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(54) Title: NOVEL PSEUDOMONAS STRAINS AND RELATED METHODS

(57) Abrégé/Abstract:
The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of Pseudomonas poae which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also plants infected with the endophyte and related methods.

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(54) Title: NOVEL PSEUDOMONAS STRAINS AND RELATED METHODS

(57) Abstract: The present invention relates to an endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas* poae which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. The present invention also plants infected with the endophyte and related methods.

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NOVEL PSEUDOMONAS STRAINS AND RELATED METHODS

Field of the Invention

The present invention relates to novel plant microbiome strains, plants infected with such strains and related methods.

Background of the Invention

Microbes represent an invaluable source of novel genes and compounds that have the potential to be utilised in a range of industrial sectors. Scientific literature gives numerous accounts of microbes being the primary source of antibiotics, immune-suppressants, anticancer agents and cholesterol-lowering drugs, in addition to their use in environmental decontamination and in the production of food and cosmetics.

A relatively unexplored group of microbes known as endophytes, which reside e.g. in the tissues of living plants, offer a particularly diverse source of novel compounds and genes that may provide important benefits to society, and in particular, agriculture.

Endophytes may be fungal or bacterial. Endophytes often form mutualistic relationships with their hosts, with the endophyte conferring increased fitness to the host, often through the production of defence compounds. At the same time, the host plant offers the benefits of a protected environment and nutriment to the endophyte.

Important forage grasses perennial ryegrass (*Lolium perenne*) are commonly found in association with fungal and bacterial endophytes. However, there remains a general lack of information and knowledge of the endophytes of these grasses as well as of methods for the identification and characterisation of novel endophytes and their deployment in plant improvement programs.

Knowledge of the endophytes of perennial ryegrass may allow certain beneficial traits to be exploited in enhanced pastures, or lead to other agricultural advances, e.g. to the benefit of sustainable agriculture and the environment.

There exists a need to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

Summary of the Invention

In one aspect, the present invention provides a substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas poae* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated. In a preferred embodiment, the *Pseudomonas poae* strain may be strain EY as described herein and as deposited with The National Measurement Institute of 1/153 Bertie Street, Port Melbourne, VIC 3207, Australia on 17th May 2019 with accession number V19/009907.

As used herein the term "endophyte" is meant a bacterial or fungal strain that is closely associated with a plant. By "associated with" in this context is meant that the bacteria or fungus lives on, in or in close proximity to a plant. For example, it may be endophytic, for example living within the internal tissues of a plant, or epiphytic, for example growing externally on a plant.

As used herein the term "substantially purified" is meant that an endophyte is free of other organisms. The term includes, for example, an endophyte in axenic culture. Preferably, the endophyte is at least approximately 90% pure, more preferably at least approximately 95% pure, even more preferably at least approximately 98% pure, even more preferably at least approximately 99% pure.

As used herein the term 'isolated' means that an endophyte is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring endophyte present in a living plant is not isolated, but the same endophyte separated from some or all of the coexisting materials in the natural system, is isolated.

As used herein the term "bioprotection and/or biofertilizer" means that the endophyte possesses genetic and/or metabolic characteristics that result in a beneficial phenotype in a plant harbouring, or otherwise associated with, the endophyte. Such beneficial properties include improved resistance to pests and/or diseases, improved tolerance to water and/or nutrient stress, enhanced biotic stress tolerance, enhanced drought tolerance, enhanced

water use efficiency, reduced toxicity and enhanced vigour in the plant with which the endophyte is associated, relative to an organism not harboring the endophyte or harboring a control endophyte such as standard toxic (ST) endophyte.

The pests and/or diseases may include, but are not limited to, bacterial and/or fungal pathogens, preferably fungal. In a particularly preferred embodiment, the endophyte may result in the production of the bioprotectant compound in the plant with which it is associated.

As used herein, the term 'bioprotectant compound' is meant as a compound that provides or aids bioprotection to the plant with which it is associated against pests and/or diseases, such as bacterial and/or fungal pathogens. A bioprotectant compound may also be known as a 'biocidal compound'.

In a particularly preferred embodiment, the endophyte produces a bioprotectant compound and provides bioprotection to the plant against bacterial and/or fungal pathogens. The terms bioprotectant, bioprotective and bioprotection (or any other variations) may be used interchangeably herein.

Thus, in a preferred embodiment, the present invention provides a method of providing bioprotection to a plant against bacterial and/or fungal pathogens, said method including infecting the plant with an endophyte as hereinbefore described and cultivating the plant.

In a particularly preferred embodiment the bioprotectant compound is poaeamide or derivative, isomer and/or salt thereof.

The endophyte may be suitable as a biofertilizer to improve the availability of nutrients to the plant with which the endophyte is associated, including but not limited to improved tolerance to nutrient stress.

Thus, in a preferred embodiment, the present invention provides a method of providing biofertilizer to a plant, said method including infecting the plant with an endophyte as hereinbefore described and cultivating the plant.

The nutrient stress may be lack of or low amounts of a nutrient such as phosphate and/or nitrogen. The endophyte may be capable of growing in conditions such as low nitrogen

and/or low phosphate and enable these nutrients to be available to the plant with which the endophyte is associated.

The endophyte may result in the production of organic acids and/or the solubilisation of phosphate in the plant with which it is associated and/or provide a source of phosphate to the plant.

Alternatively, or in addition, the endophyte may be capable of nitrogen fixation. Thus, if an endophyte is capable of nitrogen fixation, the plant with which the endophyte is associated may be capable of growing in low nitrogen conditions and/or the endophyte may provide a source of nitrogen to the plant.

In a particularly preferred embodiment, the endophyte provides the ability of the organism to grow in low nitrogen.

As used herein the term “plant of the Poaceae family” is a grass species, particularly a pasture grass such as ryegrass (*Lolium*) or fescue (*Festuca*), more particularly perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinaceum*, otherwise known as *Lolium arundinaceum*).

In another aspect, the present invention provides a plant or part thereof infected with an endophyte as hereinbefore described. In preferred embodiments, the plant or part thereof infected with the endophyte may produce a bioprotectant compound, preferably poaeamide or derivative, isomer and/or salt thereof.

Also in preferred embodiments, the plant or part thereof includes an endophyte-free host plant or part thereof stably infected with said endophyte.

The plant inoculated with the endophyte may be a grass or non-grass plant suitable for agriculture, specifically a forage, turf, or bioenergy grass, or a grain crop or industrial crop.

Preferably, the plant is a grass species plant, specifically a forage, turf, bioenergy, grain crop or industrial crop grass.

The forage, turf or bioenergy grass may be those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens,*

Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

The forage, turf or bioenergy grass may also be those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass).

The grain crop or industrial crop may be a non-grass species, for example, any of soybeans, cotton and grain legumes, such as lentils, field peas, fava beans, lupins and chickpeas, as well as oilseed crops, such as canola.

Thus, the grain crop or industrial crop species may be slected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

The grain crop or industrial crop may be a grass belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus Avena, including A.sativa (oats), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), and those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*.

A plant or part thereof may be infected by a method selected from the group consisting of inoculation, breeding, crossing, hybridisation, transduction, transfection, transformation and/or gene targeting and combinations thereof.

Without wishing to be bound by theory, it is believed that the endophyte of the present invention may be transferred through seed from one plant generation to the next. The

endophyte may then spread or locate to other tissues as the plant grows, i.e. to roots. Alternatively, or in addition, the endophyte may be recruited to the plant root, e.g. from soil, and spread or locate to other tissues.

Thus, in a further aspect, the present invention provides a plant, plant seed or other plant part derived from a plant or part thereof as hereinbefore described. In preferred embodiments, the plant, plant seed or other plant part may produce a bioprotectant compound, preferably a poaeamide, or derivative, isomer and/or salt thereof.

In another aspect, the present invention provides the use of an endophyte as hereinbefore described to produce a plant or part thereof stably infected with said endophyte. The present invention also provides the use of an endophyte as hereinbefore described to produce a plant or part thereof as hereinbefore described.

In another aspect, the present invention provides a bioprotectant compound, preferably poaeamide, produced by an endophyte as hereinbefore described, or a derivative, isomer and/or a salt thereof.

The bioprotectant compound, preferably poaeamide, may be produced by the endophyte when associated with a plant, e.g. a plant of the Poaceae family as described above.

Thus, in another aspect, the present invention provides a method for producing a bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, said method including infecting a plant with an endophyte as hereinbefore described and cultivating the plant under conditions suitable to produce the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, may also be produced by the endophyte when it is not associated with a plant. Thus, in yet another aspect, the present invention provides a method for producing a bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt

thereof, said method including culturing an endophyte as hereinbefore described, under conditions suitable to produce the bioprotectant compound.

The conditions suitable to produce the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, may include a culture medium including a source of carbohydrates. The source of carbohydrates may be a starch/sugar-based agar or broth such as potato dextrose agar, potato dextrose broth or half potato dextrose agar or a cereal-based agar or broth such as oatmeal agar or oatmeal broth. Other sources of carbohydrates may include endophyte agar, Murashige and Skoog with 20% sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar. The endophyte may be cultured under aerobic or anaerobic conditions and may be cultured in a bioreactor.

In a preferred embodiment of this aspect of the invention, the method may include the further step of isolating the bioprotectant compound, preferably poaeamide, or a derivative, isomer and/or a salt thereof, from the plant or culture medium.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The endophyte of the present invention may display the ability to solubilise phosphate.

Thus, in yet another aspect, the present invention provides a method of increasing phosphate use efficiency and/or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing phosphate levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

The endophyte of the present invention may be capable of nitrogen fixation. Thus, in yet another aspect, the present invention provides a method of growing the plant in low nitrogen containing medium, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant. Preferably, the low nitrogen medium is low nitrogen containing soil.

In yet a further aspect, the present invention provides a method of increasing nitrogen use efficiency or increasing nitrogen availability to a plant, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant.

In yet another aspect, the present invention provides a method of reducing nitrogen levels in soil, said method including infecting a plant with an endophyte as hereinbefore described, and cultivating the plant in the soil.

In a further aspect, the present invention provides a method of providing bioprotection to a plant against bacterial and/or fungal pathogens and/or providing biofertilizer to a plant, said method including infecting the plant with and endophyte as hereinbefore described. Preferably, the method includes providing bioprotection to the plant and includes production of a bioprotectant compound in the plant into which the endophyte is inoculated.

The endophyte-infected plant or part thereof may be cultivated by known techniques. The person skilled in the art may readily determine appropriate conditions depending on the plant or part thereof to be cultivated.

The production of a bioprotectant compound has particular utility in agricultural plant species, in particular, forage, turf, or bioenergy grass species, or grain crop species or industrial crop species. These plants may be cultivated across large areas of e.g. soil where the properties and biological processes of the endophyte as hereinbefore described and/or bioprotectant compound produced by the endophyte may be exploited at scale.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or nitrogen, alternatively or in addition to applied phosphate and/or nitrogen. The applied phosphate and/or applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

In preferred embodiments, the endophyte may be a *Pseudomonas poae* strain EY as described herein and as deposited with The National Measurement Institute of 1/153 Bertie Street, Port Melbourne, VIC 3207, Australia on 17th May 2019 with accession number V19/009907.

Preferably, the plant is a forage, turf, bioenergy grass species or, grain crop or industrial crop species, as hereinbefore described.

The part thereof of the plant may be, for example, a seed.

In preferred embodiments, the plant is cultivated in the presence of soil phosphate and/or applied phosphate. The applied phosphate may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

Alternatively, or in addition, the plant is cultivated in the presence of soil nitrogen and/or applied nitrogen. The applied nitrogen may be by way of, for example, fertiliser. Thus, preferably, the plant is cultivated in soil.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Brief Description of the Drawings/Figures

Figure 1 - 16S Amplicon sequence of novel bacterial strain EY (SEQ ID NO 1).

Figure 2 - Phylogeny of *Pseudomonas* spp. and novel bacterial strain EY. This maximum-likelihood tree was inferred based on 21 genes conserved among 19 genomes. Values shown next to branches were the local support values calculated using 1000 resamples with the Shimodaira-Hasegawa test.

Figure 3 - Bioprotection bioassay indicating the growth of 11 strains (including *Pseudomonas poae* novel bacterial strain EY, star) against 6 plant pathogenic fungi, *Fusarium verticillioides* (10 days post inoculation, dpi), *Bipolaris gossypina* (7 dpi), *Sclerotinia rolfsii* (5 dpi), *Drechslera brizae* (8 dpi), *Phoma sorghina* (9 dpi) and *Microdochium nivale* (6 dpi). Bars represent the mean diameter of fungal colonies from three replicate plates of each treatment. Different superscript letters indicate significant differences ($P<0.05$) between treatments.

Figure 4 - Secondary metabolite biosynthesis gene clusters in *Pseudomonas poae* novel bacterial strain EY identified using antiSMASH (Weber et al. 2015). The gene clusters have sequence homology and structure to (A) the *poaA* gene cluster and (B) the *poaB* and *poaC* gene cluster. An additional 4 genes are present in the *poaA* gene cluster in strain EY, including an ABC transporter binding protein (i), ABC transporter permease (ii), cyclodehydratase (iii) and an oxidoreductase (iv) that are all involved in microcin biosynthesis.

Figure 5 - Whole genome sequence comparison of *Pseudomonas poae* novel bacterial strain EY (top) and *Pseudomonas poae* bacterial strain RE1-1-14 (bottom). The links between genome sequences indicated percentage similarity (from 70% to 100%). Genetic variations, including non-identical regions and insertions/deletions/inversions, suggest that *Pseudomonas poae* bacterial strains EY and RE1-1-14 are genetically different. Stars represent genomic regions unique to *Pseudomonas poae* bacterial strains EY (dark grey stars) or RE1-1-14 (light grey stars).

Figure 6 - Biofertiliser activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY on Pikovskaya's Agar, which determines the ability of bacteria to solubilise inorganic phosphate.

Figure 7 - Image of 5 day old seedlings (11) inoculated with the *Pseudomonas poae* novel bacterial strain EY and an untreated control.

Figure 8 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae* (novel strain EY) and non-Pseudomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 9 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae* (novel strain EY) and non-Pseudomonads (Strain 1, 2, 3), and grown for 5 days. The * indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 10 – Agarose gel electrophoresis (2 % [w/v]) of PCR amplicons generated using the EY strain-specific primers on *Pseudomonas poae* bacterial strain EY, closely related strains (DP, HC, CT14) a negative control (NC) and a 2 kb DNA molecular ladder (M)

Figure 11 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free media. The star indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 12 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4), and grown for 4 days on nitrogen free media. The star indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 13 - Average root length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4) and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 14 - Average shoot length of barley seedlings inoculated with bacterial strains of *Pseudomonas poae*. (strain EY) and non-Pseudomonads (Strain 1, 2, 3, 4) and grown for 4 days on media containing insoluble phosphate. The star indicates significant difference in the mean at p 0.05 between the control and the bacterial strains.

Figure 15 - Average root and shoot length of barley seedlings inoculated with novel *Pseudomonas poae* bacterial strain EY at different concentrations (10^0, 10^{-1}, 10^{-2}), and grown for 7 days.

Detailed Description of the Embodiments

Isolation and characterisation of plant associated *Pseudomonas poae* novel bacterial strains providing bioprotection and biofertilizer phenotypes to plants.

The novel plant associated *Pseudomonas poae* bacterial strain EY has been isolated from perennial ryegrass (*Lolium perenne*) plants. It displays the ability to inhibit the growth of plant fungal pathogens and solubilise phosphate in plate assays. The genome of the *Pseudomonas poae* bacterial strain EY has been sequenced and is shown to be novel, related to bioprotectant *Pseudomonas poae* strains and not pathogenic Pseudomonad

bacteria. Analysis of the genome sequence has shown that the *Pseudomonas poae* novel bacterial strain EY has gene clusters for the biosynthesis of the bioprotectant compound poaeamide, genes involved in biofertilisation via the production of organic acids and the solubilisation of phosphate, while there is an absence of virulence-related genes (effectors) suggesting the strain has an endophytic life cycle. This novel bacterial strain has been used to inoculate barley (*Hordeum vulgare*) seeds under glasshouse conditions and has been demonstrated not to cause disease in these barley plants. These barley plants are also able to produce seed. Novel bacterial strain EY also enhances root and shoot growth in nitrogen limiting conditions and in insoluble phosphate. The optimal concentration of inoculum for novel bacterial strain EY is a dilution of an overnight culture (10^{-1}, 10^{-2}). Overall, novel plant associated *Pseudomonas poae* bacterial strain EY offer both bioprotectant and biofertilizer activity.

Example 1 – Isolation of Bacterial Strains

Seed associated bacterial strains

Seeds from perennial ryegrass (*Lolium perenne*) were surface-sterilised by soaking in 80% ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then plated onto sterile filter paper soaked in sterile water in sterile petri dishes. These plates were stored at room temperature in the dark to allow seedlings to germinate for 1-2 weeks. Once the seedlings were of sufficient size, the plants were harvested. In harvesting, the remaining seed coat was discarded, and the aerial tissue and root tissue were harvested. The plant tissues were submerged in sufficient Phosphate Buffered Saline (PBS) to completely cover the tissue, and ground using a Qiagen TissueLyser II, for 1 minute at 30 Hertz. A 10 µl aliquot of the macerate was added to 90 µl of PBS. Subsequent 1 in 10 dilutions of the 10^{-1} suspension were used to create additional 10^{-2} to 10^{-4} suspensions. Once the suspensions were well mixed 50 µl aliquots of each suspension were plated onto Reasoners 2 Agar (R2A) for growth of bacteria. Dilutions that provided a good separation of bacterial colonies were subsequently used for isolation of individual bacterial colonies through re-streaking of single bacterial colonies from the dilution plates onto single R2A plates to establish a pure bacterial colony.

Mature plant associated bacterial strains

Leaf and root tissue were harvested from mature plants grown in the field or grown in pots in a greenhouse. Root tissue was washed in PBS buffer to remove soil particles and sonicated (10 mins) to remove the rhizosphere. The harvested tissues were placed into sufficient PBS to completely cover the tissue and processed as per the previous section to isolate pure bacterial cultures.

Around 300 bacterial strains were obtained from sterile seedlings, and 300 strains from mature plants. The novel bacterial strain EY was collected from seed of perennial ryegrass.

Example 2 – Identification of *Pseudomonas poae* novel bacterial strain

Amplicon (16S rRNA gene) Sequencing

A phylogenetic analysis of the novel bacterial strain EY was undertaken by sequence homology comparison of the 16S rRNA gene. The novel bacterial strain EY was grown overnight in Reasoners 2 Broth (R2B) media. DNA was extracted from pellets derived from the overnight culture using a DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. The 16S rRNA gene amplification used the following PCR reagents: 14.8 µL H_2O, 2.5 µL 10X reaction buffer, 0.5 µL 10 mM dNTPs, 2.5 µL each of the 5 µM 27F primer (5'- AGAGTTTGATCMTGGCTCAG -3') (SEQ ID NO. 2) and 5 µM reverse primers 1492R (5'- GGTTACCTTGTTACGACTT -3') (SEQ ID NO 3), 0.2 µL of Immolase enzyme, and template to a final volume of 25 µL. The PCR reaction was then run in an Agilent Surecylcer 8800 (Applied Biosystems) with the following program; a denaturation step at 94°C for 15 min; 35 cycles of 94°C for 30 sec, 55°C for 10 sec, 72°C 1 min; and a final extension step at 72°C for 10 min.

Shrimp alkaline phosphatase (SAP) exonuclease was used to purify the 16S rRNA gene PCR amplicon. The SAP amplicon purification used the following reagents: 7.375 µL H_2O, 2.5 µL 10X SAP, and 0.125 µL Exonuclease I. The purification reaction was incubated at 37°C for 1 hr, followed by 15 min at 80°C to deactivate the exonuclease.

The purified 16S rRNA gene amplicon was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermofisher) with the following reagents; 10.5 µL H_2O, 3.5 µL 5X Seq buffer, 0.5 µL BigDye®, 2.5 µL of either the 3.2 µM Forward (27F) and 3.2 µM Reverse

primers (1492R), and 4.5 µL of PCR amplicon as template, to a final reaction volume of 20 µL. The sequencing PCR reaction was then run in an Agilent Surecylcer 8800 (Applied Biosystems) with the following program; denaturation step at 94°C for 15 min; followed by 35 cycles of 94°C for 30 sec, 55°C for 10 sec, 72°C 1 min; and one final extension step at 72°C for 10 min. The 16S rRNA gene amplicon from novel bacterial strain EY was sequenced on an ABI3730XL (Applied Biosystems). A 1278 bp 16S rRNA gene sequence was generated (Figure 1). The sequence was aligned by BLASTn on NCBI against the non-redundant nucleotide database and the 16S ribosomal RNA database.

BLASTn hit against database nr

Pseudomonas poae strain HTM601-1 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2361	2361	100%	0	100.00%	MG835948.1

BLASTn hit against database 16S ribosomal RNA

Pseudomonas poae strain P 527/13 16S ribosomal RNA gene, partial sequence

Max Score	Total Score	Query Coverage	E-Value	% Identity	Accession
2355	2355	100%	0	99.92%	NR_028986.1

The preliminary taxonomic identification of the novel bacterial strain EY was *Pseudomonas poae*.

Genomics

The genome of novel bacterial strain EY was sequenced. This novel bacterial strain was retrieved from the glycerol collection stored at -80°C by streaking on R2A plates. Single colonies from these plates were grown overnight in Nutrient Broth and pelleted. These pellets were used for genomic DNA extraction using the bacteria protocol of Wizard® Genomic DNA Purification Kit (A1120, Promega). A DNA sequencing library was generated for Illumina sequencing using the Illumina Nextera XT DNA library prep protocol.

The library was sequenced using an Illumina MiSeq platform or HiSeq platform. Raw reads from the sequencer were filtered to remove any adapter and index sequences as well as low quality bases using Trimmomatic (Bolger, Lohse & Usadel 2014) with the following options: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. To enable full genome assembly, long reads were generated for novel bacterial strain EY only by sequencing DNA using Oxford Nanopore Technologies (ONT) MinION platform. The DNA from the Wizard® Genomic DNA Purification Kit was first assessed with the genomic assay on Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) for integrity (average molecular weight ≥30 Kb). The sequencing library was prepared using an in-house protocol modified from the official protocols for transposases-based library preparation kits (SQK-RAD004/SQK-RBK004, ONT, Oxford, UK). The library was sequenced on a MinION Mk1B platform (MIN-101B) with R9.4 flow cells (FLO-MIN106) and under the control of MinKNOW software. After the sequencing run finished, the fast5 files that contain raw read signals were transferred to a separate, high performance computing Linux server for local basecalling using ONT's Albacore software (Version 2.3.1) with default parameters. The sequencing summary file produced by Albacore was processed by the R script minion qc (https://github.com/roblanf/minion_qc) and NanoPlot (De Coster et al. 2018) to assess the quality of the sequencing run, while Porechop (Version 0.2.3, https://github.com/rrwick/Porechop) was used to remove adapter sequences from the reads. Reads which were shorter than 300 bp were removed and the worst 5% of reads (based on quality) were discarded by using Filtlong (Version 0.2.0, https://github.com/rrwick/Filtlong).

The whole genome sequence of novel bacterial strain EY was assembled using Unicycler (Wick et al. 2017). Unicycler performed hybrid assembly when both Illumina reads and MinION reads were available. MinION reads were mainly used to resolve repeat regions in the genome, whereas Illumina reads were used by Pilon (Walker et al. 2014) to correct small base-level errors. Multiple rounds of Racon (Vaser et al. 2017) polishing were then carried out to generate consensus sequences. Assembly graphs were visualised by using Bandage (Wick et al. 2015).

A complete circular chromosome sequence was produced for the novel bacterial strain EY. The genome size for the novel bacterial strain EY was 5,469,454 bp (Table 1). The percent GC content was 60.99%. The novel bacterial strain EY was annotated by Prokka (Seemann 2014) with a custom, genus-specific protein database to predict genes and

corresponding functions, which were then screened manually to identify specific traits. The number of genes for the novel bacterial strain EY was 4,877 (Table 2).

Table 1 – Summary of properties of the final genome sequence assembly

Strain ID	Genome size (bp)	GC content (%)	Coverage Illumina reads	Coverage ONT MinION
EY	5,469,454	60.99	115×	40×

Table 2 – Summary of genome coding regions

Strain ID	Genome size (bp)	No. of tRNA	No. of tmRNA	No. of rRNA	No. of CDS	No. of gene
EY	5,469,454	69	1	16	4,791	4,877

Eighteen *Pseudomonas* spp. (*P. fluorescens*, *P. chlororaphis*, *P. syringae*, *P. putida*, *P. stutzeri*, *P. aeruginosa*, *P. oryzihabitans*) genome sequences that are publicly available on NCBI were acquired and used for pan-genome/comparative genome sequence analysis alongside the novel bacterial strain EY. A total of 21 genes that are shared by all 19 *Pseudomonas* spp. bacterial strains were identified by running Roary (Page et al. 2015). PRANK (Löytynoja 2014) was then used to perform a codon aware alignment. A maximum-likelihood phylogenetic tree (Figure 4) was inferred using FastTree (Price, Dehal & Arkin 2010) with Jukes-Cantor Joins distances and Generalized Time-Reversible and CAT approximation model. Local support values for branches were calculated using 1000 resamples with the Shimodaira-Hasegawa test. The novel bacterial strain EY clustered tightly with the bioprotectant *Pseudomonas poae* bacterial strain RE1-1-14, suggesting a close phylogenetic relationship between these two bacterial strains. Moreover, this cluster was separated from other *Pseudomonas* spp. with strong local support value (100%). This separation supports that bacterial strain EY is novel and from the species *Pseudomonas poae*.

The average nucleotide identity (ANI) was calculated for novel bacterial strain EY against *Pseudomonas poae* bacterial strain RE1-1-14. The genome sequences were aligned and compared using minimap2 (Li 2018). The ANI between bacterial strains EY and RE1-1-14 was 99.46%. Based on a species boundary of 95-96% (Chun et al. 2018; Richter & Rosselló-Móra 2009) bacterial strain EY is a novel strain of the species *Pseudomonas poae* (Müller et al. 2013).

A maximum-likelihood tree was inferred based on 21 genes conserved among 19 genomes (Figure 2).

Example 3 –Bioprotection activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY

In vitro bioassays were established to test the bioactivity of 11 plant associated bacterial strains including *Pseudomonas poae* novel bacterial strain EY, against six plant pathogenic fungi (Table 3). A plate with only the pathogen was used as a negative control (blank). The fungal pathogens were all isolated from monocot species, and were obtained from the National Collection of Fungi (Herbarium VPRI) and the AVR collection. Each bacterial strain was cultured in Nutrient Broth (BD Biosciences) overnight at 28°C in a shaking incubator (200 rpm). Each bacterial strain was drop-inoculated (20 µL) onto four equidistant points on a Nutrient Agar (BD Biosciences) plate, which was then incubated overnight at 28°C. A 6mm×6mm agar plug of actively growing mycelia from the pathogen was placed at the centre of the plate. The bioassay was incubated for at least 5 days at 28°C in the dark, and then the diameter of the fungal colony on the plate was recorded. For each treatment three plates were prepared as biological triplicates. OriginPro 2018 (Version b9.5.1.195) was used to carry out One-way ANOVA and Tukey Test to detect the presence of any significant difference ($p \leq 0.05$) between treatments.

Table 3 – Pathogens used in the bioprotection bioassay.

VPRI Accession No.	Taxonomic Details	Host Taxonomic Details	State	Collection Date
12962	*Drechslera brizae* (Y.Nisik.) Subram. & B.L.Jain	*Briza maxima* L.	Vic.	24-Oct-85
32148	*Sclerotium rolfsii* Sacc.	*Poa annua* L.	Vic.	1-Jan-05
10694	*Phoma sorghina* (Sacc.) Boerema, Dorenbosch, van Kesteren	*Cynodon dactylon* Pers.	Vic.	19-Apr-79
42586a	*Fusarium verticillioides* (Sacc.) Nirenberg	*Zea mays* L.	Vic.	27-Feb-15
42563	*Bipolaris gossypina*	*Brachiaria*	Qld	

N/A	*Microdochium nivale*	*Lolium perenne* L.	Vic	

The *Pseudomonas poae* novel bacterial strain EY inhibited the growth of all six fungal pathogens compared to the control and many of the other test bacterial strains, indicating it had broad spectrum biocidal activity (Figure 3). The *Pseudomonas poae* novel bacterial strain EY was the most active bacterial strain against *Fusarium verticillioides*, *Bipolaris gossypina*, *Sclerotium rolfsii* and *Phoma sorghina*, while it was the second most active strain against *Drechslera brizae* and *Microdochium nivale*.

Example 4 – Genome sequence features supporting the bioprotection niche of the *Pseudomonas poae* novel bacterial strain EY

Secondary metabolite biosynthesis gene clusters

The genome sequence of *Pseudomonas poae* novel bacterial strain EY was assessed for the presence of features associated with bioprotection. The annotated genome was analysed by antiSMASH (Weber et al. 2015) to identify secondary metabolite biosynthesis gene clusters that are commonly associated with the production of biocidal compounds that aid in their defence. An annotated genome was passed through antiSMASH with the following options: *--clusterblast --asf --knownclusterblast --subclusterblast --smcogs --full-hmmer*. A total of two secondary metabolite gene clusters were identified in the genome sequence of the *Pseudomonas poae* novel bacterial strain EY. (Figure 4). The two biosynthetic gene clusters (cluster 1 – *poaA*; cluster 2 – *poaB* and *poaC*) had sequence homology (99%) and structure to the poeamide gene cluster that produces the bioprotectant non-ribosomal peptide poaeamide (Figure 4). This gene cluster had the non-ribosomal peptide synthases (NRPS - *poaA*, *poaB*, *poaC*) essential for the biosynthesis of poaeamide and was similar in structure compared to the reference strain (RE1-1-14). In the *poaA* gene cluster of EY there is the presence of an additional four genes with sequence homology to genes involved in microcin biosynthesis, including an ABC transporter binding protein, ABC transporter permease, cyclodehydratase and an oxidoreductase (Figure 4A). Some of these additional genes are likely to interact with poaeamide to alter the structure and produce a slightly different compound to poaeamide.

Genome sequence alignment

The genome sequences of *Pseudomonas poae* novel bacterial strain EY and the bioprotectant *Pseudomonas poae* strain RE1-1-14 were aligned using LASTZ (Version 1.04.00, http://www.bx.psu.edu/~rsharris/lastz/) and visualised using AliTV (Ankenbrand et al. 2017) to determine the genomic similarity between the two strains. The genome sequences of the two strains were similar, but there were large genomic regions unique to the novel bacterial strain EY (red stars) or the bacterial strain RE1-1-14 (yellow stars) (Figure 5).

Example 5 - Biofertiliser activity (*in vitro*) of the *Pseudomonas poae* novel bacterial strain EY

Phosphate is an essential ion for plant growth. Phosphate is applied to fields to improve plant growth and yield. A large amount of applied phosphate is not accessible to plants. Some bacteria have been shown to have the ability to mobilise some of this inaccessible phosphate. The P-solubilisation ability of bacterial strains was detected by using the Pikovskaya's Agar (Sundar ORacand & Sinha 1963), which contains inorganic phosphate in the form of calcium phosphate (5g/L). *Pseudomonas poae* novel bacterial strain EY and *Escherichia coli* (negative control) were inoculated onto Pikovskaya's Agar at three equidistant points on a plate. All plates were then incubated for 72 hours at room temperature, and inspected visually for the formation of a clear zone around the colony. For each strain three plates were prepared as biological triplicates. The *Pseudomonas poae* novel bacterial strain EY was able to solubilise inorganic phosphate, as evidenced by a zone of clearing around the colony (Figure 6).

Example 6 – Genome sequence features supporting the biofertiliser niche of the *Pseudomonas poae* novel bacterial strain EY

A number of bacterial and fungal species have been reported to solubilise inorganic phosphate. The mechanism of inorganic phosphate solubilization is via the production of mineral dissolving compounds such as organic acids (i.e. oxalic acid, citric acid, lactic acid, gluconic acid), siderophores, protons, hydroxyl ions and CO_2 (Rodríguez & Fraga 1999; Sharma, Kumar & Tripathi 2017). Organic acids together with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release Phosphorous (Tallapragada & Seshachala 2012). A total of 4,877 genes in the annotated genome sequence of

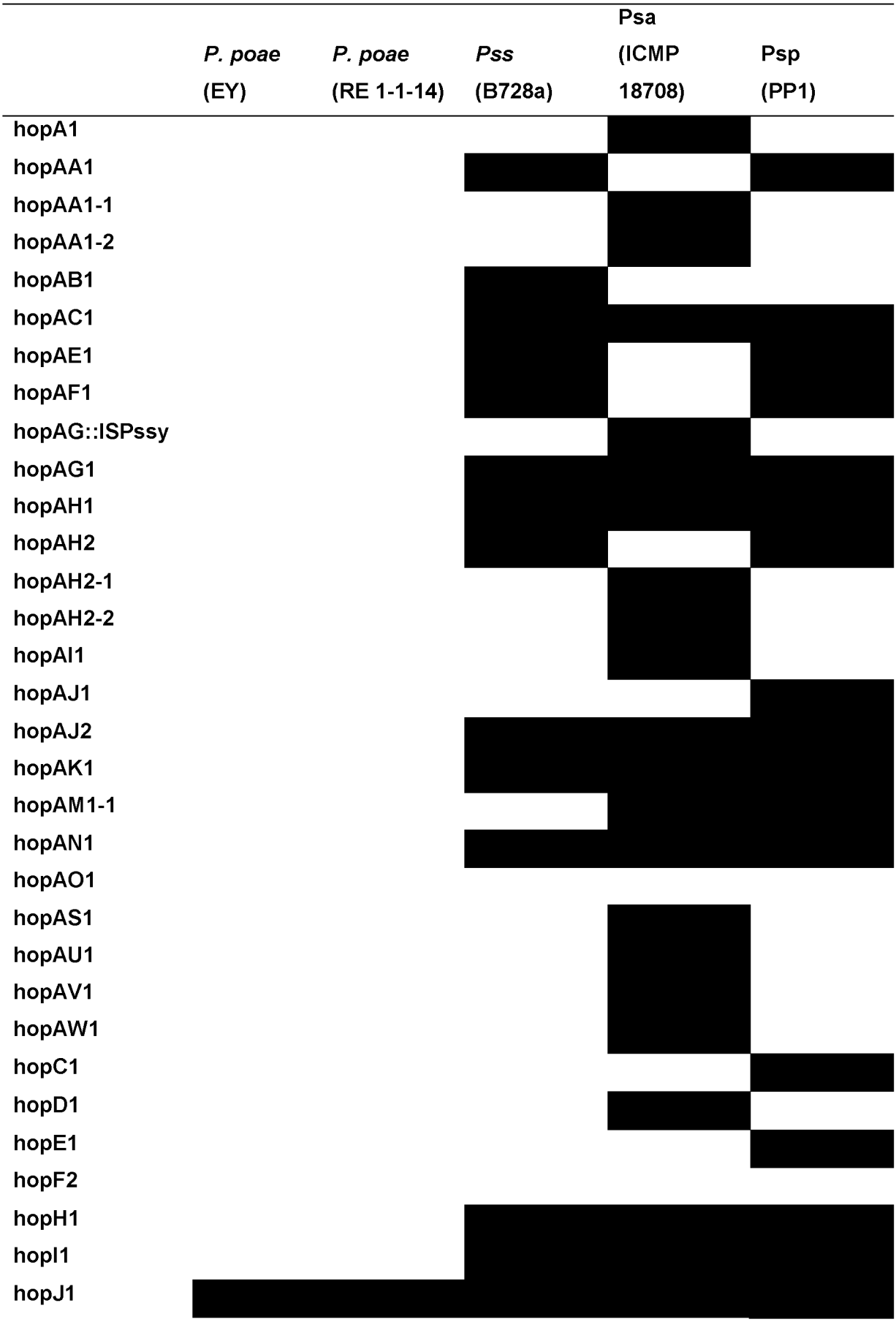
Pseudomonas poae novel bacterial strain EY were assessed for nomenclature consistent with the production of organic acids and the solubilisation of phosphate. Enzymes involved in organic acid production were identified including glucose dehydrogenases (gluconic acid), gluconate dehydrogenase (2-ketogluconic acid) and lactate dehydrogenase (lactic acid).

Example 7 – Genome sequence features supporting the endophytic niche of the *Pseudomonas poae* novel bacterial strain EY

There have been 57 virulence-related type III effector repertoires (genes) identified in *Pseudomonas syringae* pathovars that are important for the pathogenicity of this species (Lindeberg, Cunnac & Collmer 2012). These effectors are important for invading the host, suppressing the host immune system and altering host physiology for the benefit of the pathogen (Henry et al. 2017). A total of 50 type III effector repertoires were assessed for presence/absence in the genome sequence of novel bacterial strain EY (*Pseudomonas poae*), along with bacterial strains RE1-1-14 (*Pseudomonas poae*), B28a (*Pseudomonas syringae* pv. *syringae*), ICMP18708 (*Pseudomonas syringae* pv. *actinidae*) and PP1 (*Pseudomonas syringae* pv. *pisi*) through sequence homology searches (Blastp, 80% similarity, e-value 10-10) (Table 4). The *Pseudomonas poae* novel bacterial strain EY had only one of the 50 type III effector repertoires (HopJ). There was an absence of many of the key effectors involved in the pathogenicity of *Pseudomonas syringae*, including AVRE1 and HopI (Wei, Zhang & Collmer 2018).

Table 4 – Fifty type III effector repertoires (genes) identified in *Pseudomonas syringae* pathovars and *Pseudomonas poae* strains (EY and RE1-1-14)

	P. poae (EY)	*P. poae* (RE 1-1-14)	*Pss* (B728a)	Psa (ICMP 18708)	Psp (PP1)
avrB3			■		■
avrB4-1					
avrE1			■	■	■
avrPphB					
avrPto					
avrRpm1			■	■	
avrRps4					■


P. poae (EY)
P. poae (RE 1-1-14)
Pss (B728a)
Psa (ICMP 18708)
Psp (PP1)
hopA1
hopAA1
hopAA1-1
hopAA1-2
hopAB1
hopAC1
hopAE1
hopAF1
hopAG::ISPssy
hopAG1
hopAH1
hopAH2
hopAH2-1
hopAH2-2
hopAI1
hopAJ1
hopAJ2
hopAK1
hopAM1-1
hopAN1
hopAO1
hopAS1
hopAU1
hopAV1
hopAW1
hopC1
hopD1
hopE1
hopF2
hopH1
hopI1
hopJ1

	P. poae (EY)	*P. poae* (RE 1-1-14)	*Pss* (B728a)	Psa (ICMP 18708)	Psp (PP1)
hopL1			■		■
hopM1			■		■
hopN1				■	
hopQ1-1				■	
hopR1				■	■
hopS2				■	
hopW1				■	
hopX1			■		■
hopY1				■	
hopZ3			■		
hrpK1			■		■

Example 8 - *In planta* inoculations supporting endophytic niche of the *Pseudomonas poae* novel bacterial strain EY

To assess direct interactions between the *Pseudomonas poae* novel bacterial strain EY and plants, an early seedling growth assay was established in barley. A total of 4 bacterial strains (EY – *Pseudomonas poae*; Strain 1, Strain 2, Strain 3) were cultured in Lysogeny Broth (LB) overnight at 26°C. The following day seeds of barley (cultivar Hindmarsh) were surface-sterilised by soaking in 80% ethanol for 3 mins, then washing 5 times in sterile distilled water. The seeds were then soaked in the overnight cultures for 4 hours at 26°C in a shaking incubator. For control seedlings, seeds were soaked in LB without bacteria for 4 hours at 26°C in a shaking incubator. The seeds were planted into a pot trial, with three replicates (pots) per strain/control, with a randomised design. A total of 20 seeds were planted per pot, to a depth of 1 cm. The potting medium contained a mixture of 25% potting mix, 37.5% vermiculite and 37.5% perlite. The plants were grown for 5 days and then removed from the pots, washed, assessed for health (i.e. no disease symptoms) and photographed. The lengths of the longest root and the longest shoot were measured. Data was statistically analysed using a one-way ANOVA and Tukey test to detect the presence of any significant difference ($p \leq 0.05$) between treatments using OriginPro 2018 (Version b9.5.1.195).

Seedlings inoculated with the *Pseudomonas poae* novel bacterial strain EY were healthy with no disease symptoms recorded on leaves or roots (Figure 7). The length of the shoots inoculated with the *Pseudomonas poae* novel bacterial strain EY were equivalent to the control (Figure 8). The length of the roots of inoculated with the *Pseudomonas poae* novel bacterial strain EY were significantly shorter than the control (Figure 9).

Example 9 – *In planta* inoculations supporting colonisation and localisation of the *Pseudomonas poae* novel bacterial strain EY in wheat and perennial ryegrass

Strain-specific primers were designed for *Pseudomonas poae* novel bacterial strain EY targeting the 3440768-3441879 bp region of the genome, which related to an insertion the *paoA* gene of the poaeamide biosynthetic gene cluster of EY (EY-F TGTTAAACACGCAACTCGCC;(SEQ ID NO. 4) EY-R AAAGGTGCACTCACAACCTCTG; (SEQ ID NO 5) 5'→3'). An *in silico* analysis using Primer-BLAST indicated that the primers were strain-specific.

The strain-specific primer for EY was evaluated on cultures of strains *Pseudomonas poae* novel bacterial strain EY, along with closely related strains (DP, HC, CT14). Initially, bacterial cultures were grown in nutrient broth (BD Bioscience) and grown overnight at 22°C in the dark in a shaking incubator. The Promega Wizard® genomic DNA purification kit was used with the following modifications: initial centrifugation of 1 mL of overnight culture at 13,000–16,000 × g for 2 mins was performed twice to pellet bacterial cells; incubations were conducted at -20ºC for 10 mins to enhance protein precipitation; DNA pellets were rehydrated in 50 mL rehydration solution at 65°C for 10 mins followed by overnight incubation at 4°C. Final DNA concentration was measured using a Quantus™ Fluorometer and stored at 4°C until further processing. The 25 µL reaction mixture contained: 12.5 µL of OneTaq™ Hot Start 2 × master mix with standard buffer (New England BioLabs®), 2µL of each primer (10µM/µL), 8.5µL of nuclease-free water and 2 µL of template DNA sample. The thermocycling conditions were: initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min, elongation at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were separated at 120 V in a 2% (w/v) agarose gel containing 0.05 µL mL-1 SYBR safe stain in 1×TAE running buffer and visualized under UV light next to a 2kb DNA ladder. The strain-specific primer generated an amplicon of the correct size (1112 bp) for *Pseudomonas poae* novel bacterial strain EY and DP (likely duplicate of EY) (Figure 10).

The strain-specific primer for EY was evaluated on wheat plants inoculated with *Pseudomonas poae* novel bacterial strain EY. Initially, wheat seeds were sterilized in 70% ethanol for 3 minutes, followed by rinsing with sterilized distilled water (SDW) for three times. The bacterial strain was cultured in nutrient broth (BD Bioscience) overnight, while seeds were imbibed in nutrient broth overnight in the dark. Seeds and the bacterial culture were combined for 4 hours in dark in a shaking incubator. For the controls, seeds were not inoculated with bacteria. A total of three seeds were sown per pot into potting mix and grown in a glasshouse. For wheat, plants were harvested at only one time point (7 days after planting, DAP). For wheat inoculated with EY 10 replicates were maintained. For the uninoculated control treatments (wheat) 5 replicates were maintained for each time point. At harvest, plants were uprooted, washed thoroughly (roots only) and then sectioned into roots, pseudostem and leaves (wheat - 7 DAP). Each section comprised three pieces (~0.5 cm^2) of plant tissue, which was placed into collection microtubes (2 mL) and stored at -80°C. The Qiagen® MagAttract® 96 DNA plant core kit (Qiagen®, Hilden, Germany) was utilized to extract plant DNA using the Biomek® FXP lab automation workstation linked to Biomek software version v. 4.1 and Gen 5 (v. 2.08) software (Biotek Instruments, USA) with the following modifications to the manufacturer's instructions: to each well of the 96 well microplate, a 33 µL aliquot of RB buffer and 10 µL of resuspended MegAttract suspension G was added. A touch-down PCR (TD-PCR) was performed to enhance the sensitivity and specificity of primers *in planta*, compared to *in vitro* pure cultures. The PCR reaction mixture was prepared as per *in vitro* cultures. Touch-down PCR amplification was performed in two phases. In phase I, initial denaturation was carried out at 94°C for 1 min, followed by 10 cycles of denaturation at 94°C for 30 sec, annealing for at 65 - 55°C (dropping 1C for each cycle) and 72°C for 2 mins. In phase II, it was 20 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. For wheat, the presence of the *Pseudomonas poae* novel bacterial strain EY was detected at 7 DAP, with the highest rates of incidence recorded in roots (80%), followed by pseudostem (30%), however it was not detected in the leaves (0%) (Table 9). Overall, *Pseudomonas poae* novel bacterial strain EY appears to inoculate into wheat, where it colonises subterranean and aerial tissue, but appears to preferentially colonise roots.

Table 9 – Incidence of *Pseudomonas poae* novel bacterial strain EY in wheat at one harvest time point. The incidence is indicated as the number of plants showing the presence of EY per total number of replicates inoculated or uninoculated (R - roots; P - pseudostem; L - leaves).

	7 DAP		
	R	**P**	**L**
EY	8/10	3/10	0/10
Control	0/5	0/5	0/5

Example 10 – *In planta* inoculations supporting the biofertilizer (nitrogen) niche of the *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Pseudomonas poae* novel bacterial strain EY to aid growth under nitrogen limiting conditions. Initially, bacterial strains (5, including EY were cultured in 20 mL nutrient broth (BD Bioscience) overnight at 26°C whilst rotating at 200 RPM. The following day cultures were pelleted via centrifugation at 4000 RPM for 5 minutes, washed three times in 10 mL Phosphate Buffered Saline (PBS), resuspended in 20 mL PBS, quantified via spectrophotometry (OD600) and diluted (1:10). Barley seeds were sterilized in 70% ethanol for 5 minutes, followed by rinsing with sterilized distilled water (SDW) for five times. These sterile seeds were submerged in the dilution for 4 hours in a dark incubator at room temperature whilst rotating at 200 RPM. The seeds were subsequently transferred to moistened sterile filter paper and allowed to germinate for three days. The three-day-old seedlings were individually transferred to 60mm plates with semi-solid Burks media (HiMedia) (5 g/L Agar). Seedlings were allowed to grow for a further 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including EY; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P<0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain EY and grown under nitrogen limiting conditions was significantly greater than the control ($P<0.05$), with an

average increase of 28.6% (Figure 11). The shoot growth of seedlings inoculated with novel bacterial strain EY was not significantly greater than the control ($P<0.05$), despite increasing shoot growth by 12.5% (Figure 12). Overall, results indicate that novel bacterial strain EY can aid in the growth of seedlings grown under nitrogen limiting conditions.

Example 11 – *In planta* inoculations supporting the biofertilizer (phosphate solubilisation) niche of the *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in barley to evaluate the ability of *Pseudomonas poae* novel bacterial strain EY to aid growth under conditions with insoluble phosphate. Initially, bacterial strains (5, including EY) were cultured in 30mL R2B overnight at 26°C whilst rotating at 200 RPM. The following day the barley seeds were sterilized in 70% ethanol for 5 minutes, followed by rinsing with SDW for five times. These sterile seeds were submerged in the overnight cultures for 4 hours in a dark incubator at room temperature whilst rotating at 200 RPM. The seeds were subsequently transferred to moistened sterile filter paper to be allowed to germinate for three days. These three-day-old seedlings were individually transferred to 60mm plates with semi-solid Pikovskaya media which contains yeast extract (0.5 g/L), D-glucose (5.0 g/L), calcium phosphate (5.0 g/L), ammonium sulphate (0.5 g/L), potassium chloride (0.2 g/L), magnesium sulphate (0.1 g/L), manganese sulphate (0.1 mg/L), ferrous sulphate (0.1 mg/L) and agar (5.0 g/L). These seedlings were allowed to grow for another 4 days, before the shoots and roots were measured for each seedling. There was a total of 6 treatments (5 bacterial strains including EY; 1 blank media control) containing 10 seedlings per treatment. Statistical analysis (One-way ANOVA and Tukey Test) was conducted using OriginPro 2018 (Version b9.5.1.195) to detect the presence of any significant difference ($P<0.05$) between treatments.

The root growth of seedlings inoculated with novel bacterial strain EY and grown under conditions with insoluble phosphate was significantly greater than the control ($P<0.05$), with an average increase of 62.5% (Figure 13). The shoot growth of seedlings inoculated with novel bacterial strain EY was significantly greater than the control ($P<0.05$), with an average increase of 46.2 (Figure 14). Overall, results indicate that novel bacterial strain EY can aid in the growth of seedlings grown under conditions with insoluble phosphate.

Example 12 – *In planta* inoculations identifying optimal concentrations of *Pseudomonas poae* novel bacterial strain EY

An *in planta* biofertilizer assay was established in perennial ryegrass to evaluate the optimal concentration in which *Pseudomonas poae* novel bacterial strain EY would support seedling growth. Initially, the bacterial strain was cultured overnight in 20 mL nutrient broth (BD Bioscience) at 26°C whilst rotating at 200 RPM. The following day the culture was pelleted via centrifugation at 4000 RPM for 5 minutes, washed three times in 10 mL PBS, resuspended in 20 mL PBS, quantified via spectrophotometry (OD600). The culture was diluted (1:10) twice to create three concentrations (10^{0}, 10^{-1} and 10^{-2}). The perennial ryegrass seeds were sterilized in 70% ethanol for 5 minutes, followed by rinsing five times with SDW. These sterile seeds were submerged in the dilutions for 4 hours in a dark incubator at room temperature whilst rotating at 200 RPM. After inoculation, 10 seeds were transferred to moistened sterile filter paper for germination from each dilution. After seven days, the roots and shoots were measured.

Root growth of seedlings inoculated with novel bacterial strain EY was greatest with the 10^{-1} dilution, which was 4.4% greater than 10^{-2} dilution and 14.0% greater than the 10^{-0} dilution (Figure 15). Shoot growth of seedlings inoculated with novel bacterial strain EY was greatest with the 10^{-2} dilution, which was 13.3% greater than 10^{-1} dilution and 16.7% greater than the 10^{-0} dilution. Overall, results indicate that novel bacterial strain EY has the greatest effects on root and shoot growth at lower concentrations.

It is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to be in any way limiting or to exclude further additives, components, integers or steps.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could reasonably be expected to be combined by a person skilled in the art.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO:
Professor German Carlos Spangenberg
AgriBio, Centre for AgriBioscience,
5 Ring Road, La Trobe University,
Bundoora, Victoria 3083, Australia

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY:
***Pseudomonas poae* (EY)**	**V19/009907**

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☒ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on **17th May 2019** (date of the original deposit) [1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V INTERNATIONAL DEPOSITARY AUTHORITY

Name: NATIONAL MEASUREMENT INSTITUTE Address: 1/153 BERTIE STREET PORT MELBOURNE VICTORIA, AUSTRALIA, 3207 Phone: +61 3 9644 4888 Facsimile: +61 3 9644 4999	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s) **Dean Clarke** Date: **21st May 2019**

1 Where Rule 6.4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Appendix 3
page 21

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO:
Professor German Carlos Spangenberg
AgriBio, Centre for AgriBioscience
5 Ring Road, La Trobe University,
Bundoora, Victoria, Australia 3083

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name: **Professor German Carlos Spangenberg** Address: **AgriBio, Centre for AgriBioscience** **5 Ring Road, La Trobe University,** **Bundoora, Victoria, Australia 3083**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **V19/009907** Date of the deposit or of the transfer [1]: **17th May 2019**
III VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on **17th May 2019** [2] On that date, the said microorganism was ☒ [3] viable ☐ [3] no longer viable	

6. Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2. In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

3. Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
Deposit was grown on R2A (agar) at 21^{0}C for 24-48 hours	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: NATIONAL MEASUREMENT INSTITUTE Address: 1/153 BERTIE STREET, PORT MELBOURNE VICTORIA AUSTRALIA 3207 Phone: +61 3 9644 4888 Facsimile: +61 3 9644 4999	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) **Dean Clarke** Date: **21st May 2019**

4 Fill in if the information has been requested and if the results of the test were negative

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially purified or isolated endophyte strain isolated from a plant of the Poaceae family, wherein said endophyte is a strain of *Pseudomonas poae* which provides bioprotection and/or biofertilizer phenotypes to plants into which it is inoculated.

2. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype includes production of a bioprotectant compound in the plant into which the endophyte is inoculated.

3. An endophyte according to claim 2, wherein the bioprotectant compound is poaeamide or a derivative, isomer and/or salt thereof.

4. An endophyte according to claim 1, wherein the bioprotection and/or biofertilizer phenotype is selected from the group consisting of production of organic acids, solubilisation of phosphate and nitrogen fixation in the plant into which the endophyte is inoculated.

5. An endophyte according to any one of claims 1 to 4, wherein the endophyte is *Pseudomonas poae* strain EY as deposited with The National Measurement Institute on 17th May 2019 with accession number V19/009907.

6. An endophyte according to any one of claims 1 to 5, wherein the plant from which the endophyte is isolated is of the Poaceae family is a pasture grass.

7. An endophyte according to claim 6, wherein the pasture grass is from the genus Lolium or Festuca.

8. An endophyte according to claim 7, wherein the pasture grass is from the species *Lolium perenne* or *Festuca arundinaceum.*

9. An endophyte according to any one of claims 1 to 8, wherein the plant into which the endophyte is inoculated includes an endophyte-free host plant or part thereof stably infected with said endophyte.

10. An endophyte according to any one of claims 1 to 9, wherein the plant into which the endophyte is inoculated is an agricultural plant species selected from one or more of forage grass, turf grass, bioenergy grass, grain crop and industrial crop.

11. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a forage, turf or bioenergy grass selected from the group consisting of those belonging to the genera *Lolium* and *Festuca*, including *L. perenne* (perennial ryegrass), *L. arundinaceum* (tall fescue) and *L. multiflorum* (Italian ryegrass), and those belonging to the *Brachiaria-Urochloa* species complex (panic grasses), including *Brachiaria brizantha, Brachiaria decumbens, Brachiaria humidicola, Brachiaria stolonifera, Brachiaria ruziziensis, B. dictyoneura, Urochloa brizantha, Urochloa decumbens, Urochloa humidicola*, *Urochloa mosambicensis* as well as interspecific and intraspecific hybrids of *Brachiaria-Urochloa* species complex such as interspecific hybrids between *Brachiaria ruziziensis* x *Brachiaria brizantha*, *Brachiaria ruziziensis* x *Brachiaria decumbens*, [*Brachiaria ruziziensis* x *Brachiaria decumbens*] x *Brachiaria brizantha*, [*Brachiaria ruziziensis* x *Brachiaria brizantha*] x *Brachiaria decumbens*.

12. An endophyte according claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of those belonging to the genus *Triticum*, including *T. aestivum* (wheat), those belonging to the genus *Hordeum*, including *H. vulgare* (barley), those belonging to the genus Avena, including A.sativa (oats), those belonging to the genus *Zea*, including *Z. mays* (maize or corn), those belonging to the genus *Oryza*, including *O. sativa* (rice), those belonging to the genus *Saccharum* including *S. officinarum* (sugarcane), those belonging to the genus *Sorghum* including *S. bicolor* (sorghum), those belonging to the genus *Panicum*, including *P. virgatum* (switchgrass), those belonging to the genera *Miscanthus*, *Paspalum*, *Pennisetum*, *Poa*, *Eragrostis* and *Agrostis*,

13. An endophyte according to claim 10, wherein the plant into which the endophyte is inoculated is a grain crop or industrial crop selected from the group consisting of wheat, barley, oats, chickpeas, triticale, fava beans, lupins, field peas, canola, cereal rye, vetch, lentils, millet/panicum, safflower, linseed, sorghum, sunflower, maize, canola, mungbeans, soybeans, and cotton.

14. A plant or part thereof infected with one or more endophytes according to any one of claims 1 to 13.

15. A plant, plant seed or other plant part derived from a plant or part thereof according to claim 14 and stably infected with said one or more endophytes.

16. Use of an endophyte according to any one of claims 1 to 13 to produce a plant or part thereof stably infected with said one or more endophytes.

17. A bioprotectant compound produced by an endophyte according to any one of claims 1 to 13, or a derivative, isomer and/or a salt thereof, preferably the bioprotectant compound is poaeamide or derivative, isomer and/or salt thereof.

18. A method for producing a bioprotectant compound, or a derivative, isomer and/or a salt thereof, said method including infecting a plant with an endophyte according to any one of claims 1 to 13 and cultivating the plant under conditions suitable to produce the bioprotectant compound.

19. A method for producing a bioprotectant compound, or a derivative, isomer and/or a salt thereof, said method including culturing an endophyte according to any one of claims 1 to 13 under conditions suitable to produce the bioprotectant compound.

20. A method according to claim 19, wherein the conditions include a culture medium including a source of carbohydrates.

21. A method according to claim 20, wherein the source of carbohydrates is selected from one or more of the group consisting of a starch/sugar-based agar or broth, a cereal-based agar or broth, endophyte agar, Murashige and Skoog with 20% sucrose, half V8 juice/half PDA, water agar and yeast malt extract agar.

22. A method according to any one of claims 18 to 21, wherein the method further includes isolating the bioprotectant compound from the plant or culture medium.

23. A method of providing bioprotection to a plant against bacterial and/or fungal pathogens and/or providing biofertilizer to a plant, said method including infecting the plant with and endophyte according to any one of claims 1 to 13 and cultivating the plant.

24. A method according to claim 23, wherein the method includes providing bioprotection to the plant and includes production of a bioprotectant compound in the plant into which the endophyte is inoculated.

25. A method according to claim 24, wherein the bioprotectant compound is poaeamide or a derivative, isomer and/or salt thereof.

26. A method according to claim 23, wherein the method includes providing biofertilizer to the plant and includes production of organic acids, increased phosphate use efficiency, increased solubilisation of phosphate, increased nitrogen use efficiency and/or increased nitrogen availability, in the plant into which the endophyte is inoculated.

27. A method according to claim 26, wherein the method includes increasing phosphate use efficiency or increasing phosphate solubilisation in the plant, and wherein the plant is cultivated in the presence of soil phosphate and/or applied phosphate.

28. A method according to claim 27, wherein the applied phosphate includes phosphate applied by fertiliser.

29. A method according to claim 26, wherein the method includes increasing nitrogen use efficiency or nitrogen availability, and wherein the plant is cultivated in a low nitrogen medium, preferably low nitrogen soil.

30. A method of increasing phosphate use efficiency or increasing phosphate solubilisation by a plant, said method including infecting a plant with an endophyte according to any one of claims 1 to 13, and cultivating the plant.

31. A method according to claim 30, wherein the plant is cultivated in the presence of soil phosphate and/or applied phosphate.

32. A method according to claim 31, wherein the applied phosphate includes phosphate applied by fertiliser.

33. A method of growing a plant in a low nitrogen medium, said method including infecting a plant with a bioprotectant compound -producing endophyte according to any one of claims 1 to 13, and cultivating the plant.

34. A method according to any one of claims 30 to 33, wherein the plant is cultivated in soil.

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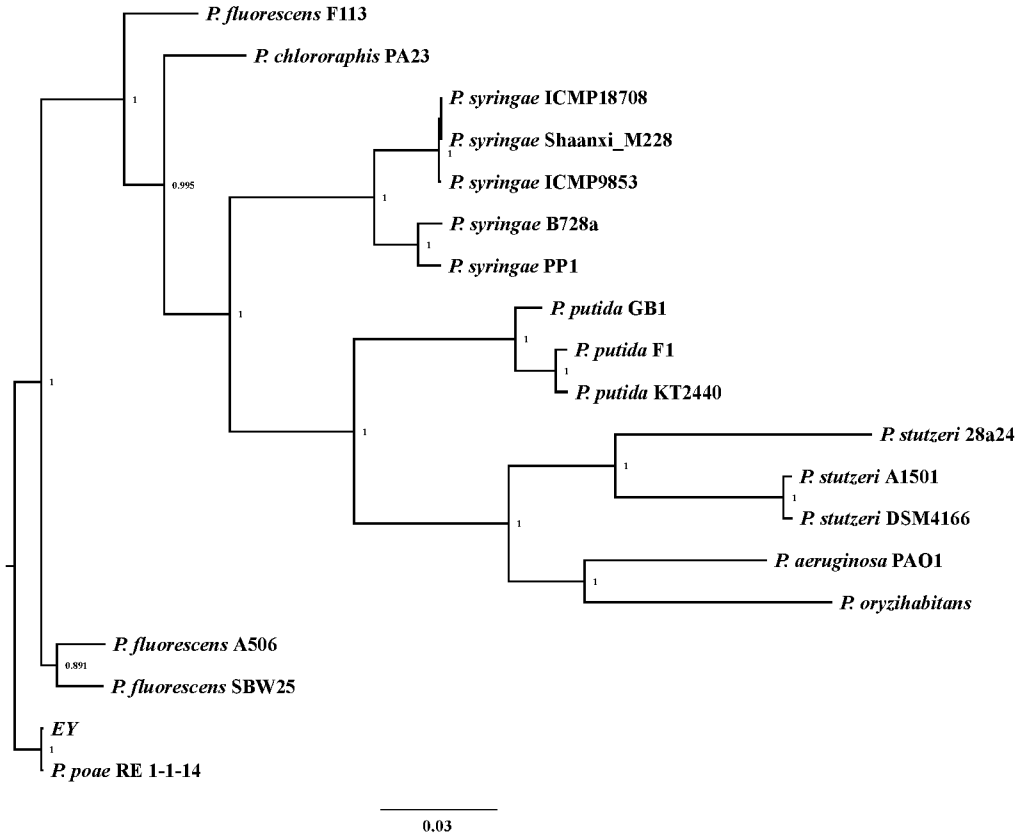
Figure 1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P. syringae PP1
P. putida GB1
P. putida F1
P. putida KT2440
P. stutzeri 28a24
P. stutzeri A1501
P. stutzeri DSM4166
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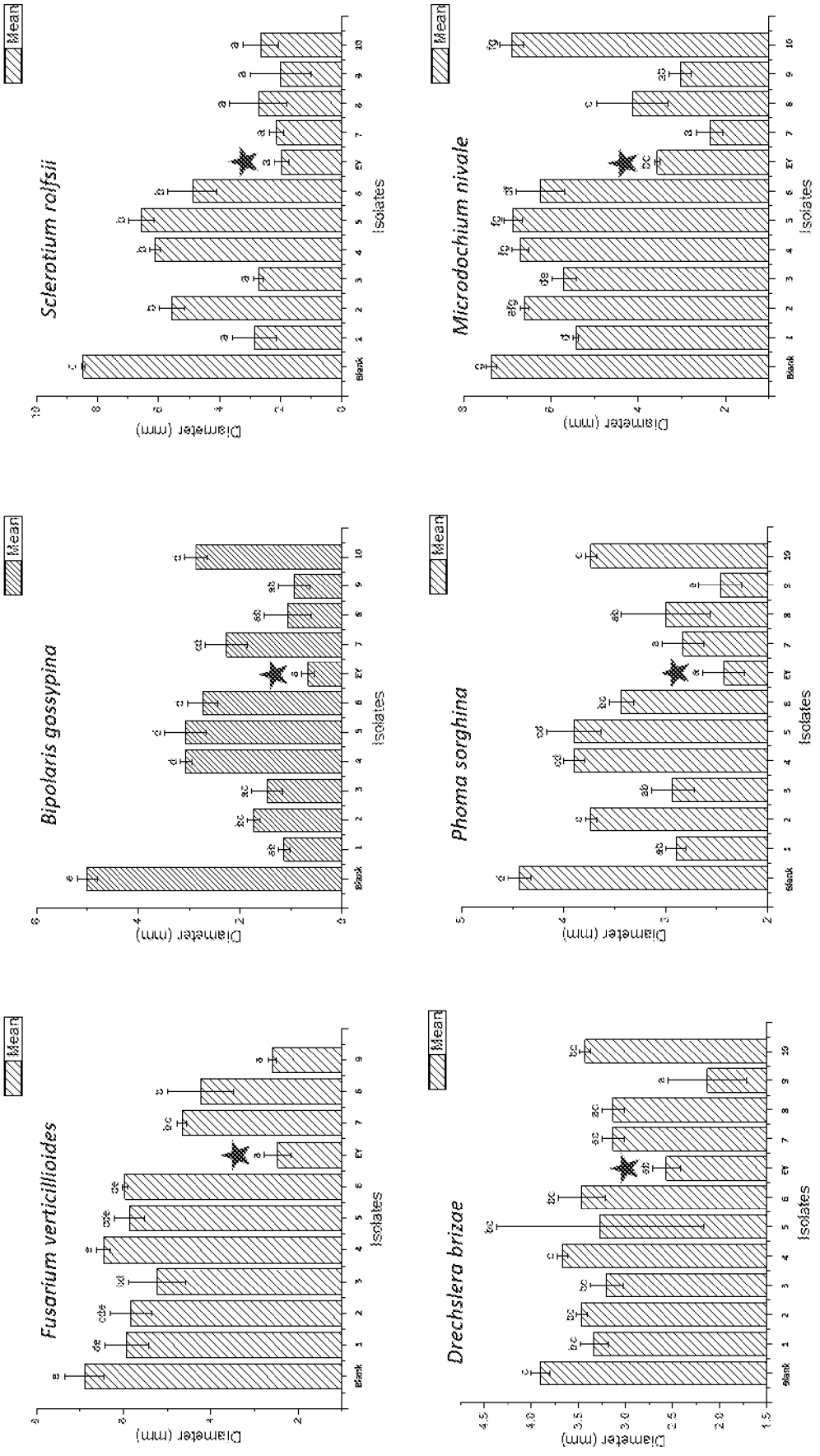
Figure 2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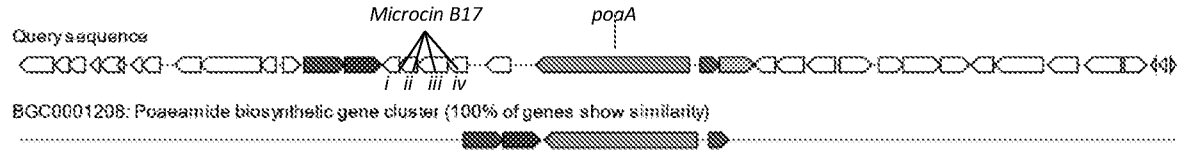
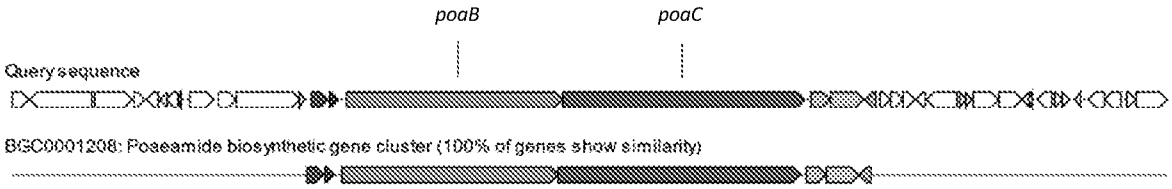
Phoma sorghina
Fusarium verticillioides
Drechslera brizae
Mean
Diameter (mm)
Isolates
Blank
EY

Figure 3

A. *poaA* Gene Cluster



B. *poaB* and *poaC* Gene Cluster



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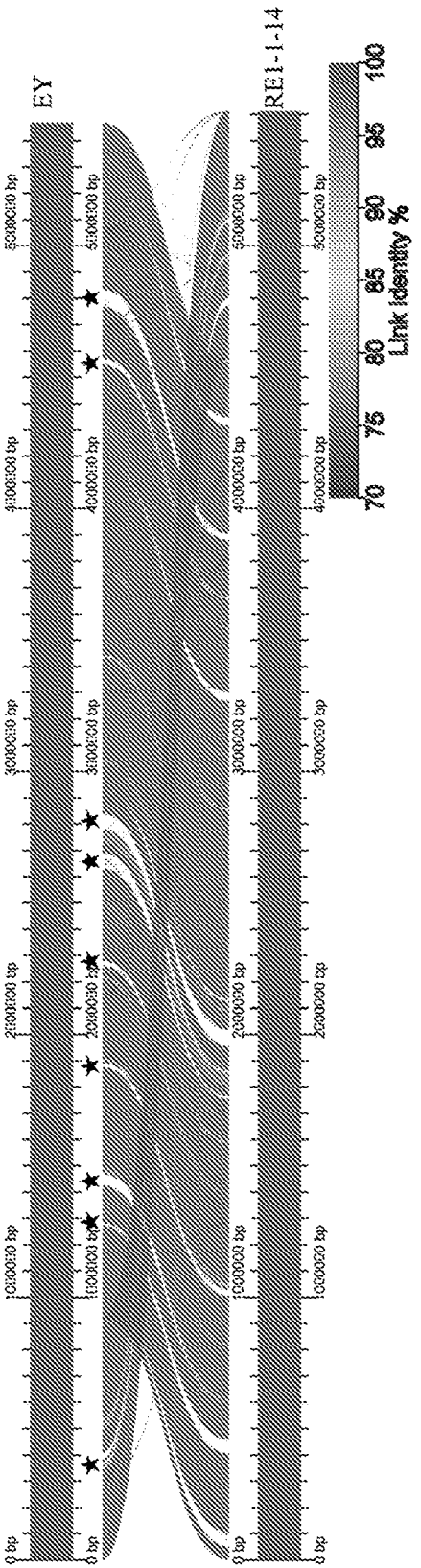
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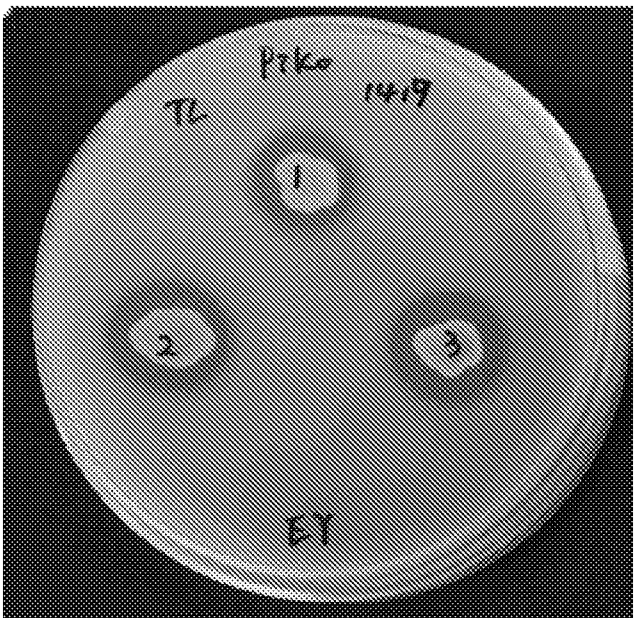
Figure 5



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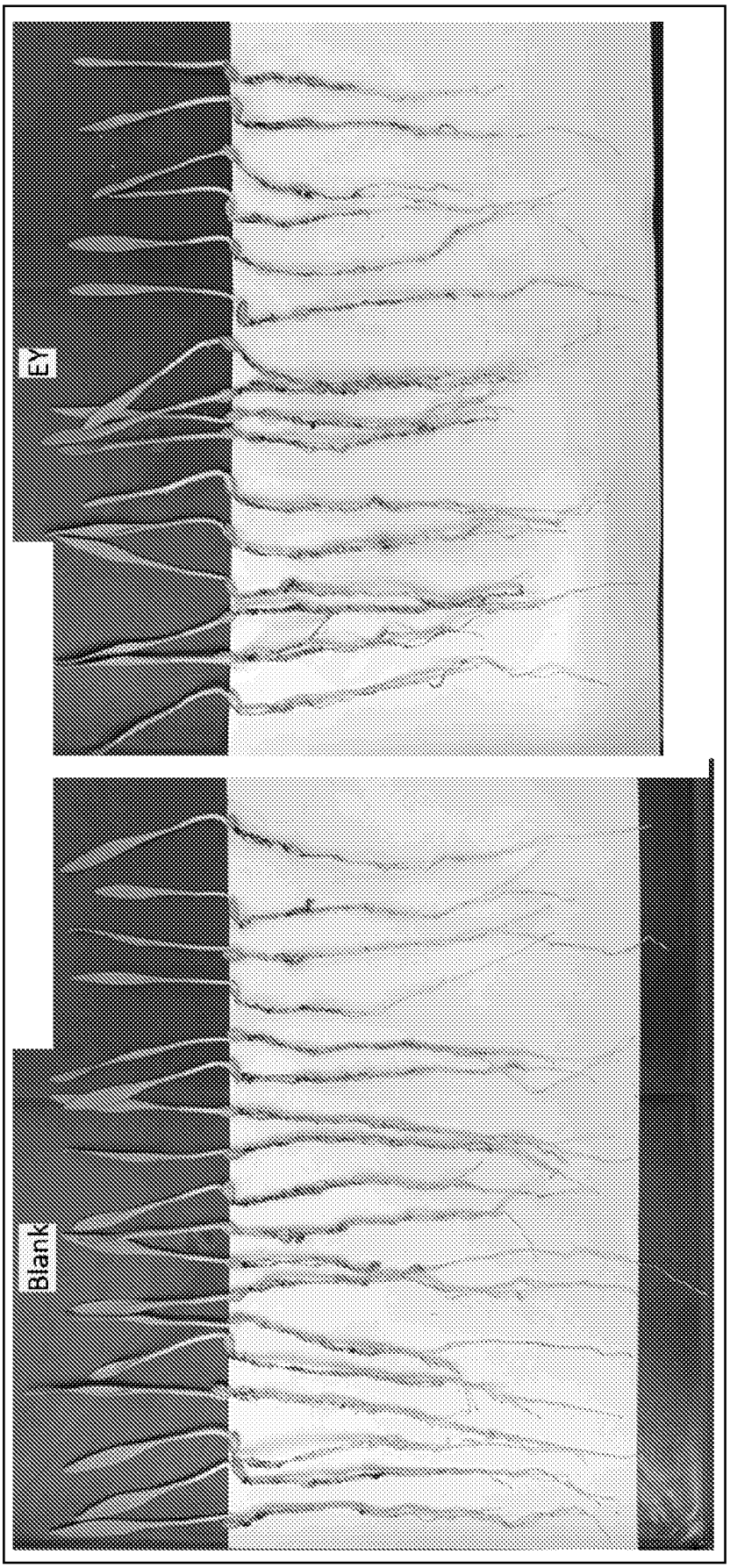
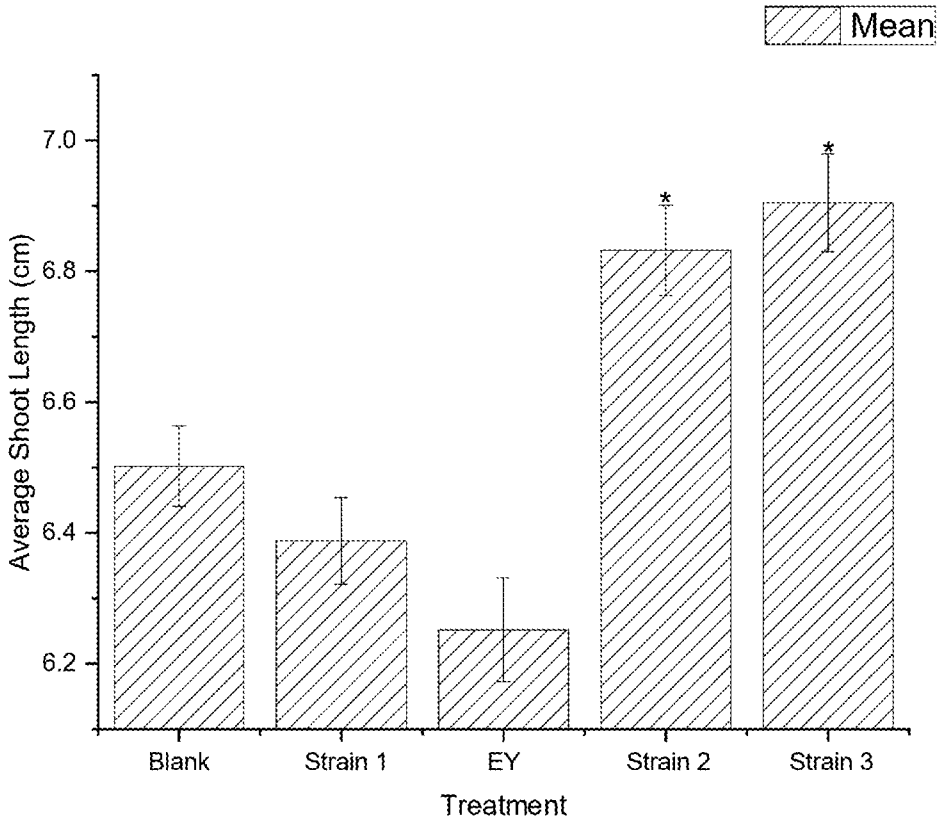


Figure 7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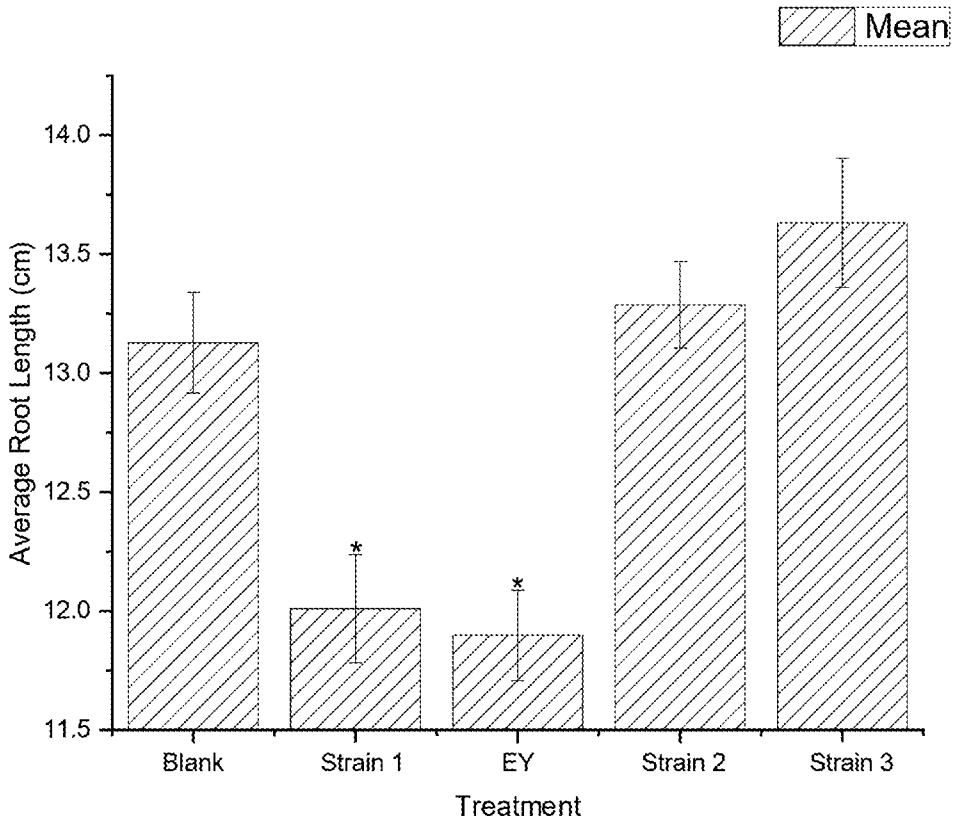
6.2
Blank
Strain 1
EY
Strain 2
Strain 3
Treatment

Figure 8


Mean
Average Root Length (cm)
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12.0
11.5
*
*
Blank
Strain 1
EY
Strain 2
Strain 3
Treatment

Figure 9

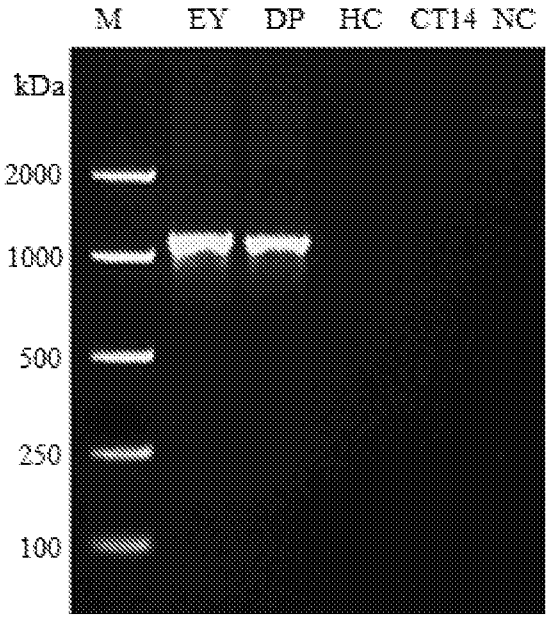


Figure 10

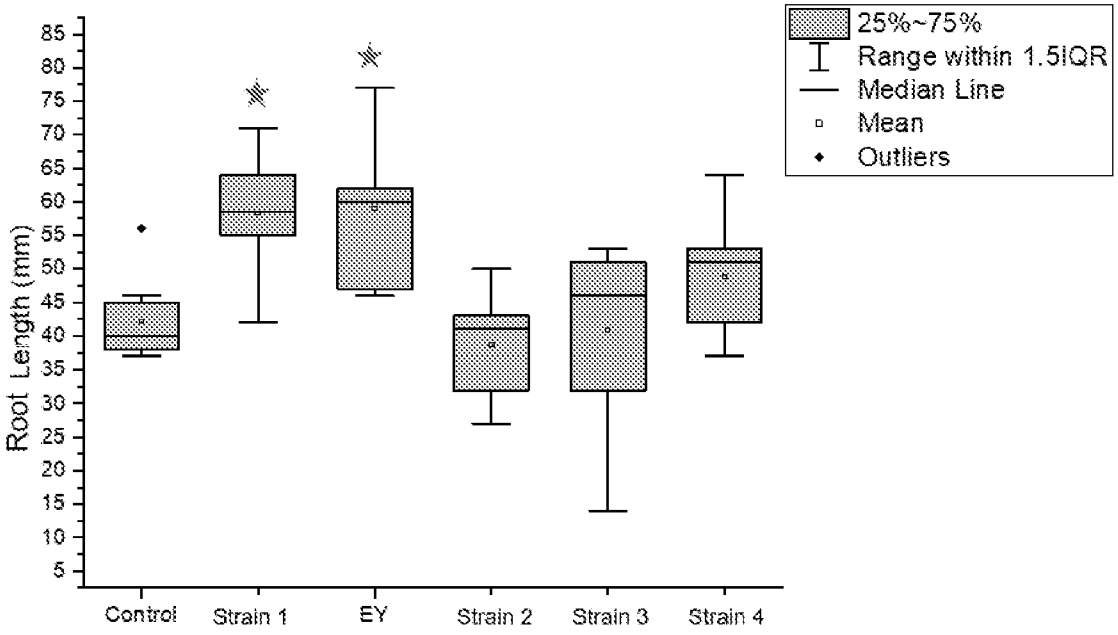
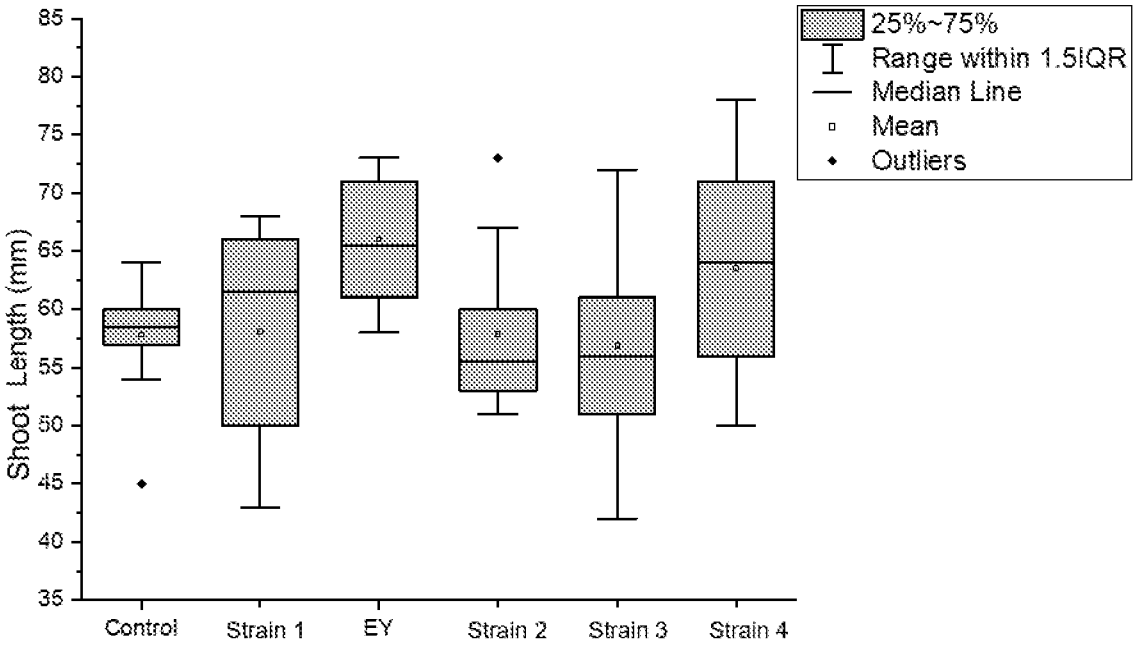

25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4

Figure 11


Shoot Length (mm)
85
80
75
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50
45
40
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Control
Strain 1
EY
Strain 2
Strain 3
Strain 4
25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers

Figure 12

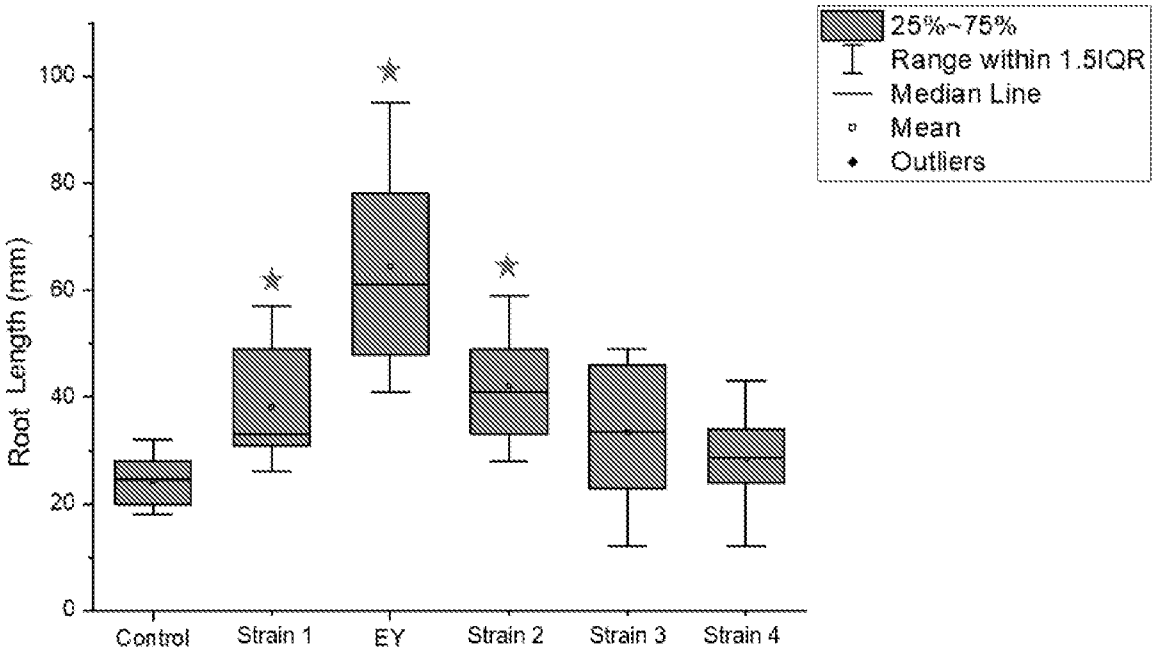
25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers
Root Length (mm)
0
20
40
60
80
100
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4

Figure 13

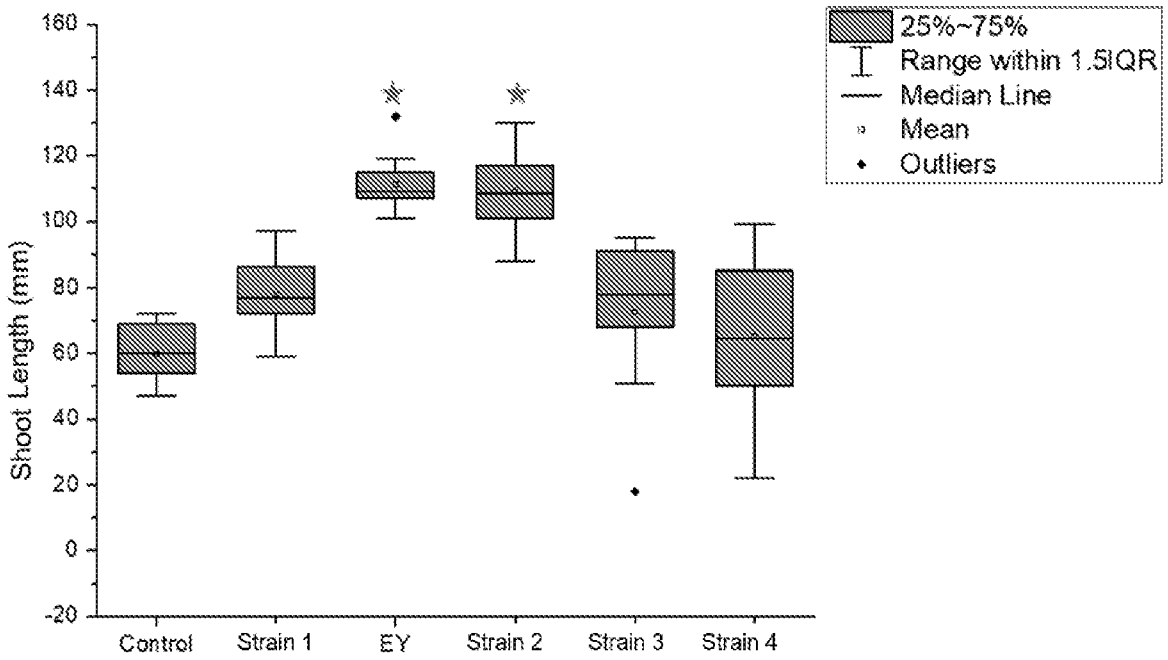
160
140
120
100
80
60
40
20
0
-20
Shoot Length (mm)
Control
Strain 1
EY
Strain 2
Strain 3
Strain 4
25%~75%
Range within 1.5IQR
Median Line
Mean
Outliers

Figure 14

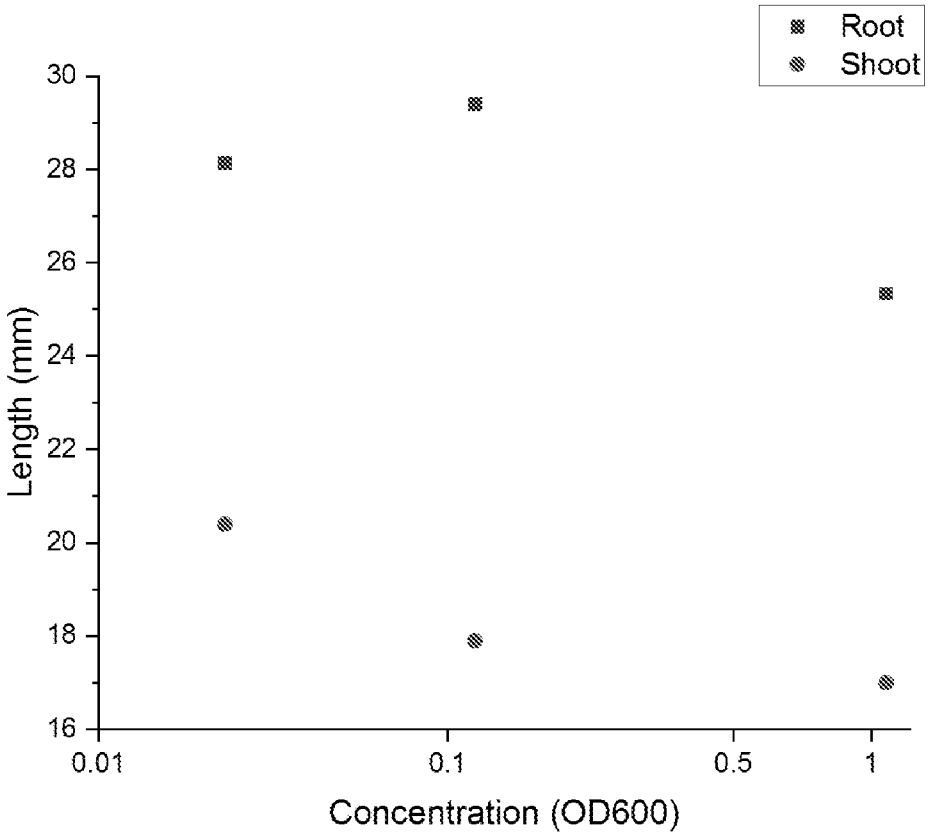
Root
Shoot
30
28
26
24
22
20
18
16
Length (mm)
0.01
0.1
0.5
1
Concentration (OD600)

Figure 15