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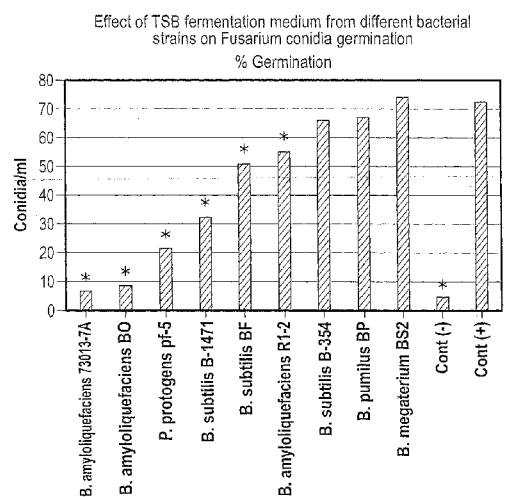
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(54) Title: COMPOSITION AND METHOD FOR REDUCING FUNGAL INFECTIONS IN CROPS

(57) Abstract: A composition useful for application to plants, seeds
or soil to inhibit fungal infections comprises a protein source inocu-
lated with from 5×10^7 cfu to 5×10^9 cfu of a *Bacillus amyloliquefa-*
ciens strain per gram of the protein source.



Cont (-) untreated with any bacterial strain or germination medium

Cont (+) treated with germination medium

Treatments with a strain ID were treated with bacteria broth and conidia germination medium

Bars marked with a * are statistically reduced relative to Cont (+) at $p \leq 0.05$

FIG. 1

**COMPOSITION AND METHOD FOR REDUCING FUNGAL
INFECTIONS IN CROPS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Application No. 15/886,124, filed February 1, 2018, which is incorporated herein by reference.

FIELD OF THE DISCLOSURE

[0002] This disclosure relates to methods and compositions for reducing or eliminating fungal infections of growing and/or harvested crops using non-pathogenic bacteria.

BACKGROUND OF THE DISCLOSURE

[0003] Members of the species *Bacillus* have been reported to be useful for preventing infections and/or promoting the growth of crops. For example, *Bacillus pumilus* strain QST2808, *Bacillus pumilus* strain GB34, *Bacillus Subtilis* strain QST713, *Bacillus Subtilis* strain GB03, various strains of *Bacillus thuringiensis* and *Bacillus firmus*, and *Bacillus amyloliquefaciens* strain FZB42 have been used in commercially available biocontrol products.

[0004] While the known natural, non-pathogenic, biological fungicides have achieved some commercial success, there remains a need for safe and highly effective biological fungicides.

SUMMARY OF THE DISCLOSURE

[0005] This disclosure relates to a safe (non-toxic, non-pathogenic) biological fungicide composition containing a protein source inoculated with from 5×10^7 cfu to 5×10^9 cfu of a *Bacillus amyloliquefacias* strain per gram of the protein source. The combination of a protein source and a biocidal strain of a *Bacillus amyloliquefacias* has been found to be

more effective at reducing and/or inhibiting fungal infections in crops than either component alone, providing an unexpected and synergistic improvement.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Figure 1 is a bar chart showing the inhibitory effect of fermentation medium effluent from different bacteria strains on *Fusarium oxysporum* conidia germination.

[0007] Figure 2 is a bar chart showing the inhibitory effect of fermentation medium effluent from different bacteria strains on *Fusarium oxysporum* conidia numbers.

[0008] Figure 3 is a bar chart showing the inhibitory effect of adding a composition containing a protein source inoculated with a strain of *Bacillus amyloliquefaciens* to a soil in which a plant is grown.

[0009] Figure 4 is a bar chart showing that the addition of the *Bacillus amyloliquefaciens* strain alone, without the protein source, to soil in which a plant is grown does not effectively inhibit *Fusarium oxysporum*.

[0010] Figure 5 is a bar chart showing that soybean flour inoculated with a strain of *Bacillus amyloliquefaciens* is much more effective than soybean flour alone at inhibiting *Fusarium oxysporum* in soil.

DETAILED DESCRIPTION OF THE DISCLOSED EMBODIMENTS

[0011] Based on experiments in which various *Bacillus* species were screened for their ability to promote growth of basil plants treated with a protein source selected from cotton seed meal, soybean flour and hydrolyzed feather meal, it was determined that the growth response to cotton seed meal and soybean flour was very poor regardless of the species of *Bacillus* and amount of *Bacillus* used. It was also determined that the growth response to hydrolyzed feather meal was good regardless of the amount or species of *Bacillus* used. This suggested to the inventors that the soybean flour and cotton seed meal was being consumed by bacteria and incorporated in the mass of the bacteria. The bacteria responsible for metabolizing the protein sources could be native bacteria, added bacteria (the protein

source inoculant), or a combination of both inoculant and native bacteria. This suggested to the inventors that certain protein sources, such as soybean flour and cotton seed meal, could be used to supplement the growth of a biocontrol organism, such as a fungicidal bacteria.

[0012] Based on separate experiments in which various *Bacillus* strains were screened for their ability to inhibit germination of conidia in water and their ability to lyse conidia in water, it was determined that the effluent from tryptic soy broth (TSB) media used for cultivating the various *Bacillus* strains tested typically contained secreted substances capable of inhibiting germination of conidia from *Fusarium oxysporum*. However, as shown in Figure 1, there were exceptions, such as *B. pumilus* strain BP, which did not appreciably inhibit *Fusarium oxysporum*. Also, as shown in Figure 1, *B. amyloliquefaciens* strains OBT 730 (deposited as ATCC Accession No. PTA-124660), and OBT 712 (deposited as ATCC Accession No. PTA-122189) were particularly effective at inhibiting *Fusarium* conidia germination.

[0013] Figure 2 shows that the ability of effluents from strains OBT 730 and OBT 712 to lyse *Fusarium oxysporum* conidia were particularly good, with over 70% of the conidia disappearing. Among the remaining conidia, percent germination was much lower for the effluents from strains OBT 730 and OBT 712 than observed when conidia were treated with effluent from other cultures.

[0014] Strain OBT 712 is a stock strain sold in most *Bacillus*-based products sold by Osprey Biotechnics, Sarasota, Florida, and strain OBT 730 was isolated by Osprey Biotechnics from a soil sample.

[0015] Strain OBT 712 was selected for further analysis. Specifically, experiments were conducted to determine the ability of strain OBT 712 to reduce *Fusarium* conidia concentrations in soil, both with and without soybean flour. Soybean flour was inoculated with 5×10^8 cfu *B. amyloliquefaciens* strain OBT 712 per gram of soybean flour. The inoculated soybean flour was added to soil in amounts of zero, 0.33%, 1% and 5%, and *Fusarium oxysporum* conidia was added at 5×10^5 conidia per gram of soil. The addition of *B. amyloliquefaciens*-inoculated soybean flour very substantially reduced *Fusarium* that could be recovered from the soil, as shown in Figure 3.

[0016] The inventors conducted several other studies to determine whether the OBT 712 strain could reduce *Fusarium* counts in the absence of soy flour and whether the effect on

Fusarium was due to soy flour alone or if the bacteria was required. One percent soy flour inoculated with 5×10^8 cfu strain OBT 712 per gram of soybean flour would deliver 5×10^6 cfu strain OBT 712 per gram of soil. Neither this concentration nor 5×10^5 cfu strain OBT 712 per gram of soil appeared to appreciably affect *Fusarium* (Figure 4). However, when compared to soy flour alone, soy flour plus strain OBT 712 reduced *Fusarium* recovery by approximately 80% (Figure 5).

[0017] The inventors have concluded from the above experiments that a soil amendment comprising soy flour inoculated with *Bacillus amyloliquefaciens* can be used to reduce *Fusarium* infections in crops. Soy flour appears to have an advantage over other nitrogen sources tested. The amount of soy required to observe a beneficial effect on *Fusarium* recovery is much lower than other organic nitrogen supplies. At 0.5% soy flour, the nitrogen content of the potting soil is approximately 375 ppm. Typically one would want a nitrogen concentration in the 100-200 ppm range, but given that *B. amyloliquefaciens* is growing on the substrate and assimilating nitrogen it is conceivable that free nitrogen would be much lower than 200 ppm and that detrimental effects to the plant would not be observed.

[0018] Alternatively, fermentation broth in itself could make a suitable anti-fungal product. One could use routine experimentation to determine the percentage of the material that could be used as a fungicide. The secreted products that are likely responsible for the anti-fungal activity of the *B. amyloliquefaciens* strains are lipopeptides. Examples being iturin, surfactin, and fengycin. Genes encoding for enzymes required for the synthesis of these compounds are present in the strain OBT 712 genome. These peptides have been shown to be haemolytic and as a result have not been commercialized.

[0019] The compositions (inoculated protein sources and effluents from cultures) disclosed herein can be used to inhibit fungal infections in plants by applying the compositions to the plants, to seeds from which the plants are grown, or to soil in which the plants are grown. Soil concentrations generally refer to concentrations within soil that the roots of the plant contact. Application to seeds refers to incorporating the disclosed compositions in a seed covering. "Effluents," refer to filtered liquids obtained from media in which the disclosed bacteria are cultured.

[0020] The described embodiments are preferred and/or illustrated, but are not limiting. Various modifications are considered within the purview and scope of the appended claims.

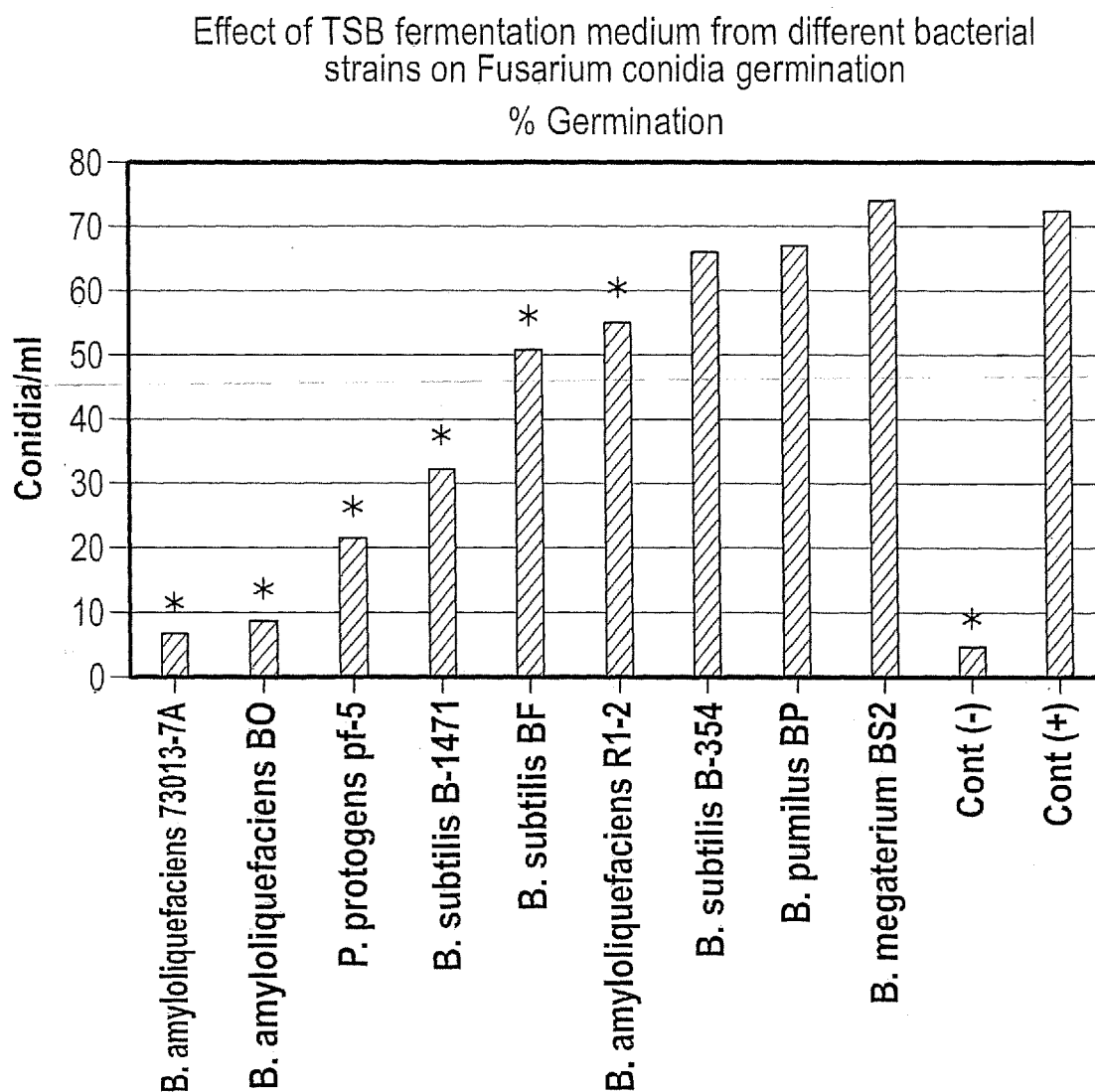
WHAT IS CLAIMED IS:

1. A composition comprising:
a protein source inoculated with from 5×10^7 to 5×10^9 cfu of a *Bacillus amyloliquefaciens* strain per gram of the protein source.
2. The composition of claim 1, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 712, deposited as ATCC Accession No. PTA-122189.
3. The composition of claim 1, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 730, deposited as ATCC Accession No. PTA-124660.
4. The composition of claim 1, wherein the protein source is soybean flour.
5. The composition of claim 1, wherein the protein source is cotton seed meal.
6. A composition comprising:
effluent from a *Bacillus amyloliquefaciens* strain grown in a cultivation medium.
7. The composition of claim 6, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 712, deposited as ATCC Accession No. PTA-122189.
8. The composition of claim 6, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 730, deposited as ATCC Accession No. PTA-124660.
9. The composition of claim 6, wherein the cultivation medium is tryptic soy broth.

10. A process for inhibiting fungal infections in a plant, comprising:
applying to a seed from which the plant grows, or to a place where the plant is grown, a composition including a protein source inoculated with from 5×10^7 cfu to 5×10^9 cfu of a *Bacillus amyloliquefaciens* strain per gram of the protein source.
11. The process of claim 10, wherein the composition is added to the soil in which the plant is grown, such that the composition is present in the soil at an amount of from about 0.33% to about 5% of the mass of the soil.
12. The process of claim 10, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 712, deposited as ATCC Accession No. PTA-122189.
13. The process of claim 10, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 730, deposited as ATCC Accession No. PTA-124660.
14. The process of claim 10, wherein the protein source is soybean flour.
15. The process of claim 10, wherein the protein source is cotton seed meal.
16. The process of claim 10, wherein the plant is infected with a strain of *Fusarium oxysporum*.
17. A process for inhibiting fungal infections in a plant, comprising:
applying to the plant, or to a place where the plant is grown, effluent from a *Bacillus amyloliquefaciens* strain grown in a cultivation medium.

18. The process of claim 17, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 712, deposited as ATCC Accession No. PTA-122189.
19. The process of claim 17, wherein the *Bacillus amyloliquefaciens* strain has the identifying characteristics of a bacterium designated strain OBT 730, deposited as ATCC Accession No. PTA-124660.
20. The process of claim 17, wherein the cultivation medium is tryptic soy broth.

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Cont (-) untreated with any bacterial strain or germination medium

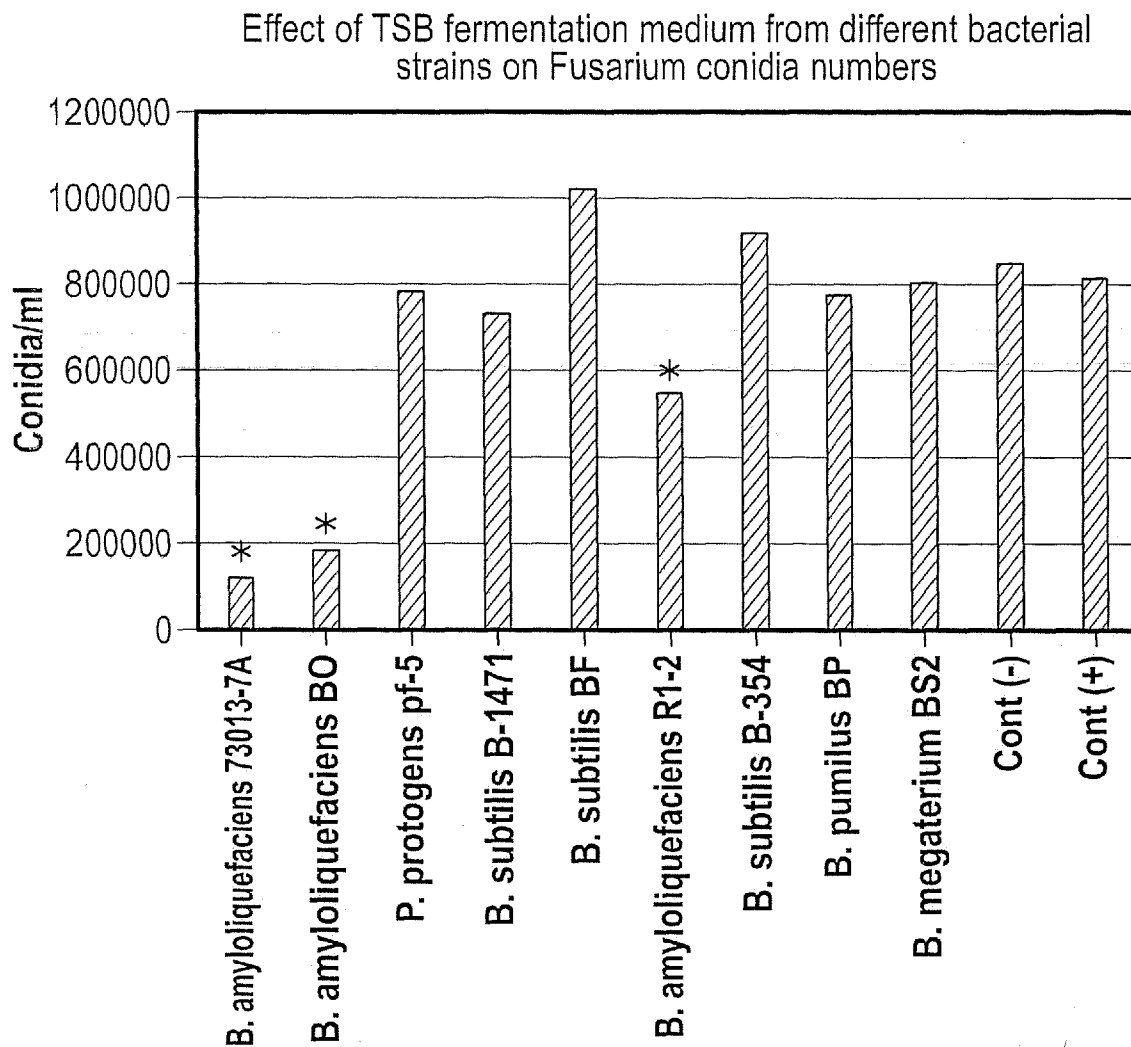
Cont (+) treated with germination medium

Treatments with a strain ID were treated with bacteria broth and conidia germination medium

Bars marked with a * are statistically reduced relative to Cont (+) at $p \leq 0.05$

FIG. 1

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Cont (-) untreated with any bacterial strain or germination medium

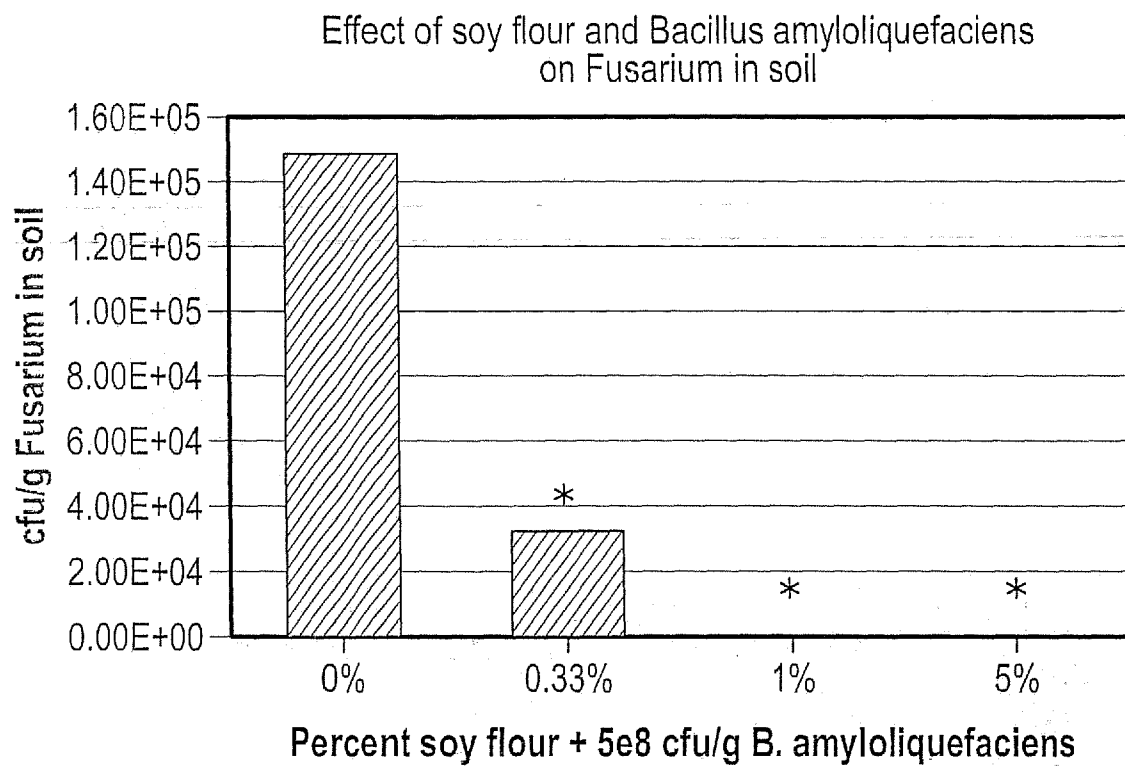
Cont (+) treated with germination medium

Treatments with a strain ID were treated with bacteria broth and conidia germination medium

Bars marked with a * are statistically reduced relative to Cont (-) at $p \leq 0.05$

FIG. 2

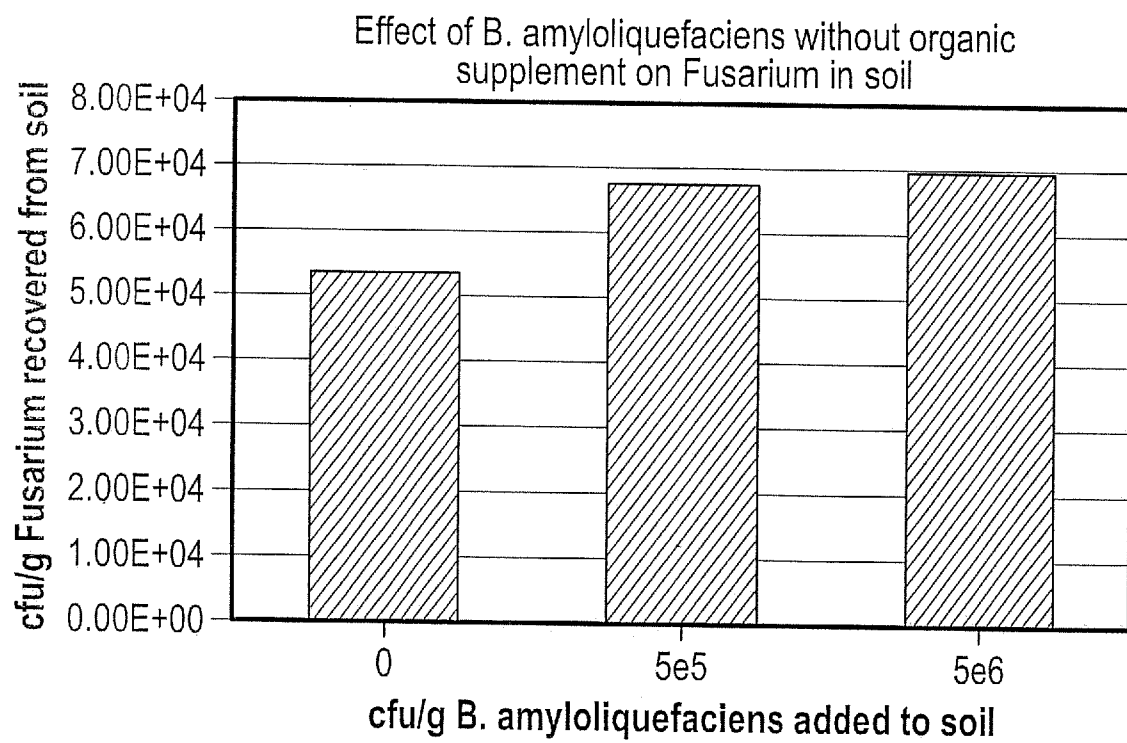
3/5



Each treatment was repeated 3 times. * indicates that the treatment significantly $P < 0.05$ reduced recovered *Fusarium* from the soil when compared to the 0% soy flour treatment

FIG. 3

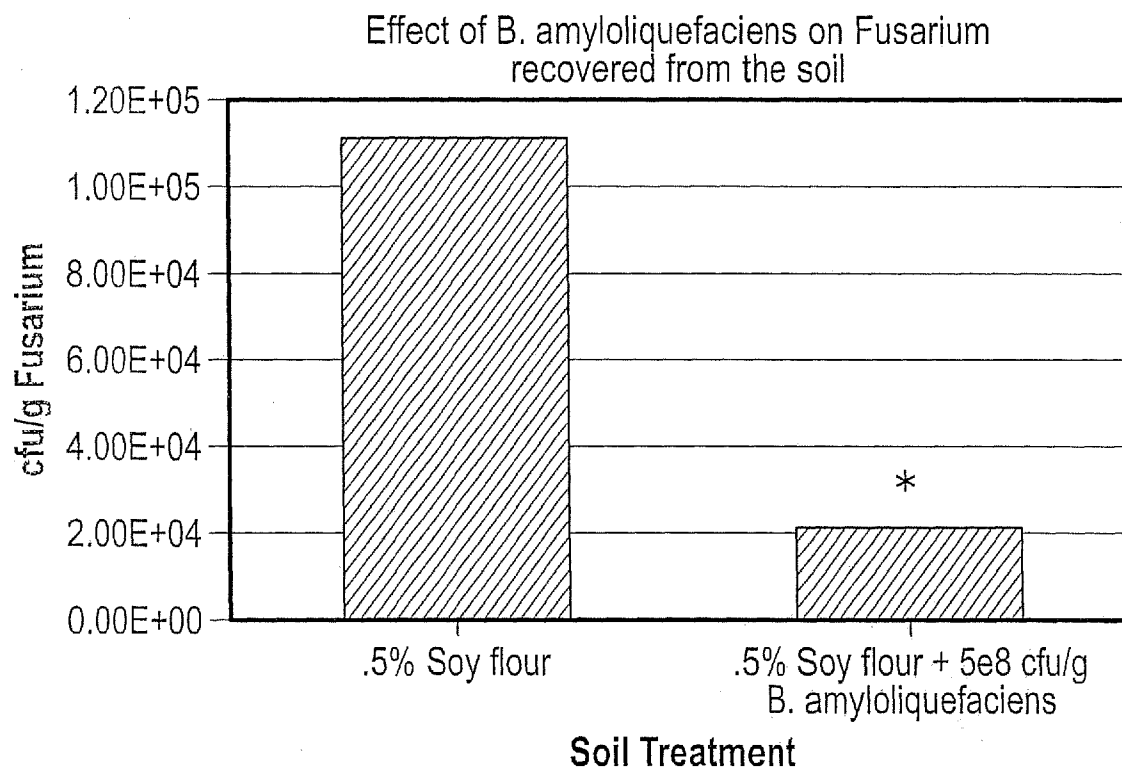
4/5



Each treatment was repeated 3 times. *B. amyloliquefaciens* at either concentration did not affect recovery of *Fusarium*

FIG. 4

5/5



Each treatment was repeated 8 times. * indicates that the treatment significantly $P < 0.05$ reduced recovered Fusarium from the soil when compared to .5% soy flour only

FIG. 5

A. CLASSIFICATION OF SUBJECT MATTER**A01N 63/00(2006.01)i, A01N 65/20(2009.01)i, C09K 17/32(2006.01)i, C05F 11/08(2006.01)i**

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

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
A01N 63/00; A23B 4/22; A61K 35/74; C12N 1/20; A01N 65/20; C09K 17/32; C05F 11/08Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: Bacillus amyloliquefaciens, OBT 712, OBT 730, fungal infection, inhibition, fungicide**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015-0258150 A1 (OSPREY BIOTECHNICS, INC.) 17 September 2015 See paragraphs [0022], [0024]; and claims 9, 16.	1,2,4-7,9
Y		10-12, 14-18, 20
A		3, 8, 13, 19
Y	KHAN, N. et al., `Combating Fusarium Infection Using Bacillus-Based Antimicrobials`, Microorganisms, 22 November 2017, Vol.5, 75, pp.1-13 See abstract; and page 2.	10-12, 14-18, 20
X	US 2015-0257400 A1 (OSPREY BIOTECHNICS, INC.) 17 September 2015 See abstract; and claims 1, 7, 14-19.	1,4-6,9
A	CN 107338202 A (ZHEJIANG SCI-TECH UNIVERSITY) 10 November 2017 See the whole document.	1-20
A	YUAN, J. et al., `Antifungal Activity of Bacillus amyloliquefaciens NJN-6 Volatile Compounds against Fusarium oxysporum f. sp. cubense`, Applied and Environmental Microbiology, August 2012, Vol.78, No.16, pp.5942-5944 See the whole document.	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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"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

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2019/015951Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

US 2015-0258150 A1

17/09/2015

None

US 2015-0257400 A1

17/09/2015

WO 2015-138539 A1

17/09/2015

WO 2015-138539 A4

15/10/2015

CN 107338202 A

10/11/2017

None