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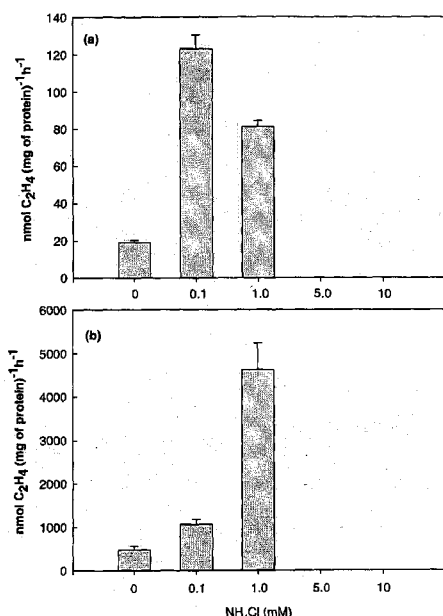
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- (54) **Title:** NITROGEN-FIXING BACTERIAL INOCULANT FOR IMPROVEMENT OF CROP PRODUCTIVITY AND REDUCTION OF NITROUS OXIDE EMISSION



- (57) **Abstract:** The present invention relates to methods of reducing chemical fertilizer usage and greenhouse gas nitrous oxide emission and to methods of improving plant growth rate and seed productivity in agriculture through the application of a novel artificially manufactured formula containing a nitrogen-fixing bacterium that efficiently colonizes non-legume plants in aerial parts and the root system. The bacteria inocula and methods are particularly suitable for plants in the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Ricinus*, *Oryza* and *Manihot*.



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NITROGEN-FIXING BACTERIAL INOCULANT FOR IMPROVEMENT OF CROP PRODUCTIVITY AND REDUCTION OF NITROUS OXIDE EMISSION

SEQUENCE SUBMISSION

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577211PCTSequenceListing.txt, created on 25 January 2013 and is 49 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to methods of reducing chemical fertilizer usage and greenhouse gas nitrous oxide (N₂O) emission and to methods of improving plant growth rate and seed productivity in agriculture through the application of a novel artificially manufactured formula containing a nitrogen-fixing bacterium that efficiently colonizes non-legume plants in aerial parts and the root system. The bacteria inocula and methods are particularly suitable for plants in the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Oryza*, *Ricinus* and *Manihot*.

[0003] The rapid rise in fossil fuel price, the fast diminishment of global fuel and the concern about rapid global warming resulted from the accumulation of atmospheric greenhouse gas have served as the three catalysts in the recent biofuel boom (Chang, 2007). The environmental benefit of biofuel consumption is attributed from the widely believed outcome of reduced greenhouse gas (CO₂) emission.

[0004] Recently, a new issue that has been brought up for debate regarding the benefit of biofuels. Nitrous oxide is produced naturally in the soil during the microbial processes of nitrification and denitrification. A significant percentage of the nitrogen fertilizer used in biofuel production is converted to reactive nitrogen N₂O, a greenhouse gas which has 310 times the ability to trap heat in the atmosphere (Barton and Atwater, 2002). The benefit of CO₂ mitigation through biofuel consumption will be cancelled out if the use of N-fertilizer is not controlled (Galloway et al., 2008; Melillo et al., 2009; Crutzen et al., 2008).

[0005] Application of nitrogen fertilizer has become an essential practice in modern agriculture as it is vital to maintain competitive crop productivity. It has been well known that legumes require much less input of nitrogen fertilizer owing to the presence of nitrogen-fixing structure called nodules, in which nitrogen-fixing microorganisms (diazotrophs), mostly belonging to species in the *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium* genera (Jourand et al., 2004; Kaneko et al., 2000; Stacey et al., 1991; Gottfert et al., 2001), form a mutually beneficial symbiotic relationship with the bacteria supplying nitrogen source to the

plants while drawing carbon source from the plants cells (Long, 1996; Young and Johnston, 1989). Some exception has been found recently. For example, *Methylobacterium nodulans* is the root nodule-inducing agent for some species in the genus *Crotalaria*, a leguminous plant (Renier et al., 2011; Jourand et al., 2005) and nitrogen-fixing nodules may develop in stem tissues of some legumes (Eaglesham and Szalay, 1983; Dreyfus and Dommergues, 1981). *Frankia*, Gram-positive soil bacteria also induce the formation of nitrogen-fixing root nodules in a few species in the *Rosaceae* family (Moir et al., 2011).

[0006] The formation of symbiotic nodules involves with complex genetic and chemical interactions between the diazotroph and host. For example, the symbiosis between *Sinorhizobium meliloti* and its plant hosts begins when the plant secretes an array of betaines and flavonoids into the rhizosphere: 4,4'-dihydroxy-2'-methoxychalcone, chrysoeriol, cynaroside, 4',7-dihydroxyflavone, 6''-O-malonylononin, liquiritigenin, luteolin, 3',5-dimethoxyluteolin, 5-methoxyluteolin, medicarpin, stachydrine, trigonelline. These compounds attract *S. meliloti* to the surface of the root hairs of the plant where the bacteria begin secreting nodulation factor (Peters et al., 1986; Maxwell et al., 1989). As a consequence, symbiotic nodule-forming nitrogen-fixation is found almost exclusively in legume species. Bradyrhizobia and rhizobia share characteristics with plant growth promoting rhizobacteria (PGPR). Nodule inducing bacteria, like other PGPR, are capable of colonizing the roots of non-legume plants (Antoun et al., 1998).

[0007] Diazotrophs have been found in free-living bacteria, among which *Azotobacter vinelandii* is the best studied. An increasingly number of diazotrophic species, e.g., *Azospirillum*, *Herbaspirillum*, *Burkholderia*, *Gluconacetobacter*, have been reported to form atypical symbiotic relationship with plants (Zehr, 2011; Raders et al., 2004; Pedraza, 2008). They often grow on the surface root system (rhizobacteria) although some are able to infect plant tissues (endophytic bacteria) and perform nitrogen fixation, which is also able promote plant growth. For example, US patent 7,393,678 B2 describes strains of *Klebsiella pneumonia*, which colonize the root surface (Liu et al., 2011), are able to promote growth of cereals (wheat and corn) either in the presence or absence of chemical nitrogen application. However, only specially isolated mutant strains of *Klebsiella pneumonia* were able to perform satisfactory plant growth promoting function as the nitrogen-fixation activity is low in majority of strains. Perhaps, the best examples of endophytic nitrogen-fixation can be found in sugarcane and wild rice, with *Herbaspirillum*, *Gluconacetobacter*, *Enterobacter*, *Azospirillum*, *Swaminathania* and *Acetobacter* being the possible contributors of nitrogen-fixing species (Pedraza, 2008; Boddey et

al., 1995; Baldani et al., 2002; Elbeltagy et al., 2001; Saravanan et al., 2008). Photosynthetic Bradyrhizobia are the natural endophytes of the African wild rice *Oryza breviligulata* while the intercellular colonization and growth-promoting effects of a *Methylobacterium* sp. was observed in common rice *Oryza sativa* L. Cv CO-43 although the later was believed to resulted from phytohormone secretion of the bacterium (Senthilkumar et al., 2009; Chaintreuil et al., 2000).

[0008] A study of *Azospirilla* indicates that, similar to nodulation and nitrogen-fixation in legumes, the endophytic colonization process is genetically controlled and exopolysaccharide production inhibits both endophytic colonization and nitrogen fixation in common wheat seedlings (Kennedy et al., 1997). In addition, a mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* (Borthakur et al., 1986). Therefore, the ability of a diazotroph to promote plant growth via nitrogen-fixation is not predictable from its performance in other plants.

[0009] *Jatropha curcas* is a small woody plant belonging to the *Euphorbiaceae* family. Several unique characteristics make it an ideal plant for biodiesel production (Fairless, 2007; Gaydou et al., 1982; Openshaw, 2000). These include the ability to grow on marginal land; low requirement for water; a non-food crop status and fast oil production in 0.5-2 years after planting compared to more than 3 years for oil palm. Accordingly, several Asian countries, particularly Indonesian and India, have made ambitious plans to promote jatropha plantation. Several other plants have also attracted strong interest as alternative crop for biofuel production. These include castor bean, sorghum, and sweet sorghum.

[0010] As *Jatropha* is targeted to marginal land where soil nutrient is low, the requirement for nitrogen fertilizer will be higher than other crops. Therefore, any technology that reduces nitrogen fertilizer usage will be highly desirable. Today there is little research on naturally occurring diazotrophs in *Jatropha* nor the application of these microbes for improvement of plant productivity. Similar situations can be found in other crops, such as Sorghum, castor bean and cassava.

SUMMARY OF THE INVENTION

[0011] The present invention relates to methods of reducing chemical fertilizer usage and greenhouse gas nitrous oxide emission and to methods of improving plant growth rate and seed productivity in agriculture through the application of a novel artificially manufactured formula containing a nitrogen-fixing bacterium that efficiently colonizes non-legume plants in aerial

parts and root system. The bacteria inocula and methods are particularly suitable for plants in the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Oryza*, *Ricinus* and *Manihot*.

[0012] In a first aspect, the present invention provides a biologically pure culture of a bacterial species selected from the *Enterobacter*, *Methylobacterium*, *Sphingomonas*, and *Pleomorphomonas* genera as described herein. In some embodiments, the bacterial species of the present invention are able to efficiently reduce atmospheric N₂ to ammonia as evidenced by the acetylene reduction assay (AR assay) *in planta*. In some embodiments, the N₂ reduction occurs on the leaf surface. In other embodiments, the N₂ reduction occurs inside the leaf surface. In additional embodiments, the N₂ reduction occurs on the surface of the roots or root system. In further embodiments, the N₂ reduction occurs inside the root tissue. In some embodiments, the bacterial species is able to self-propagate efficiently on the leaf surface, root surface or inside plant tissues without inducing a noticeable plant defense reaction, such as cell death. In other embodiments, bacterial species of the present invention can be isolated by culturing a plant tissue extract or leaf surface wash in a medium with no added nitrogen source as described herein.

[0013] In one embodiment, the genomic DNA of the bacterial species shares least 97% and preferably at least 98% identity to SEQ ID NO:10. In some embodiments, the bacterial species is an *Enterobacter* species containing a set of genes required for nitrogen-fixation. In some embodiments, the *Enterobacter* species produces substantial amounts of extracellular polysaccharide (EPS), or endoglucanase. In another embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity to SEQ ID NO:11. In some embodiments, the bacterial species is a *Pleomorphomonas* species in which the nitrogen-fixing capability is stimulated by about 0.1 mM to about 0.5 mM of NH₄⁺ ion. In an additional embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity homology to SEQ ID NO:12. In some embodiments, the bacterial species is a *Sphingomonas* species that is able to grow in nitrogen-free medium. In a further embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity to SEQ ID NO:13 or SEQ ID NO:14. In some embodiments, the bacterial species is a *Methylobacterium* species.

[0014] In a second aspect, the present invention provides an artificially formulated inoculant comprising at least one biologically pure culture of a bacterial species selected from the *Enterobacter*, *Methylobacterium*, *Sphingomonas*, and *Pleomorphomonas* genera as described herein. In one embodiment, the inoculant comprises a mixture of at least two biologically pure

cultures of these bacterial species. In some embodiments, the preparation of the inoculant comprises large-scale production of the bacterial species described herein in a medium, optionally dehydrated in a suitable manner that maintains cell viability and the ability to artificially inoculate and colonize host plants. In some embodiments, the inoculant is optionally supplemented with trace metal ions. In one embodiment, the trace metal ions are selected from the group consisting of molybdenum (Mo^{2+}) ions, iron (Fe^{2+}) ions, manganese (Mn^{2+}) ions, or any combination of these ions. In another embodiment, the concentration of the trace metal ions in the inoculant is between about 0.1 mM and about 50 mM. In some embodiments, the inoculant is formulated with a carrier. Any suitable carrier can be used and examples of suitable carriers include, but are not limited to, beta-glucan, carboxymethyl cellulose (CMC), bacterial EPS, sugar and animal milk. In some embodiments, bacterial species that can be used for the inoculants of the present invention are those bacterial species described herein. In other embodiments, bacterial species that can be used for the inoculants of the present invention can be isolated by culturing a plant tissue extract or leaf surface wash in a medium with no added nitrogen source or sourced from a microbial collection centre based on taxonomy classifications and the characteristics of the bacterial species described herein. In some embodiments, the inoculant is effective in promoting plant growth, maintaining high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight and reducing NO_2 emission due to reduced nitrogen fertilizer usage. In other embodiments, these properties are evidenced in plant species of the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Oryza*, *Ricinus* and *Manihot*.

[0015] In a third aspect, the present invention provides a method for promoting plant productivity in a non-legume plant species. In some embodiments, the plant productivity is plant growth, maintenance of high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight or any combination of these characteristics. In other embodiments, plant productivity is also a reduction in NO_2 emission which results from reduced nitrogen fertilizer use. In some embodiments, the non-legume plant species is selected from species of the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Ricinus*, *Oryza* and *Manihot*. In accordance with the present invention, the method comprises inoculating plants with a bacterial inoculant described herein. In one embodiment, the inoculant is sprayed on the plant aerial parts. In another embodiment, the inoculant is applied to the roots by inserting into furrows in which the plant seeds are planted, watering to the soil or dipping the roots in a suspension of the inoculant. In an additional embodiment, the inoculant is applied as a seed coating. In some embodiments, the inoculant comprises the trace metal ions described herein.

In other embodiments, the plant is sprayed with a trace metal ion solution that contains the trace metal ions described herein in addition to an inoculant which may not contain the trace metal ions.

BRIEF DESCRIPTION OF THE FIGURES

[0016] Figures 1a and 1b show the effect of ammonium chloride addition on nitrogenase activity of *Methylobacterium* sp. strain L2-4 (Fig. 1a) and *Enterobacter* sp. strain R4-368 (Fig. 1b). Nitrogenase activity was measured for cultures grown in 125-ml serum bottles containing N-free medium supplemented with different levels of ammonium chloride at 30 °C for 48h. Error bars indicate $\pm$ SD.

[0017] Figure 2 shows the effect of nitrogenase cofactor (Mo/Fe/Mn) addition on nitrogenase activity of *Enterobacter* sp. strain R4-368 (top) and *Methylobacterium* sp. strain L2-4 (bottom). Nitrogenase activity was measured for cultures grown in 125-ml serum bottles containing N-free medium supplemented with different levels of Mo/Fe/Mn at 30 °C for 48h.

[0018] Figure 3 shows the nitrogen-fixing activity in *Jatropha* of selected nitrogen-fixing strains in the presence of essential metals (Mo/Fe/Mn). Ethylene emission from *Jatropha* seedlings added with 15% acetylene (v/v) into the headspace was determined with and without inoculation of N-fixing root isolates. Acetylene reduction activity was calculated from the difference between inoculated with and without N-fixing root isolates. Values given are the means $\pm$ standard deviations for triplicate determinations.

[0019] Figures 4a and 4b show the nitrogen-fixation gene cluster in *Klebsiella pneumonia* (Genbank No X13303) (Fig. 4a) and the homologous proteins as identified by BLASTP in *Enterobacter* sp R4-368 (SEQ ID NO:3) (Fig. 4b).

[0020] Figure 5 shows the putative proteins encoded in SEQ ID NO:4 as identified by BLASTP.

[0021] Figures 6a and 6b show the nitrogenase activity of *Pleomorphomonas jatrophae* strain R5-392. Fig. 6a: Strain R5-392^T grown in N-free medium and growth was recorded as OD_{600nm} at different time intervals. For measurement of nitrogenase activity, grown on N-free liquid cultures by injecting purified 15% acetylene (v/v) and incubate at 30°C and then analyzed for ethylene production by GC. Fig. 6b: Nitrogenase switch-off by ammonium ions (top panel) and response to addition of nitrogenase cofactor Fe and Mo (bottom panel) in *Pleomorphomonas jatrophae* strain R5-392.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The present invention relates to novel clean technologies for agriculture and environment managements. In particular, the practice of this invention leads to reduced dependence on chemical nitrogen fertilizer; faster plant growth; improves crop productivity and reduced greenhouse gas NO₂ emission in agriculture. In some embodiments, the present invention relates to methods of reducing chemical fertilizer usage and greenhouse gas nitrous oxide emission and to methods of improving plant growth rate and seed productivity in agriculture through the application of a novel artificially manufactured formula containing a nitrogen-fixing bacterium that efficiently colonizes non-legume plants in aerial parts and root system. The bacteria inocula and methods are particularly suitable for plants in the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Oryza*, *Ricinus* and *Manihot*.

[0023] As used herein "bacterial inoculant," "inoculant" or "inoculum" refers to a preparation that includes one or more bacterial species described herein.

[0024] The term "biologically pure culture" or "substantially pure culture" shall be deemed to include a culture of a bacterial species described herein containing no other bacterial species in quantities sufficient to interfere with the replication of the culture or be detected by normal bacteriological techniques.

[0025] The term "plant productivity" refers generally to any aspect of growth or development of a plant that is a reason for which the plant is grown. For example, when referring to food crops, such as grains or vegetables, crop productivity generally refers to the yield of grain or fruit, etc., harvested from a particular crop. Thus, for purposes of the present invention, improved plant productivity refers broadly to improvements in yield of grain, fruit, flowers, or other plant parts harvested for various purposes, improvements in growth of plant parts, including stems, leaves and roots, promotion of plant growth, maintenance of high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight, reducing NO₂ emission due to reduced nitrogen fertilizer usage and similar improvements of the growth and development of plants.

[0026] In a first aspect, the present invention provides a biologically pure culture of a bacterial species selected from the *Enterobacter*, *Methylobacterium*, *Sphingomonas*, and *Pleomorphomonas* genera as described herein. In some embodiments, the bacterial species of the present invention are able to efficiently reduce atmospheric N₂ to ammonia as evidenced by the AR assay *in planta*. In some embodiments, the N₂ reduction occurs on the leaf surface. In other embodiments, the N₂ reduction occurs inside the leaf surface. In additional embodiments,

the N₂ reduction occurs on the surface of the roots or root system. In further embodiments, the N₂ reduction occurs inside the root tissue. In some embodiments, the bacterial species is able to self-propagate efficiently on the leaf surface, root surface or inside plant tissues without inducing a noticeable plant defense reaction, such as cell death. In other embodiments, bacterial species of the present invention can be isolated by culturing a plant tissue extract or leaf surface wash in a medium with no added nitrogen source, using suitable methods such as those described herein or similar methods well known to the skilled artisan or obvious modifications thereof.

[0027] In one embodiment, the genomic DNA of the bacterial species shares least 97% and preferably at least 98% identity to SEQ ID NO:10. In some embodiments, the bacterial species is an *Enterobacter* species containing a set of genes required for nitrogen-fixation. In some embodiments, the *Enterobacter* species produces substantial amounts of EPS, or endoglucanase. In another embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity to SEQ ID NO:11. In some embodiments, the bacterial species is a *Pleomorphomonas* species in which the nitrogen-fixing capability is stimulated by about 0.1 mM to about 0.5 mM of NH₄⁺ ion. In an additional embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity homology to SEQ ID NO:12. In some embodiments, the bacterial species is a *Sphingomonas* species that is able to grow in nitrogen-free medium. In a further embodiment, the genomic DNA of the bacterial species shares 97% and preferably at least 98% identity to SEQ ID NO:13 or SEQ ID NO:14. In some embodiments, the bacterial species is a *Methylobacterium* species.

[0028] A biologically pure culture of *Enterobacter* species R4-368 and *Pleomorphomonas jatrophae* R5-392 were deposited on January 6, 2012 under terms of the Budapest Treaty with the AGRICULTURAL RESEARCH SERVICE CULTURE COLLECTION (NRRL) (International Depositary Authority), National Center for Agricultural Utilization Research Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604 U.S.A., and assigned Accession Number NRRL B-50631 and NRRL B-50630, respectively. These strains can be cultured in R₂A medium (Yeast extract 0.5 g/l; Proteose Peptone 0.5 g/l; Casamino acids 0.5 g/l; Glucose 0.5 g/l; Soluble starch 0.5 g/l; Na-pyruvate 0.3 g/l; K₂HPO₄ 0.3 g/l; MgSO₄ *7 H₂O 0.05 g/l; agar 15 g/l, pH7.2).

[0029] A biologically pure culture of *Methylobacterium* sp. L2-4; *Methylobacterium* sp. L2-76 and *Sphingomonas* sp. S6-274 were deposited on January 6, 2012 under terms of the Budapest Treaty with the AGRICULTURAL RESEARCH SERVICE CULTURE

COLLECTION (NRRL) (International Depository Authority), National Center for Agricultural Utilization Research Agricultural Research Service, U.S. Department of Agriculture 1815 North University Street, Peoria, Illinois 61604 U.S.A., and assigned Accession Number NRRL B-50628, NRRL B-50629 and NRRL B-50632, respectively. These strains can be cultured in 869 medium (Tryptone 10.0g/l; Yeast extract 5.0g/l; NaCl 5.0g/l; D-glucose 1.0g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.345g/l; pH 7.0).

[0030] It is anticipated that certain mutants of the bacterial species described herein may also enhance plant growth comparable to the non-mutated forms. Mutants of the bacterial species described herein may include both naturally occurring and artificially induced mutants. Certain of these mutants will be found to be useful using the assays described herein. Others mutants may be induced by subjecting the bacterial species described herein to known mutagens, such as N-methyl-nitrosoguanidine, using conventional methods. See, for example, U.S. Patent No. 4,877,738 and U.S. Patent No. 5,552,138, each incorporated herein by reference.

[0031] In a second aspect, the present invention provides an artificially formulated inoculant comprising at least one biologically pure culture of a bacterial species selected from the *Enterobacter*, *Methylobacterium*, *Sphingomonas*, and *Pleomorphomonas* genera as described herein. In one embodiment, the inoculant comprises a mixture of at least two biologically pure cultures of these bacterial species. In some embodiments, the preparation of the inoculant comprises large-scale production of the bacterial species described herein in a medium, optionally dehydrated in a suitable manner that maintains cell viability and the ability to artificially inoculate and colonize host plants. In some embodiments, the concentration of bacterial species in the inoculant is 10^8 to 10^{10} CFU/ml. In some embodiments, the inoculant is optionally supplemented with trace metal ions. In one embodiment, the trace metal ions are selected from the group consisting of molybdenum (Mo^{2+}) ions, iron (Fe^{2+}) ions, manganese (Mn^{2+}) ions, or any combination of these ions. In another embodiment, the concentration of the trace metal ions in the inoculant is between about 0.1 mM and about 50 mM.

[0032] In some embodiments, the inoculant is formulated with a carrier. Any suitable carrier can be used. Examples of suitable carriers include, but are not limited to, beta-glucan, CMC, bacterial EPS, sugar and animal milk. Alternatively, peat or planting materials can be used as a carrier. In addition, biopolymers can be used as a carrier in which the inoculant is entrapped in the biopolymer. See, for example, U.S. Patent No. 4,828,600, U.S. Patent No. 5,061,490, U.S. Patent No. 5,951,978 and U.S. Patent No. 7,393,678, each incorporated herein by reference.

[0033] In some embodiments, bacterial species that can be used for the inoculants of the present invention are those bacterial species described herein. In other embodiments, bacterial species that can be used for the inoculants of the present invention can be isolated by culturing a plant tissue extract or leaf surface wash in a medium with no added nitrogen source or sourced from a microbial collection centre based on taxonomy classifications and the characteristics of the bacterial species described herein. In some embodiments, the inoculant is effective in promoting plant growth, maintaining high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight and reducing NO₂ emission due to reduced nitrogen fertilizer usage. In other embodiments, these properties are evidenced in plant species of the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Ricinus*, *Oryza* and *Manihot*.

[0034] In a third aspect, the present invention provides a method for promoting plant productivity in a non-legume plant species. In some embodiments, the plant productivity is plant growth, maintenance of high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight or any combination of these characteristics. In other embodiments, plant productivity is also a reduction in NO₂ emission which results from reduced nitrogen fertilizer use. In some embodiments, the non-legume plant species is selected from species of the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Ricinus*, *Oryza* and *Manihot*. In accordance with the present invention, the method comprises inoculating plants with a bacterial inoculant described herein. In one embodiment, the inoculant is sprayed on the plant aerial parts. In another embodiment, the inoculant is applied to the roots by inserting into furrows in which the plant seeds are planted, watering the soil or dipping the roots in a suspension of the inoculant. In an additional embodiment, the inoculant is applied as a seed coating. In some embodiments, the inoculant comprises the trace metal ions described herein. In other embodiments, the plant is sprayed with a trace metal ion solution that contains the trace metal ions described herein in addition to an inoculant which may not contain the trace metal ions.

[0035] In one embodiment, the bacterial inoculant can be applied to plant seeds through the use of a suitable coating mechanism or binder prior to the seeds being sold into commerce for planting. The process of coating seed with such an inoculum is generally well known to the skilled artisan. For example, the bacterial species described herein may be mixed with a porous, chemically inert granular carrier, such as described by U.S. Patent No. 4,875,921, incorporated herein by reference. In another embodiment, the bacterial inoculant may be prepared with or without a carrier and sold as a separate inoculant to be used directly by the grower, such as by

inserting directly into the furrows into which the seed is planted or by spraying the plant leaves. The process for inserting such inoculants directly into the furrows during seed planting is also generally well known to the skilled artisan, as is spraying the plants with the bacterial inoculant.

[0036] In general, the density of inoculation of the bacterial species-onto seed, into furrows, or wetted on roots should be sufficient to populate the sub-soil region adjacent to the roots of the plant with viable bacterial growth. Similarly, the density of inoculation of the bacterial species sprayed onto plants should be sufficient to populate the leaves of the plant with viable bacterial growth. An effective amount of bacterial inoculant should be used. An effective amount is that amount sufficient to establish sufficient bacterial growth so that the plant productivity is improved.

[0037] It will be appreciated by the skilled artisan that a bacterial inoculant of the type described herein offers several significant potential advantages over the chemical inoculants or growth hormones or similar agents commonly used in agriculture today. By the very nature of the bacterial inoculant, the bacterial species are self-sustaining in a continuous fashion once the plants have been inoculated by any of the methods described herein. Therefore, there is no need for retreatment of the plants during the crop season. The bacterium grows in cultivation along with the plants and should continue to exhibit its beneficial effect on the plant throughout the agricultural season. This is in strong contrast to chemical growth agents or fungicides which must be retreated periodically to have a continuing effect on inhibition of the fungus in question or to help improve the plant growth throughout its life cycle. Since the bacterial inoculant of the present invention can be inoculated onto the seeds using a dry or wet formulation, the application of this technique is relatively simple to the farmer since the seeds can be inoculated prior to distribution. Since the bacterial inoculant of the present invention can be sprayed onto plants, the application of this technique is relatively simple to the farmer. In these ways, a significant economic advantage is achievable.

[0038] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0039] The present invention is described by reference to the following Examples, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Isolation of Diazotrophic Endophytes

[0040] *Jatropha* (*Jatropha curcas* L.) cultivars were sampled from the research plots of Agrotechnology Experimental Station located at Lim Chu Kang, Singapore. Plants were sampled from three trees of different cultivars that included origin from Indonesia, China and India. The root samples (~1.2 - 2 cm dia) with adhering soil were carefully removed from root

with a trowel and collected in sterile plastic bags. Stem cuttings (~1.5 to 2.5 cm diameter) were removed from the plants with clippers and matured third leaves (non-pathogenic) from healthy branches that were placed on ice for shipment from the field to laboratory and processed immediately. Root, stem cuttings (~50g) and leaf (~15-20g) samples were thoroughly washed in distilled water, surface sterilized for 1 min in 90% ethanol followed by 10 min in 15% H₂O₂ and rinsed 3-5 times in sterile distilled water. A 100- μ l sample of the water from the third rinse was plated on rich medium to verify the efficiency of sterilization. After sterilization, the roots and shoots were macerated in 10 ml 10 mM MgSO₄ using a blender under sterile conditions. Serial dilutions were made, and 100- μ l samples were plated on non-selective media in order to test for the presence of the endophytes and their characteristics. The bacteria were isolated from spread plate technique (100- μ l) using different media with agar/phytagel such as N-free media (Nfb) (Baldani et al., 1980) for diazotrophs, rich media (M869) (Barac et al., 2004); for total heterotrophs and minimal medium (AMS) (Whittenbury et al., 1970); for methylotrophic bacteria. For isolation of diazotrophs, the suspension (10^{-3} to 10^{-5}) were placed in duplicate tubes containing semi-solid N-free media and incubated for 96 h at 30 °C. Growth pellicles in semi-solid media were streaked on to N-free agar media for single colony preservation and for further use.

EXAMPLE 2

Bacterial Inoculants and Culture Conditions

[0041] *Methylobacterium* strains were grown in liquid medium 869 (Barac et al., 2004) with 1% methanol as the carbon source. Root isolates were cultivated in liquid medium 869 or 2xYT without methanol. All cultures were incubated at 30°C and grown until early stationary phase. Just before inoculation, cells were harvested (10000rpm, 8min), washed once and resuspended in SDW or 10 mM MgSO₄ solution, the optical density at 600 nm (OD_{600nm}) was adjusted, and inoculated as follows: for root inoculation, equal volume of cell suspension (OD_{600nm} = 1.0 or 10^8 cfu ml⁻¹) of each isolate was mixed and added at the time of sowing and at 15 days after sowing (DAS); for leaf inoculation, mixed or individual isolate was given as foliar spray till the wetting of leaves at 15 and 30 DAS (volume varied depending on the plant size and leaf number).

EXAMPLE 3

nifH Amplification and Sequencing

[0042] The presence of the *nifH* gene was determined by a PCR as described by Pinto-Tomás et al. (2009). To amplify *nifH* gene using universal primers nif-Fo (5'-AAA GGY GGW ATC GGY AAR TCC ACC AC-3'; SEQ ID NO:1) and nif-Re (5'-TTG TTS GCS GCR TAC ATS GCC ATC AT-3'; SEQ ID NO:2) using the stringent PCR conditions described by Widmer et al. (1999) in order to reduce the emergence of falsely positive amplifications, including the following cycling conditions: 95 °C/5min, 40 cycles of 94 °C/11s, 92 °C/15s, 54 °C/8s, 56 °C/30s, 74 °C/10s and 72 °C/10s, and final extension for 10 min/72 °C. PCR products were eluted with QIAquick gel extraction kit prior to preparing sequencing reactions with the BigDye reaction mix and loaded into an Applied Biosystems 3700 automated DNA sequencing instrument. *nifH* sequences from this study and close reference sequences obtained from the NCBI database by BLAST analysis.

EXAMPLE 4

Nitrogenase Assays *in vitro*

[0043] The acetylene-reduction (AR) assay was performed on free-living cultures as well as on cultures in association with crop plants. Nitrogen-fixing ability was determined by growing strains in 40 ml nitrogen-free medium (DSMZ medium no. 3) contained in a 125 ml serum bottle (Wheaton Industries Inc., USA). The medium contained the following (in 1 l distilled water): 5.0 g glucose, 5.0 g mannitol, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 5.0 mg Na₂MoO₄·2H₂O, 0.9 g K₂HPO₄, 0.1 g KH₂PO₄, 0.01 g FeSO₄·7H₂O, 5.0 g CaCO₃ and 1 ml trace element mixture. The trace element mixture (SL-6, in DSMZ medium no. 27) contained the following (l⁻¹ distilled water): 0.1 g ZnSO₄·7H₂O, 0.03 g MnCl₂·4H₂O, 0.3 g H₃BO₃, 0.2 g CoCl₂·6H₂O, 0.01 g CuCl₂·2H₂O and 0.02 g NiCl₂·6H₂O. Acetylene reduction was performed for all liquid cultures by injecting purified acetylene into appropriate containers sealed with gas-tight serum stoppers to yield 15% acetylene (v/v); this was followed by incubation for up to 96 h at 30 °C. At intervals gas samples (0.5 ml) were removed by PTFE-syringe (Hewlett-Packard, USA) and analysed by Gas Chromatograph (GC 6890N, Agilent Technologies Inc., USA) with an flame ionization detector operated as follows: carrier gas, He; 35ml/min; detector temperature, 200 °C; column, GS-Alumina (30 m x 0.53 mm I.D.); pressure, 4.0 psi. Standard curve was prepared with ethylene (C₂H₄, Product Number: 00489, Sigma-Aldrich) injected in duplicate, the concentration ranged from 1 to 1000 n moles and calibrated (peak height vs nmol of C₂H₄). The

protein concentration was determined by a modified Lowry method with bovine serum albumin as standard.

EXAMPLE 5

Nitrogenase Assay *in planta*

[0044] Acetylene-reduction (AR) assay was performed 2-3 weeks after inoculation, and the viable bacterial counts on shoot/roots were determined by serial dilution technique. Samples from each replication were collected from the glass house and most of the adhering soil was removed by shaking. Shoot/root/entire seedlings were inserted into the 125 ml glass bottles, closed with a 20 mm red stopper sleeve. After removing an equivalent volume of air, acetylene was injected into these bottles to give a final concentration of 15% and incubated at 30 °C for 4-12h. Gas Chromatograph operating conditions was described by earlier. All values expressed were obtained after deducting the ethylene values for a blank treatment without samples.

EXAMPLE 6

Enumeration of Bacterial Populations

[0045] Triplicate plant samples were randomly picked on each sampling, and each replicate consists of root and aerial parts from three plants. For all bacterial enumerations, the homogenates were serially diluted using sterile distilled water and plated on to AMS media with 0.5% methanol to determine the *Methylobacterium* population or to M869 plates for total bacterial population. Bacterial colonies were counted after incubating the plates for 5 days at 30 °C.

EXAMPLE 7

In vitro and *in planta* AR Activity of Selected Diazotrophic Isolates

[0046] Many diazotrophic species were isolated from jatropha plant or rhizosphere. The nitrogen-fixing capability was confirmed by the presence of nifH-like gene by PCR, AR activity *in vitro* and *in planta*. We found that nitrogenase activity in isolate bacteria culture is significantly different from their performance observed *in planta*. Table 1 lists the activity of selected strains.

TABLE 1
AR Activity of Selected Diazotrophic Isolates

Selected strains	Medium used	Nitrogenase activity in pure culture (nmol C ₂ H ₄ h ⁻¹ mg protein ⁻¹)	<i>In planta</i> AR activity (nmol C ₂ H ₄ h ⁻¹ plant ⁻¹)	nifH gene*	Endoglucanase Activity
<i>Methylobacterium</i> sp. L2-4	JNFb	661.3 ± 65.31	94.17 ± 9.52	+(420 nt)	+
<i>Methylobacterium</i> sp. L2-76	JNFb	272.2 ± 20.15	32.70 ± 5.21	+	+
<i>Methylobacterium</i> sp. L7-515	JNFb	223.3 ± 32.14	61.57 ± 7.62	+	+
<i>Enterobacter</i> sp. R4-368	DSMZ**	1060.7 ± 110.6	220.08 ± 20.08	+(432 nt)	+
<i>Enterobacter</i> sp. R5-362	DSMZ	960.5 ± 110.5	77.54 ± 8.54	+	+
<i>Enterobacter</i> sp. R4-390	DSMZ	1012.8 ± 62.8	71.13 ± 10.07	+	+
<i>Pleomorphomonas jatrophae</i> R5-392	DSMZ	92.79 ± 4.8	Nd	+(409 nt)	-
<i>Sphingomonas</i> sp. S6-274	DSMZ	16.14 ± 1.2	Nd	+	+

Note: *nifH gene – amplified using PCR and the products are sequenced for further confirmation. ** DSMZ N-free medium no.3; Acetylene reduction was performed for all liquid cultures by injecting purified acetylene into appropriate containers sealed with gas-tight serum stoppers to yield 15% acetylene (v/v); this was followed by incubation for up to 96 h at 30 °C. Ethylene peak is not detected in control bottle without addition of 15% acetylene (v/v) into the headspace. Each value is the mean ± SD of three replications per treatment. Nd: not determined.

EXAMPLE 8

Effect of Ammonium Ion on Nitrogen-Fixing Activity

[0047] Resistance to ammonia is a critical feature of nitrogen-fixing bacteria inoculants for this invention. To demonstrate this, we assay the AR activity of two best candidates of our inoculants under the conditions with various concentration of NH₄Cl. Surprisingly, both *Enterobacter* sp. R4-368 and *Methylobacterium* sp. L2-4 showed not only strong activity up to 1 mM but also significantly enhanced activity by the NH₄⁺ ion although inhibitory effect was observed at about 5 mM (Figures 1a and 1b).

EXAMPLE 9

Effect of Metal Ions on N-Fixation

[0048] We assayed nitrogenase activity in media supplemented Na_2MoO_4 or $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at various concentrations (0, 0.1, 0.5, 1, 10mM) in N-free medium. Nitrogenase activity was clearly stimulated by Fe^{2+} and molybdenum (Mo^{2+}) ions (Figures 2 and 3).

EXAMPLE 10

Agronomical Performance of *Jatropha curcas* Inoculated with N-Fixing Strains

[0049] To confirm the efficacy of nitrogen-fixing bacterial inoculants in plants, selected strains were inoculated to *Jatropha curcas* seedlings either in a single isolates or mix isolates. Plants were grown in the open air in plastic pots (30 cm in diameter) after bacterial inoculation. From the time of flowering to harvesting, commercial NPK fertilizers was applied at half of the recommended dose (0.5g /plant) once in 15 days intervals. As shown in Table 4, significant improvements in chlorophyll content, leaf AR activity, seed yield and single seed weight were observed. Noticeably, mixed inoculation of root and leaf isolates lead to best chlorophyll content and AR activity. Flowering time was also shortened, particularly in treatments with L2-4 strains. The relatively high AR activity in control plants may result from naturally occurring N-fixing species from the environment. Importantly, total seed yields were increased to about 4-fold when inoculated with a single leaf isolate L2-4 or mix root isolates (Table 2).

TABLE 2

Effect of N-Fixing Strains on *Jatropha curcas*

Treatments	Relative Chlorophyll content	AR-activity (nmol C_2H_4 h^{-1} g leaf $^{-1}$)	Average time to flowering (DAP)	No. of fruits per treatment	Number of seeds/plant	Total seed weight per treatment (g)	Single seed weight (g)
Control	33.29 $\pm$ 2.44	18.11 $\pm$ 2.1	137	26 $\pm$ 2.87	75 $\pm$ 8.14	36.25	0.483
L2-4	38.17 $\pm$ 4.10	22.31 $\pm$ 3.3	110	101 $\pm$ 7.91	261 $\pm$ 20.78	134.89	0.517
L2-76	36.47 $\pm$ 4.01	20.95 $\pm$ 4.0	125	58 $\pm$ 4.37	157 $\pm$ 11.41	81.56	0.519
Leaf isolates (LI)	37.66 $\pm$ 4.17	24.00 $\pm$ 4.0	118	43 $\pm$ 2.72	119 $\pm$ 8.13	62.63	0.526

Root isolates (RI)	38.12 ± 3.30	19.97 ± 2.0	124	98 ± 9.00	286 ± 25.47	145.27	0.508
RI+LI (MI)	40.07 ± 5.14	36.67 ± 5.7	115	73 ± 5.03	182 ± 12.41	92.05	0.506

Note: Each value is the mean ± SD of eight replications (n=8). Leaf isolates (LI): L2-4 (*Methylobacterium* sp.), L2-76 (*Methylobacterium* sp.) and S6-274 (*Sphingomonas* sp.); Root isolates (RI): R4-368 (*Enterobacter* sp.), R5-362 (*Enterobacter* sp.), R5-431 (*Klebsiella* sp.), R7-601 (*Sinorhizobium* sp.) and R1-99 (*Rhizobium* sp.). For treatments that include leaf isolates, the cultures were applied using a hand sprayer (25 ml per pot; 1×10^9 CFU ml⁻¹ of culture or wetting of all the leaves) at 30 and 90 days after planting (DAP). Chlorophyll content and AR-activity measured on 30 days after inoculation (DAI). Mature fruits were collected continuously and dried at 37 °C for 2 days. Seeds were collected from dried fruits and stored in 4 °C for further analysis. Fruit and seed yield data was recorded over a period of 7 months.

[0050] To demonstrate that the bacteria species are able to self-propagate in plant tissues, non-surface sterilized homogenates were serially diluted using sterile distilled water and MPN techniques were performed with 3 tubes containing Nfb media using from 10^{-3} to 10^{-5} dilutions and incubated at 30 °C for 7 days. For endophytic population before homogenization, leaf and root tissues were surface-sterilized by immersion in 70% ethanol, 1 min; 15% H₂O₂ for 1 min followed by 5-10 thorough rinsing in sterile distilled water. Serial dilutions were also plated on agar N-free media. Bacterial colonies were counted after incubating the plates for 5 days at 30°C. Results are shown in Table 3. For heterotrophic and methlotrophic population were showed the same trend as higher population of leaf associated bacteria than endophytic populations (data not shown).

TABLE 3

Diazotrophic Bacterial Populations (MPN Techniques)

Treatments	Population (Log cfu/g sample)			
	Leaf sample		Root sample	
	Leaf associated	Endophytic	Root associated	Endophytic
Control	3.97 ± 0.97	2.48 ± 0.18	2.56 ± 0.16	2.46 ± 0.36
L2-4	5.38 ± 0.38	2.96 ± 0.46	3.51 ± 0.41	2.81 ± 0.11
L2-76	5.61 ± 0.51	2.82 ± 0.72	3.16 ± 0.36	2.64 ± 0.34

Leaf isolates (LI)	5.46 ± 0.26	3.10 ± 0.30	3.24 ± 0.54	2.59 ± 0.19
Root isolates (RI)	4.12 ± 0.12	3.05 ± 0.25	4.32 ± 0.32	3.65 ± 0.25
RI+LI (MI)	5.72 ± 0.62	3.21 ± 0.31	4.66 ± 0.66	3.88 ± 0.43

Note: Leaf associated or endophytic populations from both surface and non-surface sterilized leaves were measured in Nfb media at the time of fruit harvesting stage i.e. 6 months after 2nd inoculations.

EXAMPLE 11

Promotion of *Jatropha* Seedling Growth

[0051] *Jatropha* (cv. MD44) seeds were surface sterilized by immersion in 70% ethanol, 1 min; 2% NaOCl for 30 sec followed by 5-10 thorough rinsing in sterile distilled water. For plant inoculation experiments, *Methylobacterium* strains (leaf isolates, L2-4 and L7-515) were grown in liquid 2xYT medium (root isolates R4-368, R5-362, R5-431, R7-601 and R1-99 were grown in without methanol) containing methanol (30 mM) to midexponential growth phase, centrifuged, washed, resuspended in sterile distilled water and stored in 4 °C until use. The bacteria were adjusted to an OD_{600nm} of 1.2 (10⁸ cfu per ml) and used for plant inoculation after sterilization of *Jatropha* seeds. For mixed inoculation, equal volumes of five cultures were mixed and then used for seed imbibition (in RT at 6h for 60 rpm), root application (2ml per pot after sowing) and phyllosphere spray till wetting of leaves.

[0052] Under glass house growth conditions, the analysis that was carried out at 45 DAS showed that for most of the plant parameters examined, there was a significant increase in N-fixing bacteria-treated plants compared to non-inoculated control plants (Table 4). In *Jatropha*, individual inoculation of N-fixer (LI and RI) or its mixed-inoculation produced significant increases in number of leaves, chlorophyll content, seedling vigour, rate of germination and biomass compared to control plants or plants individually-inoculated with LI or RI. Under greenhouse condition, the AR activity in the control plants are much lower, a likely consequence of less cross-contamination.

TABLE 4

Seedling Vigour Index, Number of Leaves and
Germination Rate of N-Fixer-Treated *Jatropha* seeds (45 DAS)

Treatments [†]	No. of Leaves	Relative Chlorophyll	Nitrogenase activity [‡]	SVI	RG	Biomass (g)
Control	6.19 ± 0.29	34.91 ± 3.91	4.15 ± 0.25	901.7 ± 46.7	23.50 ± 2.5	46.12 ± 6.12
Root isolates (RI)	6.58 ± 0.48	36.08 ± 4.08	8.56 ± 1.06	1054.1 ± 60.18	24.55 ± 2.55	62.0 ± 4.0
Leaf isolates (LI)	6.47 ± 0.42	38.81 ± 4.31	16.79 ± 2.79	986.2 ± 65.22	23.57 ± 2.57	61.4 ± 3.4
RI+LI	6.72 ± 0.42	35.31 ± 3.81	11.52 ± 1.72	1037.6 ± 67.51	25.41 ± 2.41	63.0 ± 4.0

Note: [†]Average of 150 *Jatropha* seedlings. [‡]Value expressed in nmol C₂H₄ day⁻¹ seedlings⁻¹. Each value represents the means of four replicates per treatment. Seedling vigour index (SVI) was calculated using the formula: SVI = % germination × seedling length (shoot length + root length) in cm (Baki and Anderson, 1973). Rate of germination (RG) was calculated using the following formula, $RG = \sum Ni/Di$ where Ni is the number of germinated seeds in a given time, and Di is the time unit (day) (Madhaiyan et al., 2004).

[0053] The plant growth promoting effect can be observed in extended period in Ø35 cm pots. Significant improvements in plant height, stem diameter, leaf number and stem volume were observed (Table 5). Soil with poor nutrient content was used and a commercial NPK fertilizer was applied at half of the recommended dose (0.5 g/plant) once in 15 days intervals.

TABLE 5

Combined Inoculation of N-Fixer on the Early Growth of *Jatropha* (90 DAS)

Treatments	Plant height (cm)	Stem Diameter	No. of leaves	Relative chlorophyll content	Stem Volume
Control	32.23	25.55	11.58	26.20	167.41
RI	40.37	27.72	20.58	33.40	244.08
RI+LI	43.97	27.44	24.50	38.16	261.59

Note: Each value is the mean of 12 plants per treatment (n=12). Leaf isolates (LI): L2-4 (*Methylobacterium* sp.) and L7-515 (*Methylobacterium* sp.); Root isolates (RI): R4-368 (*Enterobacter* sp.), R5-362 (*Enterobacter* sp.), R5-431 (*Klebsiella* sp.), R7-601 (*Sinorhizobium* sp.) and R1-99 (*Rhizobium* sp.).

EXAMPLE 12

Effects of *Methylobacterium* sp Inoculation in Cotton, Castor Bean, and Sorghum

[0054] Seeds were surface sterilized by immersion in 70% ethanol, 1 min; 2% NaOCl for 30 sec followed by 5-10 rinsing in sterile distilled water. For plant inoculation experiments, *Methylobacterium* strain L2-4 was grown in liquid 2xYT medium containing 30 mM methanol (v/v) to mid-exponential growth phase, centrifuged, washed, resuspended in sterile distilled water and stored in 4°C until use. For seed imbibitions, the surface sterilized seeds were soaked in a double volume of bacterial culture ($OD_{600nm} = 1.2$ or 10^9 cfu ml⁻¹ of culture). After 6 h soaking, the culture was drained, and the seeds were dried in shade for 30 min before being allowed to sprout for another 24 h prior to sowing. For phyllosphere spray, the cultures were applied using a hand sprayer (approximately 25 ml per plant; $OD_{600nm} = 1.2$) at 15 DAS.

[0055] Under greenhouse conditions, *Methylobacterium* strain L2-4 showed good AR activity in leaves of cotton, castor, sorghum, and rice (Table 6). The effects on crop growth are shown in Table 7.

Table 6

Nitrogenase (or Acetylene-Reduction) Activity of Various Crops Inoculated with *Methylobacterium* sp. strain L2-4 (20 DAI)

Crops	AR-activity (nmol C ₂ H ₄ d ⁻¹ seedlings ⁻¹)	AR-activity (nmol C ₂ H ₄ d ⁻¹ g ⁻¹ sample (DW))
Cotton	176.67 ± 21.67	70.7 ± 11.72
Sorghum	259.61 ± 59.61	177.8 ± 28.77
Castor bean	25.78 ± 5.78	19.2 ± 5.20
Rice	17.54 ± 2.54	140.8 ± 15.77
Jatropha (MD-44)	288.45 ± 78.45	114.5 ± 19.46

Note: Each value is the mean ± SD of three replications per treatment.

TABLE 7

Effect of Inoculation of *Methylobacterium* sp. L2-4 on Crop Growth

Crops	Plant height (cm)		No. of leaves (#)		Relative chlorophyll content*		No. of branches (#)		Number flower (#)	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Cotton	101.5±4.45	103.4±3.08	40 ±2.16	44.25 ±1.89	35.95 ±1.12	39.195 ±1.89	5.75 ±0.50	8.75 ±0.96	9.25 ±1.5	15.75 ±1.26
Castor bean	105.8 ±12.21	115.8 ±3.92	10 ±2.16	12.25 ±1.50	49.95 ±4.28	54.575 ±1.97	ND	ND	0	2 (4)
Sorghum	146.5 ±31.41	138.3 ±23.45	17 ±2.16	19.25 ±4.99	51.08 ±4.04	61.15 ±3.61	ND	ND	1.5 ±1.0	1.5 ±0.58
Jatropha	70.8 ±1.71	72.0 ±2.45	16.75 ±0.5	20.75 ±4.50	30.66 ±0.97	34.18 ±1.62	ND	ND	ND	ND

Note: Each value is the mean ± SD of four replications per treatment. Relative chlorophyll content was measured 5 leaves from top of the plants. Results were recorded at 75 days after inoculation.

EXAMPLE 13

AR Activity and Taxonomy Classification data of *Pleomorphomonas jatrophae*

[0056] The N-fixation ability could be proved by a multidisciplinary approach. Strain R5-392^T was subjected to a *nifH*-specific PCR amplification. The expected 409 bp amplification product was observed with strain R5-392 tested. In the case of strain R5-392, this PCR product was purified and sequenced. The comparison of the resulting sequence with the EMBL database revealed a similarity with the *nifH* gene of *P. oryzae* of 98%. The resulting amino acid (130 deduced amino acids) sequence had 100% similarity to the ATP-dependent reductase or nitrogenase iron-protein (nitrogenase component II, dinitrogenase reductase, *nifH* protein) of *P. oryzae*. The nitrogenase activity of the strain R5-392 was tested by the acetylene-reduction assay method. Strain R5-392^T grown in N-free medium, effectively reduced acetylene and exhibited highest nitrogenase activity of >300 nmol C₂H₄ ml⁻¹ in the headspace gas sample after 96 h incubation at 30 °C with the population increase of >6 log CFU ml⁻¹ (Figure 6a). However, close relatives of R5-392 showed less than 10 nmol C₂H₄ produced per mg of protein (data not shown). Lower concentrations of nitrogen (0.1 mM NH₄Cl) in N-free medium have triggered higher nitrogenase activity than higher concentrations or no NH₄Cl in the medium (Figure 6b). Nitrogenase system was switched-off using NH₄Cl at concentrations >1mM in the N-free

medium. Nitrogenase activity was measured by supplementing $\text{Na}_2\text{MoO}_4/\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at various concentrations (0, 0.1, 0.5, 1, 10 mM) in N-free medium and injected with 15% acetylene (v/v) after inoculation. Nitrogenase activity was higher in 0.1 mM Fe concentration and reduced with increasing concentrations of Fe. No nitrogenase activity was recorded in N-free medium without Fe (Figure 6b).

EXAMPLE 14

Genome Analysis for *Enterobacter* sp R4-369 and *Methylobacterium* sp. L2-4

[0057] Total DNA was extracted for *Enterobacter* sp R4-369 and *Methylobacterium* sp. L2-4 and a partial genomic sequence was obtained for short-gun sequencing by the 454 pyrosequencing. Using the *Klebsiella pneumonia* nif gene cluster as a query (Genbank No 13303), a full set of nif genes were identified (Figure 4). The sequence for *Methylobacterium* sp. L2-4 is set forth in SEQ ID NO:3. In contrast, no such genes can be identified in either *Enterobacter cloacae* (GenBank Accession Nos. NC_014121.1 and NC_014618.1) nor the endophytic plant growth-promoting *Enterobacter* sp. 638 (GenBank Accession No. NC_009436.1).

[0058] Surprisingly, no clear homologue was found in the genome of *Methylobacterium* sp. L2-4 nor the published *Methylobacterium radiotolerans* JCM 2831 genome (GenBank Accession Nos. CP001001 - CP001009), which share about 99% sequence identity in 16S rDNA and total sequence. A Blast search using the *K. pneumonia* nifH protein sequence as query identified distantly related proteins, GenBank Accession Nos. YP_001755485.1 and YP_001754523.1 that share about 35% amino acid identity and was predicted to encode the chlorophyllide reductase iron protein subunit X and protochlorophyllide reductase iron-sulfur ATP-binding protein respectively. This is much lower than that between the *K. pneumonia* nifH and cyanobacteria nifH (GenBank Accession No. ACC79194.1). The genome also lack obvious nif gene cluster. SEQ ID NO:7 encode a putative nifH orthologue. Interestingly, the neighboring putative coding sequences for protochlorophyllide reductase subunits share significant homology to nifD and nifE (Figure 5). As L2-4 is able to fix nitrogen, it is probable that these genes are critical for nitrogen-fixation in *Methylobacterium*.

EXAMPLE 15

Effect of EPS Production on N-Fixation in *Enterobacter*

[0059] N-fixing defective mutants were identified by Tn5 mutagenesis. EPS mutants were identified as small non-motile colonies on N-free solid medium. All EPS mutants have negligible nitrogenase activity as determined by AR assay. Some of the mutants are summarized in Table 8.

TABLE 8

EPS Mutants

Mutant ID	Gene inserted	Tn5 flanking sequence (SEQ ID NO:)
S238	Colanic acid polymerase	cgtaaactcaatttggtcagcatcaaacagtttggtat (5)
S236	EPS exporter	gcgttgcccagggatgtgctgaacgaaccgtg (6)
S210	Colanic acid acetyltransferase WcaF	ccccgcaggtgctgtatcgctggcgcgcatt (7)
S237	GDP-L-fucose synthetase	ctgggtgctgcgttcccgcgaggagctgaacc (8)
S300	Colanic acid biosynthesis UDP-glucose lipid carrier transferase WcaJ	cgcttgaacgttgaatcgcggcacgacgagga (9)

EXAMPLE 16

Genome Sequencing and 16S rRNA Gene Amplification and Sequencing

[0060] Genomic DNA extractions were carried out according to standard protocol (Wilson, 1997) and purified using DNeasy mini spin column (Qiagen Cat. No.: 69106) and sequenced using Genome Sequencer FLX Titanium (GS-FLX Titanium) technology at Macrogen (Republic of Korea). Shotgun sequencing and mate-pair end sequencing (3kb) were performed and sequence assembly of quality filtered reads was performed using a GS De Novo assembler (v 2.6).

[0061] 16S rRNA gene was amplified using universal primers 27F and 1492R were used (Delong, 1992). Cycling conditions were as follows: initial denaturation for 10 min at 95°C; then 30 cycles of 1.5 min at 95°C, 1.5 min at 55°C and 1.5 min at 72°C; and a final extension for 10 min at 72°C. PCR products were ligated into a plasmid vector pGEM-T Easy (Promega, Madison, USA) and transformed into competent cells of *Escherichia coli* strain XL-Blue by using a pGEM-T Easy Vector System (Promega). After the transformants were cultured overnight at 37°C on Luria-Bertani (LB) agar plates containing ampicillin (100 µg ml⁻¹), positive transformants were cultured overnight on LB liquid medium. Plasmids were prepared

by using QIAprep Miniprep (Qiagen). Sequences of the 16S rRNA genes were obtained by using primers 27F (5'-AGAGTTTGATCMTGGCTC-3'; SEQ ID NO:15), 1492R (5'-TACCTTGTACGACTT-3'; SEQ ID NO:16), 785F (5'-GGATTAGATACCCTGGTA-3'; SEQ ID NO:17), 518R (5'-GTATTACCGCGGCTGCTGG-3'; SEQ ID NO:18), 1100R (5'-GGGTTGCGCTCGTTG-3'; SEQ ID NO:19) and their nucleotide sequences were determined with an Applied Biosystems 3730 XL DNA sequencer (AB Applied Biosystems, HITACHI). The 16S rDNA sequences were aligned by using the Megalign program of DNASTAR. Sequence similarity analyzed by the EzTaxon server (<http://www.eztaxon.org/>) (Chun et al., 2007).

EXAMPLE 17

Effect of Inoculation of Enterobacter and Methylobacterium Sp on Oil Palm

[0062] Seedling of micropropagated interspecies oil palm hybrid clones which were potted in non-sterilized soil were inoculated by pouring into the soil (for enterobacter R4-368) or sprayed until dripping to the leaves (for Methylobacterium sp L2-4). Plants were grown in the open air. Compared with mocked inoculated plants, plant height was significantly improved when inoculated both strains. For leaf number, all treatment gave a significantly higher leaf numbers (Table 8). These results were associated with a higher endophytic nitrogen fixing bacteria counts in the root and on the upper leaf surface (Table 10).

Table 9

Plant Height and Leave Number at 150 Days After Inoculation

Plant Height												
Plant series No.	P1	P2	P3	P4	P5	P6	P7	P8	P9	Avr	STDEV	P value
Mock inoculated	60	55	56	35	61	54	59	60	55	55.00	7.94	
L2-4	42	55	52	69	53	68	57	67	51	57.11	9.16	
R4-368	57	54	58	65	61	61	60	69	59	60.44	4.42	0.09
L2-4+R4-368	56	63	66	68	48	61	69	65	71	63.00	7.21	0.04
Leave Number												
Plant series No.	P1	P2	P3	P4	P5	P6	P7	P8	P9	Avr	STDEV	
Mock inoculated	10	10	9	11	10	10	11	10	10	10.11	0.60	
L2-4	12	13	13	13	13	12	12	13	13	12.67	0.50	0.0005
R4-368	11	11	12	12	12	12	12	11	11	11.56	0.53	0.0005
L2-4+R4-368	13	13	11	12	12	12	12	11	11	11.89	0.78	0.0005

Table 10

Nitrogen Fixing Bacteria Counts at 150 Days After Inoculation

Treatments	Root population ¹	STDEV	leaf population ²	
	cfu per g		cfu/cm2	STDEV
Mock inoculated	1.27 x10 ⁶	0.20	0	0
L2-4	Nd		7.33	0.88
R4-368	3.68 x10 ⁶	0.05	Nd	
L2-4+R4-368	1.85 x10 ⁷	0.22	8.00	1.73

Note: 1. root were surface-sterilized before gliding. 2. Non-sterilized upper leaf surface were imprinted on agar plates and the red-pigmented colonies were scored 3 days after incubation in 30°C.

EXAMPLE 18

Effect Of Nitrogen-Fixation Genes on Growth Promotion and Leaf N Content

[0063] Knockout mutant of *nifH*, *nifD* and *nifK* genes were created in *Enterobacter* R4-368 by electroporation of the respective knockout construct containing about 1 kb flanking sequences fused at both sides of a synthetic mini-Tn5 transposon (Kanamycin resistant). Clean knockout mutants were confirmed by PCR and Southern blotting. The mutant and wildtype strains were inoculated to the root system of plants derived from surface sterilized *Jatropha curcas* seeds and potted in sterilized sand and vermiculite (1:1) mix. Inoculation was done by pouring 100 ml cell preparation (1 OD600 in water) into each pot. Plants were grown in open air in a greenhouse. Plant height, number of leaves and dry biomass were measured at 45 days after inoculation. Dried leaf biomass (~ 2 mg) from 4 plants was also subjected to nitrogen content analysis, which was done with the Elementar Vario Micro Cube (ELEMENTAR Analysensysteme, Germany). As can be seen in Table 11, all knockout mutants showed similar plant height, leaf number, biomass and N content to mock inoculated plants whereas significant improvements were observed in plants inoculated with wildtype *Enterobacter* sp. R4-368.

Table 11

Effect of *nifHDK* Gene Deletion in *Enterobacter* sp. R4-368 at 45 Days After Inoculation

strains	Plant height (cm)*	No. of leaves*	Shoot biomass (g)*	Root Biomass (g)*	Leaf N content (%)#
Wildtype	34.0±5.8	10.0±2.4	7.1±0.7	1.4±0.1	1.968±0.323
<i>nifHΔ</i>	25.3±4.5	8.1±1.2	4.1±1.3	0.7±0.1	1.055±0.013
<i>nifDΔ</i>	23.1±4.5	7.1±1.2	4.4±0.4	0.6±0.1	0.878±0.019

<i>nifKΔ</i>	22.6±3.9	7.3±1.3	4.4±0.5	0.8±0.1	1.263±0.025
mock	17.9±1.3	6.1±1.2	2.6±0.5	0.6±0.0	0.903±0.025

*n=7; # n=4

EXAMPLE 19

Effect of EPS Gene Cluster on Plant Colonization

[0064] *Jatropha* seed (cultivar MD-44) coats were removed, seed kernel were sterilized with 75% ethanol (v/v) for 1 min followed by 10% H₂O₂ (v/v) for 60 min followed by five subsequent rinses in sterile distilled water (SDW) and immersed in SDW overnight at 28° C in darkness. The endosperm-free embryos were germinated on hormone-free seed germination medium (1/2 MS salt, B5 Vitamins, 5 g/L sucrose, 0.5 g/L MES and 2.2 g/L phytagel, pH 5.6) and cultured in a tissue culture room, at 25° C ± 2° C in a 16h light (100 μmol/m²s)/8h dark cycle. Strains used are wild-type *Enterobacter* sp. R4-368, Tn5 mutant S300 which contains a Tn5 transposon in the EPS gene cluster and produce very little EPS and the knockout mutant that has the whole EPS gene cluster deleted. The mutant and wildtype strains were inoculated to the root system of plants derived from surface sterilized *Jatropha curcas* seeds and potted in sterilized sand and vermiculite (1:1) mix. Inoculation was done by pouring 100 ml cell preparation (1 OD₆₀₀ in water) into each pot. Plants were grown in open air in a greenhouse with regular watering of water or nutrient solutions. Both the Tn5 mutant and knockout mutant of the EPS genes showed significant reduction (p<0.05) in plant heights and biomass (Table 12).

Table 12

Effect of *Enterobacter* or EPS Genes on *Jatropha* Growth Promotion

Strains	Plant height (cm) ¹	Dry biomass (g) ²	
		Shoot	Root
<i>Enterobacter</i> sp. R4-368	28.1±2.90	9.50±0.57	1.74±0.04
Knockout EPS 130-1	24.9±2.90	6.75±0.76	1.15±0.17
sTn5 mutant S300	26.0±1.50	6.90±0.31	1.14±0.12
Mock inoculated	23.3±2.30	7.57±0.30	0.96±0.20

Note: Each value represents the mean and SD of 7 plants. 1. 30 days after inoculation. 2. 45 days after inoculation.

[0065] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to

cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if the range 10-15 is disclosed, then 11, 12, 13, and 14 are also disclosed. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0066] It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A biologically pure culture of a bacterial species selected from the group consisting of:
 - (a) *Enterobacter* species R4-368 (NRRL B-50631);
 - (b) *Pleomorphomonas jatrophae* R5-392 (NRRL B-50630);
 - (c) *Methylobacterium* sp. L2-4 (NRRL B-50628);
 - (d) *Methylobacterium* sp. L2-76 (NRRL B-50629); and
 - (e) *Sphingomonas* sp. S6-274 (NRRL B-50632).
2. A bacterial inoculant for application to plants, said inoculant comprising an effective quantity of a biologically pure culture of at least one bacterial species selected from bacterial species of an *Enterobacter* genus, *Methylobacterium* genus, *Sphingomonas* genus and *Pleomorphomonas* genus, wherein the bacterial species reduces acetylene to ethylene or reduces atmospheric nitrogen (N_2) to ammonia (NH_3).
3. The bacterial inoculant of claim 2, wherein the reduction occurs on the leaf surface, inside the leaf surface, inside the root tissue or on the surface of the root system.
4. The bacterial inoculant of claim 3, wherein the reduction occurs in association with a plant selected from the group of plant species of a *Jatropha* genus, *Sorghum* genus, *Gossypium* genus, *Elaeis* genus, *Ricinus* genus, *Oryza* genus and *Manihot* genus.
5. The bacterial inoculant of any one of claims 2-4, wherein the bacterial species is selected from the bacterial species of claim 1 or any combination thereof.
6. The bacterial inoculant of claim 2, wherein the bacterial species secretes exopolysaccharide (EPS) or endoglucanase.
7. The bacterial inoculant of claim 2, wherein the *Enterobacter* species contains a DNA sequence sharing at least 97% and preferably at least 98% identity to SEQ ID NO:10 and wherein the *Enterobacter* species contains a set of genes required for nitrogen-fixation.

8. The bacterial inoculant of claim 2, wherein the *Pleomorphomonas* species contains a DNA sequence sharing least 97% and preferably at least 98% identity to SEQ ID NO:11 and wherein the nitrogen-fixing capability of the *Pleomorphomonas* species is stimulated by about 0.1 to about 0.5 mM of NH_4^+ ion.
9. The bacterial inoculant of claim 2, wherein the *Sphingomonas* species contains a DNA sequence least 97% and preferably at least 98% identity to SEQ ID NO:12 and wherein the *Sphingomonas* species able to grow in nitrogen-free medium.
10. The bacterial inoculant of claim 2, wherein the *Methylobacterium* species contains a DNA sequence sharing least 97% and preferably at least 98% identity to SEQ ID NO:13 or SEQ ID NO:14.
11. The bacterial inoculant of any one of claims 2-10, wherein the bacterial inoculant further comprises a carrier.
12. The bacterial inoculant of any one of claims 2-11, wherein the bacterial inoculant further comprises trace metal ions selected from the group consisting of molybdenum (Mo^{2+}) ions, iron (Fe^{2+}) ions, manganese (Mn^{2+}) ions, or any combination of these ions.
13. The bacterial inoculant of claim 12, wherein the concentration of the trace metal ions in the inoculant is between about 0.1 mM and about 50 mM.
14. A method of improving plant productivity comprising inoculating a plant with an effective quantity of the bacterial inoculant of any one of claims 2-13.
15. The method of claim 14, wherein the bacterial inoculant is sprayed on aerial plant parts.
16. The method of claim 14, wherein the bacterial inoculant is applied by inserting into a planting furrow, watering the plant root system in the soil, dipping the roots in the bacteria inoculant or coating seeds

17. A method of improving plant productivity comprising inoculating a plant with an effective quantity the bacterial inoculant of any one of claims 2-12 and inoculating a plant with trace metal ions selected from the group consisting of molybdenum (Mo^{2+}) ions, iron (Fe^{2+}) ions, manganese (Mn^{2+}) ions, or any combination of these ions.
18. The method of claim 17, wherein the concentration of the trace metal ions in the inoculant is between about 0.1 mM and about 50 mM.
19. The method of claim 17 or 18, wherein the bacterial inoculant and trace metal ions are sprayed on aerial plant parts.
20. The method of any one of claims 14-19, wherein the plant productivity is plant growth, maintenance of high chlorophyll content in leaves, increasing fruit or seed numbers, increasing fruit or seed unit weight or any combination of these characteristics.
21. The method of any one of claims 14-20, wherein the plant is a species of a *Jatropha* genus, *Sorghum* genus, *Gossypium* genus, *Elaeis* genus, *Ricinus* genus, *Oryza* genus or *Manihot* genus.

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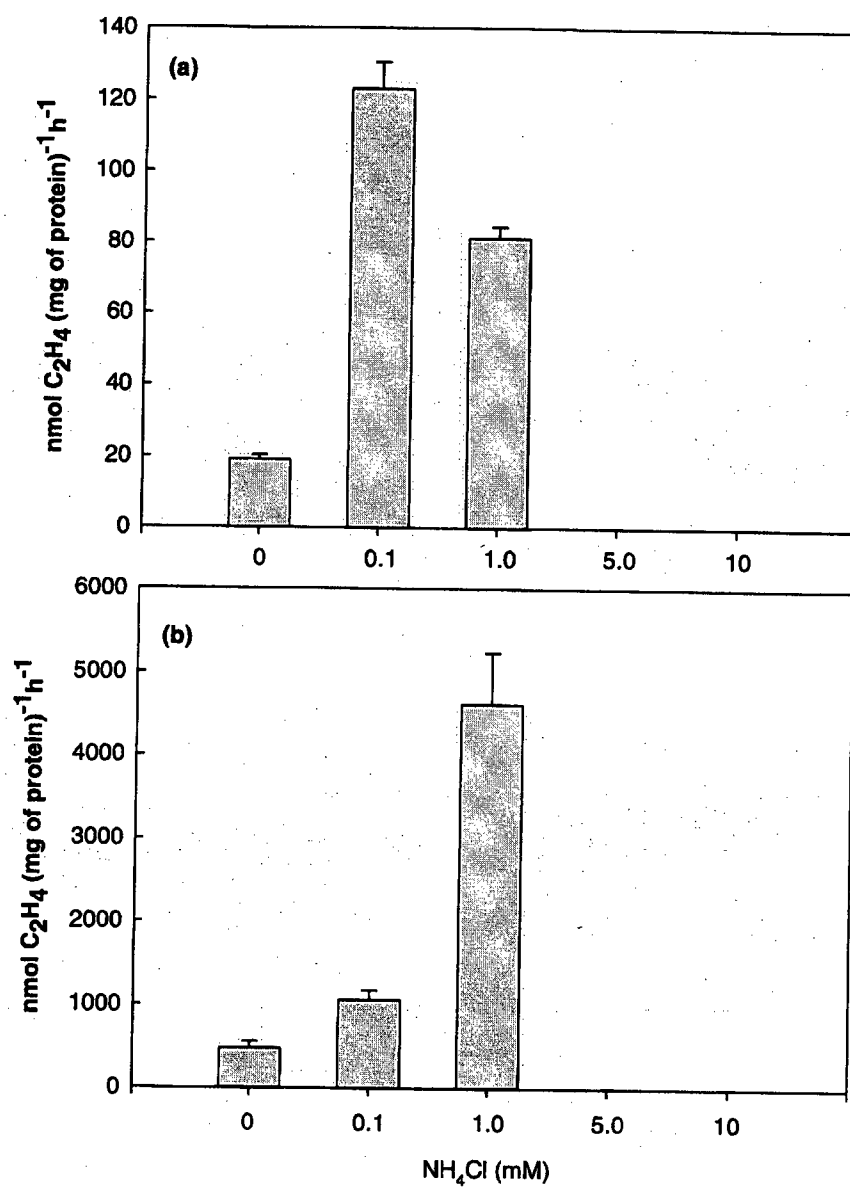


FIGURE 1

2/5

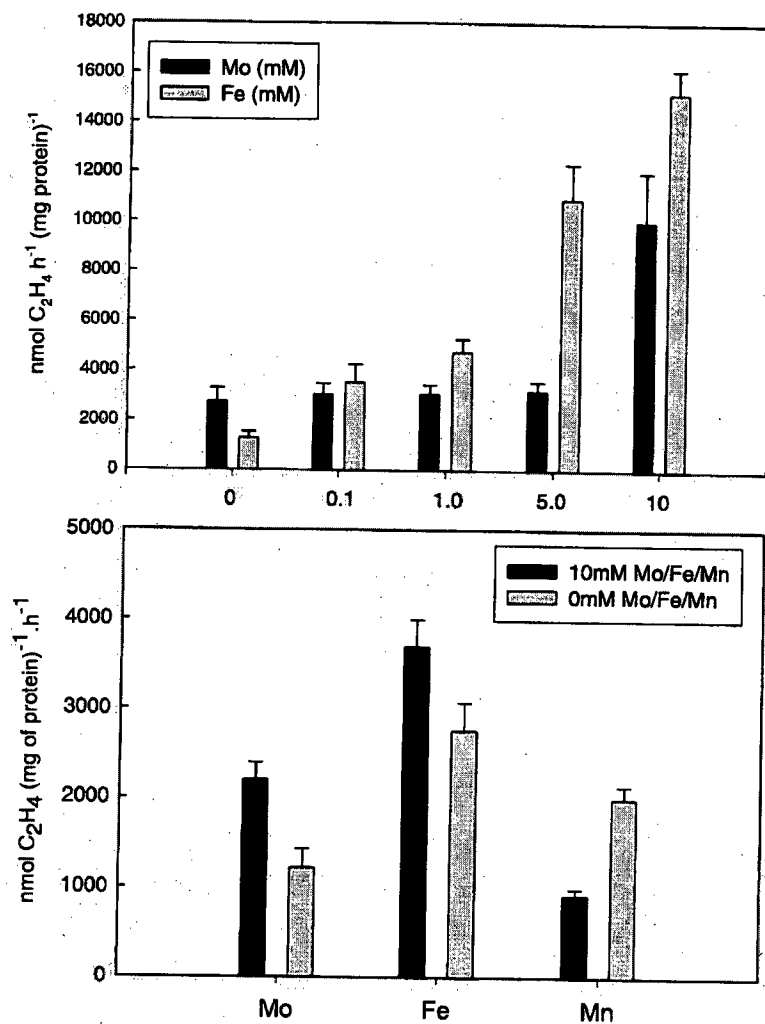


FIGURE 2

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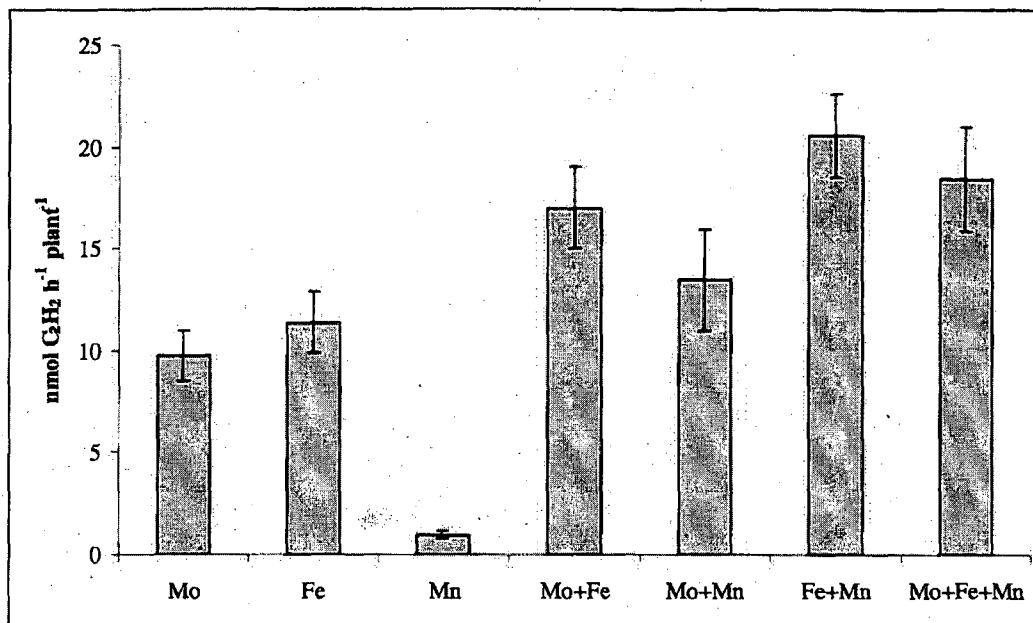


FIGURE 3

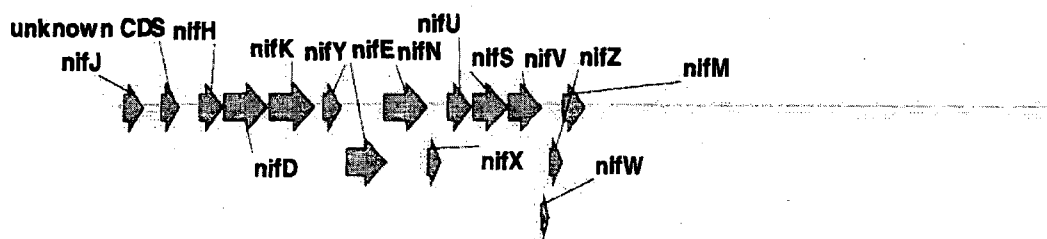


X13303

24206 bp

FIGURE 4a

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R4-368 Contig 27 NifCluster

31707 bp

FIGURE 4b



L2-4 contig 00028 nif-like genes

14762 bp

FIGURE 5

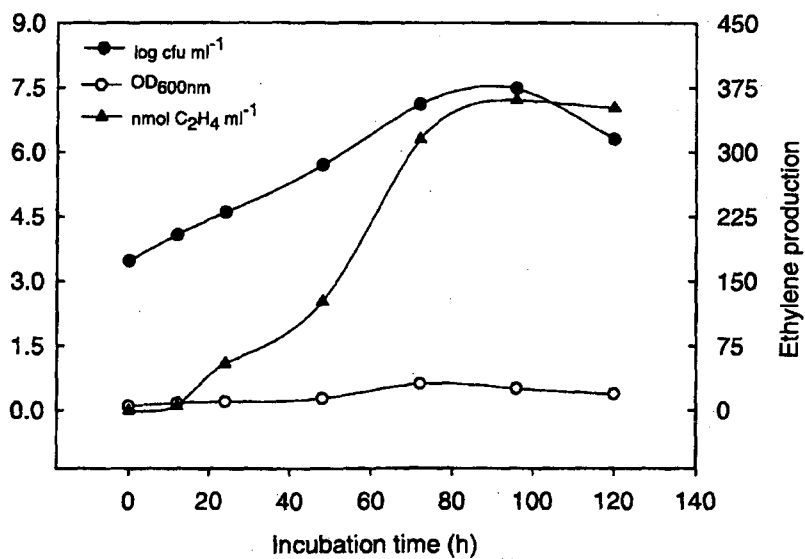


Figure 6a

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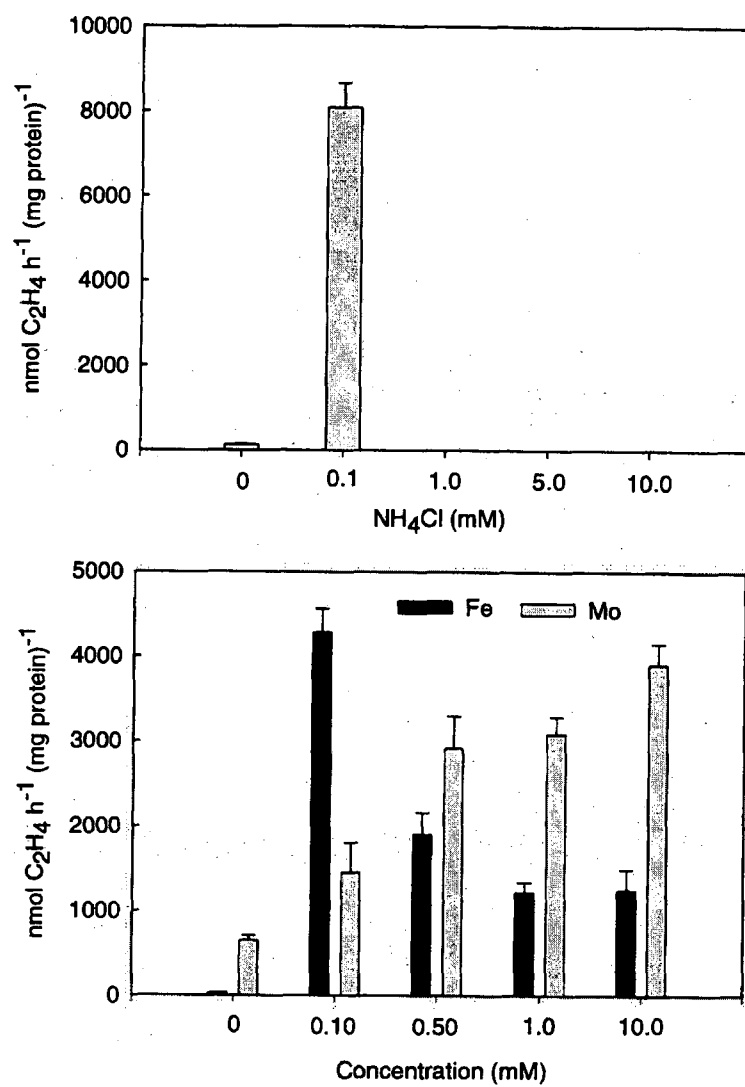


FIGURE 6b

A. CLASSIFICATION OF SUBJECT MATTER**C12N 1/20 (2006.01) A01N 63/00 (2006.01)**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: WPI, EPODOC, MEDLINE, HCAPLUS, BIOSIS, AGRICOLA. Keywords: The bacterial strain accession numbers of claim 1, Enterobacter, Pleomorphomonas, Methylobacterium, Sphingomonas, nitrogen fixing, nitrogen reducing, inoculation, Jatropha, Sorghum and like terms.

Genomequest: SEQ ID NO:10-14 searched at 96% identity.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 3 May 2013		Date of mailing of the international search report 03 May 2013	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au Facsimile No.: +61 2 6283 7999		Authorised officer David Bennett AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6222 3651	

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☒ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purposes of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Box No. IV Text of the Abstract (Continuation of item 5 of the first sheet)

The present invention relates to methods of reducing chemical fertilizer usage and greenhouse gas nitrous oxide emission and to methods of improving plant growth rate and seed productivity in agriculture through the application of a novel artificially manufactured formula containing a nitrogen-fixing bacterium that efficiently colonizes non-legume plants in aerial parts and the root system. The bacteria inocula and methods are particularly suitable for plants in the genera *Jatropha*, *Sorghum*, *Gossypium*, *Elaeis*, *Ricinus*, *Oryza* and *Manihot*.

Specific bacterial strains disclosed include *Enterobacter* species R4-368 (NRRL B-50631), *Pleomorphomonas jatrophae* R5-392 (NRRL B-50630), *Methylobacterium* species L2-4 (NRRL B-50628), *Methylobacterium* species L2-76 (NRRL B-50629) and *Sphingomonas* species S6-274 (NRRL B-50632).

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2013/000112
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Madhaiyan, M. et al., "Nodulation and plant-growth promotion by methylotrophic bacteria isolated from tropical legumes", Microbiological Research. 2009 vol. 164 pages 114-120. Page 115 column 2 paragraph 2, Table 2, Figure 1	1-21
X	Cardoso, J.D. et al., "Polyphasic approach for the characterization of rhizobial symbionts effective in fixing N ₂ with common bean (<i>Phaseolus vulgaris</i> L.)", Applied Microbiology and Biotechnology. 2012 vol. 93 pages 2035-2049. Published online 9 December 2011. Abstract	1-21
X	Lee, H.S. et al., "Physiological enhancement of early growth of rice seedlings (<i>Oryza sativa</i> L.) by production of phytohormone of N ₂ -fixing methylotrophic isolates", Biology and Fertility of Soils. 2006 vol. 42 pages 402-408. Abstract, Figure 3	1-21
X	Adhikari, T.B. et al., "Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice", Canadian Journal of Microbiology. 2001 vol. 47 pages 916-924. Abstract, Tables 1-3, page 917 paragraph spanning columns 1-2	1-21
A	US 7214509 B2 (SCHNOOR et al.) 08 May 2007 SEQ ID NO:1 (shares 99.78% identity with present SEQ ID NO:14), column 13 lines 42-44	10
A	US 2011/0028321 A1 (TANI et al.) 03 February 2011 SEQ ID NO:7 (shares 99.18% identity with present SEQ ID NO:14), table 9	10
A	Xie, C.H. et al., "Pleomorphomonas oryzae gen. nov., sp. nov., a nitrogen-fixing bacterium isolated from paddy soil of <i>Oryza sativa</i> ", International Journal of Systemic and Evolutionary Microbiology. 2005 vol. 55 pages 1233-1237. Page 1233	8
A	Madhaiyan, M. et al., "Regulation of ethylene levels in canola (<i>Brassica campestris</i>) by 1-aminocyclopropane-1-carboxylate deaminase-containing <i>Methylobacterium fujisawaense</i> ", Planta. 2006 vol. 224 pages 268-278. Table 1	10
P,X	Madhaiyan, M. et al., "Pleomorphomonas diazotrophica sp. nov., a novel endophytic N-fixing bacterium isolated from the root tissue of <i>Jatropha curcas</i> L.", International Journal of Systemic and Evolutionary Microbiology. Epub 07 December 2012. Abstract	1-6, 8, 11-21
P,X	GenBank accession no. JQ346801 10 July 2012 Pleomorphomonas sp. R5-392 16S ribosomal RNA gene, partial sequence. Sequence shares 100% identity with SEQ ID NO:11	1-6, 8, 11-21
P,X	GenBank accession no. JQ660218 01 April 2012 Sphingomonas yunnanensis strain S6-274 16S ribosomal RNA gene, partial sequence. Sequence shares 100% identity with SEQ ID NO:12	1-6, 9, 11-21
P,X	GenBank accession no. JQ659310 01 April 2012 Methylobacterium sp. L2-4 16S ribosomal RNA gene, partial sequence. Sequence shares 98% identity with SEQ ID NO:13	1-6, 10-21
Form PCT/ISA/210 (fifth sheet) (July 2009)		

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2013/000112
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GenBank accession no. JQ659313 01 April 2012 Methylobacterium populi strain L2-76 16S ribosomal RNA gene, partial sequence. Sequence shares 100% identity with SEQ ID NO: 14	1-6, 10-21
P,X	GenBank accession no. JQ659688 01 April 2012 Enterobacter sp. R4-368 16S ribosomal RNA gene, partial sequence. Sequence shares 99% identity with SEQ ID NO:10	1-7, 11-21

Form PCT/ISA/210 (fifth sheet) (July 2009)

Supplemental Box

Continuation of: Box III

The specification does not comply with Section 40(4) because the claims do not relate to one invention only. I have found different inventions based on the following features that separate the claims into distinct groups:

- Invention 1: Claim 7 (in full) and claims 1-6, 11-21 (all in part). The feature of *Enterobacter* species R4-368 (NRRL B-50631) is specific to this group of claims.
- Invention 2: Claim 8 (in full) and claims 1-6, 11-21 (all in part). The feature of *Pleomorphomonas jatrophae* R5-392 (NRRL B-50630) is specific to this group of claims.
- Invention 3: Claims 1-6, 10-21 (all in part). The feature of *Methylobacterium* species L2-4 (NRRL B-50628) is specific to this group of claims.
- Invention 4: Claims 1-6, 10-21 (all in part). The feature of *Methylobacterium* species L2-76 (NRRL B-50629) is specific to this group of claims.
- Invention 5: Claim 9 (in full) and claims 1-6, 11-21 (all in part). The feature of *Sphingomonas* species S6-274 (NRRL B-50632) is specific to this group of claims.

Unity of invention is only fulfilled when there is at least one “special technical feature” present in the claims that both:

- provides a technical relationship among all the claims; and,
- makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified distinguishing features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2013/000112	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
US 7214509 B2 08 May 2007 US 2005054030 A1 10 Mar 2005 US 2011/0028321 A1 03 Feb 2011 US 7214509 B2 08 May 2007 US 2008032382 A1 07 Feb 2008 WO 2005019136 A2 03 Mar 2005 EP 2239319 A1 13 Oct 2010 US 2011028321 A1 03 Feb 2011 WO 2009093675 A1 30 Jul 2009			
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			