(54) HOPANOIDS PRODUCING BACTERIA AND RELATED BIOFERTILIZERS, COMPOSITIONS, METHODS AND SYSTEMS

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See application file for complete search history.

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OTHER PUBLICATIONS


ABSTRACT
Hopanoids, hopanoids-producing nitrogen-fixing bacteria, and related formulations, systems and methods are described herein. In particular, hopanoids alone or in combination with hopanoids-producing nitrogen-fixing bacteria can be used as biofertilizer to stimulate plant growth and yield with enhanced tolerance to diverse stresses found in plant-microbe symbiotic microenvironments.

26 Claims, 14 Drawing Sheets
Specification includes a Sequence Listing.
FIG. 1
A) 

Hopanoid 

Tetrahymanol 

\[ R_1 = H \text{ or } CH_3; \ R_2 = OH \text{ or } NH_2 \]

B) 

\[
\begin{align*}
\text{equa} & \rightarrow \text{Shc} \rightarrow \text{diploptene} \rightarrow \text{HpnP} \rightarrow \text{2-methyldiploptene} \\
& \rightarrow \text{HpnH} \rightarrow \text{adenosyl hopane}
\end{align*}
\]

C) 

FIG. 2
FIG. 3
FIG. 4
FIG. 5

- WT
- ΔhpnP
- ΔhpnH

25°C vs 40°C
A) Aerobic - 30°C
B) Aerobic - 37°C
C) Microaerobic - 30°C
D) pH = 6

E) Control
   - WT
   - ΔhpnP
   - ΔhpnH

   Stationary
   - NaCl
   - Inositol
   - Bile Salts
   - EDTA

F) Inhibition Zone (cm)
   - SDS
   - H₂O₂
   - HCl

G) CFU/ml
   - WT
   - ΔhpnP
   - ΔhpnH

FIG. 7
FIG. 8
FIG. 11
FIG. 12
FIG. 13
FIG. 14
HOPANOIDS PRODUCING BACTERIA AND RELATED BIOFERTILIZERS, COMPOSITIONS, METHODS AND SYSTEMS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No. 62/243,418, entitled “Using Hopanoids to Improve Stress Resistance in Biological Nitrogen Fixation” filed on Oct. 19, 2015, which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT GRANT

This invention was made with government support under Grant No. NNX12AD93G awarded by NASA, under Grant No. CHE1224158 awarded by the National Science Foundation, and under Grant No. 06434 awarded by the Howard Hughes Medical Institute (HHMI). The government has certain rights in the invention.

FIELD

The present disclosure relates to hopanoids, hopanoids producing bacteria and related biofertilizers, microorganisms, compositions, methods and systems comprising hopanoid-producing microorganisms and related formulations, and uses as biofertilizer in agriculture industry.

BACKGROUND

The exploitation of beneficial microbes as a biofertilizer has become of paramount importance in agriculture sector for their potential role in food safety and sustainable crop production. However, there remains however a challenge for developing eco-friendly and economically feasible alternatives to chemical fertilizers, which can improve soil fertility, health of the environment, and agricultural productivity particularly under different soil conditions.

SUMMARY

Provided herein are hopanoid-producing bacteria, and related biofertilizers, microorganisms, formulations, and methods which can comprise one or more particular types of hopanoids and/or one or more hopanoid-producing nitrogen-fixing bacteria. In several embodiments, the microorganisms, and related biofertilizers, compositions methods and systems described herein can improve stress-resistance during the progression of plant-microbe symbiosis and in particular of a legume-microbe symbiosis.

According to a first aspect, a biofertilizer for a leguminous plant is described. The biofertilizer essentially consists of one or more nitrogen-fixing rhizobia capable of producing C35 hopanoids. The biofertilizer, the one or more nitrogen-fixing rhizobia capable of producing C35 hopanoids are in a form suitable for administration to one or more leguminous plant or seed, and/or for administration to a soil surrounding the one or more leguminous plant or seed. In some embodiments, the biofertilizer is in combination with carrier allowing increased stability, viability and/or effectiveness of the nitrogen-fixing bacteria gas exchange of the one or more nitrogen-fixing rhizobia.

According to a second aspect, a method to provide a biofertilizer for a leguminous plant, is described. The method comprises rhizobia naturally capable of producing C35 hopanoids and/or rhizobia naturally incapable of producing C35 hopanoids. The method further comprises detecting among the one or more candidate nitrogen fixing rhizobia strains, at least one rhizobia strain capable of producing C35 hopanoids in a form suitable for administration to a leguminous plant or seed or soil surrounding a leguminous plant or seed, and thus providing a biofertilizer for the leguminous plant. In some embodiments, the method further comprises providing the at least one rhizobia strain capable of producing C35 hopanoids in combination with carrier allowing increased stability, viability and/or effectiveness of the nitrogen-fixing bacteria gas exchange of the one or more nitrogen-fixing rhizobia.

According to a third aspect, a method to provide a biofertilizer for a leguminous plant, is described. The method comprises genetically engineering a nitrogen fixing rhizobia strain incapable of producing C35 hopanoids to introduce C35 synthesis genes thus providing a genetically engineered nitrogen fixing rhizobia strains capable of producing C35 hopanoids. The method further comprises providing the genetically engineered nitrogen fixing rhizobia strains in a form suitable for administration to a leguminous plant or seed, and/or a soil surrounding leguminous plant or seed thus providing the biofertilizer for the leguminous plant.

According to a fourth aspect, a biofertilizer composition for a leguminous plant is described. The biofertilizer composition comprises one or more biofertilizers essentially consisting of one or more nitrogen-fixing rhizobia capable of producing C35 hopanoids and an acceptable vehicle. In some embodiments, the biofertilizer composition can further comprise one or more C35 hopanoids. In the biofertilizer composition the one or more biofertilizers and the vehicle are formulated for administration to a leguminous plant and/or for administration to a leguminous seed. In some embodiments the vehicle comprises at least one carrier allowing increased stability, viability and/or effectiveness of the nitrogen-fixing bacteria gas exchange of the one or more nitrogen-fixing rhizobia.

According to a fifth aspect, a biofertilizer composition is described. The biofertilizer composition comprises one or more nitrogen-fixing rhizobia capable of producing C35 hopanoids and one or more C35 hopanoids. In some embodiments the composition can be formulated for administration to a leguminous plant, a leguminous seed and/or soil surrounding the leguminous plant or leguminous seed. In some embodiments the composition can be rhizobia naturally capable of producing C35 hopanoids and genetically modified to produce C35 hopanoids.

According to a sixth aspect, a genetically modified nitrogen-fixing rhizobium is described. The genetically modified nitrogen-fixing rhizobium is a rhizobium naturally incapable of producing C35 hopanoids and genetically engineered to produce C35 hopanoids.

According to a seventh aspect, described herein is a leguminous seed coated and/or inoculated with a biofertilizer, and/or biofertilizer composition herein described, optionally in combination with one or more C35 hopanoids. In the leguminous coated and/or inoculated leguminous seed, the nitrogen-fixing rhizobia in the biofertilizer and/or biofertilizer composition can be rhizobia naturally capable of producing C35 hopanoids and/or rhizobia naturally incapable of producing C35 hopanoids and genetically modified to produce C35 hopanoids.
According to an eighth aspect, a method of fertilizing leguminous plants is described. The method comprises applying one or more biofertilizer and/or biofertilizer compositions herein described to a leguminous plant or soil surrounding a leguminous plant for a time and under conditions to allow symbiosis of the nitrogen-fixing rhizobia with the leguminous plant. In some embodiments, the biofertilizer and/or biofertilizer compositions can be applied in combination with one or more C_{35} hopanoids. In these embodiments, applying the biofertilizer and/or biofertilizer compositions and applying one or more C_{35} hopanoids are performed for a time and under conditions allowing interaction of the one or more C_{35} hopanoids with the nitrogen-fixing rhizobia in the administered biofertilizer and/or biofertilizer compositions.

According to a ninth aspect, a method of fertilizing leguminous plants is described. The method comprises coating and/or inoculating one or more seeds of the leguminous plant with one or more biofertilizer and/or biofertilizer compositions herein described. In some embodiments, the method further comprises coating and/or inoculating the one or more seeds of the leguminous plant with one or more C_{35} hopanoids before the coating and/or inoculating the one or more seeds of the leguminous plant with one or more biofertilizer and/or biofertilizer composition.

According to a tenth aspect, a system to fertilize leguminous plants is described. The system comprises one or more biofertilizer and/or biofertilizer compositions herein described and one or more C_{35} hopanoids for simultaneous sequential or combined use in fertilizing a leguminous plant as herein described.

According to an eleventh aspect, a system to fertilize a leguminous plant is described. The system comprises one or more leguminous seeds coated with one or more biofertilizer and/or biofertilizer composition herein described and one or more C_{35} hopanoids for simultaneous sequential or combined use in fertilizing a leguminous plant herein described.

According to a twelfth aspect, a method of storing a biofertilizer herein described is set forth. The method comprises performing for a time and under conditions allowing interaction of the one or more C_{35} hopanoids with the nitrogen-fixing rhizobia in the administered biofertilizer and/or biofertilizer compositions.

According to a thirteenth aspect, a method of storing a biofertilizer for a soil is described. The method comprises providing a biofertilizer herein described, the biofertilizer comprising nitrogen-fixing bacteria that are naturally producing C_{35} hopanoids and/or have been genetically modified to produce C_{35} hopanoids, and storing the biofertilizer formulation at a temperature between 22° C. and 37° C.

According to a fourteenth aspect, a method of storing a biofertilizer for a leguminous plant is described. The method comprises providing a biofertilizer herein described and one or more C_{35} hopanoids for simultaneous sequential or combined use in fertilizing a leguminous plant herein described.

According to a fifteenth aspect, a method of storing a biofertilizer for a leguminous plant or soil is described. The method comprises providing a biofertilizer herein described, applying the biofertilizer and/or biofertilizer composition herein described and one or more C_{35} hopanoids for simultaneous sequential or combined use in fertilizing a leguminous plant or soil as herein described.

The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the present disclosure and, together with the detailed description and the examples, serve to explain the principles and implementations of the disclosure.
FIG. 3 shows in some embodiments the endosymbiotic context of *B. diazoefficiens* within root nodules of *A. afraspera*. *B. diazoefficiens* exists as a bacteroid, a terminally differentiated enlarged, elongated and polyploid state, within infected plant cortical cells. In addition to its own membrane, each bacteroid is surrounded by a peribacteroid plant-derived membrane. The double-layered bacteroid is called a symbiosome. The infected plant cell niche is characterized by low O$_2$, low pH, hyperosmosis and oxidative stress [1, 2].

FIG. 4 shows in some embodiments the MALDI-MS analysis of lipid A from *B. diazoefficiens* strains. Lipid A from WT and ΔhpnP is composed of a mixture of penta-acylated and hexa-acylated species, whereas ΔhpnH lipid A is mainly hexa-acylated. A C$_{35}$ hopanediolic acid is ester-linked to hexa-acylated and hepta-acylated lipid A in WT and ΔhpnP. ΔhpnH does not contain any lipid A-bound hopanoids.

FIG. 5 plots whole cell membrane fluidity measurements by fluorescence polarization, which show that rigidity decreases for all strains as temperature increases and that the ΔhpnH membrane is less rigid than that of WT or ΔhpnP (**p<0.01 by Student’s 2-Tailed t-test). Error bars represent the standard deviation from three biological replicates (22 technical replicates).

FIG. 6 shows in some embodiments the CRYO-transmission electron microscopy (TEM) micrographs which show intact outer and inner membranes in all *B. diazoefficiens* strains. Scale=200 nm.

FIG. 7 shows in some embodiments the growth of *B. diazoefficiens* strains under various stress conditions. Growth of WT (circle), ΔhpnP (square) and ΔhpnH (triangle) was monitored at OD$_{600}$ in Panel A) PSY at 30°C, Panel B) PSY at 37°C, Panel C) microaerobic PSY with 0.5% O$_2$ at 30°C, Panel D) PSY at pH=6 and 50°C. Each curve represents the average of at least three biological replicates, except the microaerobic growth curves for which a representative data set out of four trials is shown. Growth of *B. diazoefficiens* strains under stress as measured in Panel E) stressor gradient plates at 50 mM NaCl, 500 mM inositol, 0.4% bile salts or 1 mM EDTA or by Panel F) disc diffusion assays with 10% SDS, 5.5 M H$_2$O$_2$ and 2 M HCl. Error bars represent standard error (n=9). *p<0.05 and **p<0.01 by Tukey’s HSD test. Panel G) NCR335 sensitivity of *B. diazoefficiens* strains.

FIG. 8 shows in some embodiments that *B. diazoefficiens* ΔhpnH mutant is impaired in symbiosis with soybean at 21 d.p.i. Panel A: Comparison of growth of plants, non-inoculated (NI) or inoculated with WT, ΔhpnH and ΔhpnP. Panel B: Quantification of acetylene reduction activity (ARA) in nickel (Ni) or inoculated with WT, ΔhpnH and ΔhpnP. Panel C: Nodulation efficiency of WT, ΔhpnH and ΔhpnP on plants. Error bars in B, C represent standard error (n=10). Based on Tukey’s HSD test differences between strains were found to be insignificant. p>0.05. Panels D-L: Aspects of nodules elicited by WT (Panels D, G, J), ΔhpnH (Panels E, H, K) and ΔhpnP (Panels F, I, L). (D, E, F) Whole roots, scale=4 mm, (Panels G, H, I) Cross-section of live nodules, scale=1 mm, (Panels J, K, L) Nodule thin sections viewed by brightfield microscopy, scale=1 mm. Panels M-R: Observation of nodules elicited by WT (Panels M, P, R) and ΔhpnH (Panels N, Q, R) and ΔhpnP (Panels O, S, R) strains by confocal microscopy using Syto9 (green, healthy bacteroids), calcifluor (blue, plant cell wall) and propidium iodide (red, infected plant nuclei and bacteroids with compromised membranes). Scale=50 µm (M, N, O) 50 µm (Panels P, Q, R), 200 µm (Panels S, T, U), 0.2 µm (Panels V, W, X).

FIG. 9 illustrates in some embodiments that a *B. diazoefficiens* ΔhpnH mutant is impaired in symbiosis with *A. afraspera* at 21 d.p.i. Panel A) Comparison of growth of plants, non-inoculated (NI) or inoculated with WT, ΔhpnH and ΔhpnP. Panel B) Quantification of acetylene reduction activity (ARA) in plants inoculated with WT, ΔhpnH and ΔhpnP. Error bars represent standard error (n=10). **p<0.01 by Tukey’s HSD test. Panel C) Number of nodules per plant elicited by WT, ΔhpnH and ΔhpnP. Panels D-M) Aspects of the nodules elicited by WT (Panels D, G, J), ΔhpnH (Panels E, H, K) and ΔhpnP (Panels F, I, L, M). (D, E, F) Whole roots, scale=1 mm, (Panels G, H, I) Cross-section of live nodules, scale=500 µm. (Panels J, K, L) Nodule thin sections viewed by brightfield microscopy, scale=500 µm. Panel M) The black arrow shows plant defense reactions (nerotic plant cells), scale=500 µm. Panel N) Aspects of the nodules elicited by ΔhpnH as observed by confocal microscopy using the live-dead kit, scale=200 µm. White arrows show plant defense reactions. Panel O) Aspects of the nodules elicited by ΔhpnH stained with lugol. Scale=500 µm. White arrows show starch granules in dark. Panels P-U) Confocal microscopy observations of nodules elicited by WT (Panels P, Q) ΔhpnH (Panels R, S) and ΔhpnP (Panels T, U) strains and stained using Syto9 (green, healthy bacteroids), calcifluor (blue, plant cell wall) and propidium iodide (red, infected plant nuclei and bacteroids with compromised membranes). Scale=200 µm (P, R, T), 20 µm (Q, S, U), 50 µm (Panels V-Z) TEM of nodules elicited by WT (Panels V, W, X) and ΔhpnH (Panels Y, Z, Z’). Panels V, Y) Black arrows show symbiosomes. Panel Z) Cell envelope of some ΔhpnH bacteroids is not well delineated (bold black arrow) and some deposits of cellular material can be observed in the peribacteroid space (black arrow). Panel Z’) The bold black arrow shows bacteroid wall breakdown. The black arrow shows cellular material of unknown origin. Scale=2 µm (V, Y), 0.5 µm (W, Z), 0.2 µm (X, Z’).

FIG. 10 shows in some embodiments kinetics of nodulation and nitrogen fixation of *A. afraspera* plants inoculated with *B. diazoefficiens*. Panel A: Number of nodules elicited by WT, ΔhpnH and ΔhpnP on plants at 9, 14 and 21 days post inoculation (d.p.i.). Panel B: The acetylene-reducing activity (ARA) in plants inoculated with WT, ΔhpnH and ΔhpnP at 9, 14 and 21 d.p.i. Error bars represent standard error (n=10). Asterisk above the error bars indicate significant differences at *p<0.05 and **p<0.01 (Tukey’s HSD test).

FIG. 11 shows in panel (a), an exemplary structure of the C$_{35}$ hopanoid 2Me-bacteriohopanetetrol (BHT). The gene she generates the pentacyclic core from squalene; the addition of a methyl group at the C$_2$ position is performed by hpnP; and the addition of a ribose-derived hydrocarbon chain at the C$_{33}$ position to form a C$_{33}$ hopanoid is performed by hpnH. Panel b) plots acetylene reduction rates for *A. afraspera* plants at 24 days post-inoculation (dpi) with wild-type (WT), ΔhpnP or ΔhpnH. B. diazoefficiens. Panel (c) shows manual cross-sections of root nodules harvested at 24 dpi for wild type and ΔhpnH mutants stained with Calcofluor white, propidium iodide (PI) or SYTO9. Panel (d) shows normalization of acetylene reduction rates by nodule dry weight for wild type and ΔhpnH mutants. All values shown are average values from 10 plants per condition. Error bars represent standard deviation.

FIG. 12 shows in some embodiments acetylene reduction rates per plant taken every four days after inoculation for WT- and ΔhpnH-inoculated plants (panel a), number of
nODULES PER PLANT, NODULE DRY WEIGHT PER PLANT AND PLANT SHOOT HEIGHTS FOR WT- AND ΔhpnH-INOCULATED PLANTS (PANEL B) AND IMAGES OF 10 WT- AND ΔhpnH-INOCULATED PLANTS AT 20 DPI AND 40 DPI (PANEL C). ALL VALUES SHOWN ARE AVERAGE VALUES PER CONDITION AND ARE POOLED FROM TWO REPLICATES OF FOUR PLANTS EACH. ERROR BARS REPRESENT STANDARD DEVIATION.

FIG. 13 SHOWS IN SOME EMBODIMENTS SAMPLE TRACKING OF MULTIPLE NODES ON A SINGLE WT PLANT (WITH NODE RECIPI) USING DIGITAL MICROSCOPY (PANEL A); SAMPLE NODE GROWTH TIME SERIES FOR WT AND ΔhpnH PLANTS (PANEL B), AND SAME NODE GROWTH PLOT FOR A WT NODE (PANELS C-E). IN PARTICULAR, PANEL C SHOWS THE RAW DATA; PANEL D SHOWS THE RAW DATA FIT TO QUADRATIC, EXPONENTIAL OR SIGMONIDAL CURVES; PANEL E SHOWS ADDITIONAL PARAMETER FITS FOR THE SIGMONIDAL FIT; AND PANEL F SHOWS A SCHEMATIC OVERVIEW OF NODULE GROWTH PARAMETERS.


DETAILED DESCRIPTION

Provided herein are hopanoids, hopanoid-producing bacteria and related biofertilizers, compositions, methods and systems that in several embodiments stimulate plant growth with enhanced tolerance to stresses encountered during the progression of plant-microbe symbioses.

The term “hopanoids” as used herein indicate bacterio-hopanepolys (BHPs), which is a class of pentacenic triterpenoids that are found in a variety of bacteria including Gram-positive and Gram-negative bacteria as cell membrane components.

Hopanoids in the sense of the disclosure include in particular amphiphilic BHP comprising a C₃₀ pentacenic triterpene hydrocarbon skeleton, derived from squalene via the enzyme squalene-hopene cyclase, that can be linked via the A-ring, or by unsaturation and/or attachment to a ribose-derived side chain (C₃₅-hopanoids) (see Examples 3 and 4) [3]. Distribution of hopanoids with different chemical structure among bacteria does not appear to follow a systematic pattern. For example, BHPs methylated at C-2 are known to be produced in abundance by cyanobacteria, but not by other bacteria.

Hopanoids producing bacteria, in the sense of the disclosure, are bacteria having a gene set allowing production of one or more hopanoids in the sense of the disclosure and in particular a set of genes encoding molecules that catalyze the production of hopanoids using squalene as the beginning molecular substrate, including but not limited to the following genes: hpnP, hpnM (also known as shc), hpnG, hpnH, and hpnO.

Hopanoid-producing bacteria comprise both free-living bacteria and symbiotic bacteria. Examples of free-living bacteria producing hopanoids include Rhodopseudomonas palustris, Bacillus spp., Synecococcus spp., and Azotobacter spp. Examples of symbiotic bacteria producing hopanoids include Bradyrhizobium spp., Frankia spp., Anaabaena spp., and Nostoc spp. For example, hopanoids comprise BHPs localized in the cytoplasmic and outer membranes of various bacteria such as Alicyclobacillus acidoaldarius, Zymomonas mobilis, Frankia sp., and Streptomyces coelicolor. In particular, hopanoids have been found in nitrogen-fixing bacteria that form root or stem nodules in symbiosis with various types of plants, where the ability to produce hopanoid biosynthesis is believed enriched in bacteria associated with plants [4]. For example, hopanoids have been found in membranes of plant-symbiotic bacteria of nitrogen-fixing Bradyrhizobium (40% of total lipid extract (TLE)) and Frankia (87%) genera [5, 6]. Studies have shown that elimination of hopanoid biosynthesis in photosynthetic Bradyrhizobium BTAlA impairs its symbiosis with the legume Aeschynomene evenia [7]. Hopanoids have not been found in other plant-associated bacteria, including ~50% of the symbiotic Rhizobiales family. Exemplary hopanoids producing bacteria that are naturally capable of producing hopanoids include strains from Acetobacter, Acidiphilium, Azotobacter vinelandii, Bacillus amylophilicus, Bacillus anhydris, Bacillus cereus, Burkholderia cenocepecia, Bradyrhizobium, Burkholderia, Frankia, Geobacter, Methylobacterium, Pelobacter, Nitrosococcus, Rhodopseudomonas, Rhodospirillum, Syn

In embodiments herein described, biofertilizer to be used to fertilize plants or soil are hopanoids producing bacteria capable of producing C₃₅ hopanoids. The term “C₃₅ hopanoids” in the sense of the disclosure include in particular amphiphilic BHP comprising a C₃₀ pentacenic triterpene
hydrocarbon skeleton, derived from squalene via the enzyme squalene-hopene cyclase, and are linked via a C—C bond to a C₅ sugar moiety derived from ribose.

In particular, in embodiments herein described, C₃₅ hopanoids are compounds of Formula (I):

![Formula (I)](image)

C22, C31, C33 and C34 have independently R or S chirality; R, R₁, R₂ and R₃ are independently selected from H, D, methyl, or ethyl groups;

R₁, R₂, R₃, and R₄ are selected from H, D, methyl, hydroxymethyl, aminomethyl, hydroxyl, or amino groups, wherein at least three of the R₁, R₂, R₃, and R₄ groups each contains hydroxymethyl, aminomethyl, hydroxyl, or amino groups;

R₂ is selected from OH, NH₂, hydroxymethyl, aminomethyl, formula (II)

![Formula (II)](image)

wherein a wavy line on the ring carbon indicates a R or S chirality of the ring carbon,

m₁ and m₂ are independently 0 or 1;

R₁, R₂, R₃, R₄ and R₅ are selected from H, NH₂, hydroxymethyl, or aminomethyl groups wherein R₁, R₂, R₃, R₄ and R₅ contain at least one NH₂ or aminomethyl groups and one of R₂, R₄ and R₅ is hydroxymethyl, or aminomethyl groups.

In some embodiments, Formula (II) can be Formula (IIa) or Formula (IIb):

![Formula (IIa)](image)

![Formula (IIb)](image)

In some embodiments, R₅ is NH₂. In some particular embodiments, the C₃₅ hopanoids are bacteriohopanetetrol (BHT) and aminobacteriohopanetriol shown in FIG. 1A.

In some embodiments, the C₃₅ hopanoids comprise aminobacteriohopanetriol, bacteriohopanetriol, 2-methyl bacteriohopanetriol, aminobacteriohopanetriol, bacteriohopanetetrol, 2Me-aminobacteriohopanetriol, adenosyl-hopane, 2Me-bacteriohopanetetrol.

C₃₅ hopanoids are produced in bacteria by a set of genes comprising at least shc, hpnH and hpnG. The set can also comprise hpnO, hpnP, and also hpnC, hpnD and hpnE, depending on the specific C35 produced as will be understood by a skilled person. For example, hpnH is required to generate adenosyl hopane, which is a C35 hopanoid, however, hpnG and hpnO are needed to make aminobacteriohopanetetrol and bacteriohopanetriol (Welander 2012 [12]). shc, hpnH and hpnG, hpnO, hpnO, hpnC, hpnD and hpnE are conserved among various C35 producing bacteria and encodes enzymes forming a hopanoid biosynthetic pathway such as squalene-hopene cyclase (sch), B12 binding radical SAM (hpnH), nucleosidase (hpnG), ornithine-oxo-acid-transaminase (hpnO), B12 binding radical SAM (hpnP), squalene synthase (hpnC), squalene synthase (hpnD), and squalene dependent FAD-dependent desaturase (hpnE) as will be understood by a skilled person. In particular among different C35 producing bacteria strains, each of the proteins encoded by shc, hpnH and hpnG, hpnO, hpnO, hpnC, hpnD and hpnE genes shows a same enzymatic activity in the different strains even if the sequences can differ from strain to strain at a polynucleotide and/or at a protein level. In particular, proteins encoded by each of shc, hpnH and hpnG, hpnO, hpnO, hpnC, hpnD or hpnE shows an amino acid sequence identity >55% at a protein level while maintaining the respective enzymatic activity indicated above (see e.g. hpnP as indicated by Ricci et al. [14]).

The term “gene” as used herein indicates a polynucleotide encoding for a protein that in some instances can take the form of a unit of genomic DNA within a bacteria, plant or other organisms.

The term “polynucleotide” as used herein indicates an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof. The term “nucleotide” refers to any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or pyrimidine base and to a phosphate group and that are the basic structural units of nucleic acids. The term “nucleoside” refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. The term “nucleotide analog” or “nucleoside analog” refers respectively to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or a with a different functional group. Accord
The term “protein” as used herein indicates a polypeptide with a particular secondary and tertiary structure that can interact with another molecule and in particular, with other biomolecules including other proteins, DNA, RNA, lipids, metabolites, hormones, chemokines, and/or small molecules. The term “polypeptide” as used herein indicates an organic linear, circular, or branched polymer composed of two or more amino acid monomers and/or fragments thereof. A polypeptide of three or more amino acids is also called a protein oligomer, peptide, or oligopeptide. In particular, the terms “peptide” and “oligopeptide” usually indicate a polypeptide with less than 100 amino acid monomers. A protein “sequence” indicates the order of the amino acids that form the primary structure.

As used herein the term “amino acid”, “amino acid monomer”, or “amino acid residue” refers to organic compounds composed of amine and carboxylic acid functional groups, along with a side-chain specific to each amino acid. In particular, alpha- or ω-amino acid refers to organic compounds composed of amine (–NH2) and carboxylic acid (–COOH), and a side-chain specific to each amino acid connected to an alpha carbon. Different amino acids have different side chains and have distinctive characteristics, such as charge, polarity, aromaticity, reduction potential, hydrophobicity, and pKa. Amino acids can be covalently linked to form a polymer through peptide bonds by reactions between the amine group of a first amino acid and the carboxylic acid group of a second amino acid. Amino acid in the sense of the disclosure refers to any of the twenty naturally occurring amino acids, non-natural amino acids, and includes both D and L optical isomers. The term “percent identity” refers to a quantitative measurement of the similarity between sequences of a polypeptide or a polynucleotide and in particular indicates the amount of characters which match exactly between two different sequences. Widely used similarity searching programs, like BLAST, PSI-BLAST [15], SSEARCH [16,17], FASTA [18] and the HMMER3 [19] programs produce accurate statistical estimates, ensuring protein sequences that share significant similarity also have similar structures.

In embodiments herein described, hopanoids producing bacteria capable of producing C35 hopanoids, and particularly C35 hopanoids of Formula (I), are nitrogen-fixing bacteria and, in particular, symbiotic nitrogen fixing bacteria, which can be used to fertilize a plant and/or a soil, as will be understood by a skilled person upon reading of the present disclosure.

The term “nitrogen-fixing bacteria” refers to microorganisms capable of transforming atmospheric nitrogen to fixed nitrogen in inorganic compounds usable by plants. Nitrogen-fixing bacteria are also called diazotrophs; some diazotrophs are capable of performing nitrogen fixation naturally in a free-living state, while others can only fix nitrogen within plant hosts. Examples of diazotrophs include non-symbiotic bacteria, such as the plant-associated soil bacterium Azorhizobium, as well as symbiotic bacteria including rhizobia (in particular Bradyrhizobium spp., Rhizobiaceae, Phyllobacteria, Sinorhizobium (Ensifer), Mesorhizobium and Azorhizobium), cyanobacteria (Anabaena spp., Nostoc spp.), Frankia spp., and others identifiable by a person of ordinary skill in the art.

The wording “symbiotic bacteria” as used herein indicates bacteria that provide fixed nitrogen to plants via direct plant-microbe association in exchange for nutrients (generally carbon sources) and include the model bacteria Sinorhizobium meliloti, Rhizobium leguminosarum, and Bradyrhizobium diazoefficiens. During symbiotic nitrogen fixation, nitrogen-fixing bacteria establish a symbiotic relationship with plants in which the plant provides the nitrogen-fixing bacteria with carbohydrates as an energy source and the nitrogen-fixing bacteria provides the plant with nitrogen in the form of ammonium. Examples of plant-microbe symbioses include rhizobia associated with leguminous plants and trees of the Acacia and Parasponia families, Frankia associated with certain dicotyledonous species (actinorhizal plants), certain Azospirillum species, associated with cereal grasses, Nostoc or Anabaena associated with ferns, palms, lichens and hornwort, and many other plant-microbe symbiotic systems identifiable by a person of ordinary skill in the art.

In particular, in embodiments herein described hopanoids producing nitrogen-fixing bacteria capable of producing C35 hopanoids are rhizobia that can be used alone or in combination with C35 hopanoids to stimulate growth of leguminous plants and/or fertilize a soil.

The term “leguminous plants” or “legumes” indicates plants in the family of Fabaceae (or Leguminosae) with taxa such as kudzu, clovers, soybeans, alfalfa, lupines, peanuts and rooibos. Examples of leguminous plants include including Vicia faba, Arachis hypogaea, Cicerc arietum, Dolichos lablab, Lupinus albus, Pisum arvense, Glycine max, Cajanus cajan, Lens esculenta, Vigna radiata, Cynopsis tetragonoloba, Vigna aconitifolia, Vigna hissuta, Trigonella foenum-graecum, Onobrychis sativa, Coronilla cretica, Ornithopus sativus, Desmodium intortum, Indigofera hissuta, Medicago sativa, Trifolium incarnatum, Lotus pedunculatus, Trifolium agrarium and Lotus bainesii and others identifiable to a person of ordinary skill in the relevant art. Plants in the legume family can form symbioses with nitrogen-fixing soil bacteria to provide a sustainable nitrogen source to improve fertility in agricultural settings. Most legumes interact optimally with nitrogen-fixing bacteria of a single genus, although the specificities of legumes for bacterial partners are largely uncharacterized. The nitrogen-fixing rhizobia that form symbioses with a given legume are referred to as legume symbionts. Examples of legume symbionts include Bradyrhizobium, Rhizobiaceae, Phyllobacteria, Sinorhizobicia (Ensifer), Mesorhizobia and Azorhizobia.

The term “rhizobia” as used herein indicates a family of Gram-negative soil bacteria that fix nitrogen in association with plants. Rhizobia form an endosymbiotic nitrogen fixing association with roots of legumes and some trees including Acacia and Parasponia. In particular, rhizobia colonize plant cells within root nodules where they convert atmospheric nitrogen into ammonia and then provide organic nitrogenous compounds such as glutamine or ureides to the plant. The plant in turn provides the bacteria with organic compounds made by photosynthesis. Most of the rhizobia species are in the Rhizobiaceae family in the alpha-proteobacteria and are in Rhizobium, Mesorhizobium, Ensifer, or Bradyrhizobium genera. There are also some other rhizobial species, presumably arisen through lateral gene transfer of symbiotic genes. In general, rhizobia consists of about 98 species in 13 genera, including Rhizobium, Mesorhizobium, Ensifer, Bradyrhizobium, Burkholderia, Phyllobacteria, Microvirga, Ochrobactrum, Methylbacterium, Cupriavi-
Detailed taxonomic information about rhizobia are identifiable by a person of ordinary skill in the Azorhizobium, art.

In particular, in embodiments here described rhizobia used in biofertilizers, and related seeds compositions, methods and systems are naturally capable of producing C_{35} hopanoids. Exemplary legume symbions naturally capable of producing C_{35} hopanoids include Bradyrhizobium BTAI1, Bradyrhizobium japonicum, Bradyrhizobium diazoefficiens, Bradyrhizobium ORS278 and Methyllobacterium nodulans.

Exemplary legume symbions naturally incapable of producing C_{35} hopanoids but capable of being engineered to produce C_{35} hopanoids include Rhizobium etli, Rhizobium leguminosarum, Mesorhizobium loti, Sinorhizobium meliloti, Azorhizobium caulinodans, and Ochrobactrum anthropi. The symbiotic relationship between these and other exemplary legume symbions and their host plants, is shown in Table 1. Table 1 also includes an indication whether the rhizobia strain contains the hpnH gene that is required for the C_{35} biosynthesis.

<table>
<thead>
<tr>
<th>Rhizobia</th>
<th>Native host(s)</th>
<th>hpnH present</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Para)Burkholderia carbonensis</td>
<td>Mimosa spp.</td>
<td>YES</td>
</tr>
<tr>
<td>(Para)Burkholderia caribensis</td>
<td>Mimosa spp.</td>
<td>YES</td>
</tr>
<tr>
<td>(Para)Burkholderia minosarum</td>
<td>Mimosa spp.</td>
<td>YES</td>
</tr>
<tr>
<td>(Para)Burkholderia nodosa</td>
<td>Aspalathus carnosus</td>
<td>NO</td>
</tr>
<tr>
<td>(Para)Burkholderia phynumun</td>
<td>Mimosa spp.</td>
<td>NO</td>
</tr>
<tr>
<td>Azorhizobium caulinodans</td>
<td>Sesbania spp., e.g. Sesbania rostrata</td>
<td>NO</td>
</tr>
<tr>
<td>Azorhizobium diazoefficiens</td>
<td>Sesbania virgata</td>
<td>NO</td>
</tr>
<tr>
<td>Bradyrhizobium BTAI1</td>
<td>Aeschynomene indica, Aeschynomene evenia</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium canariense</td>
<td>Lupinus spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium cyasii</td>
<td>Cytisus villosus</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Aeschynomene indica</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Glycine soja</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Entada koshunensis</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium japonicum</td>
<td>Glycine Max</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Pachyrhizus erosus</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Glycine spp.</td>
<td>YES</td>
</tr>
<tr>
<td>liaotengense</td>
<td>Vigna angularis</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Controllobium paraense</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium sp. ORS278</td>
<td>Aeschynomene eventa, Aeschynomene indica</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium sp. ORS285</td>
<td>Aeschynomene afraperda</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Phascolus bunanus L.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Retama spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Arachis hypogaea L.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>subterraneum</td>
<td>Lespedeza spp.</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium</td>
<td>Glycine max</td>
<td>YES</td>
</tr>
<tr>
<td>Bradyrhizobium diazoefficiens</td>
<td>Mimosa spp.</td>
<td>YES</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>Mimosa spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Burkholderia salvia</td>
<td>Mimosa spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Burkholderia tuberum</td>
<td>Aspalathus carnosus</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Cupriavidus taiwanensis</td>
<td>Mimosa spp.</td>
<td>YES</td>
</tr>
<tr>
<td>Devisia neptuniae</td>
<td>Neptunia nasans</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium abyssinicae</td>
<td>Acacia abyssinica</td>
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</tr>
<tr>
<td>Mesorhizobium allheiae</td>
<td>Althea kalkora</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium alhagi</td>
<td>Athygi sparsifolia</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium amorphae</td>
<td>Amorpha fruticosa</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium ausareticum</td>
<td>Biserrula peletins L.</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium camelhorni</td>
<td>Athygi sparsifolia</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium caraganae</td>
<td>Carex spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium characeae</td>
<td>Prosopis alba</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium ciceri</td>
<td>Cicer arriettinum L.</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium erithani</td>
<td>Lotus spp.</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium golitense</td>
<td>Glycyrrhiza uralensis, Lotus corniculatus, Onyosperis glabra and Robinia pseudoacacia</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium hawassense</td>
<td>Acacia spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium huakani</td>
<td>Thermopos lupinoides</td>
<td>NO</td>
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<tr>
<td>Mesorhizobium javensis</td>
<td>Lotus spp.</td>
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</tr>
<tr>
<td>Mesorhizobium loti</td>
<td>Lotus spp., e.g. Lotus japonicus</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium mediterraneum</td>
<td>Cicer arriettinum L.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium metalidurans</td>
<td>Anthyllis vulneraria</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium medusense</td>
<td>Cicer arriettinum L.</td>
<td>UNKNOWN</td>
</tr>
</tbody>
</table>
### TABLE 1-continued

Symbiotic relationship between exemplary legume symbionts and their host plants and whether the 
hpnH gene is present in the rhizobia strain

<table>
<thead>
<tr>
<th>Rhizobia</th>
<th>Native host(s)</th>
<th>hpnH present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesorhizobium opportunorum</td>
<td>Biserrula pelecinus L.</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium plurifarium</td>
<td>Acacia spp.</td>
<td>NO</td>
</tr>
<tr>
<td>Mesorhizobium qinghensei</td>
<td>Astragalus siniticus</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium robiniae</td>
<td>Robinia pseudoacacia</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium sangoi</td>
<td>Astragalus spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium septentrionale</td>
<td>Astragalus adsurgens</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium shangriense</td>
<td>Caragana spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium shonense</td>
<td>Acacia spp.</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium shamunense</td>
<td>Caragana spp.</td>
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</tr>
<tr>
<td>Mesorhizobium tamadaysense</td>
<td>Anagyris latifolia and Lotus berthelotii</td>
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</tr>
<tr>
<td>Mesorhizobium tarimensense</td>
<td>Glycyrrhiza uralensis, Lotus corniculatus, Onyropis glabra and Robinia pseudoacacia</td>
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</tr>
<tr>
<td>Mesorhizobium temperatum</td>
<td>Astragalus adsurgens</td>
<td>UNKNOWN</td>
</tr>
<tr>
<td>Mesorhizobium</td>
<td>Vicia spp., Trifolium spp.</td>
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</tr>
<tr>
<td>Methylbacterium nodulanus</td>
<td>Crotalaria spp.</td>
<td>YES</td>
</tr>
<tr>
<td>Microvirga lotononidis</td>
<td>Lanzia angolensis</td>
<td>NO</td>
</tr>
<tr>
<td>Microvirga lupini</td>
<td>Lupinus texensis</td>
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</tr>
<tr>
<td>Microvirga zambicensis</td>
<td>Lanzia angolensis</td>
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</tr>
<tr>
<td>Ochrobacterium ajrophilum</td>
<td>Cicer arietinum</td>
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</tr>
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<td>Ochrobacterium cicera</td>
<td>Cicer arietinum</td>
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<td>Ochrobacterium cysti</td>
<td>Cyttisus scorpionia</td>
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<td>Ochrobacterium lupini</td>
<td>Lupinus albus</td>
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<td>Phyllobacterium legumininum</td>
<td>Astragalus algerianus, Astragalus algerianus</td>
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<td>Phyllobacterium trifolii</td>
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<td>Medicago raphanica</td>
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<td>Phaseolus vulgaris</td>
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<td>Rhizobium calliandrae</td>
<td>Callandra grandiflora</td>
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<td>Rhizobium canense</td>
<td>Kammerovia stiptisulcea</td>
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<td>Rhizobium cellulosolyticum</td>
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<td>Rhizobium elis</td>
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<td>Vicia faba</td>
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<tr>
<td>Rhizobium frexeci</td>
<td>Phaseolus vulgaris</td>
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<td>Rhizobium galegae</td>
<td>Galega orientalis</td>
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<td>Rhizobium gallicum</td>
<td>Phaseolus vulgaris</td>
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<td>Rhizobium giardinii</td>
<td>Phaseolus vulgaris</td>
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<td>Rhizobium graminii</td>
<td>Dicruchus leporin, Leucineea leucoscelera and Litoria</td>
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<td>Rhizobium hainanense</td>
<td>Hainan province legumes</td>
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<td>Rhizobium halophytocola</td>
<td>Rosa rugosa</td>
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<td>Rhizobium herbae</td>
<td>Various wild legumes in China</td>
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<td>Rhizobium hussatense</td>
<td>Scabiosa herbacea</td>
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<td>Rhizobium indigoforae</td>
<td>Indigofora spp.</td>
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<td>Rhizobium jaggaria</td>
<td>Callandra grandiflora</td>
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<td>Rhizobium lagovernae</td>
<td>Vicia faba</td>
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<td>Rhizobium leguminosarum</td>
<td>Phaseolus vulgaris, Trifolium spp., Pismus sativum</td>
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<td>Rhizobium leucaenae</td>
<td>Leucaena leucodra callarum, Leucaena esculenta, Common beans (Phaseolus vulgaris) and Gliricidia sepium</td>
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<td>Callandra grandiflora</td>
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<td>Phaseolus vulgaris, strato, cowpea and Minosa pudica</td>
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<td>Rhizobium mesostichum</td>
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<td>Rhizobium multisiphonum</td>
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<td>Phaseolus vulgaris and Glycine max</td>
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<td>Rhizobium parasacera</td>
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<td>Rhizobium phaseolli</td>
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<td>Rhizobium sphaerocephalae</td>
<td>Sphaerocephalae saliola</td>
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<tr>
<td>Rhizobium succulentes</td>
<td>Hedyccyrus coronaricti L.</td>
<td>NO</td>
</tr>
</tbody>
</table>
**TABLE 1 -continued**

<table>
<thead>
<tr>
<th>Rhizobia</th>
<th>Native host(s)</th>
<th>hpnH present</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium taibaihansense</em></td>
<td><em>Kummerowia striata</em></td>
<td>UNKNOWN</td>
</tr>
<tr>
<td><em>Rhizobium alfaticum</em></td>
<td><em>Trigonella archicricis-nicola, Medicago lupulina, Medicago sativa, Melilotus officinalis, Phascolus vulgaris and Trigonella foemina-graecum</em></td>
<td>UNKNOWN</td>
</tr>
<tr>
<td><em>Rhizobium tropici</em></td>
<td><em>Phaseolus vulgaris, L. beans and Leucaena sp. trees</em></td>
<td>NO</td>
</tr>
<tr>
<td><em>Rhizobium tibeticum</em></td>
<td><em>Onyctropis glabra</em></td>
<td>UNKNOWN</td>
</tr>
<tr>
<td><em>Rhizobium vallis</em></td>
<td><em>Nepalica retans</em></td>
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<tr>
<td><em>Rhizobium vignae</em></td>
<td><em>Phaseolus vulgaris, Mimosa pudica and Indigofera spicata</em></td>
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<tr>
<td><em>Rhizobium yanglingense</em></td>
<td></td>
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</tr>
<tr>
<td><em>Sinorhizobium/Ensifer abacens</em></td>
<td></td>
<td>NO</td>
</tr>
<tr>
<td><em>Sinorhizobium/Ensifer americana</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer arctis</em></td>
<td><em>Acacia senegal and Prosopis chilensis</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer freddii</em></td>
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<td>NO</td>
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<tr>
<td><em>Sinorhizobium/Ensifer garramancus</em></td>
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<td>NO</td>
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<tr>
<td><em>Sinorhizobium/Ensifer indena</em></td>
<td></td>
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<tr>
<td><em>Sinorhizobium/Ensifer hoedentia</em></td>
<td><em>Acacia senegal and Prosopis chilensis</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer humeronioae</em></td>
<td><em>Kummerowia stipulacea</em></td>
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<td><em>Sinorhizobium/Ensifer mediace</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer mexicana</em></td>
<td><em>Acacia angustissima</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer mexicana</em></td>
<td></td>
<td>NO</td>
</tr>
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<td><em>Sinorhizobium/Ensifer monile</em></td>
<td><em>Leucaena leucocephala</em></td>
<td>UNKNOWN</td>
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<tr>
<td><em>Sinorhizobium/Ensifer numidicas</em></td>
<td><em>Argyrolobium uniflorum, Lotus creticus</em></td>
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</tr>
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<td><em>Sinorhizobium/Ensifer salvi</em></td>
<td><em>Sebania cannabina</em></td>
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<tr>
<td><em>Sinorhizobium/Ensifer sesbaniae</em></td>
<td><em>Sebania spp.</em></td>
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</tr>
<tr>
<td><em>Sinorhizobium/Ensifer sojae</em></td>
<td><em>Acacia lasto</em></td>
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| **In some embodiments, the nitrogen-fixing rhizobia naturally capable of producing C35 hopanoids can comprise Bradyrhizobia. Bradyrhizobia are Gram-negative bacilli (rod shaped) with a single subpolar or polar flagellum. Bradyrhizobia are a common soil dwelling microorganism that can form symbiotic relationships with leguminous plant species. Many members of this genus have the ability to fix atmospheric nitrogen by forming either specific or general symbioses. This means that one species of Bradyrhizobium can only be able to nodulate one legume species, whereas other Bradyrhizobium species can be able to nodulate several legume species.**

**Exemplary Bradyrhizobia naturally capable of producing C35 hopanoids and that are suitable to be used in biofertilizer, compositions, seeds, methods and systems herein described include Bradyrhizobium diazoefficiens, Bradyrhizobium elkanii, Bradyrhizobium japonicum, Bradyrhizobium jicamae, Bradyrhizobium labilabi, Bradyrhizobium liaoningense, Bradyrhizobium manansense, Bradyrhizobium neotropicale, Bradyrhizobium oligotrophicum, Bradyrhizobium pachyrhizi, Bradyrhizobium pasiliieri, Bradyrhizobium retanae, Bradyrhizobium stylosanthis, Bradyrhizobium tropiciagr. Bradyrhizobium valentinum, Bradyrhizobium viridifluriti, Bradyrhizobium yamnagense, Bradyrhizobium sp. A1a-2, Bradyrhizobium sp. ARR65, Bradyrhizobium sp. A11, Bradyrhizobium sp. BR 10245, Bradyrhizobium sp. BR 10303, Bradyrhizobium sp. BTAa1, Bradyrhizobium sp. CCBau 15544, Bradyrhizobium sp. CCBau 15635, Bradyrhizobium sp. CCBau 43298, Bradyrhizobium sp. CCGE-LA001, Bradyrhizobium sp. CCH5-F6, Bradyrhizobium sp. Cpp5.3, Bradyrhizobium sp. DFBC1-1, Bradyrhizobium sp. DOA1, Bradyrhizobium sp. DOA9, Bradyrhizobium sp. Ec3.3, Bradyrhizobium sp. err11, Bradyrhizobium sp. G22, Bradyrhizobium sp. Leaf396, Bradyrhizobium sp. LMTR3, Bradyrhizobium sp. LTSP849, Bradyrhizobium sp. LTSP857, Bradyrhizobium sp. LTSP885, Bradyrhizobium sp. LTSP899, Bradyrhizobium sp. ORS 278, Bradyrhizobium sp. ORS 285, Bradyrhizobium sp. ORS 375, Bradyrhizobium sp. S23321, Bradyrhizobium sp. STM 3809, Bradyrhizobium sp. STM 3843, Bradyrhizobium sp. th.b2, Bradyrhizobium sp. Tv2a-2, Bradyrhizobium sp. URHA0002, Bradyrhizobium sp. URHA0013, Bradyrhizobium sp. URHD0069, Bradyrhizobium sp. WSM1253, Bradyrhizobium sp. WSM1417, Bradyrhizobium sp. WSM1743,
Bradyrhizobium sp. WSM2254, Bradyrhizobium sp. WSM2793, Bradyrhizobium sp. WSM3098, Bradyrhizobium sp. WSM4349, Bradyrhizobium sp. WSM471, and Bradyrhizobium sp. YR681.

In particular, in some embodiments Bradyrhizobia that are naturally capable of producing $C_{35}$ hopanoids that can be used in biofertilizer and related seeds compositions, methods and systems can include Bradyrhizobium BTAC1, Bradyrhizobium diazoefficiens USDA 110, Bradyrhizobium japonicum USDA 6, Bradyrhizobium sp. ORS 278, and Bradyrhizobium diazoefficiens.

In some of those embodiments, the nitrogen-fixing Bradyrhizobium naturally capable of producing hopanoids that can be used in biofertilizer and related seeds compositions, methods and systems is Bradyrhizobium diazoefficiens. The Bradyrhizobium diazoefficiens is a member of the Bradyrhizobium genus, having the ability to form root nodules on leguminous plants.

In particular, in embodiments herein described B. diazoefficiens can exhibit two different life-styles, free-living in soil or symbiotic within legume root nodule cells [2, 20]. In addition to its native soybean host, B. diazoefficiens can engage in nitrogen-fixing symbioses with the stems and roots of the tropical legume Aeschynomene afraspera [21]. In both of the these hosts, development of the symbiosis progresses through a series of defined stages: (i) colonization and invasion of host root tissue; (ii) internalization of bacteria by plant cells to form an organelle-like structure called the symbiosome, comprising endosymbiotic bacterial cells termed “bacteroids” that are surrounded by a plant-derived “peribacteroid” membrane (FIG. 3); and (iii) initiation of nitrogen fixation by bacteroids, during which there is a high rate of nutrient exchange across the symbiosome membranes between plant-supplied carbon sources and fixed atmospheric nitrogen produced by bacterial nitrogenase [2, 22].

In some embodiments, nitrogen fixing $C_{35}$ producing rhizobia to be used in biofertilizer, compositions, seeds methods and systems herein described are rhizobia naturally incapable of producing $C_{35}$ hopanoids (herein also $C_{35}$ hopanoid-deficient rhizobia), and genetically engineered to include genes for production of $C_{35}$ hopanoids thus providing genetically engineered rhizobia capable of producing $C_{35}$ hopanoids herein also indicated as genetically engineered $C_{35}$ rhizobia.

In some embodiments, the rhizobia naturally incapable of producing $C_{35}$ is a bacteria closely genetically related to (i.e. within a same taxonomic order of) hopanoids-producing legume symbionts capable of producing $C_{35}$ hopanoids that can be used in biofertilizers, compositions, methods and systems herein described. For example, a rhizobium naturally incapable of producing $C_{35}$ hopanoids that can be genetically modified in the sense of the disclosure comprise Sinorhizobium meliloti, a symbiont of alfalfa (Medicago spp.), which is closely genetically related to Bradyrhizobium and to all other members of the Rhizobiales order of the alpha proteobacteria having nitrogen-fixing capability in symbioses with plants.

In embodiments herein described, nitrogen-fixing rhizobia capable of producing $C_{35}$ either naturally or following genetic modification, can be used as biofertilizer for legumes and/or soil. The term “biofertilizer” as used herein refers to a substance containing living microorganisms, which, when applied to seeds, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and/or promotes growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers in the sense of the disclosure adds nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances.

Biofertilizers herein described comprise and in particular essentially consist of nitrogen fixing rhizobia capable of producing $C_{35}$ hopanoids. In particular, in biofertilizers essentially consisting of nitrogen fixing rhizobia capable of producing $C_{35}$ hopanoids herein described, at least 70%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, and more preferably at least 95%, of the nutrient adding activity of the biofertilizer is performed by nitrogen fixing rhizobia capable of producing $C_{35}$ hopanoids. The nutrient adding activity of a bacteria and/or the biofertilizer can be quantitatively detected by detecting with techniques identifiable by a skilled person the nitrogen fixed by the plant or soil, the nitrogen solubilized in the plant or soil and/or a difference in plant growth, following addition of the biofertilizer and/or of each rhizobia strains. In general, nitrogen detection can be performed by quantitatively detecting the percentage of the nitrogen dry weight by spectrometry on a dry sample of soil and/or the plants. Additional techniques comprise use of biosensors that use a fluorescent label or other label that can be incubated with a dried sample to report on the amount of glutamine (the main form of fixed N$_2$) or other forms of fixed N$_2$ as will be understood by a skilled person.

The terms “detect” or “detection” as used herein indicates the determination of the existence, presence or fact of a compound, bacteria and/or related activity in a limited portion of space, including but not limited to a sample, a reaction mixture, and a substrate. The “detect” or “detection” as used herein can comprise determination of chemical and/or biological properties of a bacteria, plant, soil and or related compositions, including but not limited to ability to fixing nitrogen, solubilize nitrogen promote growth and additional properties identifiable by a skilled person upon reading of the present disclosure. The detection can be quantitative or qualitative. A detection is “quantitative” when it refers, relates to, or involves the measurement of quantity or amount of the compound, bacteria and/or related activity (also referred as quantitation), which includes but is not limited to any analysis designed to determine the amounts or proportions of the compound, bacteria and/or related activity. A detection is “qualitative” when it refers, relates to, or involves identification of a quality or kind of the target or s compound, bacteria and/or related activity signal in terms of relative abundance to another compound, bacteria and/or related activity, which is not quantified.

The terms “label” and “labeled molecule” as used herein refer to a molecule capable of detection, including but not limited to radioactive isotopes, fluorophores, chemiluminescent dyes, chromophores, enzymes, enzymes substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, nanoparticles, metal sols, ligands (such as biotin, avidin, streptavidin or hapten) and the like. The term “fluorophore” refers to a substance or a portion thereof which is capable of exhibiting fluorescence in a detectable image. As a consequence, the wording “labeling signal” or signal as used herein indicates the signal emitted from the label that allows detection of the label, including but not limited to radioactive activity, fluorescence, chemiluminescence, production of a compound in outcome of an enzymatic reaction and the like.

Biofertilizers herein described a biofertilizer for a leguminous plant can be produced by providing a nitrogen fixing rhizobium in a form suitable for administration to a leguminous plant or seed or soil surrounding a leguminous
plant or seed. In particular, the nitrogen-fixing rhizobium can be provided in a biofertilizer in a culture in a viable form and preferably in a culture with a percentage of live cells in a whole population that can be at least 5%, preferably at least 25% and more preferably at least 50%.

A skilled person will be able to identify different type of rhizobia and related application including specific condition for related viability. For example, B. diazoefficiens differs phylogenetically from Bradyrhizobium BTAl as for example. Scl from these two strains falls in distinct phylogenetic clades [7]. Unlike Bradyrhizobium BTAl, B. diazoefficiens is unable to photosynthesize [23, 24]. Moreover, B. diazoefficiens infects plants via a Nod-factor dependent pathway, whereas Bradyrhizobium BTAl uses alternate symbiotic strategies [25].

In some embodiments, the nitrogen fixing rhizobia can be provided in the biofertilizer in an isolated and/or purified form wherein the bacteria are provided without a detectable presence of other microorganism. A bacterial strain in isolated form can be obtained by obtaining a clonal bacterial preparation of the bacteria, for example by inoculating a bacterial sample into a culture plate, and picking a single bacterial colony grown on the plate as will be understood by a skilled person.

In some embodiments, the nitrogen fixing rhizobia can be provided in the biofertilizer in an isolated and/or purified form wherein the bacteria are provided without a detectable presence of other microorganism. A bacterial strain in isolated form can be obtained by obtaining a clonal bacterial preparation of the bacteria, for example by inoculating a bacterial sample into a culture plate, and picking a single bacterial colony grown on the plate as will be understood by a skilled person. A purified bacterial strain can be obtained for example by separating bacteria from a growth medium, with methods identifiable by a skilled person such as inoculating a bacterial culture broth with an isolated bacterial colony, growing the culture and subsequently separating the bacteria from the medium (e.g. by centrifugation and discarding the supernatant medium, while retaining the pelleted bacteria). Additional techniques to isolate or purify bacteria are identifiable by a skilled person upon reading of the present disclosure.

In some embodiments, a biofertilizer for leguminous plant can be provided from one or more candidate nitrogen fixing rhizobium strains, by a method comprising: detecting among the one or more candidate nitrogen fixing rhizobium strains, at least one rhizobia strain capable of producing C_{35} hopanoids. The method further comprises providing the at least one rhizobia strain capable of producing C_{35} hopanoids in a form suitable for use or treatment of one or more nitrogen fixing rhizobium strains, followed by isolation using methods described below.

In particular, detecting among the one or more candidate nitrogen fixing rhizobium strains, at least one rhizobia strain capable of producing C_{35} hopanoids can be performed by detecting C_{35} hopanoids production by the one or more candidate rhizobia strains. For example, C_{35} hopanoids analysis can be performed by routine extraction and analysis using various high temperature GC-MS or ultra-performance LC-MS as well as other approaches identifiable to a skilled person in the art. Detailed protocol of hopanoid analysis can be found for example in related publications such as Welander et al., 2009 [1] or Neubauer et al., 2015 [26] as well as in Example 6 of the present disclosure.

In some embodiments detecting among the one or more candidate nitrogen fixing rhizobium strains, detecting at least one rhizobia strain capable of producing C_{35} hopanoids can be performed by detecting genes for the synthesis of C_{35} hopanoids in the one or more candidate rhizobia strain.

In some embodiments, detecting genes for synthesis of C_{35} hopanoids in the one or more candidate rhizobia strains can be performed by detecting sequences of one or more of the shc, hpnH and hpnO, as well as hpnP, and also hpnC, hpnD and hpnE, in the genome, transcriptome, or proteome of the one or more candidate strains. Exemplary techniques that can be used to detecting sequences of one more genes (e.g. where the genome is known), comprises computer-based tools for comparing gene sequences, transcript sequences, or protein sequences, such as those using the Basic Local Alignment Search Tool (BLAST) or any other similar methods known to those of ordinary skill in the art.

In some embodiments, detecting genes for synthesis of C_{35} hopanoids in the one or more candidate rhizobia strains can be performed by detecting the genes and/or related transcript in the one or more candidate rhizobia. Exemplary techniques comprise wet bench approaches such as DNA sequencing, PCR, Southern blotting, DNA microarrays, or other methods of hybridization of DNA or RNA probes to DNA, wherein probes are attached to a label capable of emitting a signal such as radiolabeling, fluorescence, luminescence, mass spectroscopy or colorimetric methods. Exemplary probes that can be used comprise primers from known shc, hpnH and hpnO, as well as hpnP, and also hpnC, hpnD and/or hpnE, and/or related transcript as will be understood by a skilled person.

In some embodiments, detecting genes for synthesis of C_{35} hopanoids in the one or more candidate rhizobia strains can be performed by detecting transcripts of shc, hpnH and hpnO, as well as hpnP, and/or also hpnC, hpnD and/or hpnE. Exemplary techniques comprise RNA sequencing, PCR, quantitative PCR, Northern blotting, in situ hybridization, RNA microarrays, or other methods of hybridization of DNA or RNA probes to RNA.

In some embodiments, detecting genes for synthesis of C_{35} hopanoids in the one or more candidate rhizobia strains can be performed by detecting proteins encoded by shc, hpnH and hpnO, as well as hpnP, and/or also hpnC, hpnD and/or hpnE detecting the proteins. Exemplary techniques comprise proteomics, antibody-based methods including immunohistochemistry, immunofluorescence, western blotting, or any other method of protein detection.

In embodiments herein described, the conditions and parameters to use probes/primers to detect shc, hpnH and hpnO, as well as hpnP, and/or also hpnC, hpnD and/or hpnE, can be varied to permit lower or higher threshold or stringency of detection, to ensure hybridization within the least 55% sequence identity at gene level in view of the specific primers/probes selected. For example, use of oligonucleotides comprising one or more degenerated nucleotide bases or using an antibody that binds to more highly conserved protein regions, can require modification of the detection conditions as will be understood by a skilled person.

In some embodiments, detecting genes for synthesis of C_{35} hopanoids in the one or more candidate rhizobia strains can be performed by detecting C_{35} hopanoids in lipid fractions or other cellular fractions isolated from cells using methods mass spectrometry.

In an exemplary embodiment, the detection can be done, for example, by isolating genomic DNA from a candidate and performing PCR using primer sequences designed to...
amplify hpnH genes from known C35 hopanoid-producing rhizobia, including the primers listed in Table 4. Alternatively, RNA samples can be isolated from the candidate(s) and these transcripts can be sequenced, and expression of the hpnH gene can be detected by identification of this gene using homology-based computational identification (e.g., BLAST).

In some embodiments, providing the at least one rhizobia strain capable of producing C35 hopanoids in a form suitable for administration to a leguminous plant or seed or soil surrounding a leguminous plant or seed, can be performed by providing the at least one rhizobia strain in a culture in a viable form and preferably in a culture containing a percentage of live cells in a whole population that is at least 5%, preferably at least 25% and more preferably at least 50%. In some embodiment providing the at least one rhizobia strain capable of producing C35 hopanoids in a form suitable for administration to a leguminous plant or seed or soil surrounding a leguminous plant or seed, and providing the genetically engineered rhizobia strain incapable of producing hopanoids in a form suitable for administration to a leguminous plant or seed, and/or a soil surrounding leguminous plant or seed.

In some embodiments a biofertilizer for a leguminous plant can be provided from a rhizobium naturally incapable of producing C35 hopanoids, by a method comprising: genetically engineering a nitrogen fixing rhizobia strain incapable of producing C35 hopanoids to introduce C35 synthesis genes to provide a genetically engineered nitrogen fixing rhizobia strains capable of producing C35 hopanoids, and providing the genetically engineered nitrogen fixing rhizobia strains in a form suitable for administration to a leguminous plant or seed, and/or a soil surrounding leguminous plant or seed.

In some of those embodiments, genetically engineering a nitrogen fixing rhizobia strain incapable of producing C35 hopanoids to introduce C35 synthesis genes can be performed by providing at least one gene selected from shc, hpnI and hpnG, hpnO, as well as hnpI, hnpC, hnpD and hnpL; and introducing the at least one gene in the rhizobium incapable of producing C35 hopanoids for a time and under conditions to allow expression of the at least one gene.

In some of those embodiments, genetically engineering a nitrogen fixing rhizobia strain incapable of producing C35 hopanoids to introduce C35 synthesis genes can be performed by obtaining C35 hopanoid synthesis genes from rhizobium that naturally produces C35 hopanoid using polymerase chain reaction-based amplification and isolation of a region of genomic DNA encoding hopanoid synthesis genes and/or genomic regulatory elements, or cDNA encoding hopanoid synthesis genes (see Examples 22-27). In some embodiments, the introduction of C35 hopanoid synthesis genes into C35 hopanoid-deficient rhizobia to produce genetically engineered C35 rhizobia can be performed by transduction using a recombinant viral vector containing a C35 hopanoid synthesis gene expression construct.

Additional techniques and related vectors, methods and systems to modify a nitrogen fixing rhizobia incapable of producing C35 are identifiable by a skilled person.

In some embodiments, providing the genetically engineered nitrogen fixing rhizobia strains in a form suitable for administration to a leguminous plant or seed, and/or a soil surrounding leguminous plant or seed can be performed by providing the at least one genetically engineered rhizobia strain in a culture in a visible form and preferably in a culture containing a percentage of live cells in a whole population can be at least 5%, preferably at least 25% and more preferably at least 50%. In some embodiment providing the at least one genetically engineered rhizobia strain capable of producing C35 hopanoids in a form suitable for administration to a leguminous plant or seed or soil surrounding a leguminous plant or seed, in an isolated or purified form wherein the bacteria are provided without a detectable presence of other microorganisms.

In several embodiments, one or more biofertilizers of the present disclosure are comprised in a biofertilizer composition together with one or more suitable vehicles, wherein the term “vehicle” as used herein indicates any of various media acting usually as solvents, carriers, binders or diluents for the biofertilizer that are comprised in the composition as an active ingredient.

In some embodiments, a biofertilizer composition for a leguminous plant comprises one or more biofertilizers essentially consisting of one or more nitrogen-fixing rhizobia capable of producing C35 hopanoids and an acceptable vehicle. In some embodiments, the biofertilizer composition can further comprise one or more C35 hopanoids. In the biofertilizer composition the one or more biofertilizers and the vehicle are formulated for administration to a leguminous plant and/or for administration to a leguminous seed. In some embodiments the biofertilizer composition is formulated for administration to a soil as will be understood by a skilled person.

In particular, in some embodiments in the biofertilizer compositions the vehicle comprises one or more carriers. Incorporation of carrier materials in the biofertilizer composition herein described can enable easy-handling, long-term storage and high effectiveness of the nitrogen-fixing capability of the nitrogen-fixing bacteria comprised in the biofertilizer composition. In particular, the suitable carrier material can allow gas exchange, also have high organic material content and high water holding capacity, as well as provide a stable medium for the storage of the biofertilizer while retaining high viability of the nitrogen-fixing bacteria comprised therein as will be understood by a skilled person.

In particular, suitable carrier materials can enhance the survival of the nitrogen-fixing rhizobia on the seed surface against drying conditions until placed into soil, the survival of the bacteria during the storage period, as well as the survival of the bacteria in soil. After being introduced into the soil, the carrier material provides nutrient and/or habitable micro-pore to the inoculant bacteria for them to compete with native soil microorganisms.

Various types of carrier materials can be used for seed or soil inoculation. For preparation of seed inoculant, the carrier material can be milled to fine powder with particle size of 10-40 µm. The properties of a suitable carrier material for seed inoculation are (1) non-toxic to inoculant bacteria strain, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8) good pH buffering capacity and (9) non-toxic to plant. Further information about carrier materials can be found in “Handbook for Rhizobia” (Somasegaran and Hoben, Springer, 1994).
In some embodiments, the method comprises applying one or more biofertilizer and/or biofertilizer compositions herein described to a leguminous plant or soil surround a leguminous plant for a time and under conditions to allow symbiosis of the nitrogen-fixing rhizobia with the leguminous plant.

In some embodiments, the biofertilizer and/or biofertilizer compositions can be administered in combination with one or more C35 hopanoids. In those embodiments, administering the biofertilizer and/or biofertilizer compositions and administering one or more C35 hopanoids are further performed for a time and under conditions allowing interaction of the one or more C35 hopanoids with the nitrogen-fixing rhizobia in the administered biofertilizer and/or biofertilizer compositions.

In some embodiments, applying the biofertilizer or biofertilizer composition alone or in combination with C35 hopanoids can be performed on the roots or to the soil in which the roots are present. In particular, the applying can be performed at any time in plant growth as will be understood by a skilled person.

In some embodiments, application on legumes of C35 hopanoids, and C35 hopanoids-producing rhizobia herein described and related biofertilizer composition, can be performed under conditions allowing extensive host control of bacterioid physiology and establishment of a specific host microenvironment, defined by low oxygen, low pH, hypoxia and oxidative stress [1]. For example, in A. afraspierosa application of C35 hopanoids, and rhizobia herein described and related biofertilizer composition can be performed in connection with the plant’s ability to produce nodule-specific, cysteine-rich antimicrobial peptides (NCRs) that induce differentiation of the bacteroid into an enlarged, elongated and polyplidoid state.

In some embodiments, application on legumes of biofertilizers, biofertilizer composition alone or in combination with C35 hopanoids, can be performed for microaerobic growth and tolerance to diverse stresses of the rhizobia the symbiotic microenvironment, such as oxidative and hypoxic condition, acidophilic (pH<6), detergent, oxidative stresses (such as due to hydrogen peroxide), ambient (between 22°C and 32°C C.) and particularly higher temperatures (i.e. 37°C C.<T<32°C C.) and/or to promote to outer membrane rigidity (Examples 14-19 for B. diazoefficacies).

In some embodiments a method of fertilizing leguminous plants can comprise coating and/or inoculating one or more seeds of the leguminous plant with one or more biofertilizer and/or biofertilizer compositions herein described. In some embodiments, the seeds can be coated with bacteria and desiccated for transport/storage. It is expected that coating or inoculating with hopanoids will improve bacterial survival of desiccation and long-term storage in a desiccated state. In some embodiments the method further comprises coating and/or inoculating one or more C35 hopanoids before the coating and/or inoculating the one or more seeds of the leguminous plant with one or more biofertilizer and biofertilizer composition. In particular in those embodiments the C35 hopanoids are typically applied prior to applying the rhizobia to the seed.

In some embodiments applying the biofertilizer composition can be performed by coating and/or inoculating the leguminous seeds with the biofertilizer formulation prior to or at the time of planting. In some embodiments, applying the biofertilizer formulation can be performed by dip-dipping the seedlings of the leguminous plants in a suspension comprising the biofertilizer. Alternatively or in addition, the
biofertilizer formulation can be directly applied to soil where the seed is planted or to be planted.

In embodiments herein described, methods of fertilizing leguminous plants are described. The methods comprise administering the C$_{35}$ hopanoids, and rhizobia herein described and related biofertilizer composition to leguminous plants through seedling dipping, and/or direct-soil application, and/or seed treatment (also called seed inoculation), or other fertilizer application approaches identifiable by the skilled person.

In some embodiments, the methods of fertilizing leguminous plants comprise preparing a suspension containing one or more biofertilizer herein described and dipping the seedlings of the leguminous plants in the suspension for a certain time under a certain condition to allow the symbiotic interaction between the rhizobia, C$_{35}$ hopanoids and the leguminous plants.

In particular, a biofertilizer/inoculant suspension comprising C$_{35}$ hopanoids and C$_{35}$-hopanoids-producing nitrogen-fixing bacteria is prepared in water. The roots of seedlings are then dipped in the suspension and kept immersed for a certain period of time, typically several minutes, before being transplanted. Here, seedling refers to a young plant sporophyte developing out of a plant embryo from a seed. Seedling development starts with germination of the seed. A typical young seedling consists of three main parts: the radicle (embryonic root), the hypocotyl (embryonic shoot), and the cotyledons (seed leaves). For example, for soybean, each seedling can be treated with 1 ml of an OD$_{600}$=1.0 ($\approx$10$^9$ cells) suspension of bacteria in a nodulation medium that includes trace elements required by the nitrogenase cofactors to support nitrogen fixation.

In some embodiments, about $\approx$1 billion cells can be suspended in a nodulation medium that contains C$_{35}$ hopanoids at a concentration below its critical micelle concentration (CMC). The term "critical micelle concentration (CMC)" is used herein to characterize the aqueous solubility of a lipid compound such as C$_{35}$ hopanoids. CMC indicates the concentration above which amphiphilic molecules aggregate to form micelles. At low surfactant concentration the amphiphilic molecules arrange on the surface of the liquid, but also exist as free molecules in the solution, with the two groups exchanging with one another. As more amphiphilic molecules are added, the surface becomes saturated, and the concentration of free molecules in solutions approaches a concentration specific to the molecule and environment, at which point further addition of amphiphilic molecules will lead to formation of micelles. This concentration point above which micelles form is called the critical micelle concentration.

Many factors have effects on the CMC of a lipid compound as will be understood by a skilled person. For example, the molecular structure of the lipid, temperature, the presence of electrolyte in the solution and so on. Several empirical correlations can be used for the estimation of CMC values. For lipids with a straight and saturated single carbon tail, the CMC can be calculated from (Klevens 1953):

$$\log \text{CMC} = -4.9n$$

where $n$ is the number of carbon atoms in the hydrophobic tail, and $A$ and $B$ are temperature dependent constants for a given type of lipid. The values of $A$ and $B$ are identifiable for a skilled person in the art. These constant values can also be found in textbooks such as Kreshech 1975 [29]. It is also possible to measure the CMC of a molecule with devices such as a contact angle system, a tensiometer, a Langmuir trough, or with other equipment identifiable by a skilled person.

In some embodiment, methods of fertilizing leguminous plants herein described comprise applying one or more biofertilizer and/or related biofertilizer composition herein described alone or in combination with the C$_{35}$ hopanoids directly to the soil where the leguminous plants are grown. In particular, the biofertilizer composition comprising a biofertilizer herein described, optionally in combination C$_{35}$ hopanoids and can be directly applied to the soil before or at the time of plantation or sowing.

For example, in some embodiments, the biofertilizer or biofertilizer composition can be firstly mixed with finely powdered farm yard manure (FYM), compost, or soil at a specific ratio and then directly applied to the soil. The formed mixture can be broadcast at the time of plowing.

In some embodiments, methods of fertilizing leguminous plants comprise applying the C$_{35}$ hopanoids, C$_{35}$ hopanoid-producing rhizobia and related biofertilizer composition herein described via soil inoculation by placing the biofertilizer and related composition into the furrow under or alongside the seeds.

In particular, the biofertilizer and related compositions can further comprise a carrier material in granular form of a size about 0.5-1.5 mm. Suitable carrier material includes granular forms of peat, perlite, talcum powder, or materials that can offer nutrient and/or habitat micro-pore to the inoculants bacteria including carriers with micro-porous structure such as charcoal or soil aggregates.

In some embodiments, the methods of fertilizing leguminous plants comprise coating/inoculating the leguminous seeds with the C$_{35}$ hopanoids, C$_{35}$ hopanoid-producing rhizobia and related biofertilizer composition herein described, also referred to as seed treatment methods.

In particular, to prepare for inoculation, the biofertilizer can be firstly mixed with water to form a slurry mixture. The seeds desired to be treated are then immersed in the mixture for a certain period of time under certain conditions to form seeds coated or inoculated with the inoculum.

Leguminous plant seeds can be treated with the biofertilizer compositions comprising a carrier, C$_{35}$ hopanoids and C$_{35}$-hopanoids-producing nitrogen-fixing bacteria inoculant at a certain weight ratio identifiable to a skilled person in the art of agriculture and inoculation. In particular, the skilled person will recognize the relationship between seed size, number of C$_{35}$-hopanoids-producing nitrogen-fixing bacteria, the amount of C$_{35}$ hopanoids and weight of the inoculant. Similar to the seedling dipping method described above, about $\approx$1 billion cells can be suspended in a nodulation medium that contains C$_{35}$ hopanoids at a concentration below its CMC.

In some embodiments, the vehicle used in the biofertilizer for seed treatment is in the form of fine powder with particle size of 10-40 $\mu$m. The carrier can be a material, such as peat, vermiculite, lignite powder, clay, talc, rice bran, seed, rock phosphate pellet, charcoal, soil, paddy straw compost, wheat bran or a mixture of such materials. In common practice, for better shelf-life of biofertilizer formulation, a carrier or a mixture of such carrier materials are selected based on the viability of the microorganisms mixed with them. The carriers used in the biofertilizer for seed inoculation are typically non-toxic to inoculant bacterial strains and plant, with certain moisture absorption and pH buffering capacity, easy to process and sterilize by autoclaving or gamma-irradiation and cost-effective. In some particular cases, to achieve a tight coating of inoculant on seed
surface, adhesive material, such as gum Arabic, methylth-
ycellulose, sucrose solutions and vegetable oils, can be used as carrier. In some cases, supplementary nutrients and cell protectants such as sucrose, maltose, trehalose, molasses, glucose and glycerol can be used together with the carrier material to ensure improved cell viability and extended shelf-life.

In some embodiments, coated seeds comprising legumi-


standard techniques. Using the DNA sequences in the donor material as a guide, design of appropriate primers, PCR reagents and methods can be achieved by a person of ordinary skill in the art.

In some embodiments, after the C35 hopanoid synthesis genes and other regulatory elements are obtained, incorporation of the genes and/or other regulatory elements into a plasmid vector or other vector can be achieved using standard molecular cloning techniques. For example, the C35 hopanoid synthesis genes can be excised from surrounding genetic material by restriction endonuclease digestion to provide a C35 hopanoid synthesis gene “insert”. In parallel, restriction endonuclease digestion can be performed on a plasmid vector or other vector into which the C35 hopanoid genes are inserted. DNA ligation can then be performed to result in a plasmid containing the C35 hopanoid synthesis genes and/or regulatory sequences.

In some embodiments, the vector containing the C35 hopanoid synthesis genes can contain appropriate regulatory elements to express the C35 hopanoid synthesis genes, including but not limited to promoters, enhancers, 5' and 3' untranslated regions, exons, introns, enzyme recognition sites for appropriate processing of transcripts, and post-transcriptional and post-translational genetic elements. Exemplary regulatory elements that can be comprised in the vector can comprise elements that are naturally associated with the C35 hopanoid synthesis genes in genomic DNA of the “donor” genetic material, and/or elements comprised of “heterologous” elements that are not normally associated with the natural C35 hopanoid synthesis genes, such as promoters normally associated with other genes.

In some embodiments, vectors used to introduce C35 hopanoids synthesis genes can in addition to elements for regulating expression of C35 hopanoid synthesis genes, other genetic material comprising other regulatory sequences, including origin of replication, genes for expressing antibiotic resistance, and restriction endonuclease sites.

In some embodiments, vectors used to introduce C35 hopanoids synthesis genes comprise a plasmid vector containing an expression cassette for a single hopanoid synthesis gene. In other embodiments, vectors used to introduce C35 hopanoids synthesis genes comprise a plasmid vector containing expression cassettes for more than one hopanoid synthesis gene. In some embodiments, a recipient rhizobium can be genetically modified with one or more plasmid vectors containing one or more hopanoid synthesis gene expression cassettes, as required to provide a full set of hopanoid synthesis genes.

In some embodiments, vectors containing hopanoid synthesis genes can be introduced into bacteria, including rhizobia, by a process of transformation, whereby the bacterial cell wall is transiently opened allowing entry of the plasmid DNA into the bacterium. Several methods of transformation can be used, including electroporation, thermal shock, freeze-thaw techniques (see Examples 25-27).

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lipid content from the cultured cells, and purifying the lipid extract and performing analytical chemistry techniques to detect the C₃₅ hopanoids (including gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry) (see Example 29).

In some embodiments, detection of C₃₅ hopanoid can be performed in addition or in the alternative by detecting one or more C₃₅ hopanoids synthesis genes. Detection of C₃₅ hopanoids synthesis genes can be performed by DNA sequencing, PCR probes and/or other nucleotide amplification techniques as will be understood by a skilled person.

For example, in some embodiments, a method of screening stress-resistant rhizobia strains as biofertilizer can comprise genetically screening rhizobia strains capable of producing C₃₅ hopanoids. In those embodiments, the method can comprise providing a plurality of rhizobia strains, for each rhizobia strain detecting C₃₅ hopanoid synthesis genes (e.g. by conducting a diagnostic polymerase chain reaction or DNA sequencing methods) in the rhizobia strain, and selecting the rhizobia strain with detected C₃₅ hopanoid synthesis genes.

In method for genetic screening of rhizobia, detected presence of C₃₅ hopanoid synthesis genes can be used as a marker for the ability of particular rhizobia to produce C₃₅ hopanoids (see Example 30). In some embodiments, detecting Diagnostic PCR or DNA sequencing on genomic DNA isolated from rhizobia obtained from soil, legume nodules, or other sources can be performed to determine whether C₃₅ hopanoid synthesis genes are present in a particular sample rhizobial genome. Using available gene sequence information, PCR primers can be designed to amplify genes in the hopanoid synthesis gene cluster, whereby amplification of genes necessary for C₃₅ hopanoid synthesis indicates a rhizobial species genetically capable of producing C₃₅ hopanoids. Similarly, DNA sequencing may be used to determine whether C₃₅ hopanoid synthesis genes are present in the genome of a sample rhizobium, and therefore whether the rhizobium is genetically equipped to synthesize C₃₅ hopanoids.

Additional techniques for detecting C₃₅ hopanoids and/or C₃₅ hopanoids synthesis genes are identifiable by a skilled person.

In some embodiments, biofertilizers, biofertilizer compositions, seed and methods herein described can comprise at least one nitrogen fixing bacteria other than a rhizobia (e.g. ar rhizobiales) which is symbiotic with legumes, is incapable of producing C₃₅ hopanoids (C₃₅ deficient), and is closely genetically related to (i.e. within a same taxonomic order of) hopanoids-producing legume rhizobia symbiont capable of producing C₃₅ hopanoids. In those embodiments, the at least one nitrogen fixing bacteria other than a rhizobia can be genetically engineered to include shc, hpnH, hpnG, hpnO, as well as hpnP, hpnC, hpnD and/hor hpnE and/or other nucleotide amplification techniques as will be understood by a skilled person.

The biofertilizer, biofertilizer composition, seeds and C₃₅ hopanoids can be included in one or more compositions, and nitrogen fixing rhizobia capable of producing C₃₅ hopanoids can be in a composition together with a suitable vehicle.

In particular, the components of the kit can be provided, with suitable instructions and other necessary reagents, in order to perform the methods here described. The kit will normally contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, will usually be included in the kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials such as buffers and the like.

Further details concerning biofertilizers, and related seeds compositions methods and system, cells and formulation of the present disclosure will become more apparent hereinafter from the following detailed disclosure of examples by way of illustration only with reference to an experimental section.

EXAMPLES

The hopanoids, hopanoids-producing nitrogen-fixing bacteria, and related formulation and methods herein described are further illustrated in the following examples, which are provided by way of illustration and are not intended to be limiting.

In particular, the following examples illustrate exemplary hopanoids, hopanoids-producing nitrogen-fixing bacteria forming symbiosis with exemplary leguminous plants and related methods and systems. In particular, in the examples described herein, B. diazoefficiens is used as a model strain to study the roles of two hopanoid classes, 2Me-hopanoids and C₃₅ hopanoids, in symbiosis with leguminous plants such as Aeschynomene afraspera and soybean.

A person skilled in the art will appreciate the applicability and the necessary modifications to adapt the features described in detail in the present section, to additional hopanoids-producing nitrogen-fixing bacteria, such as other related Bradyrhizobium bacteria as described in the above disclosure of forming symbiosis with leguminous plants, and related methods and systems according to embodiments of the present disclosure.

Example 1: Hopanoids from B. diazoefficiens

FIG. 1, panel A shows exemplary chemical structures of isolated hopanoids from B. diazoefficiens. While most hopanoids are thought to occur free within membranes, the C₃₅ hopanoid, (2-Me) 34-carboxyl-bacteriohopane-32,33-diol, was found to be covalently attached to LPS lipid A in the outer leaflet of the outer membrane (OM), a well-established player in a broad range of host-microbe interactions, to form a compound called Homanoid-Lipid A (HOL.A) [7, 30] (FIG. 1, panel B). As seen in the expanded view of the OM, B. diazoefficiens makes short (C₃₁₀) hopanoids like diploptene and extended (C₃₅₃) hopanoids like bacteriohopanetetrol (BHT) and amniotriol. Penta- and hexa-acylated Lipid A contain 5 and 6 fatty acyl chains, respectively. Hepta-acylated Lipid A contains the C₃₅ hopanoid, 34-carboxyl-bacteriohopane-32,33-diol, covalently attached to hexa-acylated Lipid A. In addition to C₃₁₀ and C₃₅₃ hopanoids [5], B. diazoefficiens makes tetracyclamino, a triterpenoid with a gammacerane skeleton [31] (FIGS. 1 and 2, panel A).
Example 2: Synthesis of Hopanoids from *B. diazoefficiens*

$C_{35}$ hopanoids are biosynthesized by a hopanoid biosynthetic gene cluster in some bacteria. For example, in *B. diazoefficiens*, $C_{35}$ hopanoids can be synthesized by a hopanoid biosynthetic gene cluster shown in FIG. 2, Panel C. In particular, she (squalene hopene cyclase) catalyzes squalene cyclization to hopene, the first reaction in the hopanoid biosynthetic pathway; hpnH catalyzes addition of adenosine to hopene, the first reaction in the synthesis of $C_{35}$ hopanoids; and hpnP catalyzes C-2 methylation. Detailed description of the biosynthetic pathway and involved genes can be found in Welander 2012 [12].

Table 2 lists the $C_{35}$ hopanoid biosynthesis genes and their sequences from *R. palustris*.

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<thead>
<tr>
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<td>-----------</td>
</tr>
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<td>hpnO</td>
</tr>
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</table>
Example 3: Bacterial Strains and Growth Conditions

For microaerobic growth of Bradyrhizobium diazoefficiens in peptone-salts-yeast extract (PSY), the medium was made anaerobic by boiling under a stream of nitrogen for 10 minutes, dispensing 25 ml cooled medium in 500 ml anaerobic bottles in a nitrogen chamber, exchanging gas phase of stoppered media bottles with nitrogen for an hour, followed by autoclaving. The sterilized medium was inoculated with aerobic PSY-grown log-phase cultures at 10⁻² dilution and the gas phase exchanged with 12-14 psi 99.5/0.5% nitrogen/oxygen gas mix for 3-5 minutes every 8-16 hours (h) [32]. In all media, B. diazoefficiens strains were incubated at 30° C. with shaking at 250 rpm for aerobic cultures and 60 rpm for microaerobic cultures, unless indicated otherwise. Antibiotics were used for selection at these concentrations (µg/ml): spectinomycin, 100; kanamycin (Km), 100; tetracycline (Tc), 50.

Example 4: Sequence Analysis

The Integrated Microbial Genome (IMG) system (https://img.jgi.doe.gov/cgi-bin/w/main.cgi) was used to access DNA and protein sequences, identify orthologs and assess genomic context of genes [33].

Example 5: Mutant Construction

Fusion PCR products of ~1 Kb upstream and downstream regions of genes of interest were cloned into pKl8mobsacB to obtain deletion plasmids that were mobilized into WT and selected using Km resistance (Table 3). Subsequently, the plasmid integrants were resolved by growth in non-selective medium and segregants were obtained by 5% sucrose selection. Potential mutants were screened by PCR and verified by sequencing. Deletion of hpnA (hpnA) and the hpnCDEFG (FIG. 2) operon using pK18mobsacB- and pSUP202pol4-based plasmids (Table 3) were attempted. For the latter, a 1.2 Kb Km resistance cassette from pBSL86 was sub-cloned between ~1 Kb upstream and downstream regions of the genes in pSUP202pol4. Following selection of deletion plasmids with Km resistance, potential Km resistant and Tc sensitive mutants were screened by PCR. It was unable to isolate an sh deletion mutant with either methods in PSY at 30° C. or room temperature (23-25° C.) with and without 100 µM cholesterol or diplopterol as supplements. Counterselection of sh deletion plasmid segregants at lower sucrose concentrations (1−4%) were also carried out, but the mutant was still not obtained. Tables 3 and 4 list the strains, plasmids and primers used in constructing the mutants.

### TABLE 3

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Strains</th>
<th>Genotype, description and constructiona</th>
<th>Source or USb</th>
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<td><strong>Strains</strong></td>
<td>E. coli DH10B</td>
<td>F− endA1 recA1 galE15 galK16 supG rpsL1, A(ara, leu)7697 mcrA (min–IncB/RMS–mcr) /; DKN89</td>
<td>[34]</td>
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<td>E. coli S17-1</td>
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<tr>
<td></td>
<td>DKN1529</td>
<td>B. japonicum 110spc4 AhpAP; deletion of blr3006 in DKN1391 using pGK255</td>
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<td><strong>Plasmids</strong></td>
<td>pK18mobsacB</td>
<td>Km’ mobilizable pUC18 derivative, sacB (DKN1387)</td>
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<td>pSUP202pol4</td>
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<td>[38]</td>
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<td></td>
<td>pBSL86</td>
<td>Ap’, Km’ (DKN1388)</td>
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<td></td>
<td>pGK259</td>
<td>she deletion vector; HindIII/PstI-digested blr3004 (she) upstream and downstream fusion PCR product amplified using primers sheupforshevrev, sheupforshevnew, shevrevforshevnew and shevrev was ligated to HindIII/PstI-digested pK18mobsacB (DKN1492)</td>
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<td>pGK248</td>
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### TABLE 3 -continued

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<tr>
<td>pGK263</td>
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<tr>
<td>pGK268</td>
<td>hpnCDEFG deletion vector; blr3001 (hpnC) upstream and blr3005 (hpnG) downstream PCR products amplified using primers hpnCupfor/pK18fusion, hpnCuprev/hpnGfusion and hpnGdnfor/hpnCfusion, hpnGdnrev/pK18fusion were Gibson cloned into XbaI/PstI-cut pK18mobsacB (DKN1604)</td>
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<td>pGK269</td>
<td>NotI/XbaI-digested hpnC upstream PCR product amplified using primers hpnCupfor and hpnCuprev was ligated to NotI/XbaIdigested pSUP202pol4 (DKN1605)</td>
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<td>pGK270</td>
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<td>pGK276</td>
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<tr>
<td>pGK247</td>
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*Km, Kanamycin; Sp, Spectinomycin

*underlined sequence


### TABLE 4

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<td>fusion</td>
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*underlined sequence
TABLE 4 -continued

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<td>PstI 24</td>
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Example 6: Hopanoid Analysis

Triplicate cultures of *B. diazoefficiens* strains were grown till saturation in aerobic (100 mL PSY in 500 ml flasks) and microaerobic (25 mL PSY in 500 ml Wheaton bottles) growth media. They were centrifuged at 5000g for 20 min at 4° C. and frozen at -80° C. until extraction. Cell pellets were suspended in 2 mL water and transferred to Teflon centrifuge tubes (VWR, Bridgeport, N.J.), followed by addition of 5 ml methanol (MeOH) and 2.5 ml dichloromethane (DCM) and sonicated for 15 min at room temperature (VWR B2500A-DTH; 42-kHz radio frequency power, 85 W). Samples were centrifuged at 7000xg for 10 min at 22° C. and the supernatants transferred to new tubes. Cell pellets obtained from aerobically-grown cultures were sonicated again, centrifuged, and the supernatants combined with the first extraction. The samples were separated into two phases by adding 7.5-13 mL DCM and centrifuged at 6000xg for 10 min at 22° C. The organic phase was transferred to a new vial and evaporated in a chemical hood overnight. The total lipid extract (TLE) was resuspended in DCM at a concentration of 1 mg/mL. 100 µl of this extract was combined with 1 µl of an internal standard (500 ng/mL pregnane-acetate [40]) and evaporated at 60° C. The TLE was derivatized to acetate esters by incubation in 100 µl 1:1 acetic anhydride/pyridine for 30 min at 60° C. and then analyzed by gas chromatography/mass spectrometry (GC-MS). Peak areas of hopanoid species were integrated and compared to those from pregnane-acetate standards to obtain the yields from TLE [41].

For liquid chromatography/mass spectrometry (LC-MS), 100 µl 1 mg/mL TLE was evaporated under nitrogen, dissolved in isopropanol-acetonitrile-water (2:1:1) or DCM-MeOH (9:1) and then analyzed [26]. Hopanoid peaks were identified by comparison of retention times and mass spectra to those of *Rhodopseudomonas palustris* TIE-1 (Tables 5 at 4° C. and frozen at -80° C. until extraction. Cell pellets were suspended in 2 mL water and transferred to Teflon centrifuge tubes (VWR, Bridgeport, N.J.), followed by addition of 5 mL methanol (MeOH) and 2.5 mL dichloromethane (DCM) and sonicated for 15 min at room temperature (VWR B2500A-DTH; 42-kHz radio frequency power, 85 W). Samples were centrifuged at 7000xg for 10 min at 22° C. and the supernatants transferred to new tubes. Cell pellets obtained from aerobically-grown cultures were sonicated again, centrifuged, and the supernatants combined with the first extraction. The samples were separated into two phases by adding 7.5-13 mL DCM and centrifuged at 6000xg for 10 min at 22° C. The organic phase was transferred to a new vial and evaporated in a chemical hood overnight. The total lipid extract (TLE) was resuspended in DCM at a concentration of 1 mg/mL. 100 µl of this extract was combined with 1 µl of an internal standard (500 ng/mL pregnane-acetate [40]) and evaporated at 60° C. The TLE was derivatized to acetate esters by incubation in 100 µl 1:1 acetic anhydride/pyridine for 30 min at 60° C. and then analyzed by gas chromatography/mass spectrometry (GC-MS). Peak areas of hopanoid species were integrated and compared to those from pregnane-acetate standards to obtain the yields from TLE [41].

TABLE 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt (min)</th>
<th>Diagnostic ions (m/z)</th>
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<td>17.37</td>
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<td>17.51</td>
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<td>442, 409, 205, 189, 149, 95</td>
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<td>2-Methyltetrahymanol (VII)</td>
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TABLE 5-continued

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<th>Compounds identified by high temperature GC-MS</th>
<th>Rt (min)</th>
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<td>3.46</td>
<td>546.487</td>
<td>[M + H]^+</td>
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<tr>
<td>2- Methylaminobacteriohopanotriol (e)</td>
<td>3.26</td>
<td>536.454</td>
<td>[M + Na]^+</td>
</tr>
<tr>
<td>Bacteriohopanotriol (BHT)</td>
<td>4.56</td>
<td>529.456</td>
<td>[M + H]^+</td>
</tr>
<tr>
<td>Bacteriohopanotetrol (b)</td>
<td>5.66</td>
<td>529.454</td>
<td>[M — Na]^+</td>
</tr>
<tr>
<td>Adenylolynxane (a)</td>
<td>6.04</td>
<td>562.450</td>
<td>[M + H]^+</td>
</tr>
<tr>
<td>2-Methylbacteriohopanotetrol (f)</td>
<td>6.22</td>
<td>543.478</td>
<td>[M — H2O + H]^+</td>
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<tr>
<td>2-Methylbacteriohopanotriol (e)</td>
<td>6.20</td>
<td>583.470</td>
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TABLE 6

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<td>Bacteriohopanotriol (BHT)</td>
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<td>529.456</td>
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<td>Bacteriohopanotetrol (b)</td>
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<td>529.454</td>
<td>[M — Na]^+</td>
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<tr>
<td>Adenylolynxane (a)</td>
<td>6.04</td>
<td>562.450</td>
<td>[M + H]^+</td>
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<tr>
<td>2-Methylbacteriohopanotetrol (f)</td>
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<td>543.478</td>
<td>[M — H2O + H]^+</td>
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<td>6.20</td>
<td>583.470</td>
<td>[M — Na]^+</td>
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</table>

Example 7: Lipid A Analysis

Bacterial cells were extracted using the phenol/water method [42] and after extensive dialyses, the extracted phases were subjected to enzymatic digestion with DNases, RNases and proteases in order to remove nucleic acids and protein contaminants and recovered by ultracentrifugation (100 000g, 4°C, 24 h). Water phases were analysed through 13.5% SDS-PAGE; the lipopolysaccharide (LPS) fraction was exclusively found in water phase as suggested by the presence of the typical ladder in its migration pattern in the gel. The LPS material was further purified by a second extraction with phenol/chloroform (CHCl3)/petroleum methods to get rid of glucan contaminants, and LPS fractions were further purified by size fractionation chromatography (Sepharcl S-400 HR in 50 mM ammonium carbonate (NH4HCO3) from GE Healthcare).

LPS sugar content was determined by GLC-MS analysis of acetylated O-methyl derivatives. Methanolic hydrochloric acid (HCl) was added to dried LPS and incubated at 85°C. For 16 h, the sample was subsequently acetylated with pyridine and Ac2O, 85°C, 20 min and analysed by GLC-MS [43]. Linkage analysis was carried out by methylation analysis. The sample was hydrolyzed with 4 M trifluoroacetic acid (100°C, 4 h), carbonyl-reduced with sodium borodeuteride (NaBD4), carboxy-methylated, carboxyl-reduced, acetylated and analysed by GLC-MS [44]. Total fatty acid content was obtained by acid hydrolysis. LPS lipids were first treated with 4M HCl (4 h, 100°C) and then with 5M sodium hydroxide (NaOH, 30 min, 100°C). Fatty acids were then extracted in CHCl3, methylated with diazomethane and analysed by GLC-MS. The ester bound fatty acids were selectively released by base-catalysed hydrolysis with 0.5M NaOH/MeOH (1:1 v/v, 85°C, 2 h), then the product was acidified, extracted in CHCl3, methylated with diazomethane and analysed by GLC-MS [45].

In order to obtain lipid A, LPS was dissolved in acetate buffer (pH 4.4), and was hydrolyzed for 5 h at 100°C. Then, adequate amounts of CHCl3 and MeOH were added to the hydrolysate to obtain CHCl3/MeOH/hydrosolate 2:2:1.8 (v/v/v), and the mixture was vigorously shaken, then centrifuged [46]. The lipid A-containing CHCl3 phases were collected and washed twice with the water phase from a freshly prepared two-phase Bligh-Dyer mixture (CHCl3/MeOH/water, 2:2:1.8 (v/v/v)).

For MALDI TOF MS, a 4800 Proteomic Analyzer (AB-Sciex), MALDI TOF/TOF instrument equipped with a Nd:YAG laser at a wavelength of 355 nm with <500-ps pulse and 200-Hz firing rate was employed. External calibration was performed using an ABSciex calibration mixture. All measurements were performed in positive polarity. Approximately, 1500 laser shots were accumulated for each spectrum in the MS experiments. Samples were dissolved in CHCl3/MeOH (50:50, v/v) at a concentration of 1 mg/ml. Matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) in MeOH/0.1% trifluoroacetic acid/acetonitrile (7:2:1, by volume) at a concentration of 75 mg/ml. 1 µl of the sample/matrix solution (1:1, v/v) was deposited onto the well plate and allowed to dry at room temperature.

Example 8: Membrane Rigidity

For whole cell membrane rigidity measurements, as described in [8], PSY-grown aerobic cultures of B. diazoefficiens strains were washed once with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM HEPES, 50 mM sodium chloride (NaCl), pH 7.0) and then resuspended in the same buffer. Lipid A was extracted in CHCl3/MeOH (50:50, v/v) at a concentration of 1 mg/ml. 1 µl of the sample/matrix solution (1:1, v/v) was deposited onto the well plate and allowed to dry at room temperature.

Example 9: CRYO-TEM (Transmission Electron Microscopy)

PSY-grown aerobic cultures at an OD600 of 1 were concentrated 5 times and frozen in a Vitrobot MkIV (FEI, Hillsboro, Oreg.) as described previously [47, 48]. In brief, 2 µl of a 10 nm colloidal gold (Sigma Aldrich, St. Louis, Mo.) in 5% Bovine serum albumin (BSA) was added to 8 µl of culture. 3 µl of this suspension was placed onto a glow-discharged carbon-coated R 2/2 Quantifoil copper-finder grid in the Vitrobot maintained at 22.5°C with 95% humidity. This was followed by a 3 s blot with a pressure of 6 atm, a drain time of 1 sec, and plunge freezing in a mixture of liquid ethane (63%) and propane (37%). The frozen grids were then stored in liquid nitrogen until further use. Grids were imaged in Tecnai TEM 120 KeV (FEI, Hillsboro, Oreg.) at ~178°C using a Gatan 626 cryoholder and Gatan 2x2K CCD. Images were acquired with Digital Micrograph at 15,000x magnification [49, 50].

Example 10: Growth Curves and Stress Assays

To monitor growth in different media, triplicate cultures were inoculated at 10^2 dilution using aerobic PSY-grown log-phase (OD600=0.5-0.7) WT or mutant strains. Growth was measured at OD600 using a Spectronic 200+ (Thermo Scientific) or a Beckman Coulter spectrophotometer for microaerobic medium. Unless otherwise indicated, the incubation temperature was 30°C. Growth curves were performed in triplicates at least twice independently.

Sensitivity to high temperature (37°C) and low or high pH was measured by monitoring growth in PSY at OD600 using Spectronic 200+. Acidic (pH-6) and alkaline (pH-8)
media were prepared by buffering PSY with 100 mM MES (4-Morpholineethanesulfonic acid) and 100 mM bicine (N,N-Bis(2-hydroxyethyl)glycine) or BIS-TRIS Propane, respectively. It was unable to collect a growth curve at pH=8 because this was outside the WT growth range. Growth curves were performed in triplicates at least twice independently.

Growth in the presence of osmotic and membrane stresses was measured using gradient plates. To prepare these, 25 ml of 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered PSY agar (pH=7) with 50 mM NaCl, 500 mM inositol, 0.4% bile salts (BS, Himedia, Mumbai, India) or 1 mM ethylenediaminetetraacetic acid (EDTA) was poured in a slightly tilted square grid plate (Fisher, Pa.). The solidified plate was topped with 25 ml PSY-MOPS agar. Control plates contained 50 ml PSY-MOPS agar. 5 µl of aerobic PSY-grown log-phase cultures at 10⁻⁴ dilution were spotted on the plates. To assess stationary-phase stress, saturated instead of log-phase cultures were used for plating. The plates were incubated at 30°C for 5-7 days. Spotting assays were performed in duplicates at least two independent times.

Disk diffusion assays were used to quantify growth under oxidative, acidic and detergent stresses. For this, 4 day-old cultures of B. diazoefficiens strains grown in yeast extract-mannitol (YM) medium were washed and adjusted to an OD₆₀₀ of 1.2 ml bacterial suspensions were then mixed with 100 ml of 42°C prewarmed YM soft agar (0.8% agar) and 5 ml portions of this mixture were poured on solid YM. Filter disks were placed at the center of the plates, and 5µl of aerobic PSY-grown log-phase cultures at 10⁻⁴ dilution were spotted on the plates. The diameters of growth inhibition areas were measured after incubation at 30°C for 5 days.

**Example 11: MIC Determination**

The MIC of polymyxin B was determined by the E-test method using disk diffusion assay as described above. Strips containing a gradient of polymyxin B ranging from 0.064-1024 µg/ml (Bromélieux, Marcy-l'etoile, France) were placed in the center of the plates, which were incubated at 30°C for 7 days before recording the results. The experiment was done in triplicates.

The effect of the NCR335 peptide on cell viability was determined by spot assays. YM-grown exponential phase cultures were washed three times in 10 mM potassium phosphate buffer pH 7.0, diluted to an OD₆₀₀ of 0.01, and treated with 6 µM NCR335 for 24 h at 30°C. Samples were serially diluted in YM medium, and 5 µl aliquots of each dilution were spotted in duplicate on YM agar. CFU/ml were determined after 7 days at 30°C. The experiment was performed in triplicates.

**Example 12: Plant Cultivation and Symbiotic Analysis**

*A. afraspera* seeds were surface sterilized by immersion in sulfuric acid for 45 minutes with shaking, followed by thorough washing in sterile distilled water and incubation in the same overnight. The seeds were germinated by transferring to 0.8% agar plates for 2 days at 37°C in dark. Subsequently, plantlets were rooted in buffered nodulation medium (BNM)-filled test tubes, which were covered with aluminum foil for hydroponic culturing [51]. Plants were grown in a 28°C growth chamber with a 16 h light and 8 h dark cycle and 70% humidity. Seven days after transfer, each seedling was inoculated with a 1 ml cell suspension from a 5 day-old bacterial culture washed in BNM and adjusted to reach an OD₆₀₀ of 1.

Soybean (*Glycine max* Williams 82) seeds were cleaned with 100% ethanol for 30 seconds and sterilized with 1% bleach for 5 min. After several washes with sterile distilled water, seeds were germinated on tap-water agar plates at 28°C for 3 days. Seedlings were then transferred to magenta boxes filled with BNM, inoculated and grown hydroponically as described above for *Aeschynomene* plants. Plants were watered with BNM medium.

Infection assays were carried out three independent times with 7 and 10 plants for soybean and *A. afraspera*, respectively. At 21 d.p.i., plants were analyzed for the number of nodules and nitrogenase activity as previously described [52].

**Example 13: Cytological Analyses and Microscopy**

Cytological analyses were done on 5-10 nodules originating from 3 different plants for each condition; microscopic observations were performed for each of the 3 plant experiments, except for the TEM observations which were only done once. Semi thin nodule sections (30-40 µm) were prepared using a vibratome (VT1000S; Leica, Naanterre, France). Immediately after slicing, the sections were incubated for 20 min in live/dead staining solution (5 µM SYTO 9 and 30 µM propidium iodide (PI) in 50 mM Tris pH 7.0 buffer, Live/Dead BacLight, Invitrogen). Sections were then removed and incubated an additional 15 min in 10 mM phosphate saline buffer (PBS) containing calcifluor white M2R (Sigma, Munich) to a final concentration of 0.01% (w/v) to stain the plant cell wall [53]. After washing with PBS, the sections were mounted on microscope slides in PBS containing glycerol at a final concentration of 50% (v/v). Analyses were carried out using a confocal laser-scanning microscope (Carl Zeiss LSM 700; Jena, Germany). Calcifluor was excited at 405 nm with emission signal collection at 405 to 470 nm. For SYTO 9 and PI, an excitation wavelength of 488 nm and 555 nm was used with emission signal collection at 490 to 522 nm and 555 to 700 nm, respectively. Images were obtained using the ZEN 2008 software (Zeiss).

For TEM of the nodules, the samples were fixed in a 4% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide, dehydrated using a series of acetone washes, and embedded in TAAB 812 epoxy resin. Ultrathin sections (60 µm) were mounted on collodion carbon-coated copper grids, contrasted using uranyl acetate and lead citrate, and examined at 80 kV with a TEM (Jeol 100CX II).

**Example 14: Elimination of Shc in B. diazoefficiens**

To eliminate hopanoid production in *B. diazoefficiens*, and to test whether a requirement for hopanoids in efficient symbiosis is conserved between *B. diazoefficiens* and *Bradyrhizobium* BTAII1, deletion of the gene encoding the enzyme catalyzing the first step in hopanoid biosynthesis, squalene hopene cyclase (Shc) (FIG. 2B) was carried out. A Δshc mutant was isolated using either the pK18mobacB-based markerless gene deletion method (—400 colonies screened) or the gene replacement strategy with pSP202polI4 (1200 colonies screened) [54]. No—we were NOT able to isolate this mutant. We don’t have an shc deletion for *B. diazoefficiens*.

This suggests that Shc may be essential either because hopanoids are required for growth and survival of *B. diazo-
Example 15: B. diazoefficiens ΔhpnP and ΔhpnH Mutants

To eliminate synthesis of 2Me- or C-35 hopanoids specifically, genes predicted to encode the C-2 methylase, hpnP [55] or the first enzyme catalyzing the extension of C-30 hopanoids, hpnH [12] (FIG. 2B) were deleted.

As illustrated in FIG. 2C, no methylated hopanoids were detected in ΔhpnP TLE using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (Tables S1 and S2) [26, 41]. ΔhpnH does not make any detectable C-35 hopanoids, including aminotriol (a, c), BHP-508 (VIII, degradation product of aminotriol), bacteriohopanetetrol (b, e) and adenosylhopanone (d). In addition, ΔhpnH accumulates a 6-fold excess of the HpnP substrate [12], diploptene (IV, WT 18±2 µg/mg TLE, ΔhpnP—29±6 µg/mg TLE, ΔhpnH—111±3 µg/mg TLE).

The presence of HoLA in the mutants using MALDI-MS (FIG. 4) was also analyzed. WT and ΔhpnP lipid A are composed of a mixture of penta- to hepta-acylated species, whereas ΔhpnH lipidA is mainly hexa-acylated (FIG. 1B). In WT and ΔhpnH hepta-acylated species, a C-35 hopanediolic acid is ester-linked to hexa-acylated lipid A, and traces of a second hopanoid substitution are also detected; conversely, ΔhpnH is missing any lipid A-bound hopanoids. Not only do the results confirm the proposed roles of HpnP and HpnH, they also show that synthesis of C-35 hopanoids is required for HoLA production.

Example 16: Hopanoids Contribute to Outer Membrane Rrigidity

A fluorescence polarization method was employed by incubating the dye diphenyl hexatriene (DPH) with whole cells to determine whether 2Me- and C-35 hopanoids affect the rigidity of B. diazoefficiens membranes at 25°C and 40°C (FIG. 5). Because previous studies of whole cells of different R. palustris hopanoid mutants indicated that the majority of DPH gets incorporated into the OM, whole cell polarization values was interpreted to reflect the rigidity of the OM [8, 26].

Membranes of all strains were less rigid at higher temperature. The ΔhpnP membrane was as rigid as the WT membrane at both temperatures, whereas the ΔhpnH membrane was less rigid. Thus, C-35 hopanoids are important for maintaining membrane rigidity in B. diazoefficiens in vivo, in contrast to R. palustris, where the ΔhpnP membrane showed similar rigidity to the WT, despite the capacity of C-35 hopanoids to enhance rigidity in vitro [8]. This indicates that the fraction of C-35 hopanoids or HoLA in the OM may be greater in B. diazoefficiens than R. palustris. Despite the lack of C-35 hopanoids, the ΔhpnH membrane is morphologically indistinguishable from the WT membrane, as seen in whole cell cryo-transmission electron microscopy (TEM) micrographs (FIG. 6).

TABLE 7

<table>
<thead>
<tr>
<th>Growth</th>
<th>% Total hopanoid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>condition</td>
<td>Strain</td>
</tr>
<tr>
<td>Aerobic</td>
<td>WT</td>
</tr>
<tr>
<td>(PSY)</td>
<td>ΔhpnP</td>
</tr>
<tr>
<td>ΔhpnH</td>
<td>94 ± 0</td>
</tr>
<tr>
<td>Microaerobic</td>
<td>WT</td>
</tr>
<tr>
<td>ΔhpnP</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>ΔhpnH</td>
<td>NGa</td>
</tr>
</tbody>
</table>

*Total hopanoids = methylated and unmethylated versions of C-35 hopanoids (compounds II, III, V, VI, VII) + tetrahymanol (VII) and C-35 hopanoids (VII) (refer to table 5 for temporal names).

b% 2Me = ratio of methylated to total hopanoids

aNG, no growth
Under acidic conditions (pH=6), ΔhpnH is unable to grow (FIG. 7D). ΔhpnH is also more prone to stationary phase stress, osmotic stressors (NaCl and inositol) and membrane destabilizers (bile salts and ethylenediaminetetraacetic acid, EDTA [12]) than WT, as evidenced by a reduction in ΔhpnH growth on stressor gradient plates (FIG. 7E).

Additionally, disc diffusion assays showed that ΔhpnH is more sensitive to oxidative (hydrogen peroxide (H₂O₂)), detergent (SDS) and acidic (hydrochloric acid (HCl)) stresses than WT (FIG. 7F).

Because B. diazoefficiens is exposed to NCRs in A. afraspera, the sensitivity of ΔhpnH to two antimicrobial peptides, polymyxin B [57] and NCR335 from the legume Medicago truncatula [58] was also tested. ΔhpnH displayed a 10-fold lower MIC (48 µg/ml) for polymyxin B than WT (512 µg/ml). In addition, ΔhpnH was found to be 100-fold more susceptible than WT to NCR335 (FIG. 7G). ΔhpnP withstood all of the aforementioned stressors as well as the WT, with the exception of acidic stress, where it grew slower than WT.

Example 19: C₄₅ Hopanoids are Required to Establish an Efficient Symbiosis with A. Afraspera and with Soybean

Because hopanoids are required for microaerobic growth and stress tolerance in the free-living state, it is hypothesized that they would aid survival within the plant microenvironment. To test this, the symbiotic phenotypes of ΔhpnP and ΔhpnH on two host plants, soybean and A. afraspera were analyzed.

FIG. 8 shows the symbiotic phenotypes of ΔhpnP and ΔhpnH on soybean. On soybean, at 21 days post inoculation (d.p.i.), both mutants induced fewer nodules and displayed reduced nitrogenase activity as estimated by the acetylene reduction assay (ARA) relative to WT (FIG. 8, panels A-C). The ARA data shown in FIG. 8, panel B has a unit of µcool per plant as a result of normalization per gram. As further described with respect to FIG. 11, panels (b) and (d), the N₂-fixation deficit of ΔhpnH is due to the reduction in nodule mass and the normalization of ARA rates by nodule dry weight eliminates the N₂-fixation rate difference between wild type and ΔhpnH (see FIG. 11, panel d, upper graph vs. FIG. 11, panel b, lower-right graph). Therefore, one would expect that if the ARA data of FIG. 8, panel B is not normalized per gram, the differences between the WT and ΔhpnH mutant would be expected to be more statistically significant. Thus, under the conditions of these assays, hpnH mutation appears to have a symbiotic effect on the host plants when B. diazoefficiens infects soybean.

FIG. 9 shows the symbiotic phenotypes of ΔhpnP and ΔhpnH on A. afraspera. Plants inoculated with ΔhpnH displayed typical nitrogen starvation symptoms, including foliage chlorosis, reduced plant growth and half the ARA activity of WT- and ΔhpnP-infected plants (FIG. 9, panels A, B). Reduced nitrogen fixation was not due to a decrease in the number of nodules, which was comparable in WT- and ΔhpnP-infected plants at 9, 14 and 21 d.p.i., suggesting that the hpnH mutation does not affect nodule organogenesis (FIG. 9, panels C and 10). However, cytological analyses revealed that ΔhpnH nodules displayed several disorders in comparison to WT and ΔhpnP nodules (FIG. 9, panels D-Z).

At the cellular level, ΔhpnH nodules were smaller (FIG. 9, panels D-F) and had pink or, in ~30% of cases, even white central tissue in contrast to WT and ΔhpnP nodules, which were dark pink due to the accumulation of the O₂-carrier, leghemoglobin (FIG. 9, panels G-I). The central symbiotic tissue of ΔhpnH nodules was often disorganized and partially infected (FIG. 9, panels L, M, R), as opposed to the fully occupied tissue of WT and ΔhpnP nodules (FIG. 9, panels J, K, P, T). In some ΔhpnH nodules, the presence of necrotic regions—characterized by the accumulation of autofluorescent brown compounds—could be seen (FIG. 9, panels M, N). These are likely polyphenol compounds whose production is associated with plant defense responses [59]. Within ΔhpnH nodules, iodine staining also revealed accumulation of starch granules in the non-infected cells surrounding the symbiotic tissue, whereas such granules were rarely observed in WT and ΔhpnP nodules (FIG. 9O). Starch accumulation is indicative of an imbalance between the photosynthates furnished by the plant and the ability of the bacteria to metabolize them, a typical feature of non-fixing or underperforming strains [60, 61].

To determine whether ΔhpnH symbiotic defects stem from a problem in the bacterial differentiation process or are due to a damaged membrane, nodule sections were examined by confocal microscopy using live-dead staining [62] and TEM to analyze the ultrastructure of bacteroids. Confocal microscopy revealed that all strains, including ΔhpnH, differentiated properly into elongated bacteroids, which were, for the majority, viable, as indicated by the green Syto9 staining (FIG. 9, panels P-U). However, TEM analysis showed that the cell envelope of some ΔhpnH bacteroids was not well delineated and in a few cases, even broken (FIG. 9, panels Y-Z). Similar damage was seen in the peribacteroid membrane that surrounds bacteroids. Deposits of cellular material, possibly resulting from the release of plant or bacterial cytoplasm, were also observed in the peribacteroid space, suggesting a beginning of senescence or perhaps necrosis of symbiotic bacterial cells (FIG. 9, panels Z, Y). Such defects were not observed in the WT (FIG. 9, panels V-X) or ΔhpnP nodules.

Taken together, the data indicate that under these conditions C₄₅ hopanoids, but not 2Me-hopanoids, play an important role in facilitating the fitness of B. diazoefficiens in symbiosis with A. afraspera and soybean.

Two reasons the plant host mounts an immune response against ΔhpnH may be that the altered mutant surface layer, as seen in TEM images, is unable to suppress this response [63] and/or the host induces nodule senescence pre-maturely on detecting an under-productive symbiont [64]. Consistent with this, nitrogenase activity is reduced in ΔhpnH relative to WT, a likely consequence of poor cell viability. Similarly, the build-up of plant carbon as starch in ΔhpnH nodules might indicate slow metabolism and/or perturbation of membrane transport processes that facilitate bacteroid carbon acquisition.

Example 20: Assessment of Effects of ΔhpnH in B. diazoefficiens on A. afraspera Nodules

To assess survival rates of WT B. diazoefficiens and ΔhpnH mutants of B. diazoefficiens within A. afraspera root nodules, live cross-sections of WT and ΔhpnH within A. afraspera root nodules were stained with SYTO9 (a live cell-permeable DNA dye) and propidium iodide (a live cell-impermeable DNA dye that reports on the fraction of dead cells). Imaging these sections with confocal microscopy revealed that the density and proportion of live bacterial cells is indistinguishable between WT and ΔhpnH nodules as shown in FIG. 11, panel c. Instead, the main difference observed was a reduction in size in ΔhpnH nodules, reducing the number of infected plant cells.
To confirm the observed reduction in size, nodules were harvested from WT- and ΔhpnH-inoculated plants at 24 dpi and their dry weight determined. In particular, to calculate nodule dry weight, all nodules from each plant were harvested by hand, transferred into a pre-weighted Eppendorf tube and dried for 48 hours in a drying oven at 50°C before weighing.

As shown in FIG. 11, panel (d), lower graph, consistent with the microscopy data, the nodule dry mass per plant was ~50% less for ΔhpnH-inoculated plants. Since the normalization of acetylene reduction rates by nodule dry weight eliminates the N₂-fixation rate difference between wild type and ΔhpnH (see FIG. 11, panel d, upper graph), the results suggest that the N₂-fixation deficit of ΔhpnH is due to the reduction in nodule mass. The N₂-fixation rate per bacterium is likely similar between the two strains. Thus, the primary symbiotic defect in the ΔhpnH mutant observed at 24 dpi appears to be an inhibition of proper root nodule development.

To further test how a nodule volume reduction at 24 dpi arises, Acetylene reduction assays were performed every 4 days between 5 dpi and 40 dpi. As shown in FIG. 12, panel a, a total of 36 plants were inoculated for each strain. At each time point, 4 inoculated plants for each strain (and 1 uninoculated control plant) were chosen randomly for ARA measurements. The experiment was repeated once.

The results shown in FIG. 12, panel b indicate that by 40 dpi, the per-nodule N₂-fixation rates, number of nodules per plant, and nodule dry weight per plant are indistinguishable between the strains. These data demonstrate that a developmental arrest in ΔhpnH nodules is not sufficient to explain their low N₂-fixation rates.

Example 21: Computational Modeling of Root Nodule Development in B. diazoefficiens WT and ΔhpnH Mutant

Plants were inoculated as previously described. After 5-7 dpi, 5 plants each for WT and ΔhpnH were removed from their plant culture tubes and transferred to a plastic imaging dish containing pre-warmed, sterile plant medium. Images of plant roots were taken using a Keyence digital microscope and manually aligned. After imaging each plant was returned to its original culture tube and returned to the plant cultivation chamber. Plant roots were imaged every 2-4 days for 40 days; only nodules that were visible within 14 dpi were tracked, due to the increasing likelihood of cross-contamination over time.

For each nodule, the radius was measured and the nodule volumes were estimated by approximating nodules as spheres. FIG. 13, panel a plots raw nodule volumes over time (dpi). Multiple models were tested to identify the function that best fit the nodule growth curves. FIG. 13, panel d plots nodule volumes fit to quadratic, exponential or sigmoidal curves and panel e shows additional parameter fitting for the sigmoidal fit.

The following parameters were extracted from each fitted sigmoidal curve: dV/dt, maximum nodule growth rates; V₀, the volume of the first infected cell; t₀, the time at which a nodule is visible by eye; Vₘₐₓ, the smallest nodule volume visible by eye; tₚₐₓ, the time at which nodule growth has leveled off, and Vₘₐₓ, the volume of the nodule when nodule growth stops; dV/dt, the rate of increase in nodule volume between tₚₐₓ and tₘᵢₙ. Panel b shows sample wild-type nodule growth time course. Nodule radii are measured directly and the nodule volume is determined by approximation of nodules as spheres. Panel c plots the distribution of newly-emerged nodules over time (in dpi) for wild-type and ΔhpnH nodules from 40 plants each.

For each nodule, a sigmoidal curve, such as the sigmoidal curve shown in FIG. 13, panel f, was generated, from which dV/dt, t₀, and Vₘₐₓ were extracted. FIG. 14 panels d,e,f plot the distributions of dV/dt, predicted t₀, and Vₘₐₓ for about 75 wild-type nodules and about 50 ΔhpnH nodules.

The results shown in FIG. 14, panels d-f suggest that both reduced nodule growth rates and more variable nodule initiation times occur for ΔhpnH nodules, and preliminary computational simulations suggest they contribute equally to nodule size defects.

Example 22: Polymerase Chain Reaction-Based Cloning of Hopanoid Synthesis Genes

In the following paragraphs, an exemplary procedure is provided that is expected to provide effective genetic modification of rhizobia incapable of producing C₃₅ hopanoids (C₃₅ hopanoid-deficient rhizobia) is described.

In some cases, genetic modification of C₃₅ hopanoid-deficient rhizobia can be performed by polymerase chain reaction-based cloning of C₃₅ hopanoid synthesis genes, based on some common approaches described in related literatures such as the method in Welander et al (2012) [11] which is incorporated herein by reference in its entirety. Briefly, C₃₅ hopanoid synthesis genes including one or more of shc, hpnH, hpnG, hpnO, hpnP, hpnC, hpnD and hpnF shown in FIG. 2, panel B are cloned into a rhizobium expression plasmid vector. A suitable plasmid vector such as those described in Welander et al (2009) [11], Ledermann et al (2015) Mol Plant Microbe Interact. 28:959, or Vincze and Bowra (2006) [65] are used. For example, a plasmid with broad host range such as pPZP211 was engineered containing a spectinomycin resistance gene, an origin of replication recognized by the recipient rhizobium and E. coli, and compatible cloning sites comprising unique restriction endonuclease recognition sites. The cloning site where a C₃₅ hopanoid synthesis gene inserted downstream of a promoter recognized by the recipient rhizobium, to ensure expression of the C₃₅ hopanoid synthesis gene in the recipient rhizobium. Primers used to amplify a C₃₅ hopanoid synthesis gene comprise forward and reverse sequences complementary with 5’ and 3’ ends of a C₃₅ hopanoid synthesis gene sequence, flanked by sequences for compatible unique restriction endonuclease recognition sites that are present in the insertion site in the plasmid.

The C₃₅ hopanoid producing rhizobium Bradyrhizobium diazoefficiens can be used as donor species, from which the hopanoid biosynthesis genes are obtained. Genomic DNA from Bradyrhizobium diazoefficiens is isolated using a DNeasy Blood and Tissue Kit (Qiagen). Primers are designed based on the DNA sequences for the hopanoid synthesis gene cluster required to amplify genes required for C₃₅ hopanoid biosynthesis. For example hpnF (Genbank locus tag AV28 RS11540) as shown in Table 2,
with the addition of appropriate restriction endonuclease recognition sites at the 5’ and 3’ ends flanking the gene

HapnH for example, the C35 hopanoid synthesis gene hpnH is amplified using PCR and the resulting amplicon is analyzed by agarose gel electrophoresis. An amplicon band of the expected size is excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The purified hpnH amplicon is then cloned into the expression plasmid vector, for example pPZP211 containing an antibiotic resistance gene, for example spectinomycin to permit selection of positive clones, as follows. The plasmid and hpnH amplicon insert are digested with restriction enzymes to create compatible DNA ends for the inserting the hpnH amplicon into the pPZP211 cloning site. For ligation of the insert into the plasmid, the plasmid is mixed with the hpnH insert, ligase buffer and T4 DNA ligase, and incubated at room temperature for 30-60 minutes. One aliquot of E. coli S17-1 competent cells is transformed by electroporation with the ligation mixture, and streaked onto Luria-Bertani (LB) agar plates containing antibiotic, for example spectinomycin. 12-16 hours later, colonies are picked and used to inoculate LB broth cultures containing spectinomycin.

After cultures are grown, 1 ml of bacterial culture is used for preparing glycerol stocks, which is stored at —80° C. and used for starting subsequent cultures. The remainder of the transformed E. coli S17-1 culture is used for plasmid purification using a Qiagen Prep Spin Miniprep Kit (Qiagen). Integrity of cloned plasmids is assessed using analytical restriction endonuclease digestion and gel electrophoresis. Following identification of positive clones by analytical restriction digest analysis, clones are also analyzed by DNA sequencing, using primers designed to bind to sequences flanking the inserted amplicon in the plasmid.

Example 23: Transfer of a Plasmid Vector Comprising a hpnH Expression Plasmid into a Recipient Rhizobium That Naturally Expresses all Genes Required for C35 Hopanoid Synthesis Except hpnH, by Conjugation with E. coli S17-1 Derivatives

In one example, transfer of a plasmid vector expressing hpnH into a recipient rhizobium expressing all genes required for C35 hopanoid synthesis except hpnH is expected to be performed by conjugation with E. coli S-17 cells that are transformed with a plasmid vector containing the hopanoid synthesis genes, as follows.

Late log cultures of E. coli S17-1 (DKNI) strain (5-10 ml) and a rhizobium recipient strain (30-40 ml) are harvested by centrifugation (10 min, 5,000 rpm). The supernatant is discarded and cells are washed with 10-20 ml sterile 0.9% NaCl. The OD_{500} of this solution is measured and cells are again centrifuged. Cells are resuspended in a volume to yield an OD_{500} of approximately 4. 250 µl of E. coli donor cells are mixed with 750 µl of a rhizobium recipient strain. The cell mixture is centrifuged (1 min, 13,000 rpm, RT) and the supernatant reduced to ~50 µl in which the cells are resuspended. The resuspended cells are transferred as a drop onto a plate containing suitable growth medium, which is dried for approx. 15 min under a laminar flow bench. The plate is incubated for at least two days at 30° C. The cells paste is collected from the plate with a loop, resuspended in 2 ml 0.9% NaCl and appropriate aliquots (e.g. 50, 100, 200 µl) are streaked on selective agar plates containing suitable growth medium and appropriate selection antibiotic. E. coli S17-1 donor cells are counter-selected with 20 µg/ml chloramphenicol which does not interfere with growth of the recipient rhizobial cells.

Colonies and grown for 48 h in the presence of appropriate selection antibiotic. Plasmid DNA is isolated from the recipient rhizobium transformants using a Qiagen plasmid preparation kit, following the manufacturer’s directions. Plasmids are analyzed using restriction endonuclease digests and gel electrophoresis and DNA sequencing.

Example 24: Preparation of Transformation-Competent S. meliloti Cells

As an alternative to conjugation method in Example 23, transfer of plasmids containing hopanoid synthesis genes into recipient S. meliloti cells is expected to be performable using transformation methods. Prior to transformation, S. meliloti cells are expected to be treated to become transformation-competent, based on the protocol of Vinceze and Bowra (2006) [65] as follows. PSY medium is inoculated with S. meliloti and grown at 28° C. with vigorous shaking until it reaches the stationary growth phase.

Two milliliters of the stationary-phase cultures is used to inoculate 50 ml of PSY medium and incubated with shaking for 6 h at 28° C. Cells are harvested by centrifugation at 12,000 g for 10 min at 4° C., and the pellet is resuspended in 2 ml of ice-cold 20 mM CaCl₂ solution. The resulting cell suspension is placed into ice-cold 1.5-ml microcentrifuge tubes in aliquots of 100 ul before being snap-frozen in liquid N₂. The prepared competent cells are stored at ~80° C.

Example 25. Transformation of Competent Recipient S. meliloti Cells with Plasmid Vectors Comprising Hopanoid Synthesis Genes Using an Electroporation Method

In one example, S. meliloti are expected to be transformable with plasmid vectors containing hopanoid synthesis genes using an electroporation method, based on the method of Garg et al (1999) [66].

In particular, recipient S. meliloti cells can be grown for 72 h at 30° C. with vigorous shaking to mid-logarithmic phase (absorbance at 600 nm of 0.4 to 0.6). Cells are prepared for electroporation by a modification of the procedure of Dower et al (1988) [67]. Cells can be chilled for 15 to 30 min on ice and then harvested by centrifugation at 9,000 rpm for 10 min at 4° C. The cell pellet is washed four times with cold sterile deionized water and finally washed with 10% glycerol. The cells can be resuspended in 10% glycerol to have an approximate concentration of 10^{10} to 10^{11} colony-forming units/ml (CFU/ml) and kept on ice. The cell suspension can be distributed in aliquots of 90 µl and mixed thoroughly with plasmid vector containing hopanoid synthesis genes (2 µg) by vortexing at high speed for 10 s and then kept on ice for 30 min. The cell-DNA mixture can be loaded in a chilled electroporation cuvette with a 0.1-cm gap (BTX Inc., San Diego, Calif.) and is subjected to a single pulse of high voltage. For pulse generation, an electrocell manipulator, model 600 (BTX Inc.), may be used that is capable of generating a field strength of up to 25 kV/cm with a 0.1-cm-gap cuvette. After the pulse is delivered, the cuvettes are kept on ice for 10 min. The electroporated cells are suspended in PSY broth and incubated for 24 h at 30° C. The cell suspension is diluted and plated on selective medium containing the appropriate antibiotic.
Colonies from the electrot transformed S. meliloti can be selected and grown for 48 h in the presence of appropriate selection antibiotic. Plasmid DNA is isolated from the S. meliloti transformants using a Qiagen plasmid preparation kit, following the manufacturer’s directions. Plasmids are analyzed using restriction endonuclease digestions and gel electrophoresis and DNA sequencing.

Example 26: Transformation of Competent Recipient S. meliloti Cells with Plasmid Vectors Comprising Hapnonoid Synthesis Genes Using a Freeze-Thaw Method

In one example, transformation of competent S. meliloti cells with plasmid vectors containing haponoid synthesis genes is expected to be performable following a “freeze-thaw” method based on that of Vincze and Bowra (2006) [65]. Approximately 1 ug of a vector plasmid containing cloned haponoid synthesis genes, can be made up to a volume of 5 ul with sterile distilled water. This can be then added to a 100 ul aliquot of competent cells immediately after they are removed from -80° C. Subsequently, the mixture is kept at 37° C. for 5 min with 37° C. shaking. For the recovery phase, 1 ml of the appropriate medium is added to the transformed cells before they are transferred to 10-ml tubes and incubated at 28° C. for 2 h with shaking. To determine the actual transformation efficiency, the cell suspension is diluted and plated on nonselective agar medium to count the cells. Cells without added DNA and the appropriately diluted transformation mixture are plated on selective medium to calculate the number of spontaneous resistant colonies and transformation efficiency, respectively.

Similar to the results of Vincze and Bowra (2006), it is expected that a 6-h growth period for competent-cell preparation will be sufficient to produce transformants of fast-growing species of Sinorhizobium. The S. meliloti transformants are checked for the presence of the introduced plasmid comprising cloned haponoid synthesis genes. Colonies from the transformed S. meliloti are selected and grown for 48 h in the presence of appropriate selection antibiotic. Plasmid DNA is isolated from the S. meliloti transformants using a Qiagen plasmid preparation kit, following the manufacturer’s directions. Plasmids are analyzed using restriction endonuclease digestions and gel electrophoresis and DNA sequencing.

Example 27: Transformation of Competent Recipient S. meliloti Cells with Plasmid Vectors Comprising Hapnonoid Synthesis Genes Using a Thermal Shock Method

In one embodiment, transformation of S. meliloti with plasmid vectors containing haponoid synthesis genes is expected to be performable by a “thermal shock” method, based on a protocol by Courtois et al. (1988) [68], as follows.

The recipient S. meliloti is grown up in the appropriate medium in a rotary bath shaker (60 revolutions per min) at 30° C. to a density of 10⁶ CFU/ml, and it maintained static at 30° C. After 3 h of incubation, the cells are harvested by centrifugation at 3,000xg for 10 min and suspended in the appropriate medium to give a cell density of 10⁷ CFU/ml.

A 0.2-ml portion of this suspension can be added to 0.1 ml of vector plasmid containing haponoid synthesis genes dissolved in 0.15 M NaCl (pH 7). The mixture is chilled rapidly at 0° C. After 15 min at 0° C, it is transferred for 5 min at 37° C. then at 0° C. After 40 min, the mixture is incubated for 30 min in a 30° C. water bath. Then, to allow phenotypic expression of the drug markers, the cells are diluted in appropriate medium and streaked on selective agar plates containing appropriate medium and antibiotic. After 4 days of incubation at 30° C., S. meliloti transformant colonies are analyzed for the presence of the plasmid vector.

Colonies from the different electrot transformed S. meliloti can be selected and grown for 48 h in the presence of appropriate selection antibiotic. Plasmid DNA is isolated from the S. meliloti transformants using a Qiagen plasmid preparation kit, following the manufacturer’s directions. Plasmids are analyzed using restriction endonuclease digestions and gel electrophoresis and DNA sequencing.

Example 28: Assessment of the Ability of S. meliloti Harboring Plasmid Vectors Containing Hapnonoid Synthesis Genes to Form Nodules

In one embodiment, assessment of the ability of genetically modified S. meliloti harboring plasmid vectors containing haponoid synthesis genes to form nodules is expected to be based on the method of Vincze and Bowra (2006) [65]. It is expected that the presence of the plasmid vector will not prevent the transformed S. meliloti from forming effective nodules on legumes. To confirm the stability of the plasmid in the S. meliloti during nodule formation, bacteria and bacteroids are isolated from nodules 4 weeks after inoculation and are characterized, as follows. The bacteria are isolated on nonselective medium from the surface-sterilized nodules. The colonies are tested for antibiotic resistance on selective plates, and the restriction endonuclease digest pattern of the purified plasmid was analyzed by gel electrophoresis. It is expected that no loss of antibiotic resistance is observed, and the S. meliloti isolated from the nodules harbors the plasmid vector containing the haponoid synthesis genes.

Example 29: Analysis of C₃₅ Hapnonoid Production in Rhizobia

In some cases, screening of rhizobia for the production of C₃₅ haponoids is expected to be performed using analysis of lipids extracted from cultured rhizobia for the presence of C35 haponoids, based on some common approaches described in related literatures such as the method in Welander et al. (2012) [12], which is incorporated herein by reference in its entirety.

Briefly, rhizobial cells can be grown in YPS medium under aerobic conditions at 30° C. to stationary phase (3 days). Cells are harvested by centrifugation at 4° C. and lipids are extracted by sonication the cells for 15 minutes at room temperature in 10 ml of 10:5:4 (v:v) methanol (MeOH): dichloromethane (DCM):water, Samples are centrifuged for 10 minutes at 3000xg and the supernatant is transferred to a new tube. Cell pellets are sonicated again in 10 ml of MeOH:DCM:water (10:5:4, v/v/v), centrifuged, and the supernatant combined with the first extraction.

The samples can be separated into two phases by adding 20 ml 1:1 (v/v) DCM:water, centrifuged for 10 minutes at 3000xg, and the organic phase is transferred to a new vial. To the remaining aqueous phase, 10 ml of DCM:water (1:1, v/v) is added again, centrifuged, and the organic phase was combined with the previous extract. The organic solvents are evaporated under N₂ and the total lipid extract (TLE) is redissolved in 2 ml DCM. The TLE is divided into two 1 ml aliquots. One aliquot is separated by chromatography on a
silica gel column. Six fractions are eluted: F1: hexane; F2: hexane:DCM (4:1, v/v); F3: DCM; F4: DCM:ethyl acetate (EtOAC) (4:1, v/v); F5: EtOAc; F6: MeOH. Separation of the TLE facilitated the detection of diplopterol in fraction 4. Fractions 4, 5, and 6 and the remaining TLEs are incubated in 100 μl of acetic anhydride-pyridine (1:1, v/v) at 1 h at 70°C to derivatize alcohols into ester esters. The hydro- carbon fractions (F1 and F2), the acetylated fractions (F4, F5, and F6), and the acetylated TLEs are analyzed by high temperature gas chromatography-mass spectrometry (GC-MS) as previously described [Welander et al., 2009] [11].

The acetylated TLEs can also be analyzed by liquid chromatography-mass spectrometry (LC-MS). A Poroshell 120 EC-C18 column (2.1x150 mm, 2.7 μm; Agilent Technologies), set at 30°C, is eluted isocratically first with MeOH/water (95:5, v:v) for 2 min at a flow rate of 0.15 ml/min, then using a linear gradient up to 20% (v) of isopropanol alcohol (IPA) over 18 min at a flow rate of 0.19 ml/min, and isocratic for 10 min. The linear gradient is then set to 30% (v) of IPA at 0.19 ml/min over 10 min, and maintained for 5 min. The column is subsequently eluted using a linear gradient up to 80% IPA (v) over 1 min at a flow rate of 0.15 ml/min and isocratic for 14 min. Finally the column was eluted with MeOH/water (95:5, v:v) at 0.15 ml/min for 5 min. The APCI parameters were as follows: gas temperature 325°C, vaporizer temperature 350°C, drying gas (N2) flow 6 1/min, nebulizer (N2) flow 30 1/min, capillary voltage 1200 V, corona needle 4µA, fragmentor 150 V. Data are recorded by scanning from m/z 100 to 1600.

Identification of the hopanoids is done using their exact mass and by comparison of the retention time and the mass spectra with published data [Talbot et al., 2007; Talbot et al., 2003b] [69, 70].

Example 30: Genetic Screen of Stress-Resistant Rhizobia as a Biofertilizer

Screening of rhizobia for the production of C35 hopanoids is expected to be performed by using a method of performing a diagnostic screen for the presence of C35 hopanoid synthesis. Oligonucleotide primers are designed to amplify sequences of genes for C35 hopanoid synthesis, based on the DNA sequences of of the genes.

Briefly, genomic DNA can be isolated from rhizobia and diagnostic PCR is performed using primers to amplify C35 hopanoid synthesis genes. The resulting amplicons are analyzed by gel electrophoresis to confirm expected DNA band molecular weight. In addition, DNA sequencing is performed on genomic DNA samples to confirm the presence of hopanoid synthesis genes.

The diagnostic PCR and DNA sequencing can determine whether C35 hopanoid synthesis genes are present in the genome of a rhizobium sample, and therefore whether the rhizobium is capable of synthesizing C35 hopanoids, and thus to be used as biofertilizer with enhanced stress-resistance properties.

The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, microorganisms, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Those skilled in the art will recognize how to adapt the features of the exemplified hopanoids, hopanoids-producing nitrogen-fixing bacteria, leguminous plants and related formulation and uses to others according to various embodiments and scope of the claims.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the disclosure pertains.

The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, microorganisms, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Those skilled in the art will recognize how to adapt the features of the exemplified hopanoids, hopanoids-producing nitrogen-fixing bacteria, leguminous plants and related formulation and uses to others according to various embodiments and scope of the claims.

The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, microorganisms, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Those skilled in the art will recognize how to adapt the features of the exemplified hopanoids, hopanoids-producing nitrogen-fixing bacteria, leguminous plants and related formulation and uses to others according to various embodiments and scope of the claims.

The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, microorganisms, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Those skilled in the art will recognize how to adapt the features of the exemplified hopanoids, hopanoids-producing nitrogen-fixing bacteria, leguminous plants and related formulation and uses to others according to various embodiments and scope of the claims.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the disclosure pertains.

The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, microorganisms, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Those skilled in the art will recognize how to adapt the features of the exemplified hopanoids, hopanoids-producing nitrogen-fixing bacteria, leguminous plants and related formulation and uses to others according to various embodiments and scope of the claims.
ranges and all subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure. Any one or more individual members of a range or group disclosed herein may be excluded from a claim of this disclosure. The disclosure illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein.

A number of embodiments of the disclosure have been described. The specific embodiments provided herein are examples of useful embodiments of the invention and it will be apparent to one skilled in the art that the disclosure can be carried out using a large number of variations of the devices, device components, methods steps set forth in the present description. As will be obvious to one of skill in the art, methods and devices useful for the present methods may include a large number of optional composition and processing elements and steps.

In particular, it will be understood that various modifications may be made without departing from the spirit and scope of the present disclosure. Accordingly, other embodiments are within the scope of the following claims.

REFERENCES


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**Sequence 26**

**Sequence 27**

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The invention claimed is:

1. A biofertilizer for a leguminous plant essentially consisting of one or more nitrogen-fixing rhizobia naturally incapable of producing C$_{35}$ hopanoids and genetically engineered to include a set of genes of a nitrogen-fixing rhizobia naturally capable of producing C$_{35}$ hopanoids, enabling production of C$_{35}$ hopanoids by the one or more nitrogen-fixing rhizobia naturally incapable of producing C$_{35}$ hopanoids, in a form suitable for administration to one or more leguminous plant or seed, and/or for administration to a soil surrounding the one or more leguminous plant or seed, wherein the set of genes comprises the gene encoding a squalene-hopene cyclase, hpnH gene encoding a B12 binding radical SAM, and gene hpnG encoding for a nucleosidase.

2. The biofertilizer of claim 1, wherein the one or more leguminous plant or seed comprises at least one selected from the group consisting of Rhizobium etli, Rhizobium leguminosarum, Mesorhizobium loti, Sinorhizobium meliloti, Azorhizobium caulinodans, and Ochrobactrum anthropi.

3. The method to provide biofertilizer for a leguminous plant of claim 1, the method comprising

   - genetically engineering a nitrogen-fixing rhizobia strain incapable of producing C$_{35}$ hopanoids to introduce C$_{35}$ synthesis genes of a nitrogen-fixing rhizobia naturally capable of producing C$_{35}$ hopanoids, and
   - providing the genetically engineered nitrogen-fixing rhizobia strain in a form suitable for administration to one or more leguminous plants or seed thus providing the biofertilizer for the leguminous plant.

5. A biofertilizer composition for a leguminous plant, the one or more biofertilizers essentially consisting of one or more genetically engineered nitrogen-fixing rhizobia capable of producing C$_{35}$ hopanoids of claim 1 together with an acceptable vehicle.

6. The biofertilizer of claim 5, wherein the vehicle comprises at least one Bradyrhizobium.

8. The biofertilizer composition of claim 5, wherein the vehicle comprises at least one selected from the group consisting of peat, vermiculite, lignite powder, clay, talc, rice bran, seed, rock phosphate pellet, charcoal, soil, paddy straw compost, wheat bran, gum Arabic, methylcellulose, sucrose solutions, vegetable oils and a mixture thereof.

9. The biofertilizer composition of claim 5, wherein the one or more nitrogen-fixing bacteria further comprise at least one Bradyrhizobium.

11. The biofertilizer composition of claim 10, wherein the one or more C$_{35}$ hopanoids comprise the one or more C$_{35}$ hopanoids having a formula of (I)

\[
\begin{align*}
\text{H}_24 & \\
\text{R}_{11}, \text{R}_{12}, \text{R}_{13} & \text{are independently selected from H, D, methyl, or ethyl groups;} \\
\text{R}_1, \text{R}_3, \text{R}_4, \text{and R}_5 & \text{are selected from H, D, methyl, hydroxymethyl, aminomethyl, hydroxyl, or amino groups, wherein at least three of the R}_1, \text{R}_3, \text{R}_4, \text{and R}_5 \text{groups each contains hydroxymethyl, aminomethyl, hydroxyl, or amino groups;} \\
\text{R}_2 & \text{is selected from OH, NH}_2, \text{hydroxymethyl, aminomethyl, formula (II)}
\end{align*}
\]
for a time and under conditions allowing interaction of the
in the administered biofertilizer and/or biofertilizer compo-
in 2'-methyl bacteriohopanetriol, aminobacteriohopanetriol,
in 2Me-aminobacteriohopanetriol, bacteriohopanetetrol (BHT)
more C35 hopanoids comprise at least one C35 hopanoid
more C35 hopanoids, and wherein the applying is performed
more biofertilizer is performed in combination with one or
method comprising
18. The method of claim 17, the method further comprising
coating and/or inoculating one or more C35 hopanoids before
coating and/or inoculating the one or more seeds of the leguminous plant with the one or more biofertilizer.

19. A system to fertilize leguminous plants, the system comprising one or more biofertilizer essentially consisting of one or more nitrogen-fixing rhizobia naturally incapable of producing C35 hopanoids and genetically engineered to include a set of genes of a nitrogen-fixing rhizobia naturally capable of producing C35 hopanoids, enabling production of C35 hopanoids by the one or more nitrogen fixing rhizobia naturally incapable of producing C35 hopanoids, and/or biofertilizer compositions comprising said one or more biofertilizer and one or more C35 hopanoids for simultaneous, sequential or combined use in fertilizing a leguminous plant
wherein the set of genes comprises she gene encoding a
squalene-hopene cyclase, hpnH gene encoding a B12 binding radical SAM, and gene hpnG encoding for a nucleosidase.
20. A system to fertilize a leguminous plant, the system comprising one or more leguminous seed coated with one or more biofertilizer essentially consisting of one or more nitrogen-fixing rhizobia naturally incapable of producing C35 hopanoids and genetically engineered to include a set of genes of a nitrogen-fixing rhizobia naturally capable of producing C35 hopanoids, enabling production of C35 hopanoids by the one or more nitrogen-fixing rhizobia and/or biofertilizer composition comprising said one or more biofertilizer and one or more C35 hopanoids for simultaneous, sequential or combined use in fertilizing a leguminous plant
wherein the set of genes comprises squalene-hopene
cyclase she gene, B12 binding radical SAM hpnH gene, and nucleosidase hpnG gene.
21. The system of claim 19, wherein the one or more nitrogen-fixing rhizobia comprise at least one selected from the group consisting of Rhizobium etli, Rhizobium leguminosarum, Mesorhizobium loti, Sinorhizobium meliloti, Azorhizobium caulinodans, and Ochrobactrum anthropi.
22. The biofertilizer of claim 10, wherein the acceptable vehicle is a carrier allowing increased stability, viability and/or effectiveness of the gas exchange of the one or more nitrogen-fixing rhizobia.
23. The biofertilizer composition of claim 10, further comprising one or more bacteria selected from the group consisting of Bradyrhizobium BTA11, Bradyrhizobium japonicum, Bradyrhizobium diazoefficiens, Bradyrhizobium ORS278, and Methylbacterium nodulans.
24. The biofertilizer composition of claim 10, wherein the one or more nitrogen-fixing bacteria comprise Rhizobium etli, Rhizobium leguminosarum, Mesorhizobium loti, Sinorhizobium meliloti, Azorhizobium caulinodans, and Ochrobactrum anthropi.
25. The biofertilizer composition of claim 10, wherein the vehicle is a carrier selected from the group consisting of peat, vermiculite, lignite powder, clay, talc, rice bran, seed, rock phosphate pellet, charcoal, soil, paddy straw compost, wheat bran, gum Arabic, methylcellulose, sucrose solutions, vegetable oils and a mixture thereof.
26. The biofertilizer composition of claim 10, wherein the C35 hopanoids are covalently linked to lipid A to form HoLA.

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