

NOVEL MICROBIAL COMPOSITION FOR PLANTS

FIELD OF THE INVENTION

- 5 The present application relates to the field of agriculture, and discloses novel microbial compositions and its uses on plants.

BACKGROUND OF THE INVENTION

- 10 Microflora refers to the community of microorganism, including algae, fungi, and bacteria, that live in, on, or around another living organism or in a particular habitat. While some may be harmful to the living organism, others have been proven to at least have a mutualistic relationship with said living organism. In humans, for example, microflora found in the human gastrointestinal tract help with food digestion. The same can be applied
15 to plants, where microflora were found to promote plant growth. Nutrients are mineralised in the soil as the result of their growth and activity, which are subsequently taken up by the surrounding plants.

- While some beneficial microflora are naturally present in agricultural soil, in theory, it
20 would have a low survival rate and efficacy due to prolong monocropping and use of excessive chemicals. Biofertilizers have therefore been developed by agriculturists over time as a means of soil ecology management. The use of biofertilizers also encourage the reduction of chemical fertilizers usage, which is undesirable in the long-run as it can change the pH of the soil and affect the ecosystem of the environment.

- 25 Research has also shown that beneficial microbes in biofertilizers are capable of suppressing plant pathogens. With the harmful impact of chemical pesticides on the environment and the increasing resistance of pathogens to chemical pesticides, biofertilizers have become an attractive alternative to chemical fertilizers and/or
30 pesticides. For example, WO 20150346294 discloses the use of a strain of *Burkholderia* for exhibiting activity against armyworms and mites, while EP 2255660 discusses the use

of a combination of strains of *Bacillus* spp. for soil-borne diseases in crops such as vegetables, banana, or rice.

One particular plant pathogen which can have devastating impacts on the world is
5 *Ganoderma boninense*, which affects the oil palm tree, one of the world's major sources
of edible oil and a significant precursor of biodiesel fuel. Although other fungi can affect
and decay all types of woody plants and trees by colonizing in wounds on trunks, branches,
and roots, *Ganoderma boninense* is one of the fungi responsible for developing complex
wood rot disease. *Ganoderma boninense* causes both basal stem rot and remains South
10 East Asia's most devastating oil palm diseases with direct loss of the stand, reduced yield
of diseased palms and the resultant requirement for earlier replanting. Once young palms
show symptoms of the disease, they usually die within 1 or 2 years, while mature trees can
survive for another 3 or so years. Although it has been clearly identified as the main cause
of the disease in oil palms, strategies for the early detection and control of *Ganoderma*
15 *boninense* are still immature.

EP2790513B1 discusses the use of an isolated *Pantoea agglomerans* strain which may
suppress the development of the *Ganoderma boninense* pathogen. However, the microbial
strain is used together with an acaricide, a bactericide, a fungicide, an insecticide, a
20 microbicide, a nematocide, and a pesticide, all of which are chemical based. Similarly, EP
2854542 also discusses using a biological control agent for controlling *Ganoderma*
boninense, but the biological control agent is used alongside an insecticide.

While many attempts have been made to integrate microbial strains into compositions for
25 plants, it is clear that these attempts are disadvantaged and non-ideal. There is thus a need
for an invention which is able to sufficiently integrate microbial strains in compositions
for plants, which addresses the limitations presently faced by present attempts.

BRIEF DESCRIPTION OF THE INVENTION

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The present invention aims to address the solution by providing compositions comprising
microbial strains which promote desirable plant properties. This and other aims of the

invention, which will be apparent throughout the description, may be achieved through the novel microbial compositions disclosed in the description, and the uses for plants.

Particularly, the invention discloses a composition comprising strains of
5 *Chryseobacterium proteolyticum* and *Achromobacter deleyi* and its benefits in enhancing plant growth, plant yield, and/or reducing the possibility of an infection of a plant pathogen when used in a composition applied to a plant.

Further disclosed is a composition comprising strains of *Chryseobacterium proteolyticum*
10 and *Achromobacter deleyi*, a nutrient source, a bulking agent, and a carrier.

In some embodiments, the composition may optionally comprise one or more microbial strains selected from *Flavobacterium tistrinum*, *Chryseobacterium flavum*, *Bacillus cereus*, *Burkholderia contaminans*, *Paenibacillus taichungensis*, *Olivibacter*
15 *oleidegradans*, *Pseudomonas aeruginosa*, and a further strain of *Chryseobacterium proteolyticum*.

The invention also discloses a method for enhancing growth of a plant, promoting plant yield, reducing development of a plant pathogen, improving phosphorus and potassium
20 solubilization in soil, and fixing nitrogen in soil by applying an effective amount of the composition to the plant or the plant's surroundings.

This and other aims, advantages, and benefits of the invention will be discussed in further detail below.

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DEPOSIT OF MICROORGANISMS

Purified cultures of microbial strains described in present invention were deposited in the Korean Collection for Type Cultures (KCTC) located at Korean Research Institute of
30 Bioscience and Biotechnology (KRIBB), 181, Ipsin-gil, Jeongeup-si, Jeollabuk-do 56212 Republic of Korea in accordance with the Budapest Treaty for the purpose of patent

procedure and the regulations thereunder (Budapest Treaty). Accession numbers for these deposits are as follows:

Table 1: Microbial isolates and the accession numbers

No.	Accession Number	Species Identification
1	KCTC 15987BP	<i>Flavobacterium tistrinum</i>
2	KCTC 15984BP	<i>Chryseobacterium flavum</i>
3	KCTC 15994BP	<i>Chryseobacterium proteolyticum</i>
4	KCTC 15985BP	<i>Achromobacter deleyi</i>
5	KCTC 15996BP	<i>Bacillus cereus</i>
6	KCTC 15991BP	<i>Burkholderia contaminans</i>
7	KCTC 15988BP	<i>Paenibacillus taichungensis</i>
8	KCTC 15993BP	<i>Chryseobacterium proteolyticum</i>
9	KCTC 15989BP	<i>Olivibacter oleidegradans</i>
10	KCTC 15995BP	<i>Pseudomonas aeruginosa</i>

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DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described in a more detailed manner. However, the present disclosure is not intended to limit the invention to the precise embodiments as disclosed, but rather, done so that the disclosure is thorough and complete.

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As mentioned above, beneficial microbes [also referred to as plant-growth promoting microbes (PGPM)] are known to enhance plant growth and efforts have been made to apply these PGPM to compositions for plants. Some of the desired traits exhibited by PGPM include nitrogen fixation, phosphate solubilisation, potassium solubilisation, ACC deaminase activity, and the production of siderophores and phytohormones. While some PGPM may have more than one of the listed traits, efforts have been made to apply a

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mixture of PGPM to maximise the beneficial effect on plants, on the premise that combining several effective strains may lead to additive or synergistic effects.

However, a paper by Pierson & Weller 1994 show that the combination of PGPM in a single composition have failed because of antagonism or incompatibility between microbial co-inoculants. Additionally, Chiarini, L. *et al* 1997 investigated the effectiveness of dual strain inocula compared to single strain inocula on plant growth. Particularly, the effect of *Burkholderia cepacia* alone and in combination with other rhizospheric strains on plant growth was investigated, and the study ultimately found that dual strain inocula were not significantly more effective than single ones.

Advantageously, the inventors of the present invention have been able to successfully demonstrate the efficacy of a multi-strain composition for plants. Particularly, the inventors found that microbial strains, applied in a composition for plants, were capable of promoting beneficial plant properties.

A first embodiment of the invention therefore discloses a composition comprising equal parts of *Chryseobacterium proteolyticum* and *Achromobacter deleyi*, the strains identified and deposited as KCTC 15994BP and KCTC 15985BP, for promoting beneficial properties in plants and/or reducing development of a plant pathogen.

In a further embodiment, the composition may comprise *Chryseobacterium proteolyticum* and *Achromobacter deleyi*, and at least one or more strains selected from a group of *Flavobacterium tistrinum* (deposited as KCTC 15987BP), *Chryseobacterium flavum* (deposited as KCTC 15984BP), *Bacillus cereus* (deposited as KCTC 15996BP), *Burkholderia contaminans* (deposited as KCTC 15991BP), *Paenibacillus taichungensis* (deposited as KCTC 15988BP), *Pseudomonas aeruginosa* (deposited as KCTC 15995BP), *Olivibacter oleidegradans* (deposited as KCTC 15989BP), or a further strain of *Chryseobacterium proteolyticum* (deposited as KCTC 15993BP). In this embodiment, the strains are present in equal parts, and comprise a concentration of 10^6 colony forming unit (c.f.u), or 10^6 - 10^7 c.f.u. overall.

Another embodiment of the invention discloses a microbial composition comprising strains of *Chryseobacterium proteolyticum* and *Achromobacter deleyi*, identified and deposited as KCTC 15994BP and KCTC 15985BP, a nutrient source for the microbial strains, a bulking agent, and a carrier.

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The nutrient source in the composition serves to enhance the survivability of microbes and establish its population in soil, and may comprise compost, fertigation soil, humic acid, coffee bean waste, or any other organic material not stated herein.

- 10 The bulking agent serves as a medium which provides the physical form of the composition and is made from material capable of maintaining air spaces and allowing circulation of airflow which promotes multiplication and/or volumizing of the microbial strains. Its properties include low bulk density & a carbon-based dry material which does not add raw material or energy to the composition, and should be easily dispersed. The
- 15 bulking agent may comprise inert mineral such as sand, charcoal, perlite, zeolite, kaolin, or be silica-based, although not limited to as such.

- The carrier serves as a medium that delivers the microbial strains, of which the microorganisms are bound to, in sufficient quantities. Carriers must have good moisture
- 20 holding capacity, a large surface area, good pH buffering capacity, the ability to withstand harsh environment conditions, be cost effective, and readily available. Carriers may also be organic or inorganic, and some examples of suitable carriers may comprise vermiculite, peat, perlite, kaolin, rice husk, wheat bran, although it should not be limited to as such.

- 25 In the present embodiment, the composition comprises 2-5% carrier, 35-70% nutrient source, 28-60% of bulking agent, and microbial strains, where equal parts of the microbial strains are present in the composition in the range of 10^6 - 10^7 colony forming unit (CFU) count per gram of product.

- 30 In a further embodiment, the composition may comprise strains of *Chryseobacterium proteolyticum* and *Achromobacter deleyi*, and at least one or more strains selected from a group of *Flavobacterium tistrianum* (deposited as KCTC 15987BP), *Chryseobacterium*

flavum (deposited as KCTC 15984BP), *Bacillus cereus* (deposited as KCTC 15996BP), *Burkholderia contaminans* (deposited as KCTC 15991BP), *Paenibacillus taichungensis* (deposited as KCTC 15988BP), *Pseudomonas aeruginosa* (deposited as KCTC 15995BP), *Olivibacter oleidegradans* (deposited as KCTC 15989BP), or a further strain of *Chryseobacterium proteolyticum* (deposited as KCTC 15993BP). In this embodiment, the strains are present in equal parts.

Another embodiment of the invention discloses applying the compositions as a coating to seeds as film-coating, encrusting or pelleting, or other seed coating method to effect desirable plant properties during growth. An example of coating method includes mixing the composition and seeds in an enclosure, such as a bag, bottle, or container, or a mixer with incorporation of biopolymers/polymers and binders such as carboxymethyl cellulose (CMC), maltodextrin, polyvinylpyrrolidone (PVP), sodium alginate, glycerol, xanthan gum, gelatin, starch, gum arabic, polyethylene glycol (PEG), gellan gum, pectin, or any other biopolymers/polymers and binders not stated herein.

Another embodiment of the invention discloses a method for enhancing growth of a plant, promoting plant yield, or reducing development of a plant pathogen by applying an effective amount of the composition to the plant, or the plant's surroundings.

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Preferably, the plant pathogen is *Ganoderma boninense*.

Another embodiment of the invention discloses a method for improving phosphorus and potassium solubilisation in soil and fixing nitrogen, or improving siderophore, 1-aminocyclopropane-1-carboxylic acid deaminase, or auxin production by applying an effective amount of the composition to the plant, or the plant's surroundings.

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The plant is preferably crop, and may comprise oil palm, corn, chilli, or paddy.

In formulating the present invention, the inventors first screened soil and healthy oil palm roots sampling from various sources, including oil palm plantations, to isolate PGPM and biocontrol agents. From the soil sampling and isolation work, about 10,000 microbes strain

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were isolated. The microbes were then screened for 6 types of plant growth promoting (PGP) properties namely, nitrogen fixation, siderophore production, phosphate solubilisation, auxin production, potassium solubilisation and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production. One hundred and ten isolates having the PGP properties were then shortlisted for further testing.

In order to study the efficacy of the shortlisted 110 PGPM in promoting oil palm seedling's growth, a screening trial, namely Single Isolate Screening Trial was established, where 67 out of 110 shortlisted isolates were tested in the trial. Based on the Single Isolate Screening Trial result such as percentage of whole plant dry weight increment as compared to control, number of leaves formation, plant height, leaves chlorophyll content data, together with criteria such as number of PGP activity and types of isolates, 23 promising PGPM were further shortlisted. The 23 shortlisted PGPM were then combined with selected biocontrol agents and subjected to compatibility test for formulation. Based on the compatibility test result, 5 formulations labelled as FM1, FM2, FM3, FM4 and FM5 were established by combining strains from *Chryseobacterium proteolyticum* (KCTC 15994BP) and *Achromobacter deleyi* (KCTC 15985BP) with different combinations of the remaining PGPM and biocontrol agents listed in Table 2 below.

Table 2: Identification of PGPM and biocontrol agents by using 16S rRNA gene sequence.

No.	Microbes ID	Species identification	Function
1	KCTC 15987BP	<i>Flavobacterium tistrinum</i>	Plant growth promoting agent
2	KCTC 15984BP	<i>Chryseobacterium flavum</i>	Plant growth promoting agent
3	KCTC 15994BP	<i>Chryseobacterium proteolyticum</i>	Plant growth promoting agent
4	KCTC 15985BP	<i>Achromobacter deleyi</i>	Plant growth promoting agent

5	KCTC 15996BP	<i>Bacillus cereus</i>	Plant growth promoting agent
6	KCTC 15991BP	<i>Burkholderia contaminans</i>	Plant growth promoting agent
7	KCTC 15988BP	<i>Paenibacillus taichungensis</i>	Plant growth promoting agent
8	KCTC 15993BP	<i>Chryseobacterium proteolyticum</i>	Plant growth promoting agent
9	KCTC 15989BP	<i>Olivibacter oleidegradans</i>	Plant growth promoting agent
10	KCTC 15995BP	<i>Pseudomonas aeruginosa</i>	Biocontrol agent

Species Molecular Identification Using 16S rRNA Gene

The microbes were identified using molecular approach by sequencing the 16S rRNA gene, which is a molecular marker for identification of bacterial species. The 16S rRNA sequences of each microbe were blasted against the National Center for Biotechnology Information (NCBI) database for its taxonomical and identification. It should be noted that use of 16S rRNA gene as a molecular tool to identify bacteria is standard practice as the 16S rRNA gene is present in all bacteria and consists of highly conserved nucleotide sequences and interspersed with variable regions that are genus- or species-specific.

The bacteria DNA is extracted as per instructions provided by InstaGene™ Matrix (BioRad) and polymerase chain reaction (PCR) was conducted to amplify 16S rRNA gene to about 1,500bp using a universal forward primer, 27F (5' AGAGTTTGATCCTGGCTCAG 3') and a universal reverse primer, 1492R (5' TACGGYTACCTTGTTACGACT 3'). A 25µl PCR reaction cocktail is prepared using 1X PCR buffer, 1U *Taq* Polymerase (Invitrogen), 1.5mM MgCl₂, 0.2mM dNTP mix, 0.4mM forward primer, 0.4mM reverse primer and 10µl DNA template prepared using InstaGene™ Matrix.

Plant growth promoting property assay

i. Phosphate solubilising assay

Phosphate solubilising assay was performed using phosphate solubilisation agar (In 100 ml; 0.5g $\text{Ca}_3(\text{PO}_4)_2$, 0.01g $(\text{NH}_4)_2\text{SO}_4$, 0.025g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02g KCl, , 1g glucose, 6g Bacto-agar) prepared in petri dish. The bacteria isolate was inoculated on the agar and incubated at 30°C for 3-5 days. The assay plate was observed daily for the formation of a clear halo on the opaque agar surrounding the bacteria.

ii. Potassium solubilising assay

Potassium solubilising assay was performed using modified Alexsandrov agar (0.5% glucose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0006% FeCl_3 , 0.01% CaCO_3 , 0.2% Ca_3PO_4 , 0.3% mica powder, 2% agar) prepared in petri dish. The bacteria isolate was inoculated on the agar and incubated at 30°C for more than 4 days. The assay plate was observed daily for the formation of a clear halo on the opaque agar surrounding the bacteria.

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iii. Siderophore production assay

The bacteria isolate was grown in 150 µl of pH 7 MM9 growth medium (In 100 ml; 0.05g K_2HPO_4 , 0.1g NH_4Cl , 0.036g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g NaCl , 0.755g PIPES buffer, 1.1g glucose, 500 µl 50% gluconic acid, 0.25 malic acid, 0.05g casamino acid) at 30°C for 5 days. After the incubation period, the culture supernatant was harvested via 4000 rpm centrifugation at 4°C for 20 minutes. Ten microliter of the supernatant was transferred to a 1.5 ml microcentrifuge tube and 200 µl of CAS assay solution (In 100 ml; 6 ml 10mM HDTMA, 1.5 ml Fe(III) solution (0.006g FeCl_3 in 40 ml 10mM HCl), 7.5 ml 2mM CAS, 4.307g anhydrous piperazine, 6.25ml 37% HCl) was added into the tube. The reaction was left to equilibrate overnight at room temperature. Absorbance at 630 nm was read with a spectrophotometer. An assay standard of DFO was prepared at the concentration of 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15 µM and 0 µM. The absorbance of the blue CAS assay solution is inversely proportional to the siderophore concentration of the standards. The assay solutions should change to an intense orange colour with greater quantities of siderophores.

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iv. ACC deaminase assay

The bacteria isolate was grown in 600 μ l Bacterial Minimal Media (BMM) (In 100 ml; 1.2g glucose, 1.0g peptone, 0.15g KH_2PO_4 , 0.15g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) overnight at 30°C with 200 rpm agitation. Transfer 180 μ l of overnight culture to 1.8 ml of DF salts medium (In 100 ml; 0.4g KH_2PO_4 , 0.6g Na_2HPO_4 , 0.02g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g glucose, 400 μ l 50% gluconic acid, 0.2g citric acid, 10 μ l DF trace elements (In 100 ml; 10mg H_3BO_3 , 11mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 124mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 78mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 mg MoO_3), 10 μ l 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) adjusted to pH 7.2 and supplemented with 5 Mm ACC, incubate overnight at 30°C with 200 rpm agitation. The overnight culture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was discarded. The cells pellet was resuspended in 1 ml 1x PBS buffer (10x in 100 ml; 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 , 1.44g KH_2PO_4) and centrifuged at 12,000 rpm for 10 minutes. The PBS wash was discarded. The cells were resuspended in 1 ml of 1x PBS buffer. Five hundred microlitre of the culture was transferred to 1.5 ml microcentrifuge tube containing 0.25g of acid-washed microglass beads and vortex for 5 minutes. The lysed cells were centrifuged at 8,000 rpm for 10 minutes at 4°C. Fifty microlitre of the lysate was transferred to a 1.5 ml microcentrifuge tube and 100 μ l of 10 mM ACC was added to the tube. The mixture was incubated at 30°C for 16 hours. For the assay standard, 50 μ l of 1x PBS buffer was added to 100 μ l of different concentration of α -ketobutyrate (2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM, 0.156 mM and 0 mM). After 16 hours, 100 μ l of 2M HCL was added to the sample and standards, and vortexed. Next, 250 μ l of 0.1% 2,4-dinitrophenylhydrazine was added, vortexed and incubated for 30 minutes at room temperature. Finally, 500 μ l of 2M NaOH was added and vortexed. Two hundred microliter of the sample and standards were transferred to a 96-well microtiter plate to measure the absorbance at 540 nm with a plate reader.

v. Auxin production assay

The bacteria isolate was grown in 150 μ l of YT medium (In 100 ml; 0.8g tryptone, 0.5g yeast extract, 0.5g NaCl, 0.04g L-tryptophan) adjusted to pH 7.0. The culture was incubated at 30°C for 5 days. After the incubation period, the culture supernatant was harvested via 4000 rpm centrifugation at 4°C for 20 minutes. Fifty microliter of the supernatant was transferred to a 1.5 ml microcentrifuge tube and 100 μ l of Salkowsky's assay solution (In 250 ml water; 7.5 ml 0.5M FeCl_3 , 150 ml H_2SO_4) was added into the

tube. The reaction was left to equilibrate at room temperature for 15-20 minutes and the absorbance at 535 nm was read immediately with a spectrophotometer. An assay standard was prepared with IAA diluted in YT medium to the concentration of 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15 µM and 0 µM.

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vi. Nitrogen Fixation Assay

The bacteria isolate was inoculated on semi-solid nitrogen-free (NF) medium (In 1 litre; 4.0g KOH, 0.5g K₂HPO₄, 0.2g MgSO₄.H₂O, 0.1g NaCl, 0.02g CaCl₂, 0.005g FeSO₄.7H₂O, 0.002g Na₂MoO₄.2H₂O, 0.01g MnSO₄.7H₂O, 5.0g malic acid, 1.75g bacteriological agar, pH adjusted to 6.8 with KOH, 2ml 0.5% bromothymol blue) and incubated overnight at 30°C. The plate was observed for color change from green to blue for isolate which has the ability to fix nitrogen.

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Screening of Biocontrol Property: Dual Culture Plate Assay

15 Dual culture plate assay

Dual culture plate assay was performed by placing a *Ganoderma* agar plug measuring 5 mm x 5 mm in the middle of a potato dextrose agar (PDA) plate. One line of the microbial isolate was streaked on both sides of the *Ganoderma* at a 3 cm distance from the *Ganoderma* agar plug. A control test plate was prepared by placing a *Ganoderma* agar plug measuring 5 mm x 5 mm in the middle of a PDA plate. The plates were incubated at 30°C for 5 days. After the incubation period, diameter of the *Ganoderma* on both test (R2) and control (R1) plates was measured. **The percent inhibition of radial growth (PIRG)**, shown in **Table 3**, was calculated based on the formula:

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$$\left(\frac{(R1 - R2)}{R1} \right) \times 100$$

The PGP and Biocontrol properties (PIRG) of each microbe are listed in **Table 3**.

Table 3: Plant Growth Promoting (PGP) and Biocontrol (PIRG) Properties

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No.	Microbes ID	Nitrogen fixation	Phosphate solubiliser	Potassium solubiliser	Siderophore production	ACC deaminase	Auxin production	Biocontrol property (PIRG)
1	KCTC 15987BP	Yes	No	No	No	No	No	No
2	KCTC 15984BP	No	No	No	Yes	No	No	No
3	KCTC 15994BP	No	No	No	Yes	No	No	No
4	KCTC 15985BP	Yes	No	No	No	Yes	No	No
5	KCTC 15996BP	Yes	No	No	Yes	No	Yes	No
6	KCTC 15991BP	Yes	Yes	Yes	Yes	Yes	No	No
7	KCTC 15988BP	No	No	No	Yes	No	No	No
8	KCTC 15993BP	No	No	No	Yes	No	No	No
9	KCTC 15989BP	No	No	No	Yes	No	No	No
10	KCTC 15995BP	Yes	Yes	No	Yes	No	No	Yes

PGP Nursery Trial

i. Nursery Trial 1

This nursery trial was set-up to study the efficacy of 3 established formulations namely, FM1, FM2 and FM3 at 3 different application dosages such as 50g, 100g and 150g, against 3 commercially available products, and Control at 75% and 100% of nursery fertiliser recommendation. Due to limited number of available 3-months old seedlings after culling, the treatments were arranged in Split Plot Design and tested with unequal numbers of seedlings per replicate (between 18-27 seedlings) with a total number of 3 replications. For FM1, FM2, and FM3, germinated seeds were soaked in the 3 formulated treatment suspensions for ~ 2 hours prior to seeding. Twenty grams of booster application was applied after 1.5 months of planting. Three levels of formulated product (50g, 100g, and

150g) application were started after 3 months of planting that is during the transplanting in the planting hole and later at every 3 months interval during the nursery stage at the soil surface surrounding the seedlings base. Culling was done 3 months after planting and at every 3 months interval. The trial period was 12 months. Destructive sampling was conducted for 3, 6, 9, and 12-months seedlings for whole plant dry weight measurement with 2 seedlings per replicate per treatment. Analysis Of Variance (ANOVA) is significant at 5% confidence level for the 12-months whole plant dry weight (**Table 4**). Between the 3 formulated treatments, FM2 was the best formulation with significance at 5% confidence level for both 100g and 150g application dosage with 75% and 100% of nursery fertiliser recommendation following the Least Significant Difference (LSD) test (**Table 5**). At 150g application dosage, with 75% of nursery fertiliser recommendation, the LSD for FM2 is highly significant at 1% confidence level. Based on the result obtained, 100g dosage with 75% of nursery fertiliser application was selected for future testing. In order to reduce the formulation materials cost, FM2 application dosage has been reduced to 50g with 2x microbes concentration in future testing.

Table 4: ANOVA for 12-months seedlings whole plant dry weight

Source of Variance	Degree of Freedom	Sum Square	Mean Square	Computed F	Tabular F		
					5%	1%	Significance
Replication	2	151209.0	75604.5				
Main plot factor (F)	1	951.0	951.0	1.119	18.510	98.490	ns
Error F	2	1699.3	849.7				
Subplot factor (T)	21	48501.6	2309.6	1.746	1.684	2.080	*
F x T	21	32909.7	1567.1	1.185	1.684	2.080	ns
Error T	84	111105.0	1322.68				
Total	131	346375.5					

Table 5: Mean whole plant dry weight and LSD analysis for 12-months old seedlings

Treatment	Dosages (g)	75% of Nursery Fertiliser				100% of Nursery Fertiliser			
		Mean (g)	Difference from Control (g)	LSD 1% (78.22 g)	LSD 5% (59.03 g)	Mean (g)	Difference from Control (g)	LSD 1% (78.22 g)	LSD 5% (59.03 g)
FM1	50	330.4	38.7	ns	ns	301.3	3.4	ns	ns
	100	337.1	45.5	ns	ns	303	5.1	ns	ns
	150	329.9	38.3	ns	ns	297.7	-0.2	ns	ns
	Average	332.5	40.8			300.7	2.8		
FM2	50	338.4	46.8	ns	ns	312.2	14.3	ns	ns
	100	361.9	70.3	ns	*	371.1	73.2	ns	*
	150	377.6	86	**	*	371.6	73.7	ns	*
	Average	359.3	67.7			351.6	53.8		
FM3	50	339.2	47.6	ns	ns	324.1	26.3	ns	ns
	100	309.8	18.1	ns	ns	286.3	-11.6	ns	ns
	150	313.3	21.7	ns	ns	296.2	-1.7	ns	ns
	Average	320.8	29.1			302.2	4.3		
Commercial Product 1	Recommendation by manufacturer	302.5	10.8	ns	ns	369.3	71.5	ns	*
Commercial Product 2	As manufacturer's recommendations	317.5	25.9	ns	ns	341.8	43.9	ns	ns
Commercial Product 3	As manufacturer's recommendations	341.1	49.4	ns	ns	306.3	8.4	ns	ns
Control	Estate practice	291.6				297.9			

ii. Nursery Trial 2

- 5 This nursery trial was set-up to test FM2 and FM5. Seedlings with only 100% fertiliser application served as control treatment in the trial. All the treatments were arranged in Randomized Complete Block Design (RCBD) with 4 replications and 22 seedlings per replicate. Fifty grams of formulated product was applied at 0, 3, 6, 9 months, and extended to 13 months application when the trial was extended from 12 to 15 months period. At '0'
- 10 time, the formulated product was applied by mixing into the potting material. At subsequent month of application, the product was applied on soil surface surrounding the seedlings base. Destructive sampling was done for 15-months seedlings to measure dry mass weight.

Referring to the tabulated data (**Table 6**), all the treatments have an increased dry mass weight as compared to control treatment, especially for FM2-75 which has increased 10.75% and FM5-75 which has increased 13.14%. These results concluded that application of FM2 and FM5 has boosted the growth of oil palm seedlings.

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Table 6a: Dry mass weight difference between Treatment and Control for 15 months old seedlings.

No.	Treatment details	Weight difference (%)
1	FM2 + 75% fertiliser	10.75
2	FM5 + 75% fertiliser	13.14
3	Control (100% fertiliser)	-

It should be noted that the fertiliser used above is a conventional, commercially available chemical fertiliser, and only 75% of fertiliser was applied during nursery stage, instead of the usual 100% dosage, as seen in the control.

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iii. Nursery Trial 3

This nursery trial was set-up to test FM2. Seedlings with only 100% fertiliser application served as control treatment in the trial. All the treatments were arranged in Randomized Complete Block Design (RCBD) with 4 replications and 23 seedlings per replicate. A total of 30% sand was added to the soil used in the main nursery for better soil aeration. Fifty gram of FM2 treatment was applied at 0, 3, 6, and 9-months seedlings. At '0' time, the formulated product was added into the potting material. In subsequent rounds of application, the product was applied on soil surface surrounding the seedlings. Destructive sampling was done for 12-months seedlings to measure dry mass weight.

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Referring to the tabulated data (**Table 7**), treatments FM2-75 has an increased dry mass weight by 15.29% as compared to control treatment. The results concluded that application of FM2 can boosts the growth of oil palm seedlings.

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Table 7: Dry mass weight difference between FM2 treatment, and Control treatment for 12 months old seedlings.

No.	Treatment details	Weight % difference
1	FM2 + 75% fertiliser	15.29
2	Control (100% fertiliser)	-

iv. Nursery Trial 4

5 In this oil palm nursery trial, FM2 and FM4 were tested. Randomised complete block design (RCBD) was applied in this trial. A total of 5 replicates were included in each treatment. The nursery practice with 100% of nursery fertilizer rate were served as control in this nursery trial. The application of FM2 & FM4 formulations was at 3 months interval, i.e. 0, 3, 6 and 9 months.

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Upon completion of trial, whole plant dry weight of 40 seedlings from each treatment were taken and compared with control, treatment T3. All the treatments showed higher mean plant dry weight than control, 100% nursery fertiliser rate. The mean plant dry weight of FM2 (treatment T1) and FM4 (treatment T2) is 30% and 11% more than control (treatment T3), respectively (**Table 8**).

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Table 8: Differences between treatments and estate nursery practice with 100% inorganic fertiliser regime (treatment T3)

Treatment No.	Treatment Name	Mean plant dry weight (g)	Weight differences between treatments and control (T3) (%)
T1	FM2 + 75% fertiliser	940.88	30*
T2	FM4 + 75% fertiliser	809.49	11
T3	Control - 100% nursery fertiliser rate	726.29	-

* Duncan Multiple Range Test (DMRT) significant at 5%

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v. **Nursery Trial 5 (2 bacterial i.e. *Chryseobacterium proteolyticum* and *Achromobacter deleyi* , FM6 formulation)**

In this 4-months oil palm nursery trial, FM6 formulation was tested with 100% of nursery fertilizer rate. Oil palm seedlings with 100% of nursery fertilizer rate and without any treatment were served as control. Complete block design (CBD) was applied in this trial. The application of FM6 formulation was at 0 and 2 months after planting.

The trial was started with 20 seedlings per treatment and upon completion of trial, whole plant dry weight of seedlings from each treatment were taken and compared with control. FM6 showed higher mean plant dry weight than control. The mean plant dry weight of FM6 is 21.9% significantly higher than control (**Table 9**).

Table 9: Summary result of Nursery Trial 5

Treatments	No. of oil palm seedlings	Mean plant dry weight (g)
FM6	15	1.604**
Control	18	1.315

**Least Significant Difference (LSD) analysis significance at $p < 0.01$

The present invention further relates to a method for enhancing growth of a plant, a method for promoting plant yield, and a method for reducing development of a plant pathogen, where the plant pathogen comprises *Ganoderma boninense*. In application, an effective amount of the composition is applied to the plant, or to the plant's surroundings.

PGP field trial – Field Trial 1

The trial is conducted on matured healthy oil palms plants in the field. The field selected for the trial was in Johor, Malaysia. The criteria for field selection is: a) Palm age between 4-5 years, b) Flat land, c) Flood free area, and d) No *Ganoderma* disease infection at '0' time of the trial. Each treatment contained 8 core palms/replicate and 16 guard palms surrounding the 8 core palms. Between treatments are separated by 2 rows of guard palms.

One kilogram of FM1, FM2, and FM3 were applied through broadcast to core & guard palm based at about 0.5-1 m away from the trunk in circle at every 4 months interval, 7-10 days after NPK fertiliser application. Fresh fruit bunch (FFB) weight was taken 3 times

in a month for yield monitoring. *Ganoderma* disease incidence was also being observed. By February 2020, FFB yield recording has been conducted for 72 months (6 years).

Based on Year 3 onwards (25-72 months) fresh fruit bunch (FFB) data, FM1 with 75% of normal field NPK fertiliser, FM2 with 75% of normal field NPK fertiliser and FM3 with 75% of normal field NPK fertiliser, have shown FFB yield improvement of 7.2%, 17% and 4.0%, respectively as compared to Control or standard field practices (**Table 10**).

By end of Year 6, the percentage of *Ganoderma* incidence for FM1 with 75% of normal field NPK fertiliser, FM2 with 75% of normal field NPK fertiliser and FM3 with 75% of normal field NPK fertiliser were 8.3%, 12.5% and 25.0%, respectively as compare to 37.5% for Control or normal field practices.

Table 10: Yield comparisons between treatment and control based on Year 3 onwards fresh fruit bunch (FFB) data (25-72 months Yield recording)

No.	Treatment details	FFB % difference between treatment and control	% <i>Ganoderma</i> incidence
1.	FM1 + 75% fertiliser	7.2	8.3
2.	FM2 + 75% fertiliser	17.0	12.5
3.	FM3 + 75% fertiliser	4.0	25
4.	Control - 100% fertiliser rate	-	37.5

Vegetable Trials

Sweet Corn

The corn was planted on 4ft x 20ft of raised planting bed. During the bed preparation, 10kg of FM5 and 75% of recommended inorganic fertiliser (as recommended by seed supplier) were ploughed to the soil. For control bed, only 100% inorganic fertiliser (as recommended by seed supplier) was ploughed to the soil. Then the bed was covered with silver shine to prevent soil erosion and weeds growing.

Each planting bed consists of 69 planting hole. During planting, the corn seed was sowed directly to the planting hole. At every 10 days, application of compound fertilizer will be applied at 75% of recommended inorganic fertiliser (as recommended by seed supplier).

For control, 100% inorganic fertiliser was applied. **Table 11** summarised Batch 1 to Batch 4 result. The results from all 4 batches of trial showed that treatment is better than control.

Table 11: Batch 1 to Batch 4 corn planting

Batch	Treatment	No. of replicate	Total fresh weight (kg)		Difference (%)
			Control	Treatment	
1	FM5, 10kg/bed + 75% F	3	39	56.4	45%
2	FM5, 10kg/bed + 75% F	3	48	58.2	21%
3	FM5, 10kg/bed + 75% F	3	38.2	59.6	56%
4	FM5, 10kg/bed + 75% F	3	36.6	48.4	32%

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Chilli

The chilli seeds were germinated on a Hy-Plug tray using top soil, peat moss and sand with a ratio of 3:2:1, respectively. After 1 month, it was transplanted out to the 15” x 18” polybags. In total, 10 replicates, 4 plants per replicate for each treatment and control were planted. The treatment polybags were mixed with 50g of FM5 per polybag. Chilli fruits were harvested when the fruit turned red. The yield recording for each replicate was recorded. Yield recording has completed up to 9 months. **Table 12** summarised the yield record. Chilli plant with FM5 treatment gave 19% more yield than control.

Table 12: Yield recording of chilli trial.

No.	Treatment details	No. of fruits	Yield (g)
1.	FM5	5,159	45,775
2.	Control	4,342	38,358
Differences to control (%)		18.8%*	19.3%**

*t- test significance at $p < 0.05$

**t-test significance at $p < 0.01$

Paddy

This trial was conducted in planting box. The soil was collected from actual paddy and the condition was mimicking the actual paddy planting condition. In total, 4 boxes per m^2 with 9 plants per box, with 3 replicates, were planted. Data recorded were number of spikelet per panicle, yield per m^2 and visual observations of paddy growth condition.

The FM5, 25g, was diluted in 200ml of water before poured equally to 4 boxes. Inorganic fertiliser was applied according the standard farmers regime. For treatment, inorganic fertiliser was applied at 80% rate. Table 13 showed the result of trial. Trial has completed with 10% yield increase using FM5 comparing to farmer's practice.

Table 13: Summary result of paddy trial

Treatments	Description	Yield Components	
		Yield/m ²	Number of spikelet/ panicle
Control	Control – Farmer's standard fertiliser	1347.67	71.72
Treatment	FM5 + 80% Farmer's standard fertiliser	1483.33	84.44
Difference to control (%)		10.07 %	17.74 %

Microbial Formulation and Mixing Process

Microbial formulation (FM) consists of concoctions of microbes, vermiculite as carrier, compost as food source for microbes during field application and sand as bulking agent in the formulation. The composition of formulation is 2-5% carrier, 35% - 70% organic material and 25% - 60% bulking agent. During the preparation of microbial formulation, the concoction of microbes is first mixed with carrier in a mixer. After it is well mixed, organic material is subsequently added and mixed together. Finally, bulking agent is added for mixing.

The present invention has thus been described in specific embodiments. It should not, however, be limited to the invention as disclosed, and it will be apparent to those skilled in the art that various changes and modifications may be made to the invention without departing from the scope of the invention.

CLAIMS

1. A composition for promoting beneficial properties in plant and/or reducing development of a plant pathogen, comprising strains of *Chryseobacterium proteolyticum* and *Achromobacter deleyi*.
5
2. A composition for plants, comprising:
strains of *Chryseobacterium proteolyticum* and *Achromobacter deleyi*,
a nutrient source for the microbial strains,
10 a bulking agent; and
a carrier.
3. The composition of claims 1 or 2, wherein the strains of *Chryseobacterium proteolyticum* and *Achromobacter deleyi* are strains deposited under deposit numbers
15 KCTC 15994BP and KCTC 15985BP respectively.
4. The composition of claims 1 or 2, comprising one or more microbial strains selected from *Flavobacterium tistrinum*, *Chryseobacterium flavum*, *Bacillus cereus*, *Burkholderia contaminans*, *Paenibacillus taichungensis*, *Olivibacter oleidegradans*,
20 *Pseudomonas aeruginosa*, or *Chryseobacterium proteolyticum*.
5. The composition of claim 4, wherein the strains of *Flavobacterium tistrinum*, *Chryseobacterium flavum*, *Bacillus cereus*, *Burkholderia contaminans*, *Paenibacillus taichungensis*, *Olivibacter oleidegradans*, *Pseudomonas aeruginosa*, or
25 *Chryseobacterium proteolyticum* are strains deposited under deposit numbers KCTC 15987BP, KCTC 15984BP, KCTC 15996BP, KCTC 15991BP, KCTC 15988BP, KCTC 15989BP, KCTC 15995BP, and KCTC 15993BP.
6. The composition of claims 1, 2, or 4, comprising equal parts of microbial strains.
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7. The composition of claim 6, comprising a microbial strain concentration of 10^6 - 10^7 colony forming unit (c.f.u).

8. The composition of claim 2, comprising 2-5% of carrier, 35-70% nutrient source, 25-60% of bulking agent, and microbial strains.
9. The composition of claim 2, wherein the nutrient source for the microbial strains
5 comprises any organic material, and preferably, comprises compost.
10. The composition of claim 2, wherein the bulking agent is preferably sand.
11. The composition of claim 2, wherein the carrier comprises vermiculite, peat,
10 perlite, kaolin, rice husk, or wheat bran.
12. The composition according to any one of the preceding claims, wherein the plant is crop.
- 15 13. The composition according to claim 12, wherein the crop comprises oil palm, corn, chilli, and paddy.
14. Use of a composition of claims 1 or 2 for coating a plant seed.
- 20 15. A method for enhancing growth of a plant, promoting plant yield, or reducing development of a plant pathogen, comprising applying an effective amount of the composition of any one of claims 1-13 to the plant, or to the plant's surroundings.
16. The method of claim 15, wherein the plant pathogen is *Ganoderma boninense*.
25
17. A method for improving phosphorus and potassium solubilisation in soil and fixing nitrogen, or improving siderophore, 1-aminocyclopropane-1-carboxylic acid deaminase, or auxin production, comprising applying an effective amount of the composition of any one of claims 1-13 to the plant, or to the plant's surroundings.
30
18. Use of the composition of claims 1 or 2 in a fertiliser.