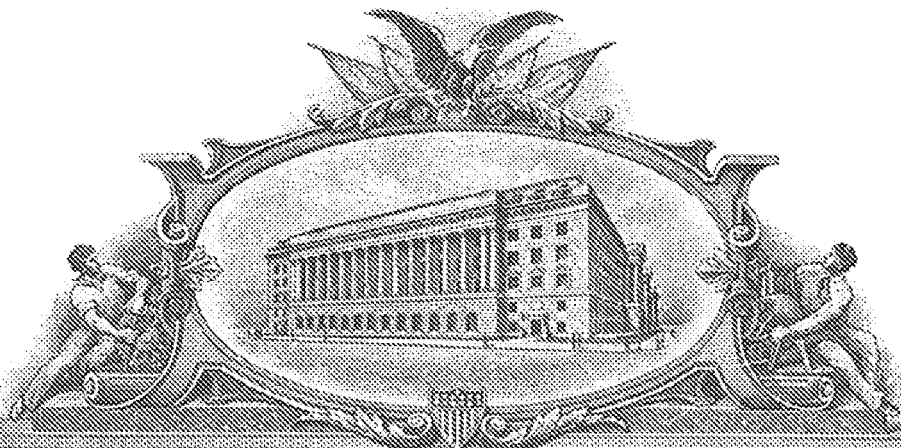


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FILING DATE: *January 31, 2014*

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	061719/439925
		Application Number	
Title of Invention	Modified Biological Control Agents and Their Use		
The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.			

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number		061719/439925	
		Application Number			
Title of Invention		Modified Biological Control Agents and Their Use			
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Application Information:

Title of the Invention	Modified Biological Control Agents and Their Use		
Attorney Docket Number	061719/439925	Small Entity Status Claimed	☒
Application Type	Provisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	1	Suggested Figure for Publication (if any)	

Filing By Reference :

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Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country i

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☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
☐ Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	061719/439925
		Application Number	
Title of Invention	Modified Biological Control Agents and Their Use		

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When referring to the current application, please leave the application number blank.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	061719/439925
		Application Number	
Title of Invention	Modified Biological Control Agents and Their Use		

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. ☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	061719/439925
	Application Number	
Title of Invention	Modified Biological Control Agents and Their Use	

Applicant 1				Remove	
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	061719/439925
		Application Number	
Title of Invention	Modified Biological Control Agents and Their Use		

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Modified Biological Control Agents and Their Uses

Field of the Invention

The invention relates to modified biocontrol agents and populations that have improved properties.

Background of the Invention

Plant diseases and pests need to be controlled to maintain the quality and quantity of food, feed, and fiber produced by growers around the world. Plant diseases are mainly caused by fungi, bacteria, viruses and nematodes. Plant pests include chewing, sucking and piercing insects from the Lepidoptera, Coleoptera, and Hemiptera, among others. Chemical pesticides are widely used in farming to protect crops from such pests and diseases. These chemical products fight crop pests, diseases, and weeds, resulting in improved yield. Without crop protection and pest control, food production and the quality of food produced would decline. However, the use of chemical pesticides does impose a level of risk as many have properties that can endanger health and the environment if not used properly.

It has long been known that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens and pests. Interactions between plant hosts and microorganisms that lead to biocontrol can include antibiosis, competition, induction of host resistance, and predation. Screening and testing isolates have yielded a number of candidates for commercialization. Microbial biopesticides represent an important option for the management of plant diseases and pests. There is a need for biological control agents that are able to compete in field conditions particularly in the presence of herbicides and fungicides that are commonly used in commercial farming and can have antibiotic effects on microorganisms.

Summary of the Invention

Compositions and methods for improving the ability of a population of biological agents or biocontrol agents to compete and survive in a field setting are provided. By improving the population of biological agents, the modified population of agents is able to grow, compete with

other microbial strains and fungi, and provide protection for plants from pathogens. In addition, modified biological control agents promote plant growth and yield. In particular, modified biological agents and modified populations of such agents that are herbicide-tolerant or –resistant; fungicide-tolerant or –resistant; pesticide-tolerant or –resistant; or tolerant or resistant to crop protection chemicals are selected or engineered. In this manner, the protection of crops from disease-causing agents or pests is enhanced.

The modified biological agents are able to grow in the presence at least one herbicide, fungicide, pesticide, or other crop protection chemical that is used in commercial farming. Such modified biological agents are able to grow and reproduce in soils where such herbicides, fungicides, pesticides, or other crop protection chemicals have been applied. The modified biological agents render the soils suppressive or resistant to disease-causing pathogens or pests. Such modified populations of biological agents can be added to soils to prevent fungal pathogens and the diseases they cause, or to inhibit feeding by insect pests or nematodes, promoting plant growth and increasing crop yield. Therefore, the present invention is useful for enhancing the competitiveness of modified biological agents particularly over other microbial agents which are not resistant to herbicides, fungicides, pesticides, or other crop protection chemicals. Therefore, compositions of the invention include selected or engineered biological agents and modified populations of biocontrol agents. These modified biological agents can be used as an inoculant or as a seed coating for plants and seeds. They can also be applied as a spray application directly to the aerial parts of plants, and can be mixed with the herbicide or other chemical to which they have been modified to become tolerant. As indicated, the presence of the modified biological agents under field conditions enhances resistance of the plants to pathogens and promotes plant growth. Such modified biological agents of the invention can be used with other agents to promote plant growth and yield.

Embodiments of the invention include:

1. A method for improving a biocontrol agent, said method comprising modifying said biocontrol agent to be resistant to at least one herbicide, fungicide, pesticide, or other crop protection chemical.

2. The method of embodiment 1, wherein said biocontrol agent is modified by growing in the presence of a herbicide, fungicide, pesticide, or other crop protection chemical to select a resistant strain.
3. The method of embodiment 1, wherein said biocontrol agent is modified by transforming said biocontrol agent with a gene that confers resistance to said herbicide, fungicide, pesticide, or other crop protection chemical.
4. The method of any one of embodiments 1-3, wherein said biocontrol agent is a bacterial biocontrol agent.
5. The method of any one of embodiments 1-3, wherein said biocontrol agent is selected from the group consisting of *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Lysobacter*, *Trichoderma*, *Paecilomyces*, *Gliocladium*, *Ampelomyces*, *Pythium*, *Metschnikowia*, *Chromobacterium*, *Penicillium*, *Coniothyrium*, *Chaetomium*, *Myrothecium*, *Aureobasidium*, *Pantoea*, *Burkholderia*, *Streptomyces*, *Variovorax*, *Pasteuria*, *Xanthomonas* genera.
6. The method of embodiment 5, wherein said bacterial biocontrol agent is a *Pseudomonas* bacterium.
7. The method of embodiment 6, wherein said *Pseudomonas* is *Pseudomonas fluorescens*.
8. The method of any one of embodiments 1-7, wherein said herbicide is selected from the group consisting of glyphosate, glufosinate (glutamine synthase inhibitor), sulfonylurea and imidazolinone herbicides (branched chain amino acid synthesis inhibitors).
9. A modified biocontrol agent wherein said biocontrol agent has been selected under herbicide, fungicide, pesticide, or other crop protection chemical pressure and is resistant to said herbicide, fungicide, pesticide, or other crop protection chemical.
10. The modified biocontrol agent of embodiment 9, wherein said modified biocontrol agent is a bacterial biocontrol agent.
11. The modified biocontrol agent of embodiment 9, wherein said biocontrol agent is selected from the group consisting of *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Lysobacter*, *Trichoderma*, *Paecilomyces*, *Gliocladium*, *Ampelomyces*, *Pythium*, *Metschnikowia*, *Chromobacterium*, *Penicillium*, *Coniothyrium*, *Chaetomium*, *Myrothecium*, *Aureobasidium*, *Pantoea*, *Burkholderia*, *Streptomyces*, *Variovorax*, *Pasteuria*, *Xanthomonas* genera.

12. The modified biocontrol agent of embodiment 10, wherein said bacterial biocontrol agent is a *Pseudomonas* bacterium.
13. The modified biocontrol agent of embodiment 12, wherein said *Pseudomonas* is *Pseudomonas fluorescens*.
14. The modified biocontrol agent of any one of embodiments 9-13, wherein said herbicide is selected from the group consisting of glyphosate, glufosinate (glutamine synthase inhibitor), sulfonyleurea and imidazolinone herbicides (branched chain amino acid synthesis inhibitors).
15. A recombinant biocontrol agent wherein said biocontrol agent has been transformed with a herbicide resistance gene rendering the biocontrol agent herbicide resistant.
16. The recombinant biocontrol agent of embodiment 15, wherein said modified biocontrol agent is a bacterial biocontrol agent.
17. The recombinant biocontrol agent of embodiment 16, wherein said bacterial biocontrol agent is selected from the group consisting of *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Lysobacter*, *Gliocladium*, *Pythium*, *Chromobacterium*, *Penicillium*, *Pantoea*, *Burkholderia*, *Streptomyces*, *Variovorax*, *Pasteuria*, and *Xanthomonas* genera.
18. The recombinant biocontrol agent of embodiment 17, wherein said bacterial biocontrol agent is a *Pseudomonas* bacterium.
19. The recombinant biocontrol agent of embodiment 18, wherein said *Pseudomonas* is *Pseudomonas fluorescens*.
20. The recombinant biocontrol agent of any one of embodiments 15-19, wherein said herbicide is selected from the group consisting of glyphosate, glufosinate (glutamine synthase inhibitor), sulfonyleurea and imidazolinone herbicides (branched chain amino acid synthesis inhibitors).
21. A modified population of biocontrol agents wherein the population substantially comprises the biocontrol agent of any one of embodiments 1-20.
22. A formulation for controlling a plant pathogen, said formulation comprising a modified population of biocontrol agents wherein said biocontrol agents are herbicide resistant.
23. The formulation of embodiment 22, wherein said population comprises modified biocontrol agents.

24. The formulation of embodiment 22, wherein said population comprises recombinant biocontrol agents.
25. A method for improving the ability of a biocontrol agent to compete in a field setting said method comprising modifying said biological agent such that said modified biocontrol agent is able to grow in the presence of a herbicide.
26. A method for promoting plant growth, said method comprising applying a composition comprising a modified population of biocontrol agents to the soil where said plant is growing.
27. The method of embodiment 26, wherein said biocontrol agents have been modified to be resistant to glyphosate.

Detailed Description of the Invention

Compositions and methods for improving biological control agents are provided. A biological agent or biocontrol agent for purposes of the present invention is used to describe a microorganism that is used to control disease-causing plant pathogens and plant pests. The biological control agents of the invention have been modified such that they are able to grow in the presence of at least one herbicide, fungicide, pesticide, or other crop protection chemical. Compositions of the invention include an isolated biological agent that has been selected for resistance to a herbicide, fungicide, pesticide, or other crop protection chemical; a recombinant biological agent that has been transformed to contain a herbicide, fungicide, pesticide, or other crop protection chemical resistant gene; a modified population of biological agents wherein the population is resistant to at least one herbicide, fungicide, pesticide, or other crop protection chemical; and compositions comprising these modified populations of biocontrol agents. The modified population may comprise microorganisms that have been selected for herbicide, fungicide, pesticide, or other crop protection chemical resistance or have been transformed with a gene that confers resistance or tolerance to such herbicide, fungicide, pesticide, or other crop protection chemical. Thus, the invention comprises substantially pure cultures, or biologically pure cultures, of such modified biocontrol agents or modified biological agents. A modified biocontrol agent includes biocontrol agents that have acquired a trait due to selection pressure

and recombinant biocontrol agents that have been transformed with a gene that confers resistance or tolerance to at least one herbicide, fungicide, pesticide, or other crop protection chemical.

By “herbicide, fungicide, pesticide, or other crop protection chemical tolerance or herbicide, fungicide, pesticide, or other crop protection chemical resistance” is intended the ability of an organism to survive and reproduce following exposure to a dose of the herbicide, fungicide, pesticide, or other crop protection chemical that is normally lethal to the wild type organism.

Biological agents or biocontrol agents of the invention include microorganisms and fungi that control disease-causing plant pathogens and promote plant health, growth, and yield. Any of these biological or biocontrol agents can be modified by selection or transformation and produce a modified biological or biocontrol agent or recombinant biological or biocontrol agent. Thus, the invention encompasses an isolated modified biocontrol agent. The modified biocontrol agents can be grown to produce a population of biocontrol agents. By “modified population of biological or biocontrol agents” is intended a population of agents that substantially comprises a culture of the selected agent or the recombinant agent having the trait of interest such as resistance to a herbicide, fungicide, pesticide, or other crop protection chemical. By substantially comprises is intended that the population has been grown and produced from the modified or the recombinant biocontrol agent. That is, the modified or recombinant biocontrol agents can be grown to produce a biologically pure culture. It is recognized that such biologically pure cultures can be used together to enhance plant health, growth, or yield.

Any biological or biocontrol agent can be used in the methods of the invention. Particular microorganisms of interest include strains of the bacteria *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Lysobacter*, *Gliocladium*, *Pythium*, *Chromobacterium*, *Penicillium*, *Pantoea*, *Burkholderia*, *Streptomyces*, *Variovorax*, *Pasteuria*, *Xanthomonas*, etc. Fungi of interest include *Aureobasidium*, *Ampelomyces*, *Beauveria*, *Metarhizium*, *Metschnikowia*, *Myrothecium*, *Lecanicillium*, *Chaetomium*, *Cordyceps*, *Coniothyrium*, *Dactylella*, *Gliocladium*, *Aspergillus*, *Paecilomyces*, *Trichoderma*, *Pisolithus*, *Glomus*, etc. See, for example, U.S. Patent Nos. 5,348,742; 5,496,547; 5,756,087; 5,955,348; 6,060,051; 6,635,425; and U.S. Patent Publication 20130142759; all of which are herein incorporated by reference. Many biocontrol agents are on the market and any of them can be modified according to the present invention. Such agents

include: *Agrobacterium radiobacter* K84; *Trichoderma atroviride*; *Bacillus subtilis* GB03; *Bacillus firmus* I-1582; *Trichoderma asperellum* (ICC 012); *T. gamsii* (ICC 080); *Bacillus pumilus* strain QST 2808; *Bacillus subtilis* strain QST 713; *B. subtilis* strain MBI 600; *Paecilomyces fumosoroseus*; *Gliocladium catenulatum*; *Trichoderma harzianum* rifai strain KRL-AG2; *Trichoderma harzianum* T-22; *Trichoderma harzianum* T-22; *Trichoderma virens* strain G-41; *Trichoderma harzianum* T-22; *Bacillus subtilis* QST 713; *Bacillus amyloliquefaciens* strain D747; *Trichoderma (Gliocladium) virens* GL-21; *Paecilomyces lilacinus*; *Paecilomyces fumosoroseus*; *Ampelomyces quisqualis*; *B. subtilis* DSM 17231; *B. licheniformis* DSM 17236; *Pythium oligandrum* DV 74; *Bacillus subtilis* GB03; *Trichoderma asperellum*; *T. gamsii*; *Pseudomonas syringae* ESC-10; *Metschnikowia fructicola*; *Trichoderma harzianum* T-22; *Pseudomonas chlororaphis* MA 342; *B. amyloliquefaciens*; *Chromobacterium subtsugae* strain PRAA4-1; *B. subtilis amyloliquefaciens* FZB24; *Penicillium bilaii*; *Paecilomyces fumosoroseus* FE 9901; *Streptomyces lydicus* WYEC 108; *P. syringae* A506; *Coniothyrium minitans*; *Paecilomyces lilacinus* strain 251; *Streptomyces lydicus* WYEC-108; *Bacillus amyloliquefaciens*; *Trichoderma virens*; *Trichoderma viride*; *Ampelomyces quisqualis*; *Chaetomium globosum*; *Pseudomonas fluorescens*; *Bacillus subtilis*; *Bacillus pumilus*; *Myrothecium verrucaria* AARC-0255; *Streptomyces actinobacterium* strain K61; *Gliocladium catenulatum* J1446; *Aureobasidium pullulans* strain DSM 14940; and *A. pullulans* strain DSM 14941. Additional biological disease control products can be found on the world wide web at: nevegetable.org/table-22-biological-disease-control-products.

Disease causing pathogens include fungi, bacteria, viruses and nematodes. Biocontrol agents of the invention are those that target any of the plant pathogens. Target pathogens include but are not limited to *Alternaria*, *Botrytis*, *Fusarium*, *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Cercospora*, *Colletotrichum*, *Cladosporium*, *-Erysiphe* spp., *Microsphaera syringae*, *Peronospora* spp., *Plasmopara* spp., *Phytophthora*, *Pythium*, *Rhizoctonia*, *Diplocarpon*, *Venturia*, *Mycosphaerella*, *Phomopsis*, *Taphrina*, *Elsinoe*, *Sclerotinia*, *Verticillium*, *Gnomonia*, *Fusicladium*, *Nectria*, *Phyllosticta*, *Diplocarpon*, *Albugo*, *Guignardia*, *Botrytis*, *Exobasidium*, *Entomosporium*, *Exobasidium*, *Pestalotia*, *Phoma*, *Cristulariella*, *Phakopsora*, *Thelaviopsis*, *Puccinia*, *Peronospora*, *Bremia*, *Pantoea*, *Clavibacter*.

Herbicide, fungicide, pesticide, or other crop protection chemical resistance is the ability of an organism to survive and reproduce following exposure to a dose of the herbicide, fungicide, pesticide, or other crop protection chemical that would normally be lethal to the wild type organism or would substantially reduce growth of the wild type organism. Resistance may be induced or identified due to selection or it may be induced through genetic engineering. To identify and produce a modified population of biocontrol agents through selection, the biocontrol agents are grown in the presence of the herbicide, fungicide, pesticide, or other crop protection chemical as the selection pressure. Susceptible agents are killed while resistant agents survive to reproduce without competition. As the biocontrol agents are grown in the presence of the herbicide, fungicide, pesticide, or other crop protection chemical, resistant biocontrol agents successfully reproduce and become dominant in the population, becoming a modified population of biocontrol agents. Methods for selecting resistant strains are known and include U.S. Patent Nos. 4,306,027 and 4,094,097, herein incorporated by reference. Therefore, the invention includes a biologically pure culture of a resistant biocontrol strain. The resistant strains of the invention have the same identification characteristics as the original sensitive strain except they are significantly more tolerant to the particular herbicide, fungicide, pesticide, or other crop protection chemical. Thus, their identification is readily possible by comparison with characteristics of the known sensitive strain.

As indicated, recombinant biocontrol agents having resistance to a herbicide, fungicide, pesticide, or other crop protection chemical can be made through genetic engineering techniques and such engineered or recombinant biocontrol agents grown to produce a modified population of biocontrol agents. A recombinant biocontrol agent is produced by introducing polynucleotides into the biocontrol host cell by transformation. Methods for transforming microorganisms are known and available in the art. See, generally, Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids *J. Mol. Biol.* 166, 557-77; Seidman, C.E. (1994) In: *Current Protocols in Molecular Biology*, Ausubel, F.M. *et al.* eds., John Wiley and Sons, NY; Choi *et al.* (2006) *J. Microbiol. Methods* 64:391-397; Wang *et al.* 2010. *J. Chem. Technol. Biotechnol.* 85:775-778. Transformation may occur by natural uptake of naked DNA by competent cells from their environment in the laboratory. Alternatively, cells can be made competent by exposure to divalent cations under cold conditions, by electroporation, by exposure to polyethylene glycol, by treatment with fibrous nanoparticles, or other methods well known in

the art.

Herbicide resistance genes for use in transforming a recombinant biocontrol agent include, but are not limited to, fumonisin detoxification genes (U.S. Patent No. 5,792,931); acetolactate synthase (ALS) mutants that lead to herbicide resistance, in particular the sulfonylurea-type herbicides, such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)); and HPPD resistance (WO 96/38576, U.S. Patent Nos. 6,758,044; 7,250,561; 7,935,869; and 8,124,846), or other such genes known in the art. The disclosures of which are herein incorporated by reference. The bar gene encodes resistance to the herbicide basta, the nptII gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the sulfonylurea herbicides including chlorsulfuron, metsulfuron, sulfometuron, nicosulfuron, rimsulfuron, flazasulfuron, sulfosulfuron, and triasulfuron, and the imadizolinone herbicides including imazethapyr, imazaquin, imazapyr, and imazamethabenz.

The modified populations of biological control agents of the invention can be formulated as wettable powders, dusts, granules, aqueous or oil based liquid products, and the like. Such formulations will comprise the modified biological control agents in addition to carriers and other agents. The formulations can be used as field inoculants for biocontrol, seed coatings, etc. That is, the modified biocontrol populations can be used in any manner known in the art, including coating seeds with an effective amount of the modified agents, in furrow application of the modified biocontrol populations directly into the soil, in foliar application, and in post-harvest disease control. Such methods are known in the art and are described, for example, in U.S. Patent No. 5,348,742 and in published European Application EP0472494 A2, both of which are herein incorporated by reference. Biocontrol includes management of resident populations of organisms and introductions of specific organisms to reduce disease.

The modified biological control agents of the invention can be used to significantly reduce disease, to promote plant growth and yield, and to reduce reliance on traditional pesticides. The modified agents of the invention can be used with other pesticides for an effective integrated pest management program. In one embodiment, the modified biocontrol populations can be mixed in formulation with known pesticides in a manner described in WO 94/10845, herein incorporated by reference.

Therefore, a further embodiment of the invention provides a method for controlling or inhibiting the growth of a plant pathogen by applying the population of modified biological control agents of the invention to an environment in which the plant pathogen may grow. The application may be to the plant, to parts of the plant, to the seeds of the plants to be protected, or to the soil in which the plant to be protected are growing or will grow. Application to the plant or plant parts may be before or after harvest. Application to the seeds will be prior to planting of the seeds.

The modified biocontrol populations are applied in an effective amount. An effective amount is an amount sufficient to control or inhibit the pathogen. The rate of application may vary according to the pathogen being targeted, the crop to be protected, the efficacy of the modified biocontrol populations, the severity of the disease, the climate conditions, and the like. Generally for a field inoculation, the rate of application is 10^{12} to 10^{16} colony forming units (CFU) per hectare. (This corresponds to about 10 g to 10 kg of active ingredient per hectare if the a.i. is 100 billion CFU per g.)

The invention further encompasses a particular modified biological control agent. Such agent includes AIP1620. AIP1620 is a *Pseudomonas* strain that has been selected for glyphosate tolerance.

AIP1620 was deposited with the Patent Depository of the National Center for Agricultural Utilization Research Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604 U.S.A. on _____ and assigned NRRL Nos. _____. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112.

The following example is offered by way of illustration and not by way of limitation.

Experimental

Example 1: Production of glyphosate resistant mutants of AIP0069.

Introduction

Biological agents are now being used in agriculture to reduce risk and improve yield. One important attribute of these biological agents is that they must be compatible with chemicals that may also be applied in commercial farming practice. Glyphosate is a chemical herbicide that accounts for about 25% of the global herbicide market and is applied at a rate of around 200 million pounds per year. This herbicide inhibits the enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) which catalyzes one step in aromatic amino acid biosynthesis in plants and many bacteria. Thus glyphosate decreases viability of organisms including any biocontrol agents that rely on EPSPS, and it has been reported to alter the plant microbial community. This report describes successful introduction of glyphosate tolerance into a *Pseudomonas fluorescens* strain used in biological control of several important fungal plant pathogens, including Pythium and Rhizoctonia, the causal agents in the agriculturally important "Damping Off" disease complex. In addition to improving chemical compatibility of this biocontrol agent, introduction of glyphosate resistance offers additional advantages in commercial production.

In addition to the example of glyphosate provided here, other agricultural chemicals may inhibit the growth of desirable biological control or plant growth promoting bacteria. Examples include the herbicides glufosinate (glutamine synthase inhibitor), sulfonyleurea and imidazolinone herbicides (branched chain amino acid synthesis inhibitors) and the antibiotics streptomycin, oxytetracycline and kasugamycin.

Materials and Methods and Results

The biological control strain *Pseudomonas fluorescens* AIP0069 was streaked onto agar plates containing 0 or 5 mM glyphosate. The basal medium consisted of 11.3 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3 g KH_2PO_4 , 1 g NH_4Cl , 10 g monosodium glutamate, 31 g molasses, 493 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.3 g thiamine per liter of deionized water. In the absence of glyphosate numerous bacterial colonies were visible after incubating overnight at

25C. In the presence of 5 mM glyphosate no colonies were visible after a similar incubation, however, after extended incubation of several days a few colonies were seen. These colonies were isolated and grown in liquid medium at multiple glyphosate concentrations. One isolate, named GlyphR1, was ten-fold more resistant to glyphosate than the parent AIP0069 strain (Figure 1). This improved strain is expected to be more competitive, and thus more effective as a biocontrol, than AIP0069 or similar glyphosate-sensitive strains in agricultural systems where glyphosate is present in the soil and crops. Also, glyphosate can be used as a selective agent during the production, formulation and/or storage of this strain to prevent contamination by other bacteria.

Example 2: Biological control activity of glyphosate resistant mutants.

Bacteria were inoculated into 50 ml of broth medium consisting of 11.3 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3 g KH_2PO_4 , 1 g NH_4Cl , 10 g Monosodium glutamate, 30 g molasses, 493 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter of deionized water. Cultures were grown in 250 ml baffled flasks in a shaking incubator at 28C, 250 rpm for 2 days. Cells were collected by centrifugation at 3500 xg for 10 minutes. The culture supernatants were discarded and the cells were resuspended in sterile deionized water to the volumes of the original cultures. AIP0323, a mutant of AIP0069 which does not have antifungal activity, was included as a negative control.

Rhizoctonia solani infested rice grain was produced as described previously (K.A. Holmes and D.M. Benson, 1994. Evaluation of *Phytophthora parasitica* var. *nicotianae* as a biocontrol for *Phytophthora parasitica* on *Catharanthus roseus*. Plant Disease, 78:193-199.) Infested rice was pulverized in a blender and screened through a #10 sieve (2 mm opening). The pulverized grain was mixed with germination medium at the rate of 2 g per liter.

Impatiens seeds were planted into the infested germination medium in size 402 plug trays, treated with 0.3 ml of resuspended bacteria per plug and grown under standard greenhouse production conditions. There were 2 replicates for each experimental treatment with 20 plugs per replicate. The number of healthy seedlings was assessed after two weeks. The data below demonstrate that the glyphosate resistant variants AIP0404 and AIP1620 retain full antifungal activity, compared to the progenitor strain, AIP0069.

Treatment	% healthy seedlings		
	Replicate 1	Replicate 2	Average
Noninoculated control	90	90	90
Inoculated control	15	20	17.5
Inoculated + AIP0069	75	80	77.5
Inoculated + AIP0323	10	15	12.5
Inoculated + AIP0404	75	80	77.5
Inoculated + AIP1620	80	75	77.5

Example 3:

Genes encoding glyphosate tolerant EPSPS enzymes may be obtained from various bacteria (A. Schulz et al, 1985. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiology Letters, 28:297-301). In particular, the EPSPS genes from *Agrobacterium tumefaciens* CP4 (G.F. Barry et al, 1992. Inhibitors of amino acid biosynthesis: Strategies for imparting glyphosate tolerance to crop plants. p. 139–145. In B.K. Singh et al. (e.) Biosynthesis and molecular regulation of amino acids in plants. Am. Soc. Plant Physiologists, Rockville, MD), and *Arthrobacter globiformis* (C.L. Peters et al, 2010, GRG23 and GRG51 genes conferring herbicide resistance. US patent 7,674,958), are highly resistant.

A suitable gene is amplified by PCR or made synthetically using techniques well known in the art. The open reading frame is cloned into the plasmid vector pKK223-3 (Pharmacia) between the *tacI* promoter and the *rrnB* transcriptional terminator. The *tacI* promoter provides strong constitutive expression of genes in *Pseudomonas*. Genomic DNA sequences from strain AIP0069 are incorporated on each side of the promoter – gene – terminator cassette to direct homologous recombination into the AIP0069 chromosome.

The resulting plasmid is mobilized from *E. coli* to *Pseudomonas fluorescens* AIP0069 by conjugation, a technique well known in the art, and selection on defined medium containing 100 mM glyphosate. The plasmid contains the narrow host range *colE1* origin of replication and thus cannot replicate in *Pseudomonas*. Glyphosate resistant colonies will be obtained when the promoter - gene - terminator cassette integrates into the *Pseudomonas* chromosome by homologous recombination. Single crossover events (where the entire plasmid is integrated into the chromosome) are distinguished from double crossover events (where only the desired promoter - gene - terminator cassette is integrated) by PCR, Southern blotting, or other techniques well known in the art. A double crossover event is selected for use.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A method for improving a biocontrol agent, said method comprising modifying said biocontrol agent to be resistant to at least one herbicide wherein said biocontrol agent is *Pseudomonas fluorescens* that has been selected for resistance to glyphosate.
2. A substantially pure culture of the biocontrol agent of claim 1.

Abstract

Methods for improving the ability of a population of biological agents to compete and survive in a field setting are provided. By improving the population of biological agents, the modified population of agents is able to grow, compete with other microbial strains and fungi, and provide protection for plants from pathogens. In particular, modified biological agents and modified populations of such agents that are herbicide tolerant or resistant are selected or engineered. In this manner, the protection from disease-causing agents is enhanced. Such modified populations of biological agents can be added to soils to prevent fungal pathogens and the diseases they cause promoting plant growth. Therefore, the present invention is useful for enhancing the competitiveness of modified biological agents particularly over other microbial agents which are not herbicide resistant. Compositions of the invention include selected or engineered herbicide resistant biological agents and modified populations of biocontrol agents. These modified biological agents can be used as an inoculant or as a seed coating for plants and seeds.

Max Growth vs. [Glyphosate]

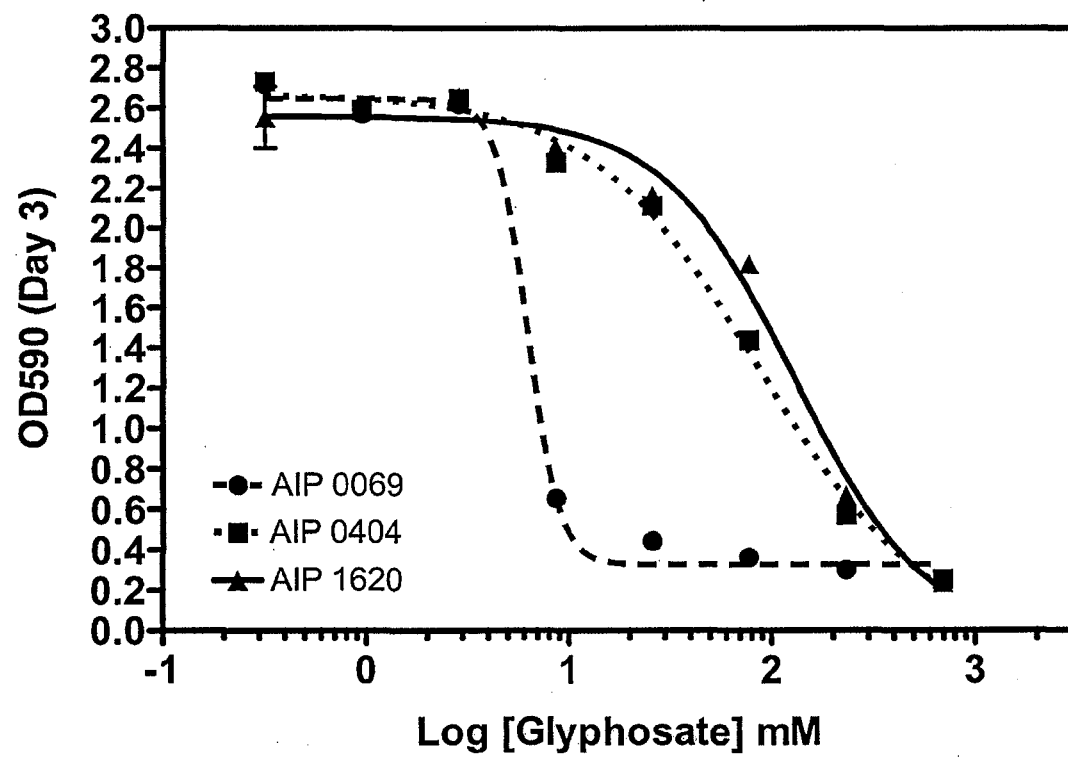


FIGURE 1

Electronic Acknowledgement Receipt

EFS ID:	18079285
Application Number:	61933954
International Application Number:	
Confirmation Number:	3886
Title of Invention:	MODIFIED BIOLOGICAL CONTROL AGENTS AND THEIR USE
First Named Inventor/Applicant Name:	Janice C. Jones
Customer Number:	826
Filer:	W. Murray Spruill/Kathryn Manginelli
Filer Authorized By:	W. Murray Spruill
Attorney Docket Number:	061719/439925
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	439925_ADS.pdf	1561279	no	7
			1fdb54b5cd0a6391a80bd8a469934f2b4e2b0e81		

Warnings:

Information:

2		439925_Application.PDF	863553	yes	16
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Multipart Description/PDF files in .zip description

	Document Description	Start	End
	Specification	1	14
	Claims	15	15
	Abstract	16	16

Warnings:

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3	Drawings-only black and white line drawings	439925_Figure.PDF	13827	no	1
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Information:

4	Fee Worksheet (SB06)	fee-info.pdf	29962	no	2
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