bioengineering and genetic engineering methods. By plant parts is meant all above ground and below ground parts and organs of plants such as shoot, leaf, blossom and root, whereby for example leaves, needles, stems, branches, blossoms, fruiting bodies, fruits and seed as well as roots, corms and rhizomes are listed. Crops and vegetative and generative propagating material, for example cuttings, corms, rhizomes, runners and seeds also belong to plant parts.

[000398] The inventive composition, when it is well tolerated by plants, has favourable homeotherm toxicity and is well tolerated by the environment, is suitable for protecting plants and plant organs, for enhancing harvest yields, for improving the quality of the harvested material. It can preferably be used as crop protection composition. It is active against normally sensitive and resistant species and against all or some stages of development.

[000399] Plants which can be treated in accordance with the invention include the following main crop plants: maize, soya bean, alfalfa, cotton, sunflower, *Brassica* oil seeds such as *Brassica napus* (e.g., canola, rapeseed), *Brassica rapa*, *B. juncea* (e.g., (field) mustard) and *Brassica carinata*, *Arecaceae* sp. (e.g., oilpalm, coconut), rice, wheat, sugar beet, sugar cane, oats, rye, barley, millet and sorghum, triticale, flax, nuts, grapes and vine and various fruit and vegetables from various botanic taxa, e.g., *Rosaceae* sp. (e.g., pome fruits such as apples and pears, but also stone fruits such as apricots, cherries, almonds, plums and peaches, and berry fruits such as strawberries, raspberries, red and black currant and gooseberry), *Ribesioidae* sp., *Juglandaceae* sp., *Betulaceae* sp., *Anacardiaceae* sp., *Fagaceae* sp., *Moraceae* sp., *Oleaceae* sp. (e.g., olive tree), *Actinidaceae* sp., *Lauraceae* sp. (e.g., avocado, cinnamon, camphor), *Musaceae* sp. (e.g., banana trees and plantations), *Rubiaceae* sp. (e.g., coffee), *Theaceae s*p. (e.g., tea), *Sterculiceae* sp., *Rutaceae* sp. (e.g., lemons, oranges, mandarins and grapefruit); *Solanaceae* sp. (e.g., tomatoes, potatoes, peppers, capsicum, aubergines, tobacco), *Liliaceae* sp., *Compositae* sp. (e.g., lettuce, artichokes and chicory – including root chicory, endive or common chicory), *Umbelliferae* sp. (e.g., carrots, parsley, celery and celeriac), *Cucurbitaceae* sp. (e.g., cucumbers – including gherkins, pumpkins, watermelons, calabashes and melons), *Alliaceae* sp. (e.g., leeks and onions), *Cruciferae* sp. (e.g., white cabbage, red cabbage, broccoli, cauliflower, Brussels sprouts, pak choi, kohlrabi, radishes, horseradish, cress and chinese cabbage), *Leguminosae* sp. (e.g., peanuts, peas, lentils and beans – e.g., common beans and broad beans), *Chenopodiaceae* sp. (e.g., Swiss chard, fodder beet, spinach, beetroot), *Linaceae* sp. (e.g., hemp), *Cannabeacea* sp. (e.g., cannabis), *Malvaceae* sp. (e.g., okra, cocoa), *Papaveraceae* (e.g., poppy), *Asparagaceae* (e.g., asparagus); useful plants and ornamental plants in the garden and woods including turf, lawn, grass and *Stevia rebaudiana*; and in each case genetically modified types of these plants.

[000400] Depending on the plant species or plant cultivars, their location and growth conditions (soils, climate, vegetation period, diet), using or employing the composition according to the present invention the treatment according to the invention may also result in super-additive ("synergistic") effects. Thus, for example, by using or employing inventive composition in the treatment according to the invention, reduced application rates and/or a widening of the activity spectrum and/or an increase in the activity better plant growth, increased tolerance to high or low temperatures, increased tolerance to drought or to water or soil salt content, increased flowering performance, easier harvesting, accelerated maturation, higher harvest yields, bigger fruits, larger plant height, greener leaf color, earlier flowering, higher quality and/or a higher nutritional value of the harvested products, higher sugar

concentration within the fruits, better storage stability and/or processability of the harvested products are possible, which exceed the effects which were actually to be expected.

[000401] At certain application rates of the inventive composition in the treatment according to the invention may also have a strengthening effect in plants. The defense system of the plant against attack by unwanted phytopathogenic fungi and/ or microorganisms and/or viruses is mobilized. Plant-strengthening (resistance-inducing) substances are to be understood as meaning, in the present context, those substances or combinations of substances which are capable of stimulating the defense system of plants in such a way that, when subsequently inoculated with unwanted phytopathogenic fungi and/or microorganisms and/or viruses, the treated plants display a substantial degree of resistance to these phytopathogenic fungi and/or microorganisms and/or viruses. Thus, by using or employing composition according to the present invention in the treatment according to the invention, plants can be protected against attack by the abovementioned pathogens within a certain period of time after the treatment. The period of time within which protection is effected generally extends from 1 to 10 days, preferably 1 to 7 days, after the treatment of the plants with the active compounds.

[000402] Plants and plant cultivars which are also preferably to be treated according to the invention are resistant against one or more biotic stresses, i.e., said plants show a better defense against animal and microbial pests, such as against nematodes, insects, mites, phytopathogenic fungi, bacteria, viruses and/or viroids.

[000403] Plants and plant cultivars which may also be treated according to the invention are those plants which are resistant to one or more abiotic stresses, i.e., that already exhibit an increased plant health with respect to stress tolerance. Abiotic stress conditions may include, for example, drought, cold temperature exposure, heat exposure, osmotic stress, flooding, increased soil salinity, increased mineral exposure, ozone exposure, high light exposure, limited availability of nitrogen nutrients, limited availability of phosphorus nutrients, shade avoidance. Preferably, the treatment of these plants and cultivars with the composition of the present invention additionally increases the overall plant health (cf. above).

[000404] Plants and plant cultivars which may also be treated according to the invention, are those plants characterized by enhanced yield characteristics, i.e., that already exhibit an increased plant health with respect to this feature. Increased yield in said plants can be the result of, for example, improved plant physiology, growth and development, such as water use efficiency, water retention efficiency, improved nitrogen use, enhanced carbon assimilation, improved photosynthesis, increased germination efficiency and accelerated maturation.

[000405] Yield can furthermore be affected by improved plant architecture (under stress and non-stress conditions), including but not limited to, early flowering, flowering control for hybrid seed production, seedling vigor, plant size, internode number and distance, root growth, seed size, fruit size, pod size, pod or ear number, seed number per pod or ear, seed mass, enhanced seed filling, reduced seed dispersal, reduced pod dehiscence and lodging resistance. Further yield traits include seed composition, such as carbohydrate content, protein content, oil content and composition, nutritional value, reduction in anti-nutritional compounds, improved processability and better storage stability. Preferably, the treatment of these plants and cultivars with the composition of the present invention additionally increases the overall plant health (cf. above).

[000406] Plants that may be treated according to the invention are hybrid plants that already express the characteristic of heterosis or hybrid vigor which results in generally higher yield, vigor, health and resistance towards biotic and abiotic stress factors. Such plants are typically made by crossing an inbred male-sterile parent line (the female parent) with another inbred male-fertile parent line (the male parent). Hybrid seed is typically harvested from the male sterile plants and sold to growers. Male sterile plants can sometimes (e.g., in corn) be produced by detasseling, i.e., the mechanical removal of the male reproductive organs (or males flowers) but, more typically, male sterility is the result of genetic determinants in the plant genome. In that case, and especially when seed is the desired product to be harvested from the hybrid plants it is typically useful to ensure that male fertility in the hybrid plants is fully restored. This can be accomplished by ensuring that the male parents have appropriate fertility restorer genes which are capable of restoring the male fertility in hybrid plants that contain the genetic determinants responsible for male-sterility. Genetic determinants for male sterility may be located in the cytoplasm. Examples of cytoplasmic male sterility (CMS) were for instance described in *Brassica* species. However, genetic determinants for male sterility can also be located in the nuclear genome. Male sterile plants can also be obtained by plant biotechnology methods such as genetic engineering. A particularly useful means of obtaining male-sterile plants is described in WO 89/10396 in which, for example, a ribonuclease such as barnase is selectively expressed in the tapetum cells in the stamens. Fertility can then be restored by expression in the tapetum cells of a ribonuclease inhibitor such as barstar.

[000407] Plants or plant cultivars (obtained by plant biotechnology methods such as genetic engineering) which may be treated according to the invention are herbicide-tolerant plants, i.e., plants made tolerant to one or more given herbicides. Such plants can be obtained

either by genetic transformation, or by selection of plants containing a mutation imparting such herbicide tolerance.

Methods for Stimulating Plant Growth and/or Promoting Plant Health and/or Controlling Plant Pathogens

[000408] A method for stimulating plant growth and/or promoting plant health and/or controlling plant pests, such as nematodes, and/or controlling plant pathogens is provided. The method comprises applying the compositions of the present invention to a plant growth medium, a plant, a plant seed, or an area surrounding a plant or a plant seed contacting the plant pest with the compositions of the present invention.

[000409] Yet another method for stimulating plant growth and/or promoting plant health and/or controlling plant pests, such as nematodes, and/or controlling plant pathogens is provided. The method comprises applying a formulation to a plant growth medium, a plant, a plant seed, or an area surrounding a plant or a plant seed. The formulation can comprise any of the formulations described herein.

[000410] In any of the methods described herein, the method can comprise applying a composition comprising an insecticide or plant growth promoting active ingredient and the recombinant *Bacillus cereus* family member, the exosporium fragments, or the formulation to the plant growth medium.

[000411] In any of the methods described herein involving the use of a plant growth medium, the plant growth medium can comprise soil, water, an aqueous solution, sand, gravel, a polysaccharide, mulch, compost, peat moss, straw, logs, clay, soybean meal, yeast extract, or a combination thereof.

[000412] The plant growth medium can comprise a fertilizer.

[000413] Any of the methods described herein can further comprise supplementing the plant growth medium with a substrate for an enzyme. Suitable substrates include, but are not limited to protein meal, casein, gelatin, albumin, or a combination of any thereof.

[000414] For example, the method can comprise applying any of the compositions of the present invention to roots of the plant.

[000415] Alternatively or in addition, the method can comprise applying the compositions of the present invention foliarly.

[000416] In any of the methods described herein, the method can comprise applying the compositions of the present invention to the plant seed.

[000417] In any of the methods described herein, the plant pest that are controlled can be phytoparasitic pests from the phylum Nematoda, for example, *Aglenchus* spp., *Anguina* spp., *Aphelenchoides* spp., *Belonolaimus* spp., *Bursaphelenchus* spp., *Cacopaurus* spp., *Criconemella* spp., *Criconemoides* spp., *Ditylenchus* spp., *Dolichodorus* spp., *Globodera* spp., *Helicotylenchus* spp., *Hemicriconemoides* spp., *Hemicycliophora* spp., *Heterodera* spp., *Hoplolaimus* spp., *Longidorus* spp., *Lygus* spp., *Meloidogyne* spp., *Meloinema* spp., *Nacobbus* spp., *Neotylenchus* spp., *Paralongidorus* spp., *Paraphelenchus* spp., *Paratrichodorus* spp., *Pratylenchus* spp., *Pseudohalenchus* spp., *Psilenchus* spp., *Punctodera* spp., *Quinisulcius* spp., *Radopholus* spp., *Rotylenchulus* spp., *Rotylenchus* spp., *Scutellonema* spp., *Subanguina* spp., *Trichodorus* spp., *Tylenchulus* spp., *Tylenchorhynchus* spp., *Xiphinema* spp.

[000418] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit increased growth as compared to plants grown in the absence of the composition under the same conditions.

[000419] In any of the methods described herein, seeds to which any of the compositions of the present invention has been applied can exhibit increased germination rates as compared to seeds to which the composition has not been applied, under the same conditions.

[000420] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit increased nutrient uptake as compared to plants grown in the absence of the composition, under the same conditions.

[000421] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit decreased susceptibility to a pest, such as nematodes, as compared to plants grown in the absence of the composition, under the same conditions.

[000422] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit decreased nematode damage, including reduced galling, reduced cysts, and/or reduced nematodes per weight of root, as compared to plants grown in the absence of the composition, under the same conditions.

[000423] In any of the methods described herein, plants or the locus in which the plant is grown, such as soil, to which any of the compositions of the present invention has been applied can exhibit reduced nematode eggs and/or reduced nematodes per volume of soil, as compared to plants grown in the absence of composition, under the same conditions.

[000424] In one embodiment, the compositons of the present invention decrease nematodes and/or nematode damage by at least about 0.5%, or by at least about 1%, or by at least about 2%, or by at least about 3%, or by at least about 5%, or by at least about 6%, or by at

least about 7%, or by at least about 8%, or by at least about 9%, or by at least about 10%, or by at least about 11%, or by at least about 12% when compared to plants produced under the same conditions but without treatment by a recombinant *Bacillus cereus* family member.

[000425] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit decreased susceptibility to a pathogen as compared to plants grown in the absence of the composition, under the same conditions.

[000426] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit decreased susceptibility to an environmental stress (e.g., drought, flood, heat, freezing, salt, heavy metals, low pH, high pH, or a combination of any thereof) as compared to plants grown in the absence of the composition, under the same conditions.

[000427] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit increased root nodulation as compared to plants grown in the absence of the composition, under the same conditions.

[000428] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit greater crop yield as compared to plants grown in the absence of the composition, under the same conditions. In one embodiment, the composition of the present invention increases yield or total plant weight by at least about 0.5%, or by at least about 1%, or by at least about 2%, or by at least about 3%, or by at least about 5%, or by at least about 6%, or by at least about 7%, or by at least about 8%, or by at least about 9%, or by at least about 10%, or by at least about 11%, or by at least about 12% when compared to plants produced under the same conditions but without treatment by the compositions of the present invention. In another embodiment, the compositions of the present invention improve some aspect of plant vigor, such as germination, by at least about 0.5%, or by at least about 1%, or by at least about 2%, or by at least about 3%, or by at least about 5%, or by at least about 6%, or by at least about 7%, or by at least about 8%, or by at least about 9%, or by at least about 10%, or by at least about 11%, or by at least about 12% when compared to plants produced under the same conditions but without treatment by a composition of the present invention.

[000429] In any of the methods described herein, plants grown in the presence of any of the compositions of the present invention can exhibit altered leaf senescence as compared to plants grown in the absence of such compositions, under the same conditions.

[000430] Having described the invention in detail, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims.

EXAMPLES

[000431] The following non-limiting examples are provided to further illustrate the present invention.

Example 1. Construction of a *Bacillus cereus* Family Member Displaying a Serine Protease or Serine Protease Variant

[000432] To construct a *Bacillus cereus* family member displaying the serine protease of SEQ ID NO: 4 or SEQ ID NO: 5 or the serine protease variant of SEQ ID NO: 6, the pSUPER plasmid was generated through fusion of the pUC57 plasmid (containing an ampicillin resistance cassette and a ColE1 origin of replication) with the pBC16-1 plasmid from *Bacillus cereus* (containing a tetracycline resistance gene, repU replication gene and oriU origin of replication). This 5.8 kb plasmid can replicate in both *E. coli* and *Bacillus* spp. and can be selected by conferring resistance to β-lactam antibiotics in *E. coli* and resistance to tetracycline in *Bacillus* spp. The basal pSUPER plasmid was modified by insertion of a PCR-generated fragment that fused the BclA promoter (SEQ ID NO: 11), a start codon, amino acids 20–35 of BclA (amino acids 20–35 of SEQ ID NO: 1) and an alanine linker sequence in frame with SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, resulting in a plasmid termed pSUPER-BclA 20–35-SEQ ID NO: 4, pSUPER-BclA 20–35-SEQ ID NO: 5, or pSUPER-BclA 20–35-SEQ ID NO: 6, respectively. This construct was transformed into *E. coli* and plated on Lysogeny broth plates plus ampicillin (100 µg/mL) to obtain single colonies. Individual colonies were used to inoculate Lysogeny broth plus ampicillin and incubated overnight at 37°C, 300 rpm. Plasmids from resulting cultures were extracted using a commercial plasmid purification kit. DNA concentrations of these plasmid extracts were determined via spectrophotometry, and obtained plasmids subjected to analytical digests with appropriate combinations of restriction enzymes. The resulting digestion patterns were visualized by agarose gel electrophoresis to investigate plasmid size and presence of distinct plasmid features. Relevant sections, such as the SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6 expression cassette, of the purified pSUPER derivatives were further investigated by Sanger sequencing.

[000433] Additionally and alternatively, a derivative plasmid of the pSUPER plasmids described above was created as follows. The pBC fragment (pBC16-1-derived section of pSUPER including BclA/serine protease variant expression cassette and tetracycline resistance) of the pSUPER plasmids described above was amplified by PCR and subsequently circularized by blunt-end ligation.

[000434] pSUPER, verified as described above, and pBC plasmid ligations were introduced by electroporation into *Bacillus thuringiensis* BT013A. Single transformed colonies were isolated by plating on nutrient broth plates containing tetracycline (10 µg/mL). Individual positive colonies were used to inoculate brain heart infusion broth containing tetracycline (10 µg/mL) and incubated overnight at 30°C, 300 rpm. Genomic DNA of resulting cultures was purified and relevant sections of the pSUPER plasmid or the pBC plasmid were re-sequenced to confirm genetic purity of the cloned sequences and, for pBC, the correct ligation site. Verified colonies were grown overnight in brain heart infusion broth with 10 µg/mL tetracycline and induced to sporulate through incubation in a yeast extract-based media at 30°C for 48 hours. Short names for BT013A carrying the above-described plasmids are described in **Table 5**, below.

[000435] *Bacillus thuringiensis* BT013A was deposited with the United States Department of Agriculture (USDA) Agricultural Research Service (ARS), having the address 1815 North University Street, Peoria, Illinois 61604, U.S.A., on March 10, 2014, and assigned accession number NRRL B-50924. *Bacillus thuringiensis* BT013A is also known as *Bacillus thuringiensis* 4Q7.

Example 2. Construction and Purification of Exosporium Fragments from a *Bacillus cereus* Family Member Expressing Serine Protease Variant

[000436] **Knock out (KO) Mutants**: To make *exsY* knockout (KO) mutant strains of *Bacillus thuringiensis* BT013A, the plasmid pKOKI shuttle and integration vector was constructed that contained the pUC57 backbone, which is able to replicate in *E. coli*, as well as the origin of replication and the erythromycin resistance cassette from pE194. This construct is able to replicate in both *E. coli* and *Bacillus* spp. A construct was made that contained the 1 kb DNA region that corresponded to the upstream region of the *exsY* gene and a 1 kb region that corresponded to the downstream region of the gene *exsY*, both of which were PCR amplified from *Bacillus thuringiensis* BT013A. For each construct, the two 1 kb regions were then spliced together using homologous recombination with overlapping regions to each other and with the pKOKI plasmid, respectively. This plasmid construct was verified by digestion and DNA sequencing. Clones were screened for erythromycin resistance.

[000437] Clones were passaged under high temperature (40°C) in brain heart infusion broth. Individual colonies were toothpicked onto LB agar plates containing erythromycin 5 µg/mL, grown at 30°C, and screened for the presence of the pKOKI plasmid integrated into the chromosome by colony PCR. Colonies that had an integration event were continued through

passaging to screen for single colonies that lost erythromycin resistance (signifying loss of the plasmid by recombination and removal of the *exsY* gene). Verified deletions were confirmed by PCR amplification and sequencing of the target region of the chromosome. Finally, the PCR-amplified, circularized pBC section of the pSUPER-BclA 20-35 SEQ ID NO: 4 plasmid, pSUPER-BclA 20-35 SEQ ID NO: 5 plasmid or pSUPER-BclA 20-35 SEQ ID NO: 6 plasmid (described above in Example 1) was transformed into this *exsY* mutant strain of BT013A.

[000438] For each *esxY*KO mutant expressing the serine protease of SEQ ID NO: 4 or 5 or the serine protease variant of SEQ ID NO: 6, an overnight culture was grown in BHI media at 30°C, 300 rpm, in baffled flasks with antibiotic selection. One milliliter of this overnight culture was inoculated into a yeast extract-based media (50 mL) in a baffled flask and grown at 30°C for 2 days. An aliquot of spores was removed and the spores were agitated by vortexing. The spores were collected via centrifugation at 8,000 x g for 10 minutes, and supernatant containing the exosporium fragments was filtered through a 0.22 μm filter to remove any residual spores. No spores were found in the filtrate.

[000439] Short names for BT013A*exsY*KO carrying the above-described plasmids are described in **Table 5**, below.

Example 3. Use of an Expression Cassette Comprising a Non-Antibiotic Selectable Marker to Express the Serine Protease Variant on the Surface of *Bacillus cerus* Family Member Spores

[000440] SEQ ID NO: 6 was cloned into a derivative of the pSUPER plasmid described in Example 1. In this derivative, the tetracycline resistance marker had previously been exchanged with a non-antibiotic selectable marker. The pBC fragment of this derivative pSUPER plasmid was created as described in Example 1. The resulting pBC ligation, referred to as pBCnam212, was introduced using electroporation into a *Bacillus thuringiensis* BT013A derivative strain that had been modified to support the use of the non-antibiotic selectable marker. Single colonies of transformations were obtained by plating on suitable selection media on petri plates. Individual colonies were used to inoculate a suitable selection media and incubated overnight at 30°C, 300 rpm. Genomic DNA of resulting cultures was purified and the pBC plasmid re-sequenced to verify genetic purity. Verified colonies were grown overnight in suitable selection media and induced to sporulate through incubation in a yeast extract-based media at 30°C for 48 hours.

Table 5. Short Names for Bacteria Carrying Various Plasmids

Shortened Name of Recombinant *Bacillus cereus* Family Member	Plasmid	Example Describing Construction of Bacteria and Whole Broth or Exosporium Fragment Preparation
BT013A-pSuper212	pSUPER-BclA 20–35-SEQ ID NO: 6	1
BT013A-pBC210	pBCtet-BclA 20–35-SEQ ID NO: 4	1
BT013A-pBC211	pBCtet-BclA 20–35-SEQ ID NO: 5	1
BT013A-pBC212	pBCtet-BclA 20–35-SEQ ID NO: 6	1
BT013A*exsY*KO-pBC210	pBCtet-BclA 20–35-SEQ ID NO: 4	2
BT013A*exsY*KO-pBC211	pBCtet-BclA 20–35-SEQ ID NO: 5	2
BT013A*exsY*KO-pBC212	pBCtet-BclA 20–35-SEQ ID NO: 6	2
BT013A-pBCnam212	pBCnam-BclA 20–35-SEQ ID NO: 6	3

Example 4: Formula for the Efficacy of the Combination of Multiple Active Ingredients

[000441] A synergistic effect of active ingredients is present when the activity of the active ingredient combinations exceeds the total of the activities of the active ingredients when applied individually. The expected activity for a given combination of two active ingredients can be calculated as follows (cf. Colby, S.R., "Calculating Synergistic and Antagonistic Responses of Herbicide Combinations," Weeds, 1967, *15*, 20-22):

If

X is the efficacy when active ingredient A is applied at an application rate of m ppm (or g/ha),

Y is the efficacy when active ingredient B is applied at an application rate of n ppm (or g/ha),

E is the efficacy when the active ingredients A and B are applied at application rates of m and n ppm (or g/ha), respectively, and

then

$$E = X + Y - \frac{X \cdot Y}{100}$$

[000442] If the actual activity exceeds the calculated value, then the activity of the combination is superadditive, i.e., a synergistic effect exists. In this case, the efficacy which was actually observed must be greater than the value for the expected efficacy (E) calculated from the above-mentioned formula.

[000443] For instance, the formula and analysis can be applied to an evaluation of plant growth promotion. Such an assay is evaluated several days after the applications to plants. 100% means plant weight which corresponds to that of the untreated control plant. Efficacy means in this case the additional % of plant weight in comparison to that of the untreated control. For example, a treatment that resulted in plant weights that were 120% compared to the untreated control plant would have an efficacy of 20%. If the plant growth promotion effect for the combination (i.e., the observed efficacy for % plant weights of plants treated with the combination) exceeds the calculated value, then the activity of the combination is superadditive, i.e., a synergistic effect exists.

[000444] The formula and analysis can also be used to evaluate synergy in disease control and insect control assays. The degree of efficacy expressed in % is denoted. 0% means an efficacy which corresponds to that of the control while an efficacy of 100% means that no disease is observed.

[000445] If the actual insecticidal or fungicidal activity exceeds the calculated value, then the activity of the combination is superadditive, i.e., a synergistic effect exists. In this case, the efficacy which is actually observed must be greater than the value for the expected efficacy (E) calculated from the above-mentioned formula.

[000446] A further way of demonstrating a synergistic effect is the method of Tammes (cf. "Isoboles, A Graphic Representation of Synergism in Pesticides" in *Neth. J. Plant Path.*, 1964, *70*, 73-80).

Example 5. Control of Soybean Cyst Nematodes

[000447] Experiments can be conducted to test the activity of a whole broth culture of BT013A-pBCnam212 or exosporium fragments of BT013A*exsY*KO-pBC212, preparation of which is described in the examples, above, either alone or in combination with fluopyram. Seed is treated with (i) 234.8 mL/100 kg of the whole broth culture of BT013A-pBCnam212, which is equivalent to 1×10^{10} colony forming units (of the recombinant cell expressing the serine protease variant) ("CFU")/100 kg seed or 234.8 mL/100 kg of the exosporium fragments of BT013A*exsY*KO-pBC212 and/or (ii) fluopyram. The concentration of each whole broth culture is 5×10^6 CFU/mL. The same volume of the exosporium fragment preparation as whole broth is applied to seeds to achieve a comparable application rate to that of the whole broth, as very little liquid is lost during the

centrifugation and filtration processes that are used to separate exosporium fragments from cells. The fluopyram is applied to the seed, alone or in combination with the whole broth culture or exosporium fragments, at or below its label rate. All treatments are planted into a sandy loam soil. Ten days post emergence, soybean plants are inoculated with 2,000 second stage juvenile soybean cyst nematodes (*Heterodera glycine*). Plants are harvested four weeks later and cysts are removed and collected using a system of sieves, centrifugation, and a sucrose solution. Cysts are then crushed to release the eggs which are enumerated by taking three sub-samples from the total solution collected from each of ten plants from each treatment. It is expected that the seeds treated with the recombinant *Bacillus thuringiensis* in combination with the fluopyram or the exosporium fragments in combination with the fluopyram will show a greater reduction in both the total number of nematode eggs and the number of eggs per gram of root, than the seed treated with only one active ingredient; i.e., a synergistic effect will be shown.

[000448] In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained. As various changes could be made in the above compositions, formulations, and methods without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

CLAIMS

What is claimed is:

1. A composition comprising:

(a) a recombinant *Bacillus cereus* family member that expresses a fusion protein comprising:

(i) an enzyme having serine protease activity comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and

(ii) a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member; and

(b) at least one insecticide or plant growth promoter,

in a synergistically effective amount.

2. A composition comprising:

(a) exosporium fragments from a recombinant *Bacillus cereus* family member that expresses a fusion protein comprising:

(i) an enzyme having serine protease activity comprising an amino acid sequence having at least 95% identity to any one of SEQ ID NOs: 4-5 or an amino acid sequence having at least 95% identity to SEQ ID NO: 6; and

(ii) a targeting sequence, exosporium protein, or exosporium protein fragment that targets the fusion protein to the exosporium of a recombinant *Bacillus cereus* family member; and

(b) at least one insecticide or plant growth promoter,

in a synergistically effective amount,

wherein the *Bacillus cereus* family member comprises a mutation that results in an exosporium that is easier to remove from the spore as compared to the exopsorium of a wild-type spore.

3. The composition of Claim 1 or 2, wherein the targeting sequence or exosporium protein comprises:

(a) an amino acid sequence having at least about 43% identity with amino acids 20–35 of SEQ ID NO: 1, wherein the identity with amino acids 25–35 is at least about 54%;

(b) amino acids 1–35 of SEQ ID NO: 1;

(c) amino acids 20–35 of SEQ ID NO: 1;

(d) SEQ ID NO: 1;

(e) SEQ ID NO: 2; or

(f) an amino acid sequence having at least 85% identity with SEQ ID NO: 3.

4. The composition of Claim 1 or 2, wherein the targeting sequence comprises the sequence X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-X_{10}-X_{11}-X_{12}-X_{13}-X_{14}-X_{15}-X_{16}, wherein:

X_1 is any amino acid or absent;

X_2 is phenylalanine (F), leucine (L), isoleucine (I), or methionine (M);

X_3 is any amino acid;

X_4 is proline (P) or serine (S);

X_5 is any amino acid;

X_6 is leucine (L), asparagine (N), serine (S), or isoleucine (I);

X_7 is valine (V) or isoleucine (I);

X_8 is glycine (G);

X_9 is proline (P);

X_{10} is threonine (T) or proline (P);

X_{11} is leucine (L) or phenylalanine (F);

X_{12} is proline (P);

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is proline (P), glutamine (Q), or threonine (T); and

X_{16} is proline (P), threonine (T), or serine (S).

5. The composition of any one of Claims 1-3, wherein the targeting sequence, exosporium protein, or exosporium protein fragment further comprises a methionine, serine, or threonine residue at the amino acid position immediately preceding the first amino acid of the targeting sequence, exosporium protein, or exosporium protein fragment.

6. The composition of any one of Claims 1-4, wherein the fusion protein further comprises an amino acid linker between the targeting sequence, the exosporium protein, or the exosporium protein fragment and the enzyme having serine protease activity.

7. The composition of Claim 6, wherein the linker comprises a polyalanine linker, a polyglycine linker, or a linker comprising a mixture of both alanine and glycine residues.

8. A composition of any one of Claims 1-7, wherein the enzyme comprises SEQ ID NO: 4.

9. A composition of any one of Claims 1-7, wherein the enzyme comprises SEQ ID NO: 5.

10. A composition of any one of Claims 1-7, wherein the enzyme comprises SEQ ID NO: 6.

11. The composition of Claim 1 or Claim 2 wherein the recombinant *Bacillus cereus* family member is derived from *Bacillus thuringiensis* BT013A.

12. The composition of Claim 2, wherein the recombinant *Bacillus cereus* family member comprises:

(i) a mutation in a CotE gene;

(ii) a mutation in an ExsY gene;

(iii) a mutation in a CotY gene;

(iv) a mutation in a ExsA gene; and

(v) a mutation in a CotO gene.

13. The composition of Claim 12, wherein the recombinant *Bacillus cereus* family member comprises a mutation in the *ExsY* gene.

14. The recombinant *Bacillus cereus* family member of Claim 13 wherein the recombinant *Bacillus cereus* family member comprises a knock-out of the *ExsY* gene.

15. The composition of any one of the preceding claims and an agriculturally acceptable carrier.

16. The composition of any one of the preceding claims wherein the insecticide is selected from the group consisting of acetamiprid, aldicarb, amitraz, beta-cyfluthrin, carbaryl, clothianidin, cyfluthrin, cypermethrin, deltamethrin, endosulfan, ethion, ethiprole, ethoprophos, fenamiphos, fenobucarb, fenthion, fipronil, flubendiamide, fluopyram, flupyradifurone, formetanate, heptanophos, imidacloprid, methamidophos, methiocarb, methomyl, niclosamide, oxydemeton-methyl, phosalone, silafluofen, spirodiclofen, spiromesifen, spirotetramat, thiacloprid, thiodicarb, tralomethrin, triazophos, triflumuron, vamidothion, *Bacillus firmus* CNMC I-1582, and *Purpureocillium lilacinum* strain 251.

17. The composition of any one of the preceding claims wherein the insecticide is clothianidin or fluopyram.

18. A plant seed treated with the composition of any one of the preceding claims.

19. A method for stimulating plant growth and/or promoting plant health and/or controlling nematodes, comprising applying the composition of any one of Claims 1-17 to a plant growth medium, a plant, a plant seed, or an area surrounding a plant or a plant seed.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/080101

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/22 A01N63/23 A01P5/00 A01P7/00 A01P21/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N A01P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2020/296960 A1 (CURTIS DAMIAN [US] ET AL) 24 September 2020 (2020-09-24) sequences 1, 2, 37, 38, 41, 42, 96, 149, 150, 175 sequences 192, 193, 198, 199, 210-212 claims 1-19 examples 1-6, 11 tables 7-9	1-16,18, 19 1-19
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Y	US 2020/029573 A1 (RIGGS JENNIFER [US]) 30 January 2020 (2020-01-30) sequence 1 claims 1-6, 13, 20, 24	1-19

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 2023	09/02/2023

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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